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Fosfoinositidy a jejich efektory v regulaci signální dráhy proteinu Wnt
Phosphoinositides and their effectors in regulation of the Wnt signaling pathway

Bakalářská práce

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Abstract

Phosphoinositides (PIs) make up only a small proportion of overall amount of lipids in cell membranes. However, their function mediated through protein effectors is indispensable for cell signaling, vesicular trafficking, cell movement and other important aspects of cellular life. In this bachelor thesis function of PIs is described in relation to Wnt signaling pathway. Proper execution of several steps of the Wnt signaling pathway requires the presence of PIs. Retrograde transport of Wntless (Wls) from the plasma membrane (PM) back to the Golgi apparatus (GA) in Wnt producing cells or internalization of Wnt receptors in Wnt receiving cells are only two examples. All processes are tightly regulated and malfunction of enzymes processing PIs can cause their deregulation resulting in disruption of the Wnt signaling pathway. As deregulated Wnt signaling is a known cause of serious diseases including cancer, understanding the crosstalk between PIs and Wnt signaling could help in designing novel strategies for therapeutic intervention.

Keywords: phosphoinositides, phosphoinositide effectors, endocytosis, Wnt signaling, signal transduction

Abstrakt

Fosfoinositidy představují pouze malou část celkového množství lipidů v buněčných membránách. Přesto je jejich činnost zprostředkovaná proteinovými efektory nezbytná pro buněčnou signalizaci, vezikulární transport, pohyb buňky a jiné důležité procesy v životě buňky. Tato bakalářská práce popisuje funkci fosfoinositidů ve Wnt signální dráze. Při Wnt signalizaci je mnoho momentů kdy je nezbytná spolupráce s fosfoinositidy. Retrográdní transport Wntless molekuly (Wls) z plazmatické membrány zpět do Golgiho aparátu nebo internalizace Wnt receptorů v buňkách citlivých vůči Wnt signalizaci jsou pouze dva příklady. Oba procesy jsou přísně regulovány a poruchy funkce enzymů zpracovávajících fosfoinositidy může způsobit deregulaci těchto procesů, eventuálně deregulaci Wnt signální dráhy. Jelikož je deregulace Wnt signální dráhy známá příčina vážných nemocí včetně rakoviny, pochopení interakcí mezi fosfoinositidy a Wnt signalizací může pomoci vývoji nových strategií v léčbě těchto chorob.

Klíčová slova: fosfoinositidy, efektory fosfoinositidů, endocytóza, Wnt signalizace, signální transdukce

Content

1 Introduction

Wnts are lipid modified molecules. Their posttranslational modifications link their functions with cell membranes. Role of phosphoinositides (PIs) in regulation of membrane activities thus logically concerns Wnt transport within both, Wnt producing and receiving cells. Direct interaction of Wnts and PIs is not likely; however, many effector proteins of PIs have been shown to be involved in Wnt signaling. Malfunction of these effectors disrupts proper PIs distribution throughout a cell and causes faulty Wnt signaling (Logan and Nusse 2004).

Phosphatidylinositol (PtdIns) is built of hydrophobic diacylglycerol (DAG) backbone bound by ester bond to hydrophilic inositol ring. Hydroxyl groups 3, 4, and 5 of inositol or their combination can be phosphorylated creating seven different phosphoinositides (PIs): three monophosphorylated PIs - phosphatidylinositol 3-phosphate (PtdIns(3)P), phosphatidylinositol 4-phosphate (PtdIns(4)P), phosphatidylinositol 5-phosphate (PtdIns(5)P), three bisphosphorylated PIs - phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P), phosphatidylinositol 3,5-bisphosphate (PtdIns(3,5)P), phosphatidylinositol 4,5-bisphosphate (PtdIns(4,5)P), and finally one trisphosphorylated PI: phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P). These PIs can be converted to each other due to the activity of specific kinases and phosphatases **(Figure 1)**.

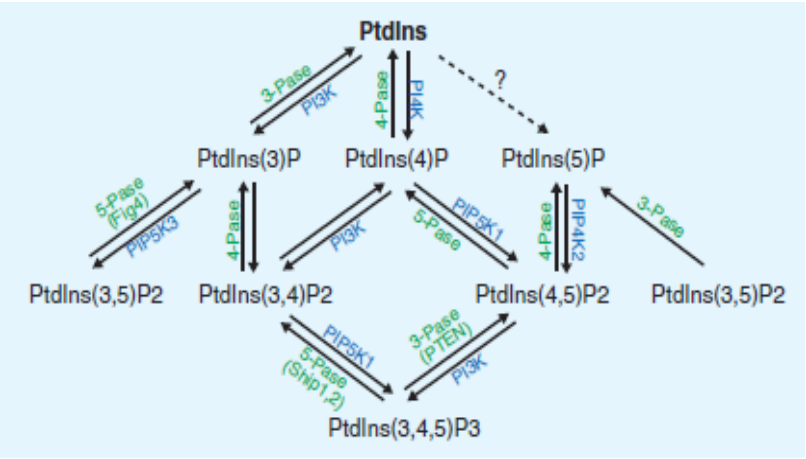

Figure 1: Known kinases (blue) and phosphatases (green) participating in creation of different PIs from PtdIns or their conversion from other PIs. (Vicinanza et al. 2008)

PIs are always located within cell membranes and their location is highly organized. Particular PI can be found in particular membrane. PtdIns(4,5)*P*, for example, is accumulated mostly in the plasma membrane (PM) and thus serves as the PM marker. Structural proteins and enzymes coordinating process of endocytosis need to be located on the cytosolic surface of the PM or in its proximity and PtdIns(4,5)*P* is used as the first point of accumulation of these proteins (Jost

et al. 1998). Location of other members of the PIs family is also known – PtdIns(3)*P* in the early endosome membrane, PtdIns(4)*P* in the Golgi apparatus (GA) membrane, PtdIns(3,5)*P* in the late endosome membrane (reviewed in Mayinger 2012). This, of course, does not mean that these PIs are found exclusively in the mentioned membranes. There is a combination of different PIs in each membrane; however, one PI is predominant and specific for a particular membrane.

PIs serve as docking platforms for many effector proteins. It is therefore necessary for all effector proteins to contain domains responsible for binding PIs. Some of these domains are well known and studied, for example, plecstrin homology (PH), epsin N-terminal homology (ENTH), phagocytic oxidase (PX) and FYVE domains which are actual structural domains (reviewed in Krauss et al. 2007). Another type of domain is known, consisting of a stretch of basic amino acids rather than of any tertiary structure (for example see Amer1 PtdIns(4,5)*P* binding site in chapter 4.1). Structural domains of proteins bind more or less specifically to different PIs. Proper proteins are thus located to proper sites by recognizing the local "PI code".

Wnts are lipid-modified secreted signal glycoproteins rich in cysteine which are well conserved throughout evolution and have important roles in both vertebrate and invertebrate development. Their function is, however, indispensable in adult individuals as well because they participate in regulation of homeostasis. Mutations of genes coding Wnts and other members of the Wnt signaling pathway can lead to developmental problems and can be even lethal in some cases (reviewed in Logan and Nusse 2004; Reya and Clevers 2006; Clevers 2006).

Similarly to other proteins designated for secretion, Wnts are cotranslationally translocated to the endoplasmic reticulum (ER) where they are modified by the activity of an enzyme called Porcupine (Kadowaki et al. 1996) at specific, conserved sites. These sites are cysteine amino acid residue (Cys77) modified with a palmitate group (Willert et al. 2003) and serine amino acid residue (Ser209) to which a palmitoleic acid is added (Takada et al. 2006). Fatty acid residues cause Wnt molecules to be strongly hydrophobic.

Next step in Wnt secretion is vesicular transport from the ER to the GA where Wntless (Wls) molecule is present (Bänziger et al. 2006). Wls is a transmembrane protein working as an intracellular receptor which is necessary for biding Wnt molecules, accumulating them and helping to transport them to the PM. Wls is then recycled from the PM through endosome 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).

Hydrophobic properties of Wnts pose a problem for their transport through extracellular space from the secreting to the receiving cells. However, it is beyond doubt that such transport works. What is not entirely explained is how exactly this transport is accomplished. Quite a few possibilities have been suggested, among them transport of Wnts from the PM of one cell to the PM of another at the site of contact, transport through a membraneous vesicle or covering insoluble parts of the Wnt molecule by a protein with amphiphilic nature (reviewed in Port et al. 2010). It seems that different cells employ different mechanisms to solve this problem.

To be able to react to the Wnt molecule and transmit the signal, it is necessary for the receiving cell to have a suitable receptor on its surface. Proteins known to fulfill this task are those of the Frizzled (Fz) family (Bhanot et al. 1996) in cooperation with a LDL receptor-related protein (LRP) (Wehrli et al. 2000). Fz is spanning seven times through the PM and has extracellular and intracellular domains. After Wnt ligand biding to the extracellular Fz N-terminal cysteine-rich domain (CRD), the intracellular part of Fz undergoes a structural change which results in Dishevelled molecule (Dvl) biding (reviewed in Logan and Nusse 2004). Dvl helps to bind another scaffold protein, Axin, preventing it from creating complex with Casein kinase-1α (CK1α) and glycogen synthase kinase-3 (GSK3). In cells without Wnt ligand bound to its Fz receptor, this complex phosphorylates β-catenin causing its degradation. Wnt signal thus serves as a factor promoting stabilization of β-catenin through signal transduction. β-catenin is transported to the cell nucleus where it forms complexes with transcription factors (TFs) such as T-cell factor (Tcf) and lymphoid-enhancer biding factor (Lef) promoting transcription of genes affected by those TFs (Cadigan and Nusse 1997). This way of Wnt signaling is termed canonical in literature. There are other known ways of so-called non-canonical Wnt signaling, e.g. planar cell polarity (PCP) and Ca^{2+} (reviewed in Widelitz 2005). However, the canonical one is much more studied and better understood.

2 Interface between PIs and effector proteins - PIs biding domains

Proteins which are meant to execute their activities in the vicinity of different membranes throughout a cell need to be able to interact with those membranes. This could be done in two ways. Effector protein can either itself contain a binding domain for PIs or can create complex with another protein which contains such a domain. Depending on the strength of the interaction between biding domain and its target PIs, it could be sufficient for effector protein recruitment to a membrane. If this interaction is not strong enough there has to be another way to support this weak linkage. One possibility is a stretch of hydrophobic amino acid residues interacting with hydrophobic region of a membrane. Second possibility is a stretch of positively charged amino acid residues interacting with the negative charge of phospholipids, including the phosphate groups of PIs. Third way is another binding domain specific for membrane protein or phospholipid. Combined interaction of two binding domains with two targets is usually strong enough. PIs binding domains are sometimes specific only for one type of PIs. However, they could also be promiscuous to some extent (Harlan et al. 1994). In this chapter, structure of three PIs binding domains - PH, PX and FYVE - will be described as an example.

PH domain was one of the first phospholipid binding domain to be discovered. It was first observed to be contained twice in the amino acid sequence of pleckstrin, a substrate of protein kinase C (PKC) in platelets (Haslam and Kolde 1993). N-terminal pleckstrin PH domain consists of approximately 100 amino acid residues and is able to bind PtdIns(4,5)*P* and to lesser extent PtdIns(4)*P* (Harlan et al. 1994). Another example of PH containing protein is phospholipase Cδ (PLCδ). PH domain of phospholipase Cδ (PLCδ-PH) also binds inositol-1,4,5-trisphosphate (Ins(1,4,5)*P*) (Lemmon et al. 1995), which is basically a head group of PtdIns(4,5)*P*. Generally, Ins(1,4,5)*P* is created from PtdIns(4,5)*P* by enzymatic activity of PLC. Increasing amount of Ins(1,4,5)*P* binds to PH domain of PLC with greater affinity than PtdIns(4,5)*P* (Hirose et al. 1999). PLC therefore dissociates from the PM to the cytoplasm. Succession of these events creates a negative loop for regulation of the amount of the secondary messengers Ins(1,4,5)*P* and diacylglycerol (DAG) in a cell.

Crystallographic data and NMR of PH domains suggests that it is folded into α-helix at its C-terminal. This α-helix is enclosed to one side of β-sandwich composed of seven β-strands. Three variable loops located opposite to the α-helix, between β-strands 1-2, 3-4 and 6-7, are positively charged due to the amino acid residues which they contain (**Figure 2**). This represents a possible area through which the negatively charged phosphate groups of PIs are

bound (Lemmon et al. 1995). With respect to the Wnt signaling, effector proteins carrying the PH domain are mainly involved in interactions with PtdIns(4,5)*P* in the PM during the process of endocytosis in both Wnt producing and Wnt receiving cells (described in more details in chapters 3.4.1 and 4.1). However, it has been shown that PIs other than PtdIns(4,5)*P* may interact with PH of some proteins (Isakoff et al. 1998). Although it is not always high-affinity binding, it can still play an important role in regulation of some cellular processes. PH domain is the most promiscuous PIs biding domain of three described in this chapter.

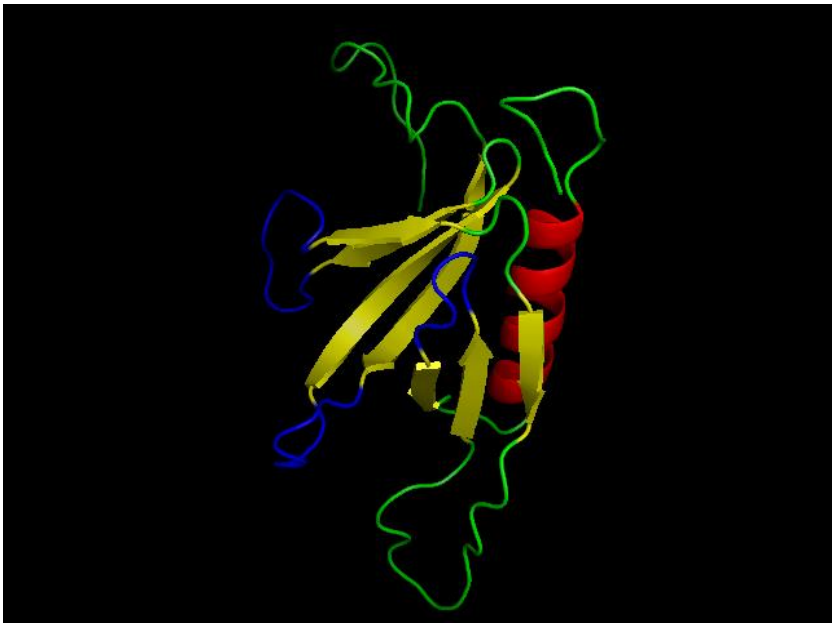

Figure 2: Structure of C-terminal PH domain of human pleckstrin. α-helix at C-terminus (red), β-strands (yellow), variable loops (green), variable loops involved in PIs binding (blue). PDB entry 1X05, NMR data (authors: Li et al. 2005). Modified in PyMOL.

PX domain containing proteins are selectively recruited to membranes containing PtdIns(3)*P*. Interaction between PtdIns(3)*P* and PX domain is specific, nevertheless, it is not always high-affinity binding. In yeast only four of fifteen tested PX domain carrying proteins bind to PtdIns(3)*P* with high affinity (Yu and Lemmon 2001). One of the high affinity interactions was observed in the yeast homolog of SNX3 which plays an important role in Wls recycling in higher organisms (discussed in more details in chapter 3.4.2). Proteins which bind to PtdIns(3)*P* with low affinity are not able to be recruited to a membrane through one PX domain only. They either have to contain another biding domain or have to be associated with other proteins in a complex. Yeast homolog of Sorting nexin 1 (SNX1), for example, forms heterodimer with yeast Vps17 protein (Horazdovsky et al. 1997). As yeast Vps17 also contains a low-affinity PX binding domain, such heterodimer would bind PtdIns(3)*P* containing membrane more readily.

PX domain consists of approximately 130 amino acid residues. Primary sequence of the domain is not conserved very well. However, secondary and tertiary structures seem to be conserved throughout PX domains in different proteins. Three β-strands create β-sheet which is positioned close to three α-helices (**Figure 3**). This structure forms a pit which is able to contain the hydrophilic part of PtdIns(3)*P*. One of the most important amino acid residue is Arg58 which interacts directly with the phosphate group at position 3 of the inositol ring (Bravo et al. 2001). Mutation of this residue leads to loss of PtdIns(3)*P* biding ability even though the overall structure of the PX domain does not seem to be changed.

In Wnt signaling, PX domain containing effector proteins are involved in the retromer complex recruitment to the membrane of early endosome (discussed in more detail in chapter 3.4.2).

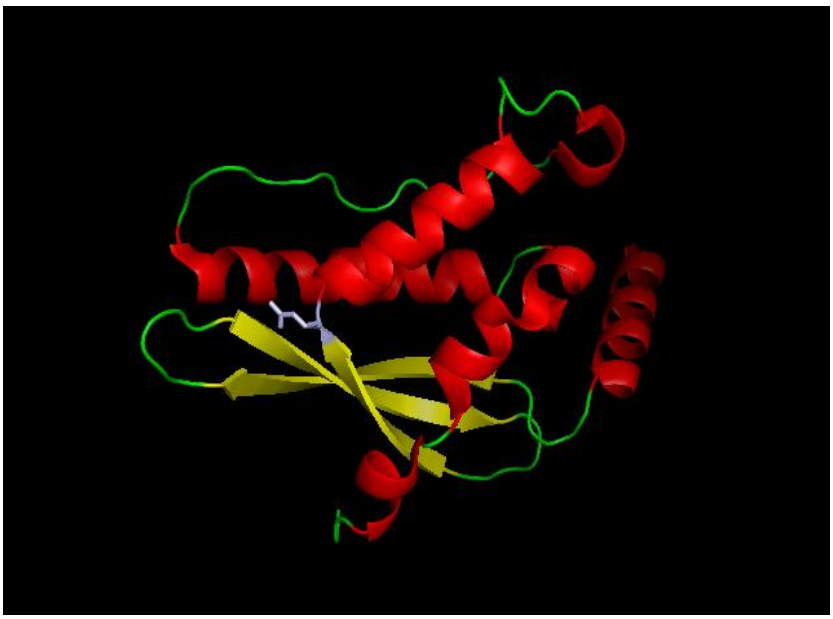

Figure 3: Structure of protein p40phox PX domain. A-helices (red), β-strands (yellow), loops (green), Arg58 specifically binding phosphate group of PtdIns(3)*P* (light blue). PDB entry 1H6H, X-ray data (authors: Bravo et al. 2001). Modified in PyMOL.

FYVE domain is different from PH and PX domains. It needs two Zn^{2+} ions to be able to interact with its target PIs (Misra et al. 1999). Name of this domain is derived from the first letters of proteins which were identified as FYVE carriers - Fab1, YOTB, Vac1, EEA1. The primary binding partner of proteins with FYVE domain is PtdIns(3)*P* (Gaullier et al. 1998). Fusion proteins containing FYVE domain showed highly specific binding to liposomes containing 2% of PtdIns(3)*P* and significantly lower binding to liposomes containing other kinds of phosphorylated inositol (Gaullier et al. 1998). FYVE domain consists of approximately 70 amino acid residues. Two sites for interaction with two Zn^{2+} ions are present. First consists of one His and three Cys residues, second consists of four Cys residues. Tertiary structure is

folded from four β-strands and one α-helix near the C-terminus (**Figure 4**). A pocket which interacts with PtdIns(3)*P* is created by six basic amino acids. Most of these basic amino acids reside in β1-strand or in its vicinity. They are termed as RRHHCR motif and are found conserved throughout FYVE domains of different proteins (Misra et al. 1998).

In order to be recruited to PtdIns(3)*P* with high affinity it is necessary for the FYVE domain to create a dimer (Gillooly et al. 2000). This goal is often achieved through homodimerization of proteins carrying the FYVE domain, as is the case for early endosome antigen-1 (EEA1) binding to the membrane of early endosome (Dumas et al. 2001) (role of EEA1 is further discussed in chapter 3.4.2).

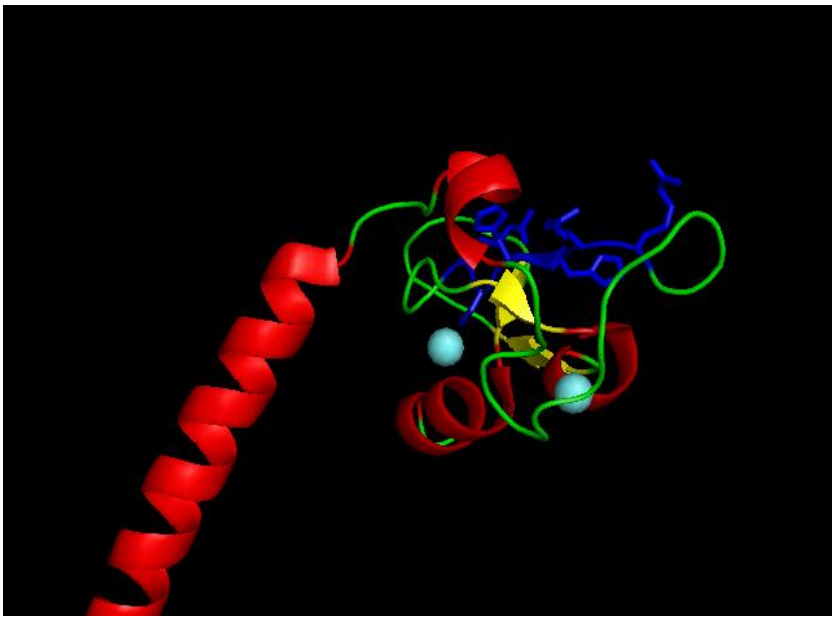

Figure 4: Structure of FYVE domain of EEA1. α-helices (red), β-strands (yellow), amino acid residues creating RRHHCR motif (blue), two Zn^{2+} (light blue spheres). PDB entry 1JOC, X-ray data (authors: Dumas et al. 2001). Modified in PyMOL.

3 PIs in Wnt producing cells

3.1 Transport of Wnt from the ER to the GA

Wnt begins its journey in the ER. After translation from mRNA it is a soluble protein in the ER lumen. It is modified here post-translationally by double acylation and several glycosylations. Exact number of glycosylations depends on the type of Wnt. Product of segment polarity gene *Porcupine* is a transmembrane protein which seems to be responsible for both glycosylation and lipidation of Wnt (Tanaka et al. 2002). Wnt folded to a correct conformation and properly modified is ready to be transported from the ER. Nature of this transport has only recently started to be uncovered. Protein family p24 seems to play a role of a cargo receptor for Wnt in the ER (Buechling et al. 2011). In *D. melanogaster* two members of this family, Emp24 and Eclair, proved to be especially important in functional Wg/Wnt exocytosis (Port et al. 2011). Emp24 and Éclair form heterodimeric complex and after binding of Wnt, this complex is targeted to the area of COPII transport vesicle formation.

COPII vesicle formation is a tightly controlled process that occurs at specific ER exit sites (ERES) of the smooth ER (Bannykh et al. 1996). Small cytosolic GTPase Sar1 is switched on by guanine-nucleotide exchange factor (GEF). Activated Sar1 binds to a membrane of the ER and helps to recruit Sec23/24 complex (Huang et al. 2001). This complex then recruits other factors which are needed for proper COPII formation. It has been suggested that PtdIns(4)*P* may play an important role in the regulation of the process described above (Blumental-Perry et al. 2006). Small GTPase Sar1 is, however, activated and binds to the ER membrane regardless of PtdIns(4)*P* presence. PtdIns(4)*P* thus clearly intervene in the COPII formation regulation at later stages. In its activated form (with bound GTP), Sar1 is able to recruit PtdIns 4-kinase type II (Blumental-Perry et al. 2006). This kinase is membrane bound due to palmitoylation in its cysteine-rich domain (Barylko et al. 2001). Its only substrate is PtdIns as is the case with all PtdIns 4-kinase family members. When PtdIns(4)*P* is sequestered by GST-Fapp1-PH (PH domain of Fapp-1 preferentially binds to PtdIns(4)*P* (Dowler et al. 2000)) and activity of PtdIns 4-kinase type II is inhibited, then recruitment of the Sec23/24 complex by Sar1 is diminished in mammalian cells. In common yeast, however, PtdIns(4)*P* depletion by a phosphatase enzyme Sac1 does not influence COPII vesicles formation (Lorente-Rodriguez et al. 2011). This stage of transport from the ER to the GA is probably not conserved and its regulation in yeast is different from that in higher organisms.

Contrary to its role in the formation of COPII vesicles in mammals, PtdIns(4)*P* is not required to be present on these transport vesicles during their fusion with the GA membrane (Lorente-Rodriguez et al. 2011). However, it is necessary that this PI is enriched in the GA. PtdIns(4)*P* recruits proteins of Soluble N-ethylmaleimide-sensitive factor Adaptor protein Receptor (SNARE) family. This recruitment subsequently enables creation of the trans-SNARE complexes between a v-SNARE and a t-SNARE (Lorente-Rodriguez et al. 2011). Similar recruitment was described for another protein of the SNARE family Vam7 (Sato et al. 1998). Vam7 contains a PX domain which binds PtdIns(3)*P* in the membrane of vacuole with high affinity (Yu and Lemmon 2001). Whether one of the SNARE proteins located in the GA membrane contains PtdIns(4)*P* biding domain is currently unknown. It would be also possible that SNARE in the GA interact with the PI through yet another protein.

Which one of PtdIns 4-kinases produce pool of PtdIns(4)*P* important for the ER to the GA transport is currently unknown. However, PtdIns 4-kinase IIIα was shown to colocalize with the ER markers in mammalian cells (Wong et al. 1997).

3.2 Transport of Wnt from the GA to the PM and its release to extracellular space

As mentioned in the introduction Wls participation is indispensable for Wnt secretion and gradient formation. Wls contains in its sequence seven hydrophobic regions long enough to pass through the lipid bilayer and can only be localized to cell membranes (Bänziger et al. 2006). It also looks like Wls facilitates Wnt transport exclusively as loss-of-function mutations in *Wls* do not seem to disrupt secretion of any other protein (Bänziger et al. 2006). Wls and Wnt has been shown to colocalize and physically interact in immunoprecipitation experiments with cell lysates (Bänziger et al. 2006). Wnt3A with cysteine changed for alanine at position 77 cannot be palmitoylated by Porcupine. Nevertheless, transport of Wnt3A without this lipid modification is still regulated by Wls (Bänziger et al. 2006). This observation proves that interaction between Wls and Wnt is not mediated by this lipid modification. Loss of glycosylation of Wnts also does not disrupt their interaction with Wls (Herr et al. 2012). Another conserved lipid modification of Wnt is palmitoleic group attached to serine amino acid residue. Contrary to the palmitate and modification by glycosylation, palmitoleic group seems to be involved in Wnt interaction with Wls (Herr et al. 2012). However, it is unknown whether palmitoleic group of Wnt interacts directly with Wls or whether it merely helps to colocalize Wnt near Wls and actual interaction is mediated through another region of Wnt.

PtdIns(4)*P* is a PI most abundant in the membrane of the GA. Its presence is necessary for series of processes associated with transport of proteins from the GA to various places in a cell. GA is an important intracellular compartment in the exocytotic pathway - pathway which is used by Wnt to get to the PM and out of the cell. Four PtdIns 4-kinases are known in mammalian cells and all of them seem to be evolutionarily conserved. The only substrate of PtdIns 4-kinases is PtdIns. Other PI kinases (such as PIPK type I or PI3K) can use PtdIns which is already phosphorylated at one or two positions of its inositol ring. PtdIns 4-kinases do not seem to be able to do it (reviewed Fruman et al. 1998). Rather than having redundant functions to each other, PtdIns 4-kinases seem to localize to different compartments of the cell and create pools of PtdIns(4)*P* destined for different tasks.

PtdIns 4-kinase type IIIβ is a cytoplasmic enzyme. Its recruitment to the GA membrane and activation is mediated by small GTPase ADP-ribosylation factor-1 (ARF1) (Godi et al. 1999). Disruption of proper function of PtdIns 4-kinase type IIIβ leads to a change in the dynamics and shape of the GA. ARF1 needs to be associated with GTP to be able to recruit PtdIns 4-kinase type IIIβ. This change of GDP for GTP is enzymatically supported by guanine-nucleotide exchange factors (GEF). In general terms, GEF themselves can be recruited to various membranes by interaction with PIs. This leads to an exchange of GDP to GTP on a small GTPase protein and a change in its conformation. Small GTPase, with bound GTP, may directly or indirectly recruit and activate PtdIns kinase or other effector proteins. Activated PtdIns kinase than produce more PIs, which recruited GEF in the first place, providing positive feedback loop. Both particular PI and particular small GTPase protein in a membrane can serve as docking sites for recruitment of specific effector proteins to specific membranes (reviewed in Di Paolo and De Camilli 2006).

Description of a role of any particular PIs and their effectors in transport of Wnt from the GA to the PM would be, at this time, pure speculation. True nature of this transport is still not clear today. It is quite possible that there is not one general way of transport used in all Wnt producing cell. In one organism different ways may be facilitated for different Wnt molecules. Few possibilities have been suggested:

- Wnt might be transported in a vesicle directly from the GA to the PM where it could dissociate from Wls. This, however, does not seem to be likely as lipid modifications of Wnt cause its hydrophobicity and thus decrease its chance to spread in the extracellular space. There would have to be some kind of enzymatically catalyzed adjustment of Wnt or its association with a mediator complex citace (discussed in more detail in the next chapter).

- Wnt might also be transported to endosome first. Similar pathway is used by transferrin receptor (Tfn-R). Transport of newly synthetized proteins from the GA is inhibited at 20°C. Protein synthesis is, however, not disrupted. When HEp.2 cells (Human epidermoid cancer cells) were incubated for 4 hours at 20°C and then shifted to 37°C, Tfn-R was shuttled to endosomes first. Ten minutes later the amount of Tfn-R in endosome was gradually decreasing and its amount in the PM was increasing (Futter et al. 1995).
- In combination of above-mentioned ways, Wnt could be secreted at the PM first, than internalized to the endosome. Whether some kind of modification of Wnt would appear after endocytosis or whether Wnt would be loaded to lipoprotein particle (suggested in Greco et al. 2001, described in more detail in the next chapter) is hard to predict. It was proved, however, that in early stages of *D. melanogaster* embryo development, Wg/Wnt is indeed endocytosed after it had been transported to the PM (Pfeiffer et al. 2002).

3.3 Wnt gradient formation, transport of Wnt through extracellular space

As is the case with Wnt transport to the PM, its spreading from Wnt producing to Wnt receiving cells seems to be ensured by different ways in different tissues. Participation of PIs in those processes is not studied very well and the following text is therefore just a very short preview of few possible ways of Wnt spreading.

Extracellular membranous particle called argosomes mediate spreading of Wg/Wnt in *D. melanogaster* imaginal discs (Greco et al. 2001). In the model proposed by Greco et al. 2001, argosomes are created by a process similar to that of exosome formation. An area of basolateral membrane is internalized into the cell and multivesicular endosome is created. Membranous particles inside endosome are loaded with Wg/Wnt. After endosome fusion with the PM, newly formed argosomes are released from the Wnt producing cell. Argosomes would, in this case, be bilayer exovesicle-like structures. Another type of particle was found to participate in long-range Wg/Wnt signaling. Instead of bilayer, these particles are formed by lipid monolayer composed of phospholipids and lipophorin (Panakova et al. 2005). In *D. melanogaster*, Wnt producing cells treated with RNAi against lipophorin were unable to form proper lipoprotein particles and this disrupted the long-range Wg/Wnt signaling. Panakova et al. 2005 still term these monolayer lipoprotein particles as argosomes. In later articles the term “argosome” is

used rather for monolayer lipoprotein particle than for bilayer exovesicle-like structures (in accordance with Panakova et al. 2005 terminology).

Genuine exosomes themselves were also proposed to participate in Wnt gradient formation. It was even shown in differential centrifugation experiments, that in *Drosophila* Kc167 cells, Wg/Wnt may colocalize with exosomes rather than with lipoproteins (Gross et al. 2012), contradicting Panakova et al. 2005 theory of argosomes. However, Gross et al. estimated that roughly 60 percent of Wnt activity is still present in cell lysates after removing exosomes by ultracentrifugation. Over half of the total amount of Wnt molecules thus seem to utilize other ways of intercellular transport than exosome vesicles.

Disrupting the process of MVB formation suspends creation of exovesicles with loaded Wnt. Gross and colleagues suggest the following model of Wnt exosome signaling: Wnt cargo receptor Wls binds to Wnt in the GA and together they are transported to the PM. Once on the PM this complex is endocytosed and enters early endosome. Here, free molecules of Wls, without bound Wnt, are recycled through the retromer complex back to the GA. Wls with bound Wnt is recognized by Endosomal sorting complexes required for transport (ESCRT) and loaded to intra-luminal vesicles (ILVs) of the MVB. Finally, fusion of MVB with the PM releases exosomes to extracellular space (Gross et al. 2012) (**Figure 5)**.

Creation of ILVs as precursors of exosomes is regulated especially by ESCRT-0 complex (Tamai et al. 2010) whose interaction with the endosome membrane is mediated via PIs. At this stage of endosomal sorting, prevalent PI is still PtdIns(3)*P*. ESCRT-0 complex subunit Hrs contains a FYVE domain in its amino acid sequence (Komada et al. 1997). Hrs FYVE domain interaction with endosome located PtdIns(3)*P* is necessary for subsequent binding of ESCRT-I complex and formation of ILVs (Katzmann et al. 2003). ESCRT itself, or yet unknown protein associated with ESCRT, thus may function as cargo receptor for Wls-Wnt complex and load this complex to the membrane of future exosomes.

Scission of inward buds off the endosome membrane and creation of ILVs seems to be the task of another ESCRT complex - ESCRT-III. Core subunit of this complex is Vps24. This protein recruits ESCRT-III to the endosome membrane through interaction with PtdIns(3,5)*P*. Amount of PtdIns(3,5)*P* starts to outbalance PtdIns(3)*P* as endosome matures. It is created by yeast PtdIns 5-kinase Fab1 and in mammalian cells by its homolog PIKfyve kinase (Ikonomov et al. 2001). These kinases contain FYVE domain through which they are recruited to the membranes rich in PtdIns(3)*P* (Sbrissa et al. 2002) which also happens to be a substrate for those kinases.

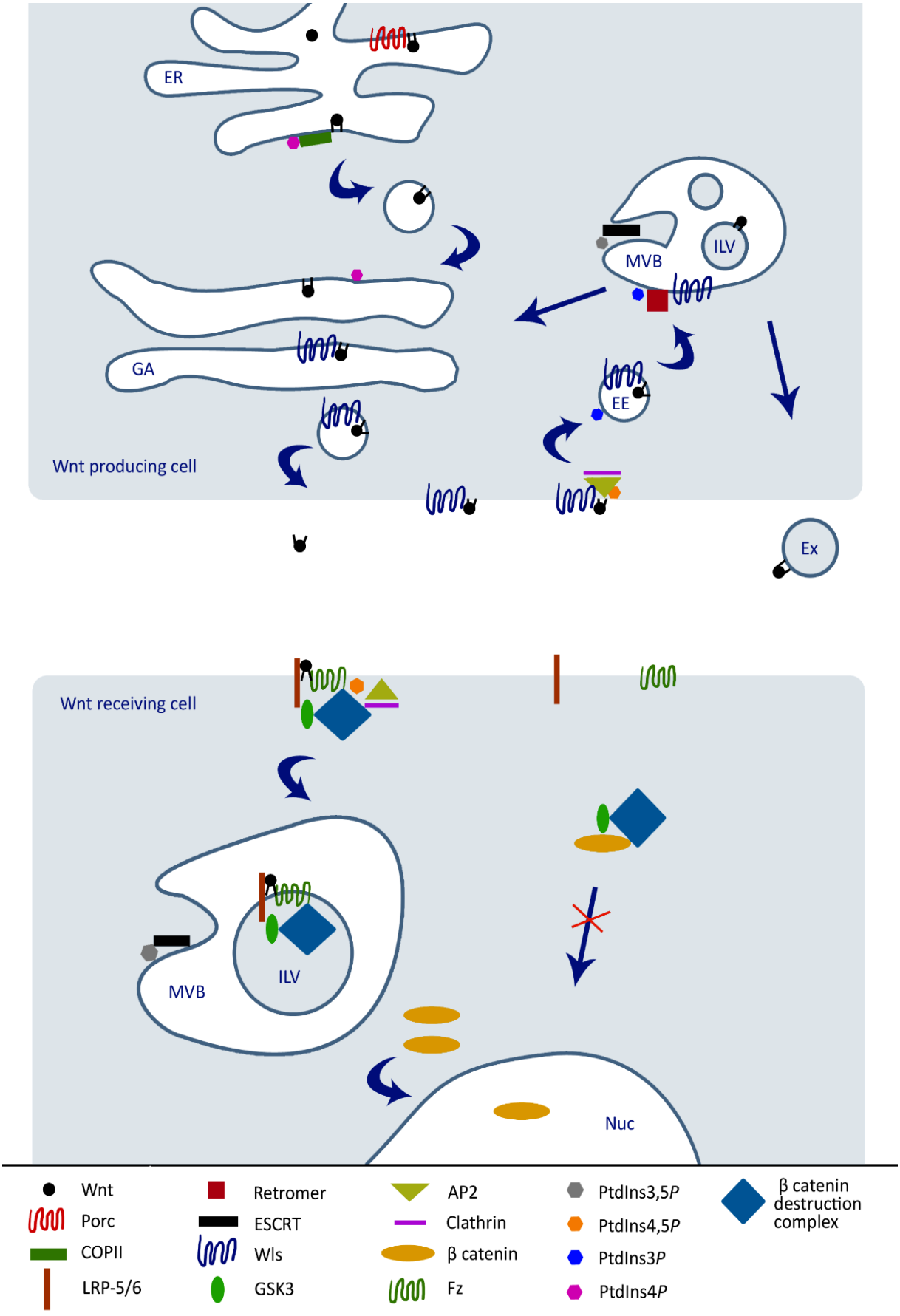
ER
GA
MVB
ILV
EE
Wnt producing cell
Ex
Wnt receiving cell
MVB
ILV
Nuc
Wnt
Porc
COPII
LRP-5/6
Retromer
ESCRT
Wls
GSK3
AP2
Clathrin
β catenin
Fz
PtdIns3,5P
PtdIns4,5P
PtdIns3P
PtdIns4P
β catenin destruction complex

Figure 5: Basic processes in Wnt producing and receiving cells and PIs involvement in those processes. Wnt producing cell: PtdIns4*P* is needed for Wnt export from the ER to the GA and from the GA to the PM. PtdIns4,5*P* is involved in Wnt-Wls complex through clathrin-mediated endocytosis. PtdIns3*P* and PtdIns3,5*P* are in charge of ILVs creation. On top of its function in ILVs creation PtdIns3*P* mediates retromer complex recruitment. Wnt receiving cell: PtdIns4,5*P* organize Wnt-LRP6-Fz-GSK3 endocytosis. PtdIns3*P* and PtdIns3,5*P* are in charge of ILVs creation. Wnt ligand causes β-catenin stabilization and its translocation to the nucleus (left bottom side of picture). In receiving cell without Wnt ligand bound to receptors, β-catenin is phosphorylated and degraded (right bottom part of picture).
Endoplasmic reticulum (ER), Golgi aparatus (GA), Multivesicular body (MVB), Intra-luminal vesicle (ILV), Nucleus (Nuc), Early endosome (EE), Exosome (Ex). Direction of Wnt and its regulators transport within Wnt producing and receiving cells is indicated by blue arrows. Created in Adobe Photoshop 7.

3.4 Wls recovery from the PM and its return to the GA

3.4.1 Endocytosis and clathrin-coat vesicle (CCV) formation

After fulfilling its role in assisting Wnt to get from the TGN to the PM, Wls has to be returned back to the TGN for more rounds of Wnt transport. The first step of this process is dynamin-mediated endocytosis of Wls. In *D. melanogaster* carrying temperature sensitive mutant of a gene coding dynamin (*shibire*^{ts}), extracellular level of fly Wg/Wnt was significantly reduced around Wg-producing cells when flies were shifted to temperature in which *shibire*^{ts} was inactive (Striginy and Cohen 2000). Wls existence and its involvement in Wg/Wnt signaling was unknown when these observations were made. Absence of Wg was interpreted as an inability of Wg to be transported from the TGN to the PM due to the lack of functional dynamin. Contrary to its extracellular level in *shibire*^{ts} mutants, Wg was accumulated in Wg producing cells, resembling *porcupine* mutant. It seems now that the lack of wild type *shibire* causes Wls molecules to be unable to enter Wg producing cell endosome through dynamin mediated endocytosis. This would lead to Wls accumulation on the cell surface (Belenkaya et al. 2008) and its lack in the GA for transport of Wnt to the PM.

There are two ways of endocytosis dependent on function of dynamin. It is either clathrin-mediated endocytosis or clathrin-independent caveolin endocytosis. Wls has been proven to colocalize with AP-2 complex (Yang et al. 2008) which is known to serve as an adaptor protein for clathrin subunits. Clathrin itself is unable to directly interact with the PM and is therefore dependent on a variety of adaptor proteins which can bind to membrane lipids or proteins as well as to clathrin (reviewed in Owen et al. 2004). In *Caenorhabditis elegans* knockdown of AP-2 complex subunits by RNAi leads to MIG-14/Wls accumulation in the PM of Wnt producing cells (Pan et al. 2008; Yang et al.2008). This was confirmed by experiments with siRNA against AP-2α in HeLa cells which causes block of Wls endocytosis and thus higher level of Wls in the PM compared to HeLa cells treated with control siRNA (Yang et al. 2008). It is therefore likely, that Wls is internalised via AP-2 mediated clathrin-dependent endocytosis. AP-2 complex consists of four different subunits, two of them large - α and β2, one of medium size-μ2 and one small- σ2 (Gaidarov et al. 1999). The large subunit α contains in its N-terminal region biding site for PIs and biding of PIs results in greater stability of the whole AP-2 heterotetramer and gives it a better opportunity to interact with clathrin and thus to start clathrin lattice assembly (Gaidarov et al. 1999). Subunit μ2 also contains biding site highly specific to PtdIns(4,5)*P* (Rohde et al. 2002) and contributes greatly to stabilization of AP-2.

In A431 human cultured cells, addition of PLCδ-PH causes lower level of receptor-bound biotinylated transferrin (BTfn) sequestration to clathrin vesicles. PLCδ-PH specifically binds PtdIns(4,5)*P* (Jost et al. 1998) lowering its availability in the PM for AP-2 and it is the lack of PtdIns(4,5)*P* that disrupts CCV formation. PtdIns(4,5)*P* has thus been shown to be an important element of this process.

It is possible to create PtdIns(4,5)*P* either by dephosphorylation of PtdIns(3,4,5)*P* or phosphorylation of PtdIns(4)*P* at its 5´ OH group or PtdIns(5)*P* at its 4´ OH group. Generally, in living cells, pool of PtdIns(4)*P* is greater than pool of PtdIns(5)*P* and in experiments with ATP radioactively labeled with ^{32}P, PtdIns(4,5)*P* are mostly radioactive at their 5´ phosphate after the pulse (reviewed in Fruman et al. 1998).This leads to a conclusion that most PtdIns(4,5)*P* in cell membranes is created by the activity of PtdIns(4)*P* kinase (PIPK). There are three known isozymes of PIPK type I in mammals called PIPK α, PIPK β and PIPK γ. They share common amino acid sequence of their kinase core domains and all of them can be bound and activated by AP-2 subunit μ2-cargo complex (Krauss et al. 2006). Subunit μ2 seems to be a very important member of AP-2 as it contains a biding site for cargo proteins, PIs biding site and site which binds and regulates the activity of PIPK and thus the amount of PtdIns(4,5)*P* in the PM and overall stability of the clathrin assembly sites (Collins et al. 2002).

When clathrin coat assembly is finished it needs to be pinched off the PM. This task is performed by activity of dynamin. I already mentioned the influence of *D. melanogaster shibire/dynamin* mutations on the intracellular and extracellular distribution of Wg/Wnt at the beginning of this chapter. Dynamin is a soluble GTPase and needs to be localized near the PM to perform a scission of clathrin coated pit (CCP). Apart from GTP biding site, this GTPase contains in its amino acid sequence a PH domain and a proline rich domain (PRD). Interaction of the PH domain and PIs promotes the GTPase activity of dynamin (Lin et al. 1997). Moreover, unlike other proposed dynamin activators, such as microtubules, PIs can do so at physiological ionic strength. PtdIns(4,5)*P* proved to be particularly potent dynamin activator (Lin et al. 1997). Mutations disrupting the ability of dynamin to bind PIs by the PH domain lead to cells not being able to conduct endocytosis (Achiriloaie et al. 1999). However, Lin himself speculates in his article (Lin et al. 1997) that it is not the interaction of PtdIns(4,5)*P* with PH domain which targets dynamin to the PM. This recruiting function seems to be a task of dynamin PRD domain which, via interaction with proteins such as those from AP-2 complex, actually recruits dynamin to the neck of a newly formed CCPs (Wang et al. 1995).

Series of processes described above leads to a creation of endocytosed CCVs. PtdIns(4,5)*P* plays a crucial role in initiation of these processes by anchoring AP-2 complex to the PM and thus AP-2 μ subunit biding to a cargo protein. AP-2 μ subunit-cargo protein complex in turn binds and activates PIPKs type I which produce more PtdIns(4,5)*P* providing a local pool of this PI and a positive loop for greater stability of the clathrin assembly site. Detachment of the newly formed CCPs is catalyzed by activity of GTPase dynamin which is also regulated by PtdIns(4,5)*P*. This main representative of PIs in the PM is a key member of endocytosis machinery and thus essential for initiation of Wls molecule recycling.

3.4.2 Retromer complex assembly and binding of Wls to this complex

Soon after scission of CCP from the PM and formation of CCV the clathrin coat subunits fall apart. PtdIns(4,5)*P* helps to stabilize clathrin coat subunits and it is obvious that this PI needs to be eliminated from CCVs. In nervous system cells, enzyme performing activity exactly opposite to the PIPK type I is PI phosphatase synaptojanin-1 and similar role in endocytosis regulation has its homolog in *S. cerevisiae,* INP51 (synaptojanin-like protein) (Guo et al. 1999). This enzyme dephosphorylates PtdIs(4,5)*P* at position 5 of its inositol ring creating PtdIns(4)*P* and thus destabilizing vesicle clathrin coat and helping its disassembly. Only vesicles stripped of their clathrin coat are able to fuse with early endosome and pass on their cargo further to endocytic pathway (Cremona et al. 1999).

Early endosome serves as crossroads for endocytosed cargo, some of which is recycled back to the PM, some continues through the late endosome all the way to the lysosome for degradation and some of it, including Wls, is incorporated into the retromer complex and returned to the GA. Retromer complex in yeast consists of three main subunits: Vps26, Vps29 and Vps35 (Seaman et al. 1998). Retromer complex was at first described as a complex returning lysosomal enzyme receptors from endosome back to the GA. Later, mutation in *C. elegans* gene encoding vps-35 subunit was found to disrupt correct function of the retromer complex leading to insufficient production of EGL-20/Wnt from Wnt producing cells (Coudreuse et al. 2006; Prasad and Clark 2006).

Main PI of early endosome is PtdIns(3)*P* and similarly to PtdIns(4,5)*P* in the PM it recruits effector proteins which are necessary for further regulation of endosomal sorting processes. Binding domains recognizing PtdIns(3)*P* are FYVE domain and PX domain. Pool of PtdIns(3)*P* on early endosome seems to originate from multiple sources by coordinated action of PI kinases and phosphatases. This process starts in the PM by activity of PI kinase type Iβ (PI3Kβ) and creation of PtdIns(3,4,5)*P* (Shin et al. 2005). Successive dephosphorylation at 4 and 5 positions of inositol ring by PI phosphatases creates PtdIns(3)*P*. Another, more direct way to create PtdIns(3)*P*, is by activity of the kinase Vps34 which phosphorylates PtdIns at its 3 position. Enzymes participating in both possible ways of PtdIns(3)*P* creation are recruited by protein Rab5 (Shin et al. 2005). Small GTPase Rab5 is typically present on early endosome membrane and it is responsible for recruitment of EEA1. EEA1 is a key component in early endosome fusion. It binds both Rab5 and PtdIns(3)*P*. Rab5 is bound through specific Rab5 biding domain and PtdIns(3)*P* through FYVE domain (Patki et al. 1998). Rab5 thus helps to recruit EEA1 to early endosome membrane directly through a binding domain and indirectly through localization of PtdIns kinase to the same membrane.

Complex of three retromer subunits Vps26, Vps29 and Vps35 serves for selection of proteins which are meant to be transported from early endosome to the GA (Seaman et al. 1998). This includes Wls which was shown to directly interact with the Vps35 subunit in fly (Belenkaya et al. 2008). Other proteins are necessary for functional retromer complex assembly, among them Sorting nexins (SNX). One of them is SNX1 which has in its amino acid sequence not only biding site for Vps35 but also a PX domain (Yu et al. 2001). This domain is a mediator of retromer complex localization to the PtdIns(3)*P*-rich early endosome membrane. SNX1, however, seems to be involved in the classical recycling of the lysosomal enzymes receptors only citace. Wls recovery to the GA was shown to be intact in SNX1 gene mutants. Rather,

SNX3 is involved in Wls recycling (Harterink et al. 2011). SNX3 also contains a PX domain and binds to PtdIns(3)*P* in a similar way as SNX1 does.

Whole retromer complex may work as a coat of a transport vesicle that is detached from the early endosome and later fused with the GA. Another way of early endosome to GA transport has been shown to exist. After formation, retromer complex may be localized on tubular structures projecting from the early endosome membrane (reviewed in Seaman 2005). Retromer with cargo protein is then transported from those structures to the GA. Which one of these two ways is utilized by Wls recovery process? Formation of tubular structures is dependent on a BAR domain of SNX (Peter et al. 2004). Wls recycling was proved to be mediated by SNX3 (Harterink et al. 2011) which, unlike SNX1, does not contain a BAR domain. Wls is thus likely transported to the GA through retromer coated vesicles. However, in *C. elegans* with mutation in gene coding for the retromer subunit VPS-29, MIG-14/Wls is only partly recovered through retromer complex coated vesicles. Great portion of MIG-14/Wls continues to a later stage of the endosomal pathway where the tubular structures are actually created by interaction of the three main retromer subunits (VPS-26, VPS-29, VPS-35) and SNX-1 containing the BAR domain. Some of MIG-14/Wls molecules are then incorporated into tubular structures and transported to the GA (Lorenowicz et al. 2014). Amount of MIG-14/Wls recovered via tubular structures is, nevertheless, insufficient to fully compensate for the loss of wild type MIG-14/Wls recycling through retromer coated vesicles (Lorenowicz et al. 2014).

Role of another PI effector in retromer complex formation and selective binding of Wls has been discovered recently in *C. elegans*. This effector is a complex of Myotubularin lipid-phosphatases MTM-6 and MTM-9. Phosphatase MTM-6 dephosphorylates PtdIns(3)*P* in early endosome membrane. It may seem that this dephosphorylation would disrupt PtdIns(3)*P* dependent formation of the retromer complex. However, it is not the case. Amount of PtdIns(3)*P* needs to be balanced and its lack as well as its excess leads to inefficient recovery of Wls from early endosome membrane (Silhankova et al. 2010). Enzymatic activity of the MTM-6/MTM-9 complex keeps the amount of PtdIns(3)*P* in early endosome membrane at levels optimal for efficient Wls recycling.

Once Wls is returned back to the GA, it is ready to transport Wnt molecules again. Protein producing is costly for cell in terms of energy and material. Recycling enables cell to save both. Wnt signaling is non-redundant and Wls recovery from early endosome to the GA is a vital step to keep this signaling functional.

4 PIs in Wnt receiving cells

4.1 Canonical Wnt signaling

After reaching target cell, Wnt ligand binds to a proper receptor and triggers Wnt signal transduction to the receiving cell. Apart from the main receptors of Wnt - LRP5, LRP6 and Frizzled, others, such as RYK and ROR have been identified, the latter two being involved rather in a non-canonical Wnt signaling. Besides binding to CRD of Fz through a Wnt domain made of hydrophobic amino acid residues, interaction ligand-receptor is also promoted by the palmitoleic modification of Wnt (Janda et al. 2013). Interaction of Wnt ligand with its receptor is probably independent of any PIs. This interaction takes place on the extracellular leaflet of the PM, while PIs are present in cytosolic leaflets of membranes.

First step after Wnt ligand binding to its Fz receptor is accumulation of a protein complex in the receiving cell. This complex is made of proteins and kinases which phosphorylate β-catenin. Sequestration of the complex from cytosol to the PM leads to stabilization of β-catenin and its translocation to the nucleus (reviewed in Logan and Nusse 2004). Kinases in the sequestered complex then phosphorylate LRP6. GSK3 and CK1 kinases were shown to perform LRP6 phosphorylation (Zeng et al. 2005). These are the same kinases that phosphorylate and thus mark β-catenin for destruction in the proteasome. It seems that clustering of LRP6 into Wnt-Fz-Dvl-Axin aggregate makes phosphorylation of LRP6 more efficient. Formation of such aggregates may be mediated by polymerization of Dvl molecule through its DIX domain (Schwarz-Romond et al. 2007a). At the beginning, binding of Wnt to Fz causes recruitment of the first Dvl molecule to intracellular part of Fz. Dvl molecules then polymerize to fibril structures via DIX domain. Dvl polymer serves as a docking site for Axin (Schwarz-Romond et al. 2007b) which is also bound to Dvl DIX domain. Thus Dvl-Axin polymer complex accumulates and phosphorylates LRP6. In this way, Wnt ligand receptors Fz as well as LRP6 are accumulated to close proximity and create hot spots for more Wnt ligand binding (Bilić et al. 2007).

Screen has been done to show whether some other kinases are also involved in LRP6 phosphorylation. As results of siRNA knockdown experiments show, there are indeed two other kinases participating in the phosphorylation process. These kinases are PtdIns 4-kinase IIα and PIP5K Iβ (Pan et al. 2008). Their substrates are PtdIns and PtdIns(4)*P* respectively and it is unlikely that they affect LRP6 phosphorylation directly. What is their exact role in Wnt signal transduction? Subsequent enzymatic activity of both kinases creates a pool of PtdIns(4,5)*P*.

Amount of this PI in the PM corresponds with the amount of phosphorylated LRP6 (Pan et al. 2008). Furthermore, when HEK293T cells are treated with siRNAs against three Dvl mRNAs (Dvl1-3), production of PtdIns(4,5)*P* as well as phosphorylation of LRP6 is abolished (Pan et al. 2008). Taken together, experiments of Pan et al. 2008 and other research groups suggest following model for LRP6 phosphorylation and aggregation:

After binding of Wnt ligand to Fz receptor Dvl is recruited to the Fz. Dvl contains in its amino acid sequence binding sites for both PtdIns 4-kinase (Pan et al. 2008) and PIP5K (Qin et al. 2009). Their interaction with Dvl activates them and local pool of PtdIns(4,5)*P* is produced. Interlink between PtdIns(4,5)*P* and LRP6 phosphorylation has not been confirmed yet. Two articles have been published recently which try to explain nature of this interlink.

First one reports the involvement of APC membrane recruitment-1 (Amer1) protein in the process of LPR6 phosphorylation (Tanneberger et al. 2011). Amer1 is able to bind to PtdIns(4,5)*P* via two binding sites at its N-terminus (Grohmann et al. 2007). Amer1 PtdIns(4,5)*P* binding site seems to be an example of PIs biding domain made of a stretch of basic amino acids. When Amer1 is translocated to the PM it recruits Axin with bound GSK3. At the PM, complex of Amer1 and Axin-GSK3 interacts with LRP6 via binding sites in the central and in the C-terminal region of Amer1. Axin is still able to bind to LRP6 in the absence of Amer1, however, such interaction is much weaker (Tanneberger et al. 2011). Apart from recruiting GSK3 to the LRP6, Amer1 also recruits CK1 (Tanneberger et al. 2011). The above mentioned process leads to stabilization of β-catenin and to activation of its downstream effectors.

Recent article describes as pivotal a role of clathrin and AP-2 in formation of phosphorylated LRP6 clusters (Kim et al. 2013). According to this article, increasing amount of PtdIns(4,5)*P* after Wnt ligand binding leads to accumulation of AP-2 via its α and μ2 subunits at the PM. AP-2 complex subunit μ2 was shown to interact with cargo protein through a specific amino acid signal sequence. This sequence is present in at least one copy in LRP5 as well as in LRP6 (Kim et al. 2013). Knockdown of mRNAs for AP-2μ or clathrin heavy chain (CHC) resulted in measurable decrease of LRP6 aggregates at the PM. Apart from its ability to bind LRP5 and LRP6, AP-2 is also able to bind to Dvl (Yu et al. 2007). This would serve as a connection between AP-2-associated LRP6 and Fz-associated Dvl. Taken together, these findings indicate the importance of PtdIns(4,5)*P* for correct transmission of the Wnt signal and emphasize the role of endocytosis in β-catenin stabilization.

It has been shown in another article that knockdown of CHC and subsequent inability to create CCVs leads to disruption of β-catenin downstream signaling (Blitzer and Nusse 2006). Yet

another article exploring this matter has been published the same year. Contrary to the findings of Blitzer and Nusse 2006 and Kim et al. 2013, it suggests that caveolin is involved in binding and internalization of LRP6 (Yamamoto et al. 2006). Nevertheless, regardless whether through clathrin or caveolin it seems that internalization is indeed necessary for proper Wnt signal transduction. Mapping of members of β-catenin destabilizing complex (Axin, Dvl, GSK3, etc.) localization suggests that this complex does not stay on the cytosolic side of the PM. Instead it is translocated to the endocytic pathway and continues to the ILVs of MVB (Taelman et al. 2010). Disruption of the ESCRT-0 complex as well as mutation in Vps4 protein, which are both involved in ILVs formation, leads to abrogation of β-catenin stabilization in cytosol (Taelman et al. 2010). Creation of ILVs by activity of ESCRT complexes is thus important in both Wnt producing cells as well as in Wnt receiving cells and as mentioned earlier, PtdIns(3)*P* and PtdIns(3,5)*P* play important roles in this process (**Figure 5)**.

4.2 Non-canonical Wnt signaling

According to the type of Wnt ligand and type of Wnt receptors it binds, results of such interaction may be very different. Previous chapter deals with canonical Wnt signaling through stabilization of β-catenin. In this chapter few aspects of non-canonical Wnt pathways that may involve PIs will be discussed.

Beginning of PCP signaling shares some aspects with the canonical pathway. After Wnt-5A ligand binding, Fz4 receptor interacts with Dvl2 in a same manner as in the canonical pathway. Dvl2 is able to bind to AP-2μ subunit and thus recruit AP-2 complex which in turn causes clathrin-mediated endocytosis (Yu et al. 2007). There are two regions in the amino acid sequence of Dvl2 which are important for the interaction of AP-2μ with Dvl2. First is a DEP domain with tertiary structure and second is YHEL motif (Yu et al. 2007). Both of them are located near the C-terminus of Dvl2. If Dvl2, like Dvl1 in canonical Wnt signaling, was able to bind PtdIns 4-kinase IIα and PIPK Iβ, it would provide a local pool of PtdIns(4,5)*P* and further increase recruitment AP-2μ subunit. Whether this is indeed the case, remains to be addressedin further research. Actual process of Fz4 clathrin-mediated endocytosis is likely to be very similar to that of Wls endocytosis described in more detail in chapter 3.4.1.

Apart from its role in establishing tissue polarity via the PCP pathway (reviewed in Gao 2012), Wnt5A has been shown to promote phosphorylation and degradation of β-catenin and

thus inhibit canonical pathway signaling promoted by other types of Wnt ligands (Topol et al. 2003).

In the cells of *Danio rerio* embryo injected with *X.laevis* Wnt5A (XWnt5A) mRNA, increased frequency of Ca^{2+} influxes has been observed (Slusarski et al. 1997a). Ca^{2+} signaling is known to be regulated by second messengers Ins(1,4,5)*P* and DAG, both of which are created by enzyme PLC. In Slusarski et al. 1997a this lipase was proposed to be activated by Wnt5A signaling through one of Fz receptors. Later that year Fz2 was identified as a binding partner for Wnt5A (Slusarski et al. 1997b). Possible negative loop regulation of production of Ins(1,4,5)*P* from PtdIns(4,5)*P* was described in chapter 2 in the section about PH domain.

5 Conclusion

Wnt signaling pathways have been studied extensively for over three decades now. Since Wnt discovery in 1982 huge amount of data, concerning journey of this morphogen, has been collected. Nevertheless, this long-lasting research of all aspects of Wnt function seems to be far from over. Every solved problem brings new stack of questions which needs to be solved. Luckily for Wnt researchers, involvement of this molecule in human diseases keeps it in the spotlight.

In this work, Wnt signaling pathway close relationship with PIs was touched. An overview of this partnership is summarized in **Figure 5**. Nature of Wnt molecule requires its close interaction with lipid membranes. Processes which lipid membranes undergo, are regulated to a great extent by membranes minor component, PIs. However, PI role is not restricted to the regulation of membrane properties and PIs are more and more recognized as important regulators of various cell functions. Even more importantly, regulation of PI metabolism is crucial for the outcome of several signaling pathways active in development and disease (Skwarek and Boulianne. 2009). This thesis has shown that the Wnt pathway is not an exception and PIs are required at several steps of Wnt signal transduction, including, among others, retrograde transport of Wls from the plasma membrane back to the Golgi apparatus in Wnt producing cells or internalization of Wnt receptors in Wnt receiving cells.

Role of all minor factors involved in production, spread and targeting of Wnt is not and probably cannot be elucidated entirely any time soon. Research dedicated to PIs, however, shows that they, together with their effectors, belong to factors of great importance for every single step of Wnt signaling pathway. Impaired Wnt signaling is known to cause numerous diseases. As described above, malfunction of Wnt signaling can be caused by defective PI metabolizing and effector proteins. Function of those effectors should be scrutinized and described in the greatest possible depth as perfect knowledge of their specific roles in Wnt signaling may help in developing new treatments for the Wnt-related diseases.

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