The Role of ER-Golgi Membrane Contact Sites and of FAPP1 in Phosphoinositide Homeostasis at the Golgi Complex

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The role of ER-Golgi membrane contact sites and of FAPP1 in phosphoinositide homeostasis at the Golgi complex

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Thesis submitted in accordance with the requirements of the Open University for the degree of Doctor of Philosophy

March 2018
To my dad
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FAPP1 is a PI4P sensor acting at ER-TGN membrane contact sites (ERTGoCS)

FAPP1 controls the activity of the oncogene GOLPH3 by regulating PI4P at the Golgi

The Golgi PI4P pool regulated by FAPP1 controls post-Golgi trafficking of specific cargoes

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Abstract

Initially considered to be a precursor for highly phosphorylated phosphatidylinositol species, phosphatidylinositol 4-phosphate (PI4P) turned out to be “active” in its own right as a pivotal regulator of multiple cellular functions including membrane trafficking, sphingolipid metabolism, autophagy, and cell migration. PI4P levels at the Golgi in control conditions are the result of the balanced activity of Golgi-localized PI4 kinases (PI4KIIB and to a lesser extent PI4KIIα) and a single, highly conserved 4-phosphatase known as SAC1, localized in the ER. To coordinate such a variety of functions, multiple layers of regulation are required to modulate PI4P levels in time and space, by directing both PI4P enzymes localization and their activity. Here I show that the phosphatidylinositol 4-phosphate adaptor protein 1 (FAPP1) acts as a PI4P sensor, binds to PI4P and regulates its levels by interacting with and stimulating SAC1 at the level of ER-TGN membrane contact sites (ERTGoCS). At these ERTGoCS, FAPP1 acts as an adaptor that directs and stabilizes SAC1 towards PI4P-rich domains to promote its activity in trans to dephosphorylate PI4P at the TGN. Consequently, FAPP1 depletion leads to a huge increase in PI4P levels at the Golgi. I found that the FAPP1-regulated PI4P pool controls the post-Golgi trafficking of specific cargoes. One of these is the β-lipoprotein ApoB-100, which is more secreted upon FAPP1 depletion, highlighting a possible physiological role of this pool of PI4P controlled at the level of ER-TGN membrane contact sites. Intriguingly, another affected cargo is the autophagy protein ATG9, whose trafficking from the Golgi is increased in FAPP1 KD cells, thus resulting in a strong induction of autophagy. Besides the regulation of specific trafficking events, I found that the pool of PI4P controlled by FAPP1 recruits and promotes the activation of the PI4P-binding oncogene GOLPH3. When PI4P levels
increase in FAPP1-KD cells, GOLPH3 is hyperactivated and promotes uncontrolled cell migration and invasion. My thesis work presents new information on the molecular mechanisms underlying the regulation of PI4P at the Golgi complex, identifying FAPP1 as a negative regulator of PI4P levels. It also sheds light on the need to keep PI4P levels within a certain range since an uncontrolled increase in PI4P can result in oncogenic potential and mis-trafficking of selective cargoes. Overall, this work provides novel insights into the homeostatic control of PI4P with an important contribution in understanding the complex picture of PI4P control and function in cell biology.
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Chapter 1

Introduction

1.1 Phosphoinositides (PIs) are determinants of organelle identity

Eukaryotic cells are organized as a complex system of membrane-bound compartments (organelles) engaged in specialized functions. Organelle identity is expressed at two levels: a morphological identity, defined by the ultrastructural architecture, and a biochemical identity, given by the enrichment of specific lipids and proteins on the cytosolic face. Among these, the phosphoinositides (PIs) together with two classes of GTPases, the Arf and the Rab proteins, appear to be crucial to provide organelles with a unique identity (Behnia & Munro, 2005). Phosphatidylinositol (PI) is the basic building block for the PIs. In vivo, the PIs concentrate at the cytosolic surface of biological membranes with their lipid portion (DAG) directly inserted into the lipid bilayer and the hydrophilic headgroup protruding into the cytosol where it is accessible for the PI regulatory enzymes and for a wide range of cytosolic proteins that use PIs to be recruited onto the membranes and perform their function (Lemmon, 2008). The headgroup of PI is subjected to reversible phosphorylation by specific PI kinases in three of the five free hydroxyl groups on the inositol ring (D3, D4 and D5) generating seven PI species, each of which has been defined as having unique functions, subcellular distribution and abundance (Fig. 1.1).
Figure 1.1 PI metabolic cycle. The inositol ring of PI can be phosphorylated in the 3, 4 and/or 5 positions, giving rise to seven different PIs. The main pathways of PI synthesis and degradation in mammalian cells are shown, with the PIKs indicated in blue, and the PI phosphatases in green (adapted from Vicinanza et al. 2008).
Although they are quantitatively minor components of cell membrane phospholipids (~10%), PIs can coordinate a variety of cellular functions because of their versatile nature, which is in turn determined by their fast metabolic interconversion operated by distinct PI kinases and phosphatases. Indeed, PI metabolism is spatially and temporally regulated through the controlled recruitment and activation of PI metabolizing enzymes, so that distinct PIs can be enriched in specific membrane compartments (Fig. 1.2).

Figure 1.2 Subcellular distribution of PIs and PI metabolizing enzymes. Schematic representation of PI kinases (blue), PI phosphatases (green) and the predominant PI species in different subcellular compartments. Note that this map is based on PI visualized with PI-
binding domains, often based on coincident recognition of PIs and proteins, and thus shows the most representative situation under steady state conditions. The use of different probes has shown that PIs are also present on other organelles under different conditions (Vicinanza et al., 2008).

1.2 Phosphatidylinositol 4-Phosphate (PI4P)

Phosphatidylinositol 4-Phosphate (PI4P), together with PI(4,5)P2, represents the most abundant phosphoinositide in the cell (Lemmon, 2008). By virtue of its amount, PI4P is the main precursor for the production of multi-phosphorylated PIs, in particular PI(4,5)P2 and PI(3,4,5)P3, which play key functions in canonical signaling pathways. Indeed, PI(4,5)P2 is the main substrate of the phospholipase C (PLC) enzymes, yielding inositol 1,4,5-trisphosphate (Ins(1,4,5)P3) and diacylglycerol (DAG), two important messengers in Ca^{++} signaling. PI(4,5)P2 also controls several types of ion channels and enzymes, such as phospholipase D (PLD), and interacts with proteins that link membranes to the actin cytoskeleton (Lemmon, 2003). PI(3,4,5)P3, generated from PI(4,5)P2 by the class I PI3-kinases, regulates apoptosis and survival, via activation of the Akt signaling pathway (Lemmon 2003). Given the importance of PI(4,5)P2 and PI(3,4,5)P3 driven signaling pathways, the role of PI4P was long thought to be solely a precursor for other phosphoinositides in the plasma membrane (PM). However, in the ‘80s, a specific PI4-Kinase activity was found to be enriched in the Golgi apparatus relative to other cellular compartments (Cockcroft, Taylor, & Judah, 1985). Moreover, studies of PI4 kinase distribution and function in yeast and mammals established that PI4P, although present in the PM, is mainly enriched at the Golgi complex and that it
could exert a role in its own right, without being phosphorylated to form PI(4,5)P2 (Li et al., 2002; Walch-Solimena and Novick, 1999). Since then, a growing body of evidence has indicated that PI4P can modulate a variety of functions, many of which are conserved across yeast, plants and mammals, acting at the Golgi Complex (GC) (De Matteis, et al., 2013). This has been confirmed by the evidence that most of the PI4P effectors, which bind PI4P to exert their function, are localized at the Golgi and that all the PI4 kinases responsible for PI4P production localize or function, at least partially, at the Golgi apparatus, with PI4KIIIβ and PI4KIIα accounting for the predominant PI4P production at the Golgi (D’Angelo et al., 2008). Important insights about the role of PI4P in cells have been inferred from the genetic and chemical manipulation of the PI4P metabolizing enzymes: the four PI4-kinases and the 4-phosphatase SAC1.

1.3 Regulation of PI4P homeostasis by the four PI4-kinases

Eukaryotes have two classes of PI4-kinases that are conserved from yeast to mammals and named type II and III, since the group of kinases originally classified as PI4K type I are actually PI3-kinases. The two classes mainly differ in their resistance to inhibitors, with type-II PI4Ks being insensitive to PI3Ks inhibitors, such as Wortmannin. In mammals, four PI4Ks have been identified: two type II kinases (PI4KIIα and PI4KIIβ), and two type III kinases (PI4KIIIα and PI4KIIIβ). Yeast have only three PI4Ks: Lsb6, Stt4 and Pik1, orthologs of PI4KIIα, PI4KIIIα and PI4KIIIβ, respectively. The PI4Ks localize in different subcellular compartments, thus accounting for the production of PI4P pools required for different functions.

1.3.1 PI4KIIIα

1.3.1.1 PI4KIIIα Function
Most knowledge about this protein comes from studies of its yeast ortholog Stt4. It was initially isolated as an important factor for resistance to the inhibitor staurosporine (Yoshida et al., 1994). Stt4 has been shown to control actin cytoskeleton organization and cell wall integrity (Audhya, Foti and Emr, 2000). A temperature sensitive Stt4 mutant (Stt4\textsuperscript{ts}) exhibits a strong decrease in the total amount of PI4P and PI(4,5)P\textsubscript{2} (Audhya, Foti and Emr, 2000), indicating that most of the PI4P pool at the PM produced by Stt4 could be used as a substrate to produce PI(4,5)P\textsubscript{2}. This is confirmed by the evidence that the expression of the only yeast PIP5-kinase, Mss4 (multicopy suppressor of Stt4 gene), is sufficient to rescue the staurosporine hyper-sensitivity and the cell wall integrity defects caused by the Stt4 mutant (Yoshida et al., 1994), indicating that the supply of PI(4,5)P\textsubscript{2} is sufficient to compensate for decrease in PI4P.

Surprisingly, Stt4 is also important for the transport of phosphatidylserine from the ER to the Golgi complex, indicating that this kinase could also have a role in these endomembrane compartments, even though the mechanism through which this occurs has not been clarified (Trotter et al., 1998).

Much less is known about the function of PI4KI\textsubscript{IIa} in higher organisms. This lack of information is probably due to the fact that, for a long time, there was an apparent discrepancy between the site of PI4KI\textsubscript{IIa} localization (the ER) and the site of PI4P production (the PM). This puzzling picture was solved relatively recently by the work of Nakatzu and colleagues, who identified a previously missed N-terminal short sequence in the PI4KI\textsubscript{IIa} gene that is responsible for its PM localization (Nakatsu et al., 2012). Similarly to its yeast ortholog, PI4KI\textsubscript{IIa} is also important for the production of a PM PI4P pool that, in most cases, is used as a precursor for PI(4,5)P\textsubscript{2}; this is particularly important for the replenishment of PI4P under activation of PLC signaling or under hormone stimulation (Balla et al., 2007). However, work by Hammond et al. identified
the existence, under steady state conditions, of a stable PM pool of PI4P that is not destined for PI(4,5)P2 production, which has a major role in establishing the negative charge that defines the inner leaflet of the plasma membrane. This pool of PI4P is important for recruitment of proteins with anionic polybasic motifs and regulation of ion channels (Hammond et al., 2012).

A role for PI4KIIIα has also been proposed at the level of ER-exit sites (ERES), as PI4KIIIα depletion significantly reduces ERES formation in HeLa cells (Farhan et al., 2008). Since it has been previously shown that the small GTPase Sar1 stimulates the production of a pool of PI4P at ERES that is necessary for the nucleation of COPII coats (Blumental-Perry et al., 2006), PI4KIIIα could be considered as being the kinase responsible for the production of this pool. However, the work by Blumental-Perry et al. shows that Sar1 induces a membrane-associated PI4K activity compatible with a typeII PI4K, since it is insensitive to Wortmannin (Blumental-Perry et al., 2006). This discrepancy may reflect cell-type specificity.

1.3.1.2 Regulation of PI4KIIIα localization

Stt4 is localized at the PM where it clusters in peculiar structures known as phosphoinositide kinase (PIK) patches that contain multiple copies of the kinase together with accessory proteins (Audhya and Emr, 2002). Stt4 is stabilized at these sites by the interaction with the scaffold protein Ypp1. Reduction of Ypp1 levels causes Stt4 redistribution to the cytosol and subsequent degradation with a consequent increase in PI4P levels (Audhya and Emr, 2002). The interaction with the transmembrane protein Efr3 allows Ypp1-Stt4 complex stabilization at the PM (Baird et al., 2008). PI4P produced by Stt4 at the PM is dephosphorylated by the ER-localized phosphatase Sac1 and this has been demonstrated to occur at the level of ER-PM
contact sites (Stefan et al., 2011). Thus, the possibility that PIK patches correspond to ER-PM contact sites is very plausible.

Mammalian PI4KIIIα localization is subjected to a similar regulation mechanism, since its recruitment to the PM is mediated by the interaction with the cytosolic protein TTC7 and the trans-membrane protein EFR3, orthologs of Ypp1 and Efr3, respectively (Nakatsu et al., 2012). However, unlike its yeast counterpart, PI4KIIIα is not enriched in specific domains and no structures similar to PIK patches have been observed in mammals. Recently, PI4KIIIα localization has been shown to be regulated also by TMEM150A, the ortholog of yeast Sfk1, also reported to be important for Stt4 localization (Chung et al., 2015; Audhya and Emr, 2002). Since TMEM150A binds PI4KIIIα only in the absence of TTC7, it has been proposed that the EFR3-TTC7-PI4KIIIα complex is required for initial PI4KIIIα localization to the PM, while TMEM150A stabilizes the kinase in the PM (Chung et al., 2015), thus explaining the absence of a patched organization.

1.3.1.3 Regulation of PI4KIIIα activity

Since most of the PI4P produced by PI4KIIIα is used as a precursor for PI(4,5)P2, this kinase can be regulated in response to canonical signaling pathways. In particular, the Drosophila ortholog of PI4KIIIα, Stt4, is regulated by Hedgehog (Hh) signaling. Yavari and colleagues propose that, under steady state conditions, the Hh receptor Patched (Ptc) blocks Stt4, and that Hh stimulation releases this block to produce a PI4P pool necessary for the translocation of the Hh tranducer Smoothened (Smo) to the plasma membrane, thus inducing the Hh intracellular cascade (Yavari et al., 2010). Increasing PI4P by Sac1 4-phosphatase depletion, produces a similar activation of Smo, confirming the importance of PI4P in this pathway (Yavari et al., 2010). Notably, in
mammalian fibroblasts, Hh-reporter genes are activated by PI4KIIIα but also PI4KIIIβ, confirming a role for PI4P in Hh signaling also in higher organisms, although with some divergence from flies (Yavari et al., 2010).

PI4KIIIα is also regulated in response to PLC stimulation, which cleaves PI(4,5)P2 at the PM to produce diacylglycerol (DAG) and IP3, necessary to trigger the intracellular signaling cascade. In this condition, intracellular Ca++ release increases the formation of ER-PM membrane contact sites (Giordano et al., 2013), and the ER localized PI transfer protein Nir2 is recruited at these structures to provide the substrate for PI4KIIIα to produce PI4P (Chang et al., 2013). This mechanism of substrate delivery ensures the replenishment of the PI(4,5)P2 pool under receptor stimulation (Chang et al., 2013).

Figure 1.3 Cellular localization and functions of PI4KIIIα. Collective data from mammalian cells, yeast and animal model systems are shown. PI4KIIIα mainly exhibits PM localization in
yeast, with enrichment on PIK patches that are likely to correspond to ER-PM contact sites. Its mammalian counterpart shows a broader PM distribution, but can also localize in the ER, where it is thought to regulate ERES formation/function. The pool produced by PI4KIIIα, both in yeast and in mammals, is mainly needed as a substrate for PI(4,5)P2, that becomes particularly important for the replenishment of PI4P under activation of PLC signaling. Adapted from Tan and Brill, 2014, see text for details.

1.3.2 PI4KIIIβ

1.3.2.1 PI4KIIIβ function

PI4KIIIβ is the better characterized PI4 kinase in terms of regulation. Its yeast ortholog, Pik1, was the first PI4K to be cloned (Flanagan and Thorner, 1992) and it has been reported to localize at the Golgi Complex and in the nucleus (Strahl et al., 2005). Like Stt4, Pik4 is also an essential gene and the two kinases show non-redundant functions in yeast (Audhya, Foti and Emr, 2000). Pik1 mutants (e.g. temperature sensitive) show a strong decrease in the cellular PI4P and PI(4,5)P2 amount (45% and 40%, respectively) (Audhya, Foti and Emr, 2000, Walch-Solimena and Novick, 1999). If the role of Pik1 in the nucleus is not understood very well, Pik1 function at the Golgi has been better characterized, and it mainly consists in the regulation of the late secretory pathway. Pik1 mutants, indeed, show ultrastructural defects in Golgi morphology and a strong impairment in invertase secretion, which is reduced by ~80% in Pik1<sup>ts</sup> mutants (Walch-Solimena and Novick, 1999). The trafficking of the vacuolar enzyme CPY from the Golgi to the vacuole is also impaired under these conditions (Audhya, Foti and Emr, 2000, Walch-Solimena and Novick, 1999). The role of Pik1 in the secretory pathway has been corroborated by a number of lines of evidence:
1) the amount of PI4P is decreased in a temperature sensitive mutant of Sec14, a Golgi localized PI transfer protein which transfers PI from the ER to the Golgi, thus providing for the substrate for Pik1 (Hama 1999); Sec14 mutants exhibit growth and secretory defects that are rescued by the expression of Pik1 or the depletion of the 4-phosphatase Sac1, indicating that they are dependent on PI4P production (Hama et al., 1999; Cleves, Novick and Bankaitis, 1989).

2) Pik1 shows genetic interaction with the small GTPase Arf1, and some phenotypes induced by Pik1 mutants, such as defects in Golgi morphology and the decreased PI4P and PI(4,5)P2 amount, were recapitulated by loss-of-function mutants of Arf1 (Audhya, Foti and Emr, 2000). Moreover, the double Pik1/Arf1 mutant, although viable, exhibits synthetic protein trafficking defects, enforcing the idea that the two proteins act in a common pathway to maintain Golgi transport activity.

3) Pik1 is synthetically lethal with the RAB-GTPase Ypt31p, the ortholog of mammalian Rab11, an important regulator of Golgi to PM trafficking. Ypt31p seems to act downstream of Pik1, since Ypt31p depletion does not alter Pik1 localization and function (Sciorra, 2004).

Similarly to its yeast ortholog, mammalian PI4KIIIβ is localized at the Golgi Complex (Wong, Meyers and Cantley, 1997; Godi et al., 1999), although a pool of PI4KIIIβ is also present in the nucleus and on lysosomes (de Graaf et al., 2002; Sridhar et al., 2013). PI4KIIIβ has a very well established role in membrane trafficking. PI4KIIIβ depletion, indeed, causes an inhibition of both intra-Golgi trafficking of influenza hemagglutinin and basolateral delivery of the vesicular stomatitis virus (VSV)-G protein in MDCK cells (Bruns et al., 2002). Golgi to PM trafficking is also regulated by PI4KIIIβ in non-
polarized cells, where the over-expression of a kinase dead PI4KIIIβ mutant impairs the post-Golgi trafficking of VSV-G, by exerting a dominant-negative effect on the endogenous PI4KIIIβ (Godi et al., 2004). PI4KIIIβ has been also found to interact with the small GTPase Rab11, which is recruited at the Golgi upon PI4KIIIβ binding and contributes to the transport of cargo proteins to the PM (de Graaf et al., 2004, Burke et al., 2014). Another role for PI4KIIIβ in cellular secretion is indicated by its interaction with the 14-3-3γ proteins, which are responsible for the stabilization of the kinase, and with the fission-controlling protein BARS (Valente et al., 2012). In this model, a PI4KIIIβ-14-3-3-BARS complex forms selectively at the TGN under a trafficking wave, and disrupting this complex results in elongated tubular carriers that do not undergo fission (Valente et al., 2012). Since PI4KIIIβ (through PI4P) regulates the initial part of carrier formation, while BARS is involved in carrier fission, the authors propose that a similar mechanism would link the fission machinery to the site of tubule binding. A role for PI4KIIIβ in regulated exocytosis has also been proposed: in pancreatic β-cells, PI4KIIIβ mediates the release of insulin under glucose stimulation, which increases PI4P levels in WT cells but not in PI4KIIIβ-depleted cells (Gromada et al., 2005; Waselle et al., 2005). However, PI4P produced by PI4KIIIβ in this process functions only as a precursor for PI(4,5)P2, which is required for the recruitment of the cytosolic adaptors CAPS, which mediate fusion events between the PM and the secretory granules. In neuronal PC12 cells, PI4KIIIβ activity is modulated in response to an increase in intracellular Ca"sup+" levels. In response to purinergic receptor stimulation, indeed, the release of Ca"sup+" from intracellular stores activates the neuronal calcium sensor-1 (NCS-1), which binds PI4KIIIβ and stimulates its activity (see below), in turn promoting cellular secretion of dense core granules (De Barry et al., 2006). PI4KIIIβ depletion prevents NCS-1 induced exocytosis in response to Ca"sup+" stimulation, but, also in this
case, the authors postulate that PI4P acts as a precursor for PI(4,5)P2, required for the binding of several proteins implicated in exocytosis (De Barry et al., 2006). Finally, PI4KIIIβ has been also described to have a role at the lysosome, where it is required to regulate lysosomal cargo sorting and prevent abnormal lysosomeal tubulation, independently of its Golgi function (Sridhar et al., 2013).

1.3.2.2 Regulation of PI4KIIIβ localization

Pik1 localizes to the Golgi via binding with frequenin (Frq1), the yeast ortholog of the Ca++ binding protein NCS-1. Frq1 localizes at the Golgi and recruits Pik1, increasing its activity in vitro (Hendricks et al., 1999). The N-terminal myristoyl group of Frq1 is responsible for the recruitment of the Frq1-Pik1 complex to membranes, while Pik1 activation induced by Frq1 occurs independently of the N-terminal myristoylation, and is probably due to an allosteric change in the protein (Strahl et al., 2007).

A similar regulation mechanism takes place in mammals, where the neuronal calcium sensor-1 (NCS-1), a myristoylated Ca++-binding protein, directly binds PI4KIIIβ and stimulates its activity in vitro (Zhao et al., 2001). Unlike its homolog Frq1, NCS-1 is not primarily localized at the Golgi, but it is distributed in the cytosol (Rajebhosale et al., 2003), and it is not known to what extent NCS-1 contributes to the recruitment of PI4KIIIβ to the Golgi in mammalian cells. Golgi recruitment of PI4KIIIβ depends on the binding to the small GTPase Arf1 (Godi et al., 1999). As a matter of fact, Arf1 has been shown to induce PI4P synthesis through the recruitment and the stimulation of PI4KIIIβ activity on Golgi membranes (Godi et al., 1999). PI4KIIIβ activation by Arf1 is important to create and preserve Golgi morphology, since a kinase dead mutant of PI4KIIIβ, although efficiently recruited at the Golgi complex, alters both Golgi structure and the dynamics of Golgi reformation after treatment with the fungal toxin Brefeldin-A (BFA),
which prevents Arf1 activation and induces the disassembly of the Golgi complex (Lippincott-Schwartz et al., 1989; Godi et al., 1999). The crosstalk between ARF and PI4KIIIβ at the TGN seems to be conserved, at least functionally, in yeast where it has been shown that Pik1 is synthetically lethal with Arf1 (Walch-Solimena and Novick, 1999) and interacts with the Arf1 exchange factor Sec7 at the late Golgi, providing a dual-signal necessary for PI4P-dependent clathrin assembly at the Golgi (Gloor et al., 2010).

Interestingly, Arf1 and NCS-1 are able to interact and this establishes a regulatory negative feedback loop in which the stimulatory effect of Arf1 or NCS-1 on PI4KIIIβ is negatively controlled by their interaction (Haynes, Thomas and Burgoyne, 2005). In fact, NCS1-mediated stimulation of PI4KIIIβ activity is markedly reduced in the presence of ARF1 and vice versa (Haynes, Thomas and Burgoyne, 2005). This important evidence suggests that membrane sites occupied by both regulators serve as PI4KIIIβ-inactive zones, so that Arf1- and Ca++-dependent pathways do not interfere with each other.

Therefore, a tight modulation of different PI4P pools is crucial also if they co-exist in the same organelle and they are produced by the same kinase.

1.3.2.3 Regulation of PI4KIIIβ activity

PI4KIIIβ activity is regulated by phosphorylation, as suggested by the fact that it was initially isolated as a phosphoprotein in Sf9 cells (Suer et al., 2001). In particular, PI4KIIIβ is phosphorylated on S294, which is in a very conserved region of the protein, by protein kinase D (PKD), a Golgi-localized serine-threonine kinase family (Hausser et al., 2005). PI4KIIIβ S294 phosphorylation by both PKD1 and PKD2 isoforms increases PI4KIIIβ lipid activity and, subsequently, boosts the Golgi-to-PM trafficking of VSV-G,
which is, indeed, enhanced by wild-type but not by the S294 non-phosphorylatable PKD mutant (Hausser et al., 2005). PKD phosphorylation of PI4KIIIβ also promotes its interaction with 14-3-3 proteins, which stabilizes the kinase in its active conformation by preventing its dephosphorylation mediated by the serine-phosphatase PP2A (Hausser et al., 2006). This can be speculated to be a mechanism to ensure a constant supply of PI4P at the Golgi membranes.

In yeast, no similar regulation of Pik1 activity has been described, since no orthologs of PKD are present. On the other hand, Pik1 interaction with 14-3-3 proteins is evolutionary conserved, since it also occurs in yeast, where the interaction of Pik1 with the yeast 14-3-3 orthologs Bmh1 and Bmh2 in the cytosol preserves Pik1 activity by preventing its dephosphorylation (Demmel et al., 2008). In yeast, the cytosolic pool of Pik1 can both localize at the TGN, where it promotes vesicular trafficking through the interaction with Frq1, and in the nucleus in a nutrient-dependent manner (Demmel et al., 2008). Under glucose deprivation, indeed, Pik1 dissociates from the TGN, increases its interaction with 14-3-3 in the cytosol and eventually re-localizes to the nucleus (Demmel et al., 2008). This has been proposed as a means to shut down secretion under nutrient limitation. As a matter of fact, 14-3-3 over-expression inhibits Golgi-to-PM secretion to a similar extent as Pik1 depletion (Demmel et al., 2008).

Finally, PI4KIIIβ activity has been reported to be modulated in response to the Hedgehog pathway (see par. 1.3.1.3)
Figure 1.4 Cellular localization and functions of PI4KIIIβ. Collective data from mammalian cells, yeast and animal model systems are shown. PI4KIIIβ shows a prominent Golgi localization, both in yeast and mammalian cells and it is able to shuttle in the nucleus, even though the role of the nuclear PI4KIIIβ pool has not been elucidated. A cytosolic pool is stabilized by the interaction with 14-3-3 proteins, that prevents PI4KIIIβ dephosphorylation. PI4KIIIβ produces a PI4P pool at the Golgi that regulates different steps of Golgi to PM trafficking. A lysosomal pool of PI4KIIIβ is required to regulate lysosomal cargo sorting. Adapted from Tan and Brill, 2014, see text for details.
1.3.3 PI4KIIα and PI4KIIβ

1.3.3.1 PI4KIIα function

According to early studies, type II PI4Kinase activity was detected in PM fractions, so it was assumed that these kinases were localized in the PM. However, when the two type II kinases were cloned it was discovered that, although a pool of PI4KIIα is indeed present on the PM (Wei et al., 2002), this kinase is mainly localized in endo-membranes, in particular the TGN and early endosomes (Balla et al., 2002; Wang et al., 2003), but also late endosomes (Minogue et al., 2006), compartments positive for the adaptor protein AP-3 (Salazar et al., 2005), synaptic vesicles (Guo et al., 2003), and autophagosomes (Wang et al., 2015). PI4KIIα is responsible for the production of ~ 50 % of cellular PI4P (Wang et al., 2003) and, in accordance with its localization, PI4KIIα have a prominent role in regulating TGN-to-endosome trafficking (Wang et al., 2003; Wang et al., 2007). The clathrin-coated vesicle (CCV) adaptor protein AP-1, indeed, localizes at the Golgi in a PI4KIIα-dependent manner (Wang et al., 2003). Defects in AP-1 recruitment observed after PI4KIIα depletion are specifically dependent on PI4P production, since they are rescued by PI4P, but not PI(4,5)P2 addition (Wang et al., 2003). PI4P is also responsible for the TGN targeting of Golgi-localized, γ -ear containing, Arf-binding proteins (GGAs), a major family of TGN-enriched adaptors (Wang et al., 2007). PI4KIIα also regulates AP-3, a less-well characterized clathrin adaptor that produces vesicles that traffic from endosomes to lysosomes. AP-3 regulates PI4KIIα localization and, at the same time, PI4KIIα depletion causes the mis-targeting of the AP3 cargo LAMP1 and alters the distribution of t-snares (Vti1b and Syntaxin8) needed for endosome-lysosome fusion (Craige, Salazar and Faundez, 2008). Finally, PI4KIIα also regulates the PM-to-late
endosome trafficking of EGF receptor (Minogue et al., 2006) and the endosomal sorting of the t-snare VAMP3, which is in turn important for efficient Transferrin receptor (Tfr) recycling (Jovic et al., 2014).

Interestingly, PI4KIIα and PI4KIIIβ account for most of the PI4P production at the Golgi (Godi et al., 2004, Wang et al., 2003, Haynes, Thomas and Burgoyne, 2005). However, while both of them localize to the TGN, they have non-overlapping roles indicating that they control pools of PI4P that play distinct functions in the same organelle by recruiting different effectors. As a matter of fact, two pools of PI4P, one showing a broader Golgi distribution and one specifically localized at the TGN, have been described as being produced by PI4KIIIβ and PI4KIIα, respectively (Weixel et al., 2005). A clear example of distinct and coordinated PI4K action at the TGN is represented by the trafficking of the lysosomal hydrolase β-glucocerebrosidase (GBA), required for efficient hydrolysis of lysosomal Glucosylceramide (GlcCer), which accumulates in lysosomes after GBA mutation and causes Gaucher disease (Reczek et al., 2007). GBA is produced in the ER, traffics through the Golgi and is delivered to lysosomes via the lysosomal receptor LIMP-2, critical for AP-3 binding and sorting to lysosomes, rather than via the canonical M6PR mediated pathway (Reczek et al., 2007). Jovic and colleagues demonstrated that, while PI4KIIIβ is required for LIMP-2/GBA exiting from the Golgi, PI4KIIα operates in a second phase, namely the trafficking of GBA between late endosomes (LE) and lysosomes (Jovic et al., 2012). GBA cannot reach the lysosome and is mis-sorted to the extracellular space under PI4KIIα depletion, but not if PI4KIIIβ is simultaneously inhibited, confirming sequential, non-overlapping roles of the two kinases in this process (Jovic et al., 2012).

Much less is known about the PI4KIIα ortholog in yeast, Lsb6, which is a non-essential gene mainly localized at the PM and on the vacuole (Han et al., 2002; Shelton et al.,
Two concomitant independent studies established that Lsb6 has a 4-kinase activity, although the contribution of Lsb6 to total cellular PI4P production is modest (Han et al., 2002; Shelton et al., 2003). The only endocytosis-related process ascribed to Lsb6 in yeast is the regulation of surface GPCR receptor internalization, mediated by actin polymerization and subsequent endosome movement (Chang et al., 2005). However, the kinase activity of Lsb6 is dispensable for its role in endocytosis, instead, seems to occur via binding to the yeast homologue of the Wiskott-Aldrich syndrome protein (WASP) Las17, a regulator of actin polymerization (Chang et al., 2005).

1.3.3.2 PI4KIIβ function

PI4KIIβ is the most elusive of the four PI4-kinases. It has been shown to localize at early and recycling endosomes (Balla et al., 2002) and, under growth factor stimulation, at the PM (Wei et al., 2002). The overlap between PI4KIIα and PI4KIIβ localization led to the assumption that PI4KIIβ could also have a role in TGN to endosome trafficking, but a formal proof was missing until Wieffer and colleagues demonstrated that PI4KIIβ is able to bind AP-1, and the two proteins regulate each other’s localization (Wieffer et al., 2013). Interestingly, the PI4P pool produced by PI4KIIβ in this context controls Wnt signaling by regulating surface expression, internalization, or endosomal sorting of the Wnt receptor Frizzled (Fz) (Wieffer et al., 2013). Increasing knowledge about PI4KIIβ function allowed the identification of distinct, non-redundant roles for PI4KIIα and PI4KIIβ. In particular, a recent work by Alli-Balogun et al. established the existence of two separate PI4P pools produced by PI4KIIα and PI4KIIβ at the interface between the TGN and endosomes, defined by the co-localization with the TGN markers syntaxin6 and TGN46, respectively (Alli-Balogun et al., 2016). In the same work, a
specific role for PI4KIβ was proposed in invadopodia formation and cancer progression (Alli-Balogun et al., 2016). PI4KIβ drives these events by inducing the mis-targeting of the metalloproteinase MT1-MMP to the PM as a result of an imbalance between the endo-lysosomal and the exocytosis trafficking routes of the protein, in favor of the latter (Alli-Balogun et al., 2016).

1.3.3.3 Regulation of PI4KIα and PI4KIβ localization

PI4KIα and β are cytosolic, and their membrane localization has been shown to be regulated by palmitoylation of a conserved stretch of cysteines within their catalytic domains (Barylko et al., 2001). PI4KIα can be palmitoylated by six different palmitoyl acyltransferases (PATs, i.e. DHH2, 3, 7, 14, 15 and 21) that all reside in the TGN (Lu et al., 2012). Intriguingly, this palmitoylation is cholesterol-dependent: after methyl-b-cyclodextrin treatment, PI4KIα is found to be less associated with Golgi membranes, and this causes a dramatic drop in PI4P levels on the Golgi (Lu et al., 2012). While PI4KIα exists mainly as an active, integral-membrane bound kinase, PI4KIβ has a significant inactive cytosolic component, which is recruited to the PM in response to growth factor stimulation (Wei et al., 2002). PI4KIβ is stabilized in the cytosol by the interaction with HSP90, which prevents its degradation (Jung et al., 2011). Platelet derived growth factor (PDGF) or epidermal growth factor (EGF) stimulation disrupts the PI4KIβ-HSP90 complex, thus inducing PI4KIβ translocation to the plasma membrane where it can be activated through palmytoylation and produce its substrate (Jung et al., 2011).

PI4KIα localization is also regulated under autophagy induction by the interaction with the γ-aminobutyric acid receptor-associated protein (GABARAP). Upon starvation, PI4KIα is recruited by GABARAP on autophagosomal membranes and produces a pool
of PI4P in loco which is required for autophagosome-lysosome fusion (Wang et al., 2015).

1.3.3.4 Regulation of PI4KIIα and PI4KIIβ activity

Both PI4KIIα and PI4KIIβ activity has been shown to be regulated in response to canonical signaling pathways activation. The activity of both PI4KIIα and PI4KIIβ has been shown to be regulated in response to the activation of canonical signaling pathways.

In particular, PI4KIIα plays a role in the Wnt pathway, as PI4KIIα depletion attenuates Wnt signaling both in cellular and animal models (Pan et al., 2008; Qin et al., 2009). Under stimulation with Wnt3a, the adaptor protein Dishvelled (Dsv) simultaneously binds PI4KIIα and PIP5K1β to promote sequential production of PI4P and PI(4,5)P2. The latter is required for the phosphorylation of the lipoprotein receptor-related protein (LRP)6, one of the earliest events in the Wnt signaling cascade (Pan et al., 2008; Qin et al., 2009). A subsequent work by Mossiger et al. demonstrated that the role of PI4KIIα in the Wnt pathway is regulated by ubiquitination. In fact, the E3 Ubiquitin ligase Itch interacts with and ubiquitinates PI4KIIα, and this regulates the internalization and the endosomal sorting of the Wnt receptor Frizzled (Fz) (Mössinger et al., 2012). A role in the Wnt cascade has been proposed also for PI4KIIβ, whose depletion attenuates the activation of Wnt signaling proteins (Wieffer et al., 2013). In PI4KIIβ-KD cells, the Wnt receptor Frizzled (Fz), instead of being correctly recycled to the plasma membrane, is mis-sorted in late endosomal/lysosomal compartments for degradation (Wieffer et al., 2013). Thus, while the pool of PI4P produced by PI4KIIα regulates Wnt signaling mainly by acting as a PI(4,5)P2 substrate, the role of PI4KIIβ in this pathway involves the production of a specific endosomal PI4P pool that plays its own role in Fz recycling.
Figure 1.5 Cellular localization and functions of PI4KIIα. Collective data from mammalian cells, yeast and animal model systems are shown. While yeast PI4KIIα is mainly localized at the PM, its mammalian counterpart also shows a prominent endomembrane-localized component, mainly divided between the TGN and the endosomal compartments. The pool of PI4P produced by PI4KIIα at the TGN mainly regulates Golgi-to-endosome trafficking via binding and activation of clathrin adaptors, but is also involved in other steps of endocytic trafficking. The pool of PI4P produced by PI4KIIα is mainly converted to PI(4,5)P2 acting downstream of surface receptors. Adapted from Tan and Brill, 2014, see text for details.
Collective data from mammalian cells, yeast and animal model systems are shown. PI4KIIβ is mainly localized in the cytoplasm, where it is stabilized by the interaction with HSP90, and it shuttles to the PM under growth factor stimulation. The pool of PI4P produced by PI4KIIβ is thought to be mainly used as a substrate for PI(4,5)P2, although recent evidence demonstrates a specific role for this pool in Wnt signaling and cancer progression (not shown in this model). Adapted from Tan and Brill, 2014, see text for details.
1.4 Regulation of PI4P homeostasis by the 4-phosphatase SAC1

Working antagonistically to the four PI4-kinases, there is just one 4-phosphatase in the cell named Sac1, an ER-localized, highly conserved trans-membrane protein.

Sac1 was identified more than 25 years ago by a genetic screen aimed to find correctors of actin cytoskeleton defects in yeast, and therefore classified as Suppressor of Actin (Sac)1 (Novick, Osmond and Botstein, 1989). A concomitant genetic screen identified Sac1 as a suppressor of mutation in the yeast secretory gene Sec14, which encodes a Golgi-localized PI transfer protein (PITP) and is essential for secretory functions (Bankaitis et al., 1990). Early studies established that Sac1 is an integral membrane protein localized to the endoplasmic reticulum (ER) and Golgi membranes in yeast and in mammalian cells (Whitters et al., 1993; Nemoto et al., 2000). Sac1 has been shown to be an inositol auxotroph, and this first established a correlation between Sac1 and inositol phospholipid metabolism (Whitters et al., 1993), later confirmed by the similarity of Sac1 with the PIP phosphatase synaptojanin (McPherson et al., 1996). As a matter of fact, Sac1 was demonstrated to possess phosphoinositide phosphatase activity (Guo et al., 1999), and, if Sac1 seems to be active on multiple PIP species in vitro, the in vivo analysis of Sac1 mutants identified PI4P as its selective substrate (Guo et al., 1999, Foti, Audhya and Emr, 2001). Indeed, when a Sac1<sup>ts</sup> mutant is shifted to the restrictive temperature, PI4P levels rapidly increase 7-fold, with small changes in the other species, indicating that Sac1 primarily controls PI4P metabolism, and the alteration of other PIP species probably occurs as an indirect consequence of PI4P imbalance (Foti, Audhya and Emr, 2001).

Topological studies in yeast identified Sac1 as a type II trans-membrane protein, with two C-terminal trans-membrane domains (TMD) (Konrad et al., 2002; Fig. 1.7). This
creates a “J” topology with both the N- and C-terminal domains exposed in the cytosol, which is conserved also in mammals (Nemoto et al., 2000). Mammalian Sac1 contains a putative leucine zipper (LZ) motif at its N-terminus and a COP-I binding motif (KEKIDD) at its extreme C-terminus, which are essential for Sac1 localization at the Golgi and ER, respectively (Rohde et al., 2003; Fig. 1.8). From the crystal structure it emerged that the first 503 amino acids of yeast Sac1 (which include the entire SAC domain) has N- and C-terminal subdomains (Manford et al., 2010). SacN (residues 1-182) mediates various protein-protein interactions while SacC (residues 183-503) comprises the P-loop and contains the conserved catalytic CX5R(T/S) motif. The crystal structure of Sac1 (Fig. 1.7) shows the presence of a peptide stretch of ~70 residues (residues 453-522) located between the catalytic motif and the first TMD (TMD1) that is predicted to be an unstructured linker region possibly used by SAC1 to act “in trans” to dephosphorylate its substrate on a different, juxtaposing membrane (Manford et al., 2010; Fig. 1.7). A subsequent study demonstrated that a significant portion of this region (461-511) is required for PI4P recognition and for catalytical activity, thus making the remaining available free portion shorter (Cai et al., 2014). In any case, this flexible linker would be able to span the distance between narrow membrane contact sites (10-12 nm), and would still allow Sac1 to dephosphorylate its substrate in trans on another juxtaposed membrane (Fig. 1.7).
Figure 1.7 Domain organization and graphical model of SAC1 function. A The first 500aa of Sac1 are divided in two sub-domains: the N-sub-domain, involved in protein-protein interactions, and the C-sub-domain, containing the conserved catalytic motif. Mutation in Cys392 is sufficient to eliminate the catalytic activity. B The SAC domain of Sac1 is shown by molecular surfaces colored cyan (the N-sub-domain) and yellow (the C-sub-domain). The catalytic site is colored red. The region between residues 460 and 522 (dashed segment) was predicted to be an unstructured linker region that allows Sac1 to dephosphorylate its substrate both in a cis and in a trans mode (Manford et al., 2010). Although a subsequent work established that a part of the linker is required for the catalytic activity (Cai et al., 2014), the remaining free unstructured portion (~7nm) would still be able to cover narrow distances.

Adapted from Manford et al. 2010 and Cai et al. 2014.
1.4.1 SAC1 function

Although the Sac1 gene in yeast is not essential for viability, ∆Sac1 mutants exhibit a variety of defects such as alteration in the actin cytoskeleton, cold sensitivity for growth and inositol auxotrophy, since Sac1 mutants are unable to produce their own inositol de novo (Novick, Osmond and Botstein, 1989; Whitters et al., 1993). ∆Sac1 shows a defective secretion of yeast invertase and delayed ER to Golgi trafficking of carboxypeptidase Y (CPY), commonly used to study protein processing and trafficking in yeast (Novick, Osmond and Botstein, 1989; Mayinger, Bankaitis and Meyer, 1995). Sac1 mutants also display the so called “bypass Sec14” phenotype, because ∆Sac1 is able to survive the lethality induced by the loss-of-function of Sec14, the major yeast PtdIns/PtdCho transfer protein (Bankaitis et al., 1990). The same work reported that Sac1 also interacts genetically with six other SEC genes (sec6, sec9, sec17, sec18, sec21, sec23) involved in trafficking between the ER, Golgi and the PM, thus confirming its role in secretion (Bankaitis et al., 1990).

Important insights into the role of Sac1 in mammals came from the work of Liu et al., which described the effect of Sac1 genetic depletion in a mouse model (Liu et al., 2008). Sac1 function is essential at the organismal level, since Sac1-KO embryos are unable to progress beyond the E.35 developmental stage and undergo pre-implantation lethality (Liu et al., 2008). HeLa cells depleted of SAC1 also show viability defects, mainly because they fail to progress through the G2/M phase of the cell cycle, as a consequence of the formation of abnormal multipolar spindles (Liu et al., 2008). Importantly, Sac1-KD cells exhibit extensive fragmentation of the Golgi complex, which, however, preserves its functionality, since VSV-G reaches the plasma membrane at a similar extent as in control cells (Liu et al, 2008). Both the Golgi and the mitotic spindle defects observed in Sac1-KD cells depend on PI4P regulation, as the
phenotypes are rescued by WT-SAC1, but not by a catalytically-inactive mutant (Liu et al., 2008).

In yeast Sac1 is implicated in sphingolipid biosynthesis. This process begins in the ER with the production of long-chain bases (LCBs), generated by the serine palmitoyltransferases, Lcb1 and Lcb2, which condense serine and palmitoyl-coenzymeA. Sac1 has been proposed to regulate sphingolipid metabolism in two ways: directly, in the ER, where it is part of the SPOTS (Serine palmitoyltransferase, Orm1/2, Tsc3, Sac1) complex, binds Lcb1/2 and negatively regulates LCB production (Breslow et al., 2010), or indirectly, at the Golgi, by modulating the availability of phosphatidylinositol (which provides inositol phosphate) via dephosphorylation of PI4P (Brice, Alford and Cowart, 2009). As a consequence, Sac1 deletion leads to elevated LCB levels and resistance to the Lcb1/2 inhibitor myriocin (Breslow et al., 2010).

In mammals, PI4P also affects sphingolipid metabolism, but it mainly occurs through the recruitment of lipid transfer proteins that control non-vesicular trafficking of lipids between membranes (see below).

Mammalian Sac1 has also been shown to be important for proper Golgi enzyme localization (Cheong 2010). In the absence of Sac1, indeed, the medial-Golgi enzymes Mann-II and N-acetylglucosaminetransferase-I (GnT-I) lose their Golgi localization, become dispersed in peripheral puncta and are mis-localized to the plasma membrane (Cheong et al., 2010). Sac1 KD-induced mis-localization of Golgi enzymes is rescued by WT-Sac1, but not by a catalytically-inactive mutant, indicating that it is dependent on PI4P, probably as a consequence of PI4P-mediated secretion (Cheong et al., 2010).
1.4.2 Regulation of SAC1 localization and activity

Under steady state conditions Sac1 is distributed between the ER and early Golgi compartments but it is virtually absent from TGN membranes (Cheong et al., 2010). This spatial segregation of Sac1 from its substrate determines the enrichment of PI4P at the trans-Golgi under steady state conditions. Sac1 cycling between ER and Golgi membranes occurs in a COP-I and COP-II dependent manner. As a matter of fact, mammalian Sac1 is able to bind the α- β- γ- and ε- subunits of the COP-I coatamer, thanks to a COP-I interaction motif (KEKIDD), and this interaction is responsible for the Golgi-to-ER retrieval of the protein. As a consequence, a Sac1 mutant containing lysine to alanine substitutions within the C-terminal ER retrieval motif (SAC1-K2A), accumulates at the Golgi complex (Rohde et al., 2003; Fig. 1.8). Conversely, a leucine-zipper (LZ) domain localized at the N-terminus of the protein is responsible for Sac1 dimerization and subsequent incorporation in COP-II vesicles to allow its ER export (Blagoveshchenskaya et al., 2008; Fig. 1.8).

![Figure 1.8 Schematic representation of SAC1 domains required for ER and Golgi localization.](image)
Blagoveshchenskaya and colleagues have shown that ER to Golgi shuttling of Sac1 is regulated in response to growth factor stimulation and is needed to control cellular secretion (Blagoveshchenskaya et al., 2008). Nutrient starvation, indeed, induces Sac1 oligomerization and subsequent accumulation at the Golgi that causes, in turn, a reduction in PI4P levels and a subsequent block of anterograde membrane trafficking from the Golgi to the PM (Blagoveshchenskaya et al., 2008; Fig. 1.9). Conversely, mitogen stimulation of the p38/MAPK pathway induces dissociation of Sac1 oligomers, and the subsequent COP-I mediated retrieval of SAC1 to the ER (Blagoveshchenskaya et al., 2008 Fig. 1.9). These experiments show that Sac1, by regulating PI4P homeostasis, controls anterograde membrane trafficking which is important for the delivery of proteins and lipids to the periphery of an expanding cell in response to growth factor stimuli. As a matter of fact, Sac1 depletion by RNA-interference leads to a substantial increase in constitutive secretion (Blagoveshchenskaya et al., 2008; Fig. 1.9). Also in yeast, Sac1 shuttles between the ER and Golgi in a nutrient-dependent manner, even though a C-terminal COP-I interacting motif is absent. During exponential cell growth, yeast Sac1 localizes to the ER by binding the dolicholphosphate-mannose synthase (Dpm1) (Faulhammer et al., 2005; Fig. 1.9). Under glucose deprivation, Sac1 binds the adaptor protein Rer1, that is thought to disrupt the interaction between Sac1 and Ddm1, and relocalizes to the Golgi complex, where it dephosphorylates a pool of PI4P produced by the PI4-kinase Pik1 (Faulhammer et al., 2005). Interestingly, glucose deprivation has an opposite effect on Pik1, which dissociates from the Golgi in this condition (Faulhammer et al., 2007; Fig. 1.9).
In yeast, under glucose starvation, the combined effects of Sac1 Golgi translocation and Pik1 relocalization in the cytosol induces a drop in PI4P levels at the Golgi and, subsequently, a slow down in secretion. In mammals, serum starvation induces Sac1 oligomerization and shuttling to the Golgi, where it down-regulates PI4P and constitutive secretion. After growth factor stimulation, MAP kinase activity is required for Sac1 oligomer dissociation and Sac1 redistribution to the ER. Reduction of Sac1 levels at the Golgi generates elevated concentration of PI4P on this organelle, thus increasing constitutive secretion. Adapted by Blagoveshchenskaya and Mayinger, 2009.

Together, these lines of evidence indicate that PI4P is controlled by multiple mechanisms to ensure a fine-tuning of secretion depending on nutrient availability.

Mammalian Sac1 has also been shown to interact with 14-3-3 proteins, thanks to a 14-3-3-phosphorylation-dependent binding motif. This interaction is required for efficient ER-export, since 14-3-3 seems to act as a bridge between Sac1 and the COP-II component Sec24 (Bajaj Pahuja et al., 2015). These observations indicate that Sac1 exit
from the ER is regulated by phosphorylation, even though the responsible kinase has not been identified yet.

The apparent contradictory observation that a phosphatase that is mainly localized in the ER can dephosphorylate its substrate in other organelles can be explained by the evidence that the ER is able to enter in close proximity with a variety of subcellular compartments, establishing the so called ER-organelle membrane contact sites (MCSs).

1.5 Regulation of PI4P metabolism at ER-organelle Membrane Contact Sites (MCSs).

1.5.1 General features of membrane contact sites

ER-organelle membrane contact sites are sites at which two organelles are in close proximity without fusing, and they have become the object of growing attention in recent years. The ER has been shown to be closely apposed to almost all membrane-bound organelles, including the PM, mitochondria, lipid droplets, Golgi, endosomes, and peroxisomes (Phillips and Voeltz, 2016; Fig 1.10). ER-organelle contact sites share some features. First of all, for an ER-organelle contact to be considered as a membrane contact site, the distance between membranes has to be very short, usually ≤ 30nm; for example electron micrograph of ER-endosome and ER-mitochondria MCSs showed an average distance of 3-15nm (Alpy et al., 2013) and 6-15nm (Csordás et al., 2006), respectively. Another feature of MCSs is the presence of complexes or proteins defined as “tethers” that are involved in their establishment and maintenance. Importantly, these proteins are often redundant, as the ablation of one or more tethers leads to a reduced MCS number, but does not completely abolish their formation. Finally, MCS formation and function is dynamically regulated in response to different stimuli (Prinz et al., 2014, Gatta and Levine, 2017, Phillips and Voeltz, 2016).
Figure 1.10 ER-membrane contact sites (MCSs). The ER spreads all over the cell as a network of sheets and tubules and establishes contact sites (dashed circles) with a variety of organelles, including Golgi, mitochondria, endosomes, lipid droplets, peroxisomes and the plasma membrane. Phillips and Voeltz, 2016.

1.5.2 Membrane contact site tethering proteins: the role of VAPs

The existence of tethering complexes responsible for membrane contact site formation has been extensively studied in yeast. Manford and colleagues identified a group of six genes (the orthologs of VAP proteins Scs2/Scs22, the tricalbins Tcb1,2 and 3 and the ion channel Its2) that, if simultaneously depleted (∆tether), abolish the formation of ~95% of contacts between the endoplasmic reticulum and the plasma membrane (Manford et al., 2012). A similar mechanism has been described for ER-mitochondria contact sites, that are maintained by the so called ERMES complex. This
complex is formed by an ER protein (Mmm1), two mitochondrial proteins (Mdm34 and Mdm10), and a further component Mdm12 that has no integral membrane region but is required for structural integrity of the complex (Kornmann et al., 2009). The deletion of any ERMES subunit leads to disintegration of the complex, which can be seen by fluorescence microscopy as a loss of the typical ERMES foci (Kornmann et al., 2009).

Apart from these oligomeric tethering examples, many proteins have been shown to be required for ER-organelle contact site maintenance, or simply use these contacts to perform their function. The Vesicle-associated membrane protein- (VAMP) associated proteins (VAPs) are among the most studied, as they are involved in the formation of membrane contact sites between the ER and almost every other organelle (Fig. 1.11 and Fig. 1.12). VAPs are type-II integral membrane proteins expressed in all eukaryotic organisms. The VAP protein family includes two members: VAPA and VAPB, both consisting of a C-terminal trans-membrane domain (TMD), a linker region (≤100 aa) partly forming a coiled coil in some species, and an N-terminal major sperm protein (MSP) domain (Murphy and Levine, 2016; Fig. 1.10).

![Figure 1.11 Schematic representation of VAPA and VAPB domain organization.](image)

VAPs have been proposed to bridge the ER with different organelles, acting as a multiple tether of membrane contact sites. In particular, the yeast orthologs of VAPs (Scs2/Scs22) have been shown to have a role in establishing ER-PM membrane contact
sites, that are reduced by 50% under Scs2/Scs22 depletion (Stefan et al., 2011). Depletion of VAP proteins in mammals determines the loss of ~30% of contact sites between the ER and mitochondria (Stoica et al., 2014). Apart from their role as direct tethers, VAPs are also involved in contact site regulation because they represent the ER determinant for proteins working at the ER-organelle interface. This has been observed at ER-endosome contact sites, where VAP binds proteins important for the formation and/or function of these sites, e.g. protrudin, which regulates late endosome (LE) translocation to the cell periphery and neurite outgrowth (Raiborg et al., 2015), ORP1L, involved in the regulation of endosomes positioning in response to sterols levels (Rocha et al., 2009), the lipid transfer protein STARD3, which transfers cholesterol form ER on endosomes (Wilhelm et al., 2017), and SNX2, important for the retrograde trafficking between endosomes and Golgi (Dong 2016). At the level of ER-Golgi membrane contact sites, VAPA binds the lipid transfer protein OSBP, required for the counter-exchange of cholesterol and PI4P at the ER-Golgi interface (Mesmin et al., 2013). VAPs interact with proteins containing the so-called FFAT motif (two phenylalanines (FF) in an acidic tract). The motif has a core with six defined elements across a stretch of seven residues: EFFDAxE. The substitution of some elements in the FFAT domain are tolerated, such that some possible variants, defined as FFAT-like domains, have been identified (Murphy and Levine, 2016). This extended the list of putative VAP binding partners, and provided a clearer picture on how these proteins can control such a variety of cellular processes at the level of membrane contact sites.
Figure 1.12 VAPs are engaged in multiple ER-organelle membrane contact sites Schematic illustration of ER-mediated membrane contact sites (MCSs). ER-resident proteins are listed on the left, and their corresponding factors on the other membrane are listed on the right. VAPs and their yeast orthologs Scs2/Scs22 (red dashed circles) are involved in the formation and maintenance of a variety of MCSs. Adapted from Zhang and Hu, 2016.

1.5.3 Regulation of PI4P homeostasis at the level of ER-organelle membrane contact sites (MCSs).

In recent years, a growing body of evidence has indicated that PI4P homeostasis is regulated at the level of membrane contact sites, and this occurs mainly, but not exclusively, through dephosphorylation of PI4P by the ER-localized phosphatase Sac1.
1.5.3.1 PI4P metabolism at ER-PM MCS

ER-plasma membrane contact sites were already described in the 1950’s and have been mainly characterized for their role in the regulation of Ca^{++} dynamics. As a matter of fact, ER-PM MCSs regulate the replenishment of ER Ca^{++} stores, called store operating calcium entry (SOCE). When calcium levels are low, the ER protein STIM1 interacts with the PM Ca^{++} channel Orai1 at ER-PM MCSs and this restores the calcium levels in the ER (Hewavitharana et al., 2008). ER-PM MCSs are also formed in response to Ca^{++} stimulation, and this involves the tethering factors Extended-synaptotagmins (E-syts) (Giordano et al., 2013).

Fundamental insights into the role of ER-PM MCSs in PI4P regulation came from the milestone work by Manford and colleagues, which demonstrated that the simultaneous ablation of six ER-resident proteins (named Δtether mutant by the authors) was able to almost completely abolish the connections between the ER and the PM in yeast. This powerful approach highlighted that ER-PM MCSs are important to maintain PI4P homeostasis, since in Δtether cells, in which just ~ 5% of the PM was connected to the ER, PI4P levels increased greater than 7-fold compared to the WT (Manford et al., 2012). Importantly, the simultaneous depletion of the 4-phosphatase Sac1 with the tethering genes abolished the increase in PI4P levels, indicating that it was due to a defective dephosphorylation by Sac1 (Manford et al., 2012).

The ER-localized Sac1 has been shown to hydrolyze its substrate at the PM in trans in this system through the action of the oxysterol-binding protein Osh3 (Stefan et al., 2011). Two concomitant studies in yeast and mammals also proposed that the PM PI4P pool is regulated at the level of ER-PM MCSs by other members of the Osh family, Osh6 and Osh7 in yeast and their mammalian orthologs ORP5 and ORP8. However, in
these cases PI4P consumption at the level of MCSs occurs through a different mechanism, which consists in the counter-exchange of PI4P in the PM with the ER lipid phosphatidylserine (PS) in the ER (Chung et al. see below).

In other cases, it has been reported that perturbation of ER-PM contact sites determines changes in PI4P levels at the PM, which is, however, mainly used to replenish the PM pool of PI(4,5)P2 after surface receptor stimulation (Chang et al., 2013; Dickson et al., 2016).

1.5.3.2 PI4P metabolism at ER-endosome MCSs

In addition to its prominent Golgi localization and the presence of a considerable plasma membrane pool, a small fraction of PI4P has been detected also in other endomembrane organelles. Among these, an endosomal component of PI4P, co-localizing with Rab7 and Rab5, has been shown to be controlled by contact sites between the ER and endosomes, established by the VAP proteins and regulated by OSBP1 (Dong et al., 2016).

VAPs, binding the retromer component SNX2 on endosomes, establishes ER-endosome contact sites and, via OSPBP1, mediate the consumption at these sites of a PI4P pool produced by PI4KIIα and PI4KIIβ (Dong et al., 2016). The authors also identified a role for the endosomal PI4P pool in the regulation of WASH-mediated actin nucleation on endosomes, which allows the correct formation of retromer tubules that, in turn, mediate endosome to Golgi trafficking (Dong et al., 2016). VAP depletion causes disruption of MCSs and the subsequent accumulation of PI4P, which is mimicked by OSBP1 or Sac1 depletion (Dong et al., 2016).
1.5.3.3 PI4P metabolism at ER-Golgi MCSs

Despite the existence of contact zones between the ER and the last trans-cisterna/Trans Golgi Network was established in the early 60’, this class of membrane contact sites is the less studied because of the position in the crowded perinuclear Golgi area which makes it difficult to resolve them by optical microscopy.

Although there is little information about the role of ER-TGN MCSs, what is available converges on the regulation of PI4P homeostasis. PI4P levels at the Golgi complex indeed seem to be regulated, both in yeast and in mammals, at the level of ER-Golgi membrane contact sites. The work of Mesmin and colleagues described for the first time that the stabilization of ER-Golgi MCSs by artificial tethers determines a reduction in PI4P levels, because the lipid transfer protein OSPBP1 exchanges it with the cholesterol in the ER (Mesmin et al., 2013). A similar mechanism for PI4P regulation at the level of ER-Golgi MCSs has also been proposed to occur in yeast, where it is mediated by the lipid transfer protein Kes1/Osh4 (von Filseck et al., 2015). Thus, the only function identified for the ER-TGN MCSs consists in the regulation of the Golgi PI4P pool, and the only mechanism proposed so far is the counter-exchange of PI4P with other lipids in the ER (see below).

1.5.3.4 Mechanisms of PI4P regulation at ER-organelles MCSs

At the level of ER-organelle membrane contact sites, PI4P metabolism can be controlled by two different co-existing mechanisms:

1. Regulation of PI4P metabolizing enzyme localization (binding of the enzyme to scaffold proteins)
2. Regulation of PI4P metabolizing enzyme function (substrate delivery or stimulation of the enzymatic activity)

1.5.3.4.1 Regulation of PI4P metabolizing enzyme localization

The only example of a PI4P metabolizing enzyme specifically localized to membrane contact sites comes from studies in neuronal cells. In particular, Dickson and colleagues demonstrated for the first time that Sac1 is able to localize at ER-PM contact sites (defined as ER-PM Sac1 puncta), and Sac1 recruitment to these structures (even in an acute fashion with rapamycin), strongly reduces the amount of PI4P at the PM (Dickson et al., 2016). Under Ca++ stimulation, the ER-localized extended-synaptotagmin proteins (E-Syt1, E-Syt2 and E-Syt3) establish ER-PM contact sites by binding PI(4,5)P2 in the PM (Giordano et al., 2013). The work by Dickson et al. demonstrates that E-Syt2 recruits Sac1 at PI(4,5)P2/E-Syt-mediated ER-PM contact sites in order to dephosphorylate PI4P on the PM. As a consequence, E-Syt2 depletion shows increased PI4P and subsequent PI(4,5)P2 levels in the PM, following ER-PM puncta ablation, demonstrating that this mechanism controls phosphoinositide metabolism in the PM (Dickson et al., 2016). Even though the data suggest that Sac1 in the ER could dephosphorylate PI4P in trans on the PM in this system, the authors do not exclude that PI4P could be delivered to the ER to allow Sac1-mediated dephosphorylation in cis.

A recent work by Zewe et al. failed to observe an enrichment of SAC1 at the level of ER-PM MCSs both under steady state condition and after stimulation, suggesting that this could be a mechanism specific for neurons, which could respond differently to Ca++ signaling (Zewe et al., 2017).
1.5.3.4.2 Regulation of PI4P metabolizing enzyme function (substrate delivery)

Many reports in the last years demonstrated that a mechanism through which the ER-localized phosphatase Sac1 can consume its substrate PI4P present on different membranes is the delivery of the latter to the ER, mediated by specialized lipid transfer proteins.

This has been shown to occur at the level of ER-PM MCSs, where the OSPB-related protein family members ORP5 and ORP8 regulate PI4P levels on the PM by mediating the counter-exchange of PI4P with phosphatidylserine (PS) in the ER (Fig. 1.13). Once in the ER, PI4P can be dephosphorylated in cis by its phosphatase Sac1. Notably, a concomitant work in yeast describes an analogous mechanism for the lipid transfer proteins Osh6 and Osh7, closely related to ORP5 and ORP8. Osh6/7, similarly to their mammalian counterparts, maintain PI4P homeostasis at the PM/ER interface by exchanging PI4P with PS and inducing PI4P consumption by Sac1 in the ER (von Filseck et al., 2015a; (Fig. 1.13).

The PI4P pool at the Golgi has been shown to be regulated through a similar mechanism at sites of apposition between the ER and the TGN. The work of Mesmin and colleagues, indeed, established that the lipid transfer protein OSPB1 acts at the ER-TGN interface where, by simultaneously binding of the ER proteins VAPs (via the FFAT domain) and PI4P at the Golgi (via the PH domain), it mediates the transfer of cholesterol from the ER to the TGN and of PI4P in the opposite direction (Fig. 1.13). Consequently, PI4P is delivered from the TGN to the ER membranes where it is dephosphorylated in cis by the phosphatase Sac1 (Mesmin et al., 2013).

PI4P/sterol metabolism is regulated at the ER-Golgi interface via a similar pathway in yeast, where the PI4P effector Osh4/Kes1 exchanges PI4P with ergosterol and delivers it to the ER for Sac1-mediated dephosphorylation (von Filseck et al., 2015b);
Importantly, Osh4/Kes1 depletion had been previously shown to increase the levels of PI4P at the Golgi (Fairn et al., 2007). Together, these results demonstrate that PI4P consumption mediated by Osh4/Kes1 at ER-Golgi MCSs is important to control Golgi PI4P amount.

Figure 1.13 Regulation of PI4P homeostasis by lipid exchange at ER-PM and ER-TGN MCSs. Sterol and PS are synthesized in the ER. A) Osh4p and Osh6/Osh7p transport ergosterol or PS from the ER to the trans-Golgi or the PM, respectively, and PI4P in the backward direction. B) A similar regulation occurs in mammals, OSBP1 and ORP5/ORP8 transfer cholesterol or PS from the ER to the TGN or the PM, respectively, in exchange for PI4P. PI-4 kinases ensure PI4P production. At the ER, Sac1 hydrolyses PI4P to PI. Maintenance of the PI4P gradient would allow the vectorial transport of sterols or PS, thereby generating a gradient for these lipids. Adapted from Drin, von Filseck and Čopič, 2016.
Another mechanism of substrate delivery that can regulate PI4P homeostasis at membrane contact sites regards the PI4P precursor PI. Upon surface receptor stimulation, PI(4,5)P2 on the PM is cleaved by phospholipase C (PLC) to produce diacylglycerol (DAG) and inositol 3,4,5 triphosphate (IP3), which stimulates the release of intracellular calcium and, subsequently, the formation of ER-PM membrane contact sites (Giordano et al., 2013). Under Ca\textsuperscript{2+} stimulation, the ER-localized PI transfer protein Nir2 is recruited to ER-PM contact sites and delivers PI to the PM, where it is used as a substrate for the production of PI4P, needed to replenish the PI(4,5)P2 pool (Chang et al., 2013). A subsequent work from the Balla group established that, at ER-PM MCSs, Nir2 acts as a bi-directional exchanger of PI and phosphatidic acid (PtdOH) (Kim et al., 2015). In particular, Nir2 couples the transfer of PI in the PM with the delivery of PtdOH (generated from DAG) to the ER, where it is needed for PI re-synthesis (Kim et al., 2015).

1.5.3.4.3 Regulation of PI4P metabolizing enzyme function (sensors)

Recent work has suggested that there are some PI4P effectors that not only bind PI4P but are also able to modulate its levels by recruiting and stimulating PI4P metabolizing enzymes activity towards a specific PI4P pool. These proteins have been defined “PI4P sensors”, and their depletion causes an increase in PI4P levels.

The best characterized example of a PI4P sensor in yeast have been provided by Stefan et al., who demonstrated that the oxysterol-binding homology (Osh) protein Osh3 stimulates the ER-localized Sac1 to dephosphorylate the PI4P pool in the PM in trans at the level of ER-PM MCSs (Stefan et al., 2011; Fig. 1.14). Sac1 function in this context also requires the contact site tethers Scs2/Scs22, homologues of VAPs protein. As a matter of fact, the depletion of Osh3 or Scs2/Scs22 caused a marked accumulation of
PI4P *in vivo* and the stimulation of Sac1 *in vitro* activity (Stefan *et al.*, 2011). Osh3, which simultaneously binds the ER-localized VAPS (via the FFAT domain) and the PM PI4P pool (via the PH domain) acts as a coincident detector of PI4P, VAPs and Sac1, and behaves as a PI4P sensor, which, in response to increased PI4P levels, induces its consumption by binding and stimulating its phosphatase Sac1 (Stefan *et al.*, 2011; Fig. 1.14).

![Figure 1.14](image)

**Figure 1.14 Regulation of PI4P homeostasis via stimulation of Sac1 activity *in trans* at ER-PM MCSs.** Osh3 acts as a PI4P sensor at the level of ER-PM MCSs. High PM PI4P levels recruit and activate Osh3 at ER/PM contact sites. Interactions between Osh3 and the VAP proteins Scs2/Sc22 activate the ER-localized Sac1, which dephosphorylates its substrate *in trans* at the PM. Adapted from Stefan *et al.*, 2011.

The above-mentioned ORP5 and ORP8 can also be considered as PI4P sensors in mammals, although acting through a different mechanism. ORP5 and ORP8, by delivering PI4P to the ER for degradation by Sac1, regulate PI4P levels on the PM, as
confirmed by the evidence that ORP5/8 depletion is responsible for increased PI4P amounts in this organelle.

Regarding the Golgi PI4P pool, a sensor in yeast can be identified in Osh4/Kes1 that exchanges PI4P for cholesterol at MCSs (von Filseck et al., 2015b). Indeed, Osh4/Kes1 depletion had been previously shown to increase the levels of PI4P at the Golgi (Fairn et al., 2007).

In mammals, while it is clear that OSBP overexpression promotes ER-TGN membrane tethering and subsequent PI4P consumption (Mesmin 2013), the effects of OSBP1 depletion on the global Golgi PI4P levels has been debated. A subsequent work by Antonny’s group showed that PI4P levels at the Golgi increase in the absence of OSBP1 (Mesmin et al., 2017), but this is in contrast with a previous report by Goto et al. according to whom OSBP1 depletion does not alter the total Golgi PI4P amount (Goto, Charman and Ridgway, 2016).

*Other PI4P sensors*

In yeast, another P4P sensor has been identified in Vps74, a GOLPH3 ortholog. Vps74 forms a complex with Sac1 in the early/medial Golgi and stimulates its activity to selectively dephosphorylate PI4P in these compartments (Wood et al., 2012). As a matter of fact, a ΔVps74 exhibits an accumulation of PI4P in the medial- but not in the trans-Golgi. Since the PI4-kinase Pik1 produces PI4P throughout the Golgi, while Vps74-mediated PI4P dephosphorylation by Sac1 occurs only in the early compartments, the authors propose that this mechanism determines the steady state restriction of PI4P at the trans Golgi cisternae (Wood et al., 2012). The mammalian ortholog GOLPH3, while preserving some functions of its yeast ortholog such as the
regulation of Golgi enzyme distribution (Ali et al., 2012; Eckert et al., 2014), has never been demonstrated to bind Sac1, nor to control PI4P levels. Thus, the formal proof that PI4P levels at the Golgi complex can be controlled by an obvious sensor in mammals is still missing.

1.6 The role of PI4P at the Golgi complex

1.6.1 Membrane trafficking

PI4P coordinates a large variety of functions by binding and recruiting its effectors at the Golgi complex, namely proteins that contain PI4P-binding modules. These modules may be either PH-domains (such as in FAPPs, CERT, OSBP), ENTH/ANTH domains (such as in epsinR) or simply exposed patches or pockets containing basic residues that can interact with the phosphates of PI4P and aromatic residues (usually a tryptophan) that can pack against the inositol ring (as in Vps74/Golph3, GGAs, Drs2p and others Fig. 1.15). Many of PI4P effectors are membrane trafficking genes, indicating that PI4P plays a pivotal role in this process (Fig. 1.15). Indeed, virtually all the steps of membrane trafficking are influenced by PI4P modulation.
Figure 1.15 PI4P effectors at the Golgi complex. Most of the effectors recruited and activated by PI4P-binding at the Golgi complex are involved in membrane trafficking. PI4P effectors that, both in yeast and mammals, have been reported to regulate Golgi-to-PM or Golgi-to-endosome trafficking are indicated with red or green circle, respectively. Modified from D’Angelo et al., 2008.

1.6.1.1 Golgi to Plasma membrane trafficking

The importance of PI4P in Golgi-to-PM trafficking is clearly demonstrated by the work of Szentpetery et al., in which the selective, acute depletion of the Golgi PI4P pool by recruiting Sac1 at the TGN with a rapamycin-inducible system virtually abolished the trafficking of cargoes to the PM (Szentpetery, Várnaí and Balla, 2010). This depends on
PI4P recruitment of a number of effectors to the Golgi that are involved in different steps of post-Golgi carrier formation, such as membrane bending/tubulation, membrane fission and translocation of carriers via cytoskeletal motors.

One such effector is the lipid transfer protein FAPP2, which localizes at the TGN via coincident detection of PI4P and ARF1 and, if depleted, impairs post-Golgi trafficking to PM both in polarized and non-polarized cells (Godi et al., 2004, Vieira et al., 2005). The mechanism by which FAPP2 regulates carrier biogenesis has not been completely clarified, but the most plausible mechanism may involve the ability of its PH domain to insert into membranes and induce membrane tubulation both in vitro and in vivo (Lenoir et al., 2010, Godi et al., 2004), even though a role in creating specific microdomains at the TGN that induces cargo sorting has also been envisaged (D’Angelo, Rega and De Matteis, 2012).

The next step in cargo transport, namely linking of the cargoes to actomyosin, is carried out by the PI4P effector GOLPH3, which simultaneously binds PI4P at the TGN and the actin cytoskeleton via the unconventional MYO18A and creates a mechanical force that induces post-Golgi carrier formation (Dippold et al., 2009). GOLPH3 depletion, indeed, impairs VSV-G arrival to the PM, while GOLPH3 overexpression drives increased Golgi-to-PM trafficking (Dippold et al., 2009, Xing et al., 2016).

Finally, the last step in post-Golgi carrier formation, membrane fission, also depends on PI4P. PI4KIIIβ itself has been demonstrated to form a complex with 14-3-3 proteins and the fission-controlling protein BARS at the TGN in a process that mediates post-Golgi carrier formation (Valente et al., 2012). Moreover, protein kinases of the PKD family (PKDs), that are critically involved in the fission of transport carriers en route to the cell surface (Liljedahl et al., 2001), phosphorylate and activate both PI4KIIIβ, thus inducing PI4P production, and the PI4P effector CERT (Fugmann et al., 2007). CERT is a
lipid transfer protein that transfers ceramide from the ER to the TGN where it is converted to sphingomyelin and diacylglycerol (DAG), which is a prerequisite for PKD activation (Hanada et al., 2003). Thus, CERT activates PKD at the TGN and in turn regulates post-Golgi trafficking, that, as a consequence, is inhibited in CERT-silenced cells (Fugmann et al., 2007). Hence, the fission of post-Golgi plasma membrane-directed carriers relies on a tightly controlled machinery containing PKDs, PI4P-producing enzymes and PI4P effectors such as CERT.

1.6.1.2 Golgi to endosomes trafficking

A very well established role of PI4P at the Golgi is the recruitment of clathrin-coated adaptors, namely AP-1 (Wang et al., 2003), GGAs (Wang et al., 2007) and Epsin-R (Mills et al., 2003), which regulate Golgi-to-endosome trafficking. The pool of PI4P involved in these processes is mainly produced by PI4KIIα, whose silencing, indeed, impairs clathrin adaptor recruitment and Golgi-to-endosome trafficking (Wang et al., 2003). Moreover, PI4P promotes the recognition of ubiquitin-sorting signals by mammalian and yeast GGAs (Wang et al.2007) and thus is involved in ubiquitin-dependent sorting at the TGN (see par. 1.3.3.1 for details). In yeast, another PI4P effector involved in these processes is the flippase Drs2, whose localization and activity depend on PI4P binding (Natarajan et al., 2009). Drs2 depletion impairs TGN-to-endosome trafficking, by translocating aminophospholipids to the cytosolic leaflet of the Golgi, thus inducing the membrane curvature necessary for vesicle budding (Natarajan et al., 2009).

1.6.2 Non vesicular trafficking of lipids

PI4P production is strongly linked to other classes of lipids, in particular sphingolipids (SL) and cholesterol. Studies in yeast, indeed, demonstrate that Sac1 is involved in
sphingolipid metabolism (see par. 1.4.1). In mammals, the role of PI4P in this pathway is mainly mediated by the ability of PI4P to recruit a class of effectors called lipid transfer proteins (LTPs) that includes CERT, FAPP2 and OSPB1 (Fig. 1.16). These proteins have the peculiar characteristic of simultaneously binding the ER (via the VAP-binding FFAT domain) and the TGN (via the PI4P-binding PH domain) and promote non-vesicular trafficking of lipids (Fig. 1.16). CERT binds ceramide via its START domain and transfers it from the ER to the TGN where ceramide is converted into sphingomyelin (SM) (Hanada et al., 2003). The activity of CERT is controlled by a phosphorylation-dephosphorylation cycle: the phosphorylation of a serine-repeat motif has been proposed to favor the interaction between the PH and START domains and thus to inhibit both the PI4P-binding and ceramide-transport by CERT, while the dephosphorylation promotes both activities of CERT (Kumagai et al., 2007; Saito et al., 2008).

FAPP2 binds GlcCer through its GLTP homology domain and transfers it from the cis-Golgi to the TGN, where GlcCer is used as a building block for glycosphingolipid (GL) synthesis (D’Angelo et al., 2007). In particular, the GlcCer delivered to the TGN by FAPP2-mediated non-vesicular trafficking is required for the selective production, in loco, of the Globo series of GLs (i.e. GB3), in contrast to the Ganglio series of GLs, which is instead produced in the Golgi cisternae and reaches the TGN via canonical vesicular trafficking (D’Angelo et al., 2013).

OSBP1 is able to bind cholesterol and mediates its trafficking from the ER to the TGN in exchange for PI4P (see par. 1.5.2.2.1). Moreover, OSBP has been shown to mediate the sterol-dependent recruitment of CERT to the Golgi apparatus and thus to increase SM synthesis (Perry et al., 2006), most likely by stimulating the activity of PI4KIIα, which is activated by cholesterol.
1.6.3 Pathogens infection

PI4P is a target of pathogen invasion since the PI4P production system is hijacked by different viruses to create a microenvironment that is permissive for their replication and secretion. Positive-sense RNA viruses, including hepatitis C virus (HCV), coxsackie virus, and poliovirus, induce the formation of PI4P-enriched “membranous webs” derived from the ER and other cellular membranes, which serve as platforms for viral replication. PI4KIIIα and PI4KIIIβ are the major targets used by viruses to divert the PI4P metabolism, although via different mechanisms: PI4KIIIα is recruited by the viral protein NS5A and is required for the integrity of the membranous web (Reiss et al., 2011); on the other hand, PI4KIIIβ is required for HCV virus secretion in a PI4P-dependent manner. Indeed, depletion of PI4KIIIβ, GOLPH3, or MYO18A leads to retention of HCV particles in the cell, as does expression of Golgi-targeted Sac1, suggesting that Golgi PI4P and its effectors are required for virus secretion (Bishé et al., 2012). Additionally, bacteria use PI4P to build their replication organelle. Upon infection, *Legionella pneumophila* establishes a replication vacuole, the Legionella-containing vacuole (LCV), and avoids fusion with lysosomes by intercepting and fusing with ER-derived vesicles, disguising the LCV with host markers (Brombacher et al., 2008). Two Legionella proteins, the Rab1 GEF SidM and its parologue SidC, are released.
into the host cytoplasm upon infection and bind PI4KIIIβ-produced PI4P on the LCV (Brombacher et al., 2008; Ragaz et al., 2008). In this way they are able to misdirect ER vesicles en route to the Golgi by binding to Rab1 and calnexin, respectively (Brombacher et al., 2008; Ragaz et al., 2008).

1.6.4 Cancer progression

PI4P is an essential substrate for the generation of two phosphoinositide species, (PI4,5)P2 and PI(3,4,5)P3, that are intimately linked with cell proliferation and migration. Thus, modulation of PI4-kinases could have a role in cancer progression by simultaneously impacting on both the downstream PLC and PI3-kinase pathways, driven by (PI4,5)P2 and PI(3,4,5)P3, respectively. Indeed, PI4KIIIα has been shown to be up-regulated in pancreatic cancer and this correlates with a metastatic phenotype (Ishikawa et al., 2003). Moreover, PI4KIIIα was identified in an RNAi screen as a mediator of resistance to cisplatin in medulloblastoma cell lines (Guerreiro et al., 2011). PI4KIIα has also been found to be upregulated in several human cancers, namely malignant melanoma, fibrosarcoma, breast cancer, bladder transitional cell carcinoma and thyroid papillary carcinoma (Li et al., 2010). In this work Li and colleagues showed that PI4KIIα overexpression leads to an alteration of the HERB2/PI3K signaling pathway ultimately resulting in an increase in HIF1 activation and angiogenesis (Li et al., 2010). PI4KIIα have been also shown to be activated in response to different signaling pathways involved in cell proliferation, such as EGF-induced Akt activation or Wnt (Chu et al., 2010; Pan et al., 2008), although the mechanisms by which these cascade promote the 4-kinase activity has not been completely elucidated.
However, in the above-mentioned examples, is very difficult to identify a role of the Golgi pool of PI4P in cancer progression that is independent on its action as a precursor for other phosphoinositides. A possible involvement of the Golgi PI4P in cancer can be inferred by the observation that PI4KIIIβ, that almost exclusively produces PI4P at the Golgi, has been found to be upregulated in breast cancer (De Matteis, Wilson and D’Angelo, 2013). The formal proof of a direct role of the Golgi PI4P pool in cancer comes from the work by Tokuda et. al, in which it was shown that Sac1 depletion and subsequent PI4P accumulation at the Golgi is responsible for decreased cell/cell adhesion and induction of cell migration. Conversely, depletion of PI4KIIIβ had an opposite effect, reducing migration and invasion (Tokuda et al., 2014). According to this work, the increase of PI4P at the Golgi complex drives tumorigenesis by inducing the recruitment and activation of the oncogene GOLPH3.

1.6.4.1 GOLPH3

GOLPH3 is a PI4P effector which localizes at the Golgi via a stretch of positively charged amino acids present in a hydrophobic pocket of the protein that mediate its binding to PI4P (Dippold et al., 2009). GOLPH3 is the first oncogene that was found to be localized on the Golgi and a large number of reports in the last years have shown that GOLPH3 is upregulated in a variety of solid tumors including melanoma, lung cancer, breast cancer, glioma, esophageal squamous cell carcinoma, colorectal cancer, prostate cancer, renal cell carcinoma, oral tongue cancer, rhabdomyosarcoma, gastric cancer, hepatocellular carcinoma, epithelial ovarian carcinoma and pancreatic ductal adenocarcinoma, and its expression levels correlate with poor prognosis (Sechi et al., 2015). GOLPH3 is localized on chromosome 5 (5p13), which is frequently amplified in several solid tumors. The overexpression of GOLPH3 significantly enhanced
xenograft growth of human melanoma (WM239A) and non-small cells lung carcinoma (A549) cell lines, thus allowing it to be classified as a bona fide oncogene (Scott et al., 2009). The mechanisms of GOLPH3 driven oncogenesis are not unequivocal.

GOLPH3 oncogenic activity has been primarily linked to the hyper-activation of the mTOR/Akt pathway (Scott et al., 2009). Scott and colleagues, who first observed this effect, hypothesized that, since a pool of GOLPH3 can localize on endosomes, it may regulate the recycling of receptors upstream of mTOR signaling, but the exact mechanism by which GOLPH3 regulates this cascade has not been elucidated. A work in glioblastoma cell lines identified a role for GOLPH3 in the recycling of the EGF receptor with subsequent hyper-activation of the downstream Akt pathway (Zhou et al., 2017).

However, several studies demonstrated that Golgi localization and functions are required for GOLPH3 to drive malignant transformation. Indeed, in the above-mentioned work by Tokuda et al., it was shown that cell migration and invasion, both in vitro and in vivo, are stimulated by the overexpression of WT-GOLPH3, but not by a GOLPH3 mutant unable to bind PI4P and thus localizing at the Golgi (Tokuda et al., 2014).

GOLPH3 is a strong regulator of Golgi morphology because it simultaneously binds PI4P and MYO18A to induce a tensile force that keeps the Golgi in its flattened conformation (Dippold et al., 2009; Fig.1.17). As a consequence, GOLPH3 overexpression induces Golgi fragmentation, while its depletion is responsible for Golgi collapse (Dippold et al., 2009, Ng et al., 2013).
Figure 1.17 GOLPH3 preserves Golgi architecture. Golph3 (green), by simultaneously binding PI4P (blue) and MYO18A (orange) links the Golgi complex with the F-actin cytoskeleton, thus exerting a tensile force required to maintain the Golgi in its flattened morphology. Adapted from Dippold et al., 2009.

It has not been demonstrated if the regulation of Golgi fragmentation is important for GOLPH3-mediated oncogenesis. The only connection between the role of GOLPH3 in oncogenesis and Golgi morphology organization so far comes from the work by Faber-Katz et al. in which it was shown that GOLPH3 is phosphorylated by DNA-PK after DNA damage and this induces GOLPH3 hyper-activation and subsequent Golgi fragmentation that is required for cell survival to DNA-damage (Farber-Katz et al., 2014).

Another cellular role of GOLPH3, conserved from yeast to mammals, is to mediate the intra-Golgi distribution of a subset of Golgi glycosyl-transferases by simultaneously binding the adaptor COP-I and the cytosolic tails of these enzymes (Tu et al., 2008; Ali et al., 2012; Eckert et al., 2014). GOLPH3-mediated regulation of glycosylation, in particular sialylation, seems to be important in cancer, as the overexpression of α2,6-
sialyltransferase (ST6Gal1), one of the enzymes mislocalized under GOLPH3 KD, was sufficient alone to restore cell migration in GOLPH3-KD cells (Isaji et al., 2014).

Finally, GOLPH3 has a role in regulating anterograde membrane trafficking, that is indeed inhibited by GOLPH3 depletion and stimulated under GOLPH3 overexpression (Dippold et al., 2009; Xing et al., 2016). This depends on PI4P, since the perturbation of PI4P levels at the Golgi also has the same effect on membrane trafficking; as a matter of fact, cellular secretion is stimulated both by PI4KIIIß overexpression (Hausser et al., 2005) and by Sac1 down-regulation (Blagoveshchenskaya et al., 2008). Along these lines, GOLPH3 has been shown to induce the reorientation of the Golgi in a cultured cell monolayer after wound scratching, and this has been shown to promote directional trafficking towards the leading edge, which is functionally important for directional cell migration (Xing et al., 2016). Indeed, GOLPH3-overexpressing cells show enhanced trafficking toward the leading edge and migrate faster than control cells, while GOLPH3 silencing produces the opposite effect (Xing et al., 2016).

Recently, it has been shown that GOLPH3 contributes to the metastatic potential of a newly identified PI4P-binding oncogene named PITPNC1 (Halberg et al., 2016). PITPNC1 not only localizes at the Golgi via binding PI4P, but it is also able to regulate PI4P levels, even though the mechanism by which this occurs has yet to be clarified (Halberg et al., 2016). PITP1C overexpression, indeed, increases PI4P levels at the Golgi, thus inducing a hyper-activation of the PI4P-GOLPH3-MYO18A pathway that, in addition to inducing Golgi fragmentation, stimulates the secretion of pro-angiogenic and pro-metastatic factors (Halberg et al., 2016).

Thus, although the picture of GOLPH3-driven oncogenesis is heterogeneous, it is clear that the perturbation of each element of the PI4P-GOLPH3-MYO18A axis leads to tumor transformation, mainly by promoting aberrant secretion (Fig. 1.18).
Figure 1.18 Many elements of the GOLPH3 pathway are cancer drivers. PITP1B provides the substrate for PI4-kinase-mediated PI4P production at the Golgi complex. PI4P recruits and activates GOLPH3, which, by simultaneously binding MYO18A, exerts a tensile force required to drive the formation of post-Golgi carriers and, in turn, the secretion of pro-migration and pro-invasion factors. The alteration of each element of this pathway drives cancer progression. Adapted from Buschman and Field, 2018.

1.6.5 Autophagy

In recent years, a role for PI4P in autophagy has been envisaged. In yeast, the PI4P effector Osh4/Kes1, which is also able to control PI4P levels at the Golgi, has been shown to regulate autophagy, since the inactivation of Atg genes is able to rescue the Kes1 mutant-induced intracellular membrane accumulation phenotype (LeBlanc and McMaster, 2010). Moreover, Wang et al. demonstrated that the Golgi PI4P pool is required for autophagy, since a temperature sensitive mutant of the Golgi localized PI4-kinase Pik1 shows defective autophagy induction under starvation conditions. In particular, Pik1 regulates the anterograde movement of Atg9, the only Atg transmembrane protein conserved from yeast to mammals, that relocalizes from the
Golgi (where it normally resides) to the pre-autophagosomal structure (PAS), under starvation, to mediate autophagosome biogenesis (Wang et al., 2012). The Pik1\textsuperscript{ts} mutant shows defective relocalization of Atg9 on the PAS under starvation, with a subsequent block of autophagy induction (Wang et al., 2012).

In mammals, PI4KII\textalpha has been shown to be recruited onto autophagosomes via binding to GABARAP and generates a pool of PI4P in loco that is required for efficient autophagosome-lysosome fusion (Wang et al., 2015). PI4P has also been found on auto-lysosomes, where it serves as a substrate for PIP5K1B to produce Pl(4,5)P2 that, together with clathrin, starts the membrane tubulation that drives lysosome reformation after autophagy induction (Rong et al., 2012). However, so far, a clear role for the pool of PI4P at the Golgi in autophagy induction has not been described.

1.7 FAPP1: a PI4P effector with an unknown function

Among the above-mentioned PI4P effectors, the phosphatidylinositol four phosphate adaptor protein 1 (FAPP1) represents a “mysterious” PI4P binding protein. It was identified almost 30 years ago in a screen for PH-containing proteins, and named FAPP1 for the ability of its PH domain to selectively bind PI4P relative to other phosphoinositide species (Dowler et al., 2000). FAPP1 is expressed from zebra fish (Danio rerio) to higher organisms. Similarly to other PI4P effectors, FAPP1 localizes at the Golgi by virtue of coincident binding to PI4P and the small GTPase ARF1, restricting its localization to the TGN and avoiding its binding to other PI4P-containing membranes in the cell (Godi et al., 2004). FAPP1 is 80% identical and 90% similar with its “sister” FAPP2 at the N-terminus (containing the PH domain) and both proteins
have a putative FFAT domain. However, FAPP1 and FAPP2 differ in the C-terminal domain, with FAPP2 possessing a GLTP domain needed to bind and promote non-vesicular trafficking of GlcCer (see par. 1.6.2). On the other hand, FAPP1, although usually listed among the PI4P transfer proteins, does not possess any lipid transfer domain nor any other particular structural organization besides the PH domain and a proline-rich domain (PRD) (Fig. 1.19).

Figure 1.19 Schematic representation of FAPP1 and FAPP2 domain organization. FAPP1 and FAPP2 share a high degree of homology in the PH domain and they both show a putative FFAT-(red box) and a proline rich-domain (PRD). However, FAPP1 does not possess any lipid transfer motif or other particular modules at the C-terminus.

Initial studies performed in Antonella De Matteis’s laboratory showed an impairment of Golgi-to-PM trafficking under simultaneous depletion of FAPP1 and FAPP2 (Godi et al., 2004). However, when the effects of FAPP1 and FAPP2 KD were addressed separately on TGN-to-PM trafficking both in HeLa and Cos7 cells, FAPP2 was found to be the only FAPP involved in this trafficking step (Vieira et al., 2005), while the biological role of FAPP1 is still unknown.
Chapter 2

Materials and Methods

2.1 Sub-cloning and large-scale preparation of DNA

2.1.1 DNA constructs

GFP-FAPP1 has been previously produced in Antonella De Matteis lab (Godi et al., 2004). TGN-46-HA-GFP and pSPORT6-hSac1 were kindly provided by Tamas Balla (NIH, Bethesda), Venus-Cb5 by Prof. Nica Borgese (CNR Institute of Neuroscience), GFP-GOLPH3 by Alberto Luini (IBP, CNR, Naples). pIRES-neo2, pmCherry-C3, pmCherry-N1, pEGFP-N1, pEGFP-C3 and pEGFP-C1 were purchased from Clontech.

All the DNA constructs were generated by Michele Santoro (Telethon institute of genetics and medicine TIGEM, Italy).

The TGN46-FRB-H-T2A-mCherry-FKBP-Cb5-pIRES-neo2 construct was generated by Michele Santoro e Laura Rega (OBPG, Rome).

The ER-Golgi membrane contact site reporter construct TGN46-FRB-HA-GFP-T2A-mCherry-FKBP-Cb5-pIRES-neo2 was generated by sequential sub-cloning into the pIRES-neo2 vector. Briefly, the ER-membrane reporter Cb5 (17 residues of the transmembrane domain of rat cytochromeb5), cloned in-frame downstream of the mCherry-FKBP construct, was cloned into pIRES-neo2 followed by in vitro primer annealing of the T2A peptide coding sequence to produce T2A-mCherry-FKBP-Cb5-IRES-neo2 (note that this cloning procedure resulted in the removal of the AgeI
restriction site at the 3’ end, which was 16 reconstituted at the 5’ end of the T2A sequence to allow the sequential cloning of the TGN46 construct). The TGN membrane protein TGN46-FRB-HA (TGN46 fused with the FKBP12-rapamycin-binding domain FRB) was amplified from TGN46-FRB-HA-GFP and cloned into T2A-mCherry-FKBP-Cb5-pIRES-neo2.

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Table 2.1 List of the primers and cloning strategies used in this study

2.1.2 Restriction and ligation

DNA (vectors and inserts) were cut with 5U/μg of the appropriate restriction enzymes in the buffer supplied with each enzyme by New England Biolabs (USA). After restriction, the enzymes were usually inactivated by incubating them at 65°C to 75°C for 10-20 min, according to the manufacturer instructions and then loaded onto 1% agarose gels. The band of interest were cut from the gel with a sterile scalpel and the DNA was extracted with QIAquick gel extraction kit (USA), according to the manufacturer instructions. The DNA was eluted in 10mM Tris-HCl, pH 8.0. To ligate the vector and the insert, ~100 ng of the vector and a ~3 fold molar amount of the insert were incubated with 1 U of T4 DNA ligase in T4-DAN-ligase buffer (NEB, UK), for 1h at RT.

2.1.3 Transformation of bacteria by heat shock

Competent bacterial cells (DH5α) were prepared by M. Santoro (Telethon institute of genetics and medicine TIGEM, Italy). The DNA plasmid of interest (10ng of plasmid)
was added to 200μl of competent bacteria. After gentle mixing, the cells were left on ice for 15min and then heat shocked for 45s at 42°C. After the addition of 400μl of LB, the bacteria were incubated under shacking (200 rpm) at 37°C for 1h. The bacteria were plated onto LB agar plate (10 g/l NaCl, 5 g/l yeast extract, 10 g/l tryptone peptone and 15 g/l agar) containing the appropriate selective antibiotic, and incubated overnight at 37°C. The next day an isolated bacterial colony was picked and used to inoculate 2.5 mL LB containing the appropriate antibiotic. The culture was incubated overnight at 37°C.

2.1.4 Large scale preparation of plasmid DNA

A single colony of XL-1 Blue E. coli bacteria transformed with the plasmid of interest was inoculated into 500 ml LB broth plus the selective antibiotic. After 15-20 h of incubation, the acteria were harvested by centrifugation at 6,000 rpm in a JA10 rotor for 10 min at 4 °C and processed according to the manufacturer instructions (Quiagen-plasmid-kit). The DNA obtained was resuspended in 10mM Tris-HCl pH 8 and stored at -20°C.

2.2 Cell culture

HeLa cells, A549, HepG2 and human fibroblasts were bought from American tissue type collection (ATCC, USA). HeLa cells stably expressing the membrane contact sites reporter TGN46-FRB-H-T2A-mCherry-FKBP-Cb5-pIRES-neo2 construct (HeLa Clone 14) was generated by Rossella Venditti (Telethon institute of genetics and medicine TIGEM, Italy). HeLa cells were transfected with the plasmid of interest and after 48h, the neomycin analog antibiotic G418 (1mg/mL) was added. When the control, untrasfected cells died, the selected cells expressing the plasmid of interest were
seeded in 96 well plates at a density allowing single cell sorting.

HeLa WT, and A549 cells were grown in Dulbecco’s Modified minimal Essential Medium (DMEM) supplemented with 4.5 g/l glucose, 2 mM L-glutamine (Thermoscientific, UK), 1 U/ml penicillin and streptomycin (Sigma Aldrich, Germany), and 10% FCS (Euroclone, UK). HF BJ-5Ta were grown in DMEM/M199 4:1 (Thermoscientific, UK) supplemented with 4.5 g/l glucose, 0.02 mg/mL hygromycinB, 1 U/ml penicillin and streptomycin, and 10% FCS. HepG2 were grown in Modified minimal Essential Medium (MEM) supplemented + sodium pyruvate, supplemented with, 1 U/ml penicillin and streptomycin, and 10% FCS.

All cell lines were grown in a controlled atmosphere in the presence of 5% CO₂ at 37°C. Cells were grown in a flask (corning, UK) to 80-90% confluence. The medium was removed and trypsin-EDTA solution (0.05% trypsin, 0.02% EDTA purchased from Sigma Aldrich, Germany) was added for 3-5 min at 37°C. The medium was added back to block the protease action, cells were collected into a plastic tube and centrifuged for 5 min at 800 x g. The pellet was resuspended in fresh medium and placed in a new plastic flask.

2.3 Cell transfection and RNA interference

2.3.1 TransIT-LT1-reagent-based cell transfection

HeLa cells were seeded into 24-well or in a 15 cm plates at a concentration suitable to have 50%-70% confluence for transfection. The transfection mixture was prepared in a polypropylene tube: for each well, 0.3 μl TransIT-LT1 (Mirus, Germany) transfection reagent was diluted in 50 μl OptiMEM culture medium. The mixture was shaken and incubated at RT for 5 min. Then 100 ng of total DNA was added to the transfection
mixture, which was shaken and kept at RT for 15 min, to allow the DNA-TransIT-LT1 (Mirus, USA) complex to form. The cells were then incubated with the transfection mixture at 37 °C in the presence of 5% CO₂ for 16-20 h.

2.3.2 siRNA duplexes

SiRNA oligonucleotides for FAPP1, GOLPH3 and PI4KIIIβ were purchased from Dharmacon (USA). Non-targeting siRNA sequences were used as controls; the siRNAs sequences are listed in Table 2.2.

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**Table 2.2** List of siRNA used in this study
2.3.3 siRNA-duplex transfection

HeLa and A549 cells were transfected using Oligofectamine (Invitrogen, USA), HF were reverse transfected with Lipofectamine RNAiMAX (Thermo Fisher Scientific). HepG2 cells were interfered with Lipofectamine 2000 (Thermo Fisher Scientific).

HeLa or A549 cells were plated in 24-well plates to subconfluent density on glass coverslips, in antibiotic-free medium. The day after, a transfection mixture was prepared: for each well, specific siRNA duplexes were diluted in 40 μl of OptiMEM (Gibco, UK) culture medium in a polypropylene tube to the final concentration of 50 nM. In a separate polypropylene tube, for each well, 1.25 μl of Oligofectamine Reagent was diluted in 8.75 μl of the same medium. The mixtures were first shaken and incubated at RT for 5 min, then combined, shaken and kept at RT for 20 min, to allow the siRNA-Oligofectamine complex to form. The cells were incubated with the transfection mixture at 37 °C in the presence of 5% CO₂ for 72 h.

A similar protocol was adopted for HepG2 cells siRNA transfection with Lipofectamine 2000. For HF reverse transfection, siRNA duplexes were diluted in 50 μl of Optimem to reach a final concentration of 50 nM. A second mix was prepared with 1μl of Lipofectamine RNAiMAX. The mixtures were first shaken and incubated at RT for 5 min, then combined and spotted into the 24 well plates. After 15’ of incubation, 50000 cells were added to each well and incubated with the transfection mixture at 37 °C in the presence of 5% CO₂ for 72 h.

SDS-PAGE, Western blotting and densitometry analyses were carried out to evaluate the efficiency of siRNA treatment for each experiment.
2.4 Cell treatments

Rapamycin, nocodazole PIK93, BrefeldinA (BFA), Bafilomycin (Baf-A1), Torin and cycloheximide were purchased from Sigma Aldrich, Germany. Digitonin was from Calbiochem, HBSS was from Termoscientific (UK).

For rapamycin-induced heterodimerisation, HeLa cells stably expressing TGN46-FRB-HA-T2A-mCherry-FKBP-Cb5-pIRES-neo2 were pre-treated with 50 μg/ml cycloheximide in serum-free medium for 30 min and then treated with 200 nM rapamycin at 37°C for 2, 4 or 15 min.

Nocodazole was added in serum-free medium at final concentration of 33 μM. Cells were incubated 5 min at 4°C and then shifted at 37°C for 3 hrs.

For PIK93 treatment to reduce the Golgi pool of PI4P, cells were treated with 250nM PIK93 at 37°C for 30 min. PIK93 during wound healing experiments was given to a final concentration of 50nM from the wound scratching until the end of the experiments (24 h).

Brefeldin A acute treatment to detach FAPP1 from the Golgi without altering Golgi structure was performed with 2,5 μg/ml BFA at 37°C for 3 min.

Bafilomycin-A1 was added in complete medium at a final concentration of 100nM. Cells were incubated at 37°C for 3 h.

To induce autophagy, cells were treated with 1μM Torin at 37°C for 2h. Alternatively, cells were washed 4 times with HBSS to remove serum and starved with HBSS for 2 h at 37°C.
2.5 Immunofluorescence Confocal Microscopy

2.5.1 Immunofluorescence procedures

HeLa, HeLa clone 14 and HF cells were grown to subconfluent density on glass coverslips and treated as follows. They were fixed with 4% paraformaldehyde for 10 min at RT (or 100% methanol for 5 min at -20°C for LC3 staining) and washed twice in PBS. Blocking buffer (0.05% saponin, 0.5% BSA, 50 mM NH4Cl in PBS) was then added to the cells for 20 min, followed by a 1-h incubation with the primary antibody in blocking reagent (see Table 2.1 for the dilutions used). When required, cells were permeabilised with 0.01% Triton-X 100 in PBS. The cells were then extensively washed with PBS and incubated with secondary antibodies (1:400) for 45 min, diluted in blocking solution. After immuno-staining, the cells were washed three times in PBS and once in sterile water, to remove salts. The coverslips were then mounted on glass microscope slides (Carlo Erba, Italy) using Mowiol.

2.5.2 Staining of Golgi PI4P pool

The staining of PI4P was performed as described by Hammond et al. (2009) with some modifications. Briefly, cells in 300 µl medium were fixed by the addition of 300 µl of paraformaldehyde 4% to a final concentration of 2%, and incubated for 15 min at RT. All steps were performed at room temperature. After removing of paraformaldehyde with three rinses in PBS containing 50 mM NH4Cl, the cells were permeabilised for 5 min by the addition of 20 µM digitonin in Buffer A (20 mM Pipes pH 6.8, 137 mM NaCl, 2.7 mM KCl) for 5 min. Digitonin was removed by three rinses in Buffer A, and cells were blocked for 45 min with buffer A supplemented with 5% (v/v) FBS and 50 mM
NH4Cl. Primary and secondary antibodies were applied in blocking buffer for 1h and 45 min, respectively. Cells underwent post-fixation for 5 min in 2% paraformaldehyde, washed with PBS containing 50 mM NH4Cl, washed once with water and then mounted with mowiol.

The antibodies used in this studies are listed in table 2.3.

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<th>Antibody</th>
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<th>Animal source</th>
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</tr>
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</table>

**Table 2.3** List of antibodies used for immunofluorescence in this study
2.5.3 Immunofluorescence analysis by laser scanning confocal microscopy

IF samples were examined under a confocal laser microscope (Zeiss LSM710, LSM700 and Leica SP5 confocal microscope systems) equipped with 63x1.4 NA oil objective. Optical confocal sections were taken at 1 Airy unit with a resolution of 1024×1024 pixels. Images were composed using Microsoft Office Power Point.

2.5.4 Immunofluorescence analysis by super resolution microscopy

IF samples were examined using LSM880 equipped with 63x1.4 NA oil objective and images exported as TIFF and composed using power point.

2.5.5 Image processing and quantification of signal on fixed cells

For quantification experiments, 10-15 randomly chosen fields each containing 8-10 cells were scanned with the same microscope settings (i.e. laser power and detector amplification) below pixel-saturation. The mean intensity per cell was determined using the ImageJ software. For the quantification of Golgi PI4P stained with the anti-PI4P Ab, a mask using the TGN marker Golgin97 was generated for each cell, and the mean intensity of both PI4P and Golgin97 were measured in those regions. After background subtraction, the PI4P values were normalized singularly using their own Golgin97 values. All experiments were repeated at least three times.

2.6 Transport assays

2.6.1 VSV-G transport assay

HeLa cells were infected with VSV-G 1 hr at 32°C in serum free DMEM buffered with HEPES. Cells were then washed three times in DMEM and incubated at 40°C for 3 h in
growth medium supplemented with HEPES. Subsequently, cells were incubated at 20°C for additional 2 h in order to accumulate VSV-G at the TGN in presence of cycloheximide (50 µg/ml). Finally, cells were shifted at 32°C in presence of cycloheximide (50 µg/ml) for the indicated times.

2.6.2 PC-I transport assay

To follow PC-I in Human Fibroblasts, cells were incubated for 3 hrs at 40°C in DMEM supplemented with 1% serum and 20mM HEPES pH 7.2 (Euroclone, UK), then shifted to 32°C in the presence of cycloheximide (100 µg/ml) and ascorbate (50 µg/ml) for the indicated times.

2.6.3 Shiga toxin internalization

Cells were equilibrated in 0.1% BSA HEPES-buffered DMEM for 5 min at 4°C and incubated with 14 µg/mL shiga toxin-Alexa-Fluor-568 in 0.1% BSA HEPES-buffered DMEM for 30 min. Cells were then washed three times in cold PBS and incubated in growth medium at 37°C for 30, 60, 120 and 240 minutes.

2.7 SDS-PAGE and Western blot analysis

To assembly polyacrylamide gels, two 16 x 15 cm glass plates were assembled to form a chamber using two 1.5 mm plastic spacers lined on the lateral edges of the plates. This chamber was fixed using two clamps and mounted on a plastic base that sealed the bottom (Hoefer Scientific instruments, Germany). The polyacrylamide gel was then prepared using one solution of the desired polyacrylamide concentration.

Samples were prepared by adding sample buffer (0.125 M Trizma base, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol, pH 6.8) and boiling for 5 min at 95 °C
before loading onto the gel. The end wells were loaded with 3 μl of pre-stained molecular weight standards (Sigma, USA). The chamber was then assembled into the electrophoresis apparatus (Hoefer Scientific Instruments, Germany) and electrophoresis was carried out under a constant current of 8 mA (for overnight runs) or 30-40 mA (for 4 h runs).

To perform Western blot analysis the polyacrylamide gel was soaked for 15 min in transfer buffer, placed on a sheet of 3 MM Whatman paper and covered by a nitrocellulose filter (Schleicher & Schuell, USA). The filter was covered by a second sheet of 3 MM paper to form a "sandwich" that was subsequently assembled into the blotting apparatus (Hoefer Scientific Instruments, Germany). Protein transfer occurred at 400 mA for 4-5 h. At the end of the run, the “sandwich” was disassembled and the nitrocellulose filter was soaked in 0.2% (w/v) red Ponceau in 5% (v/v) acetic acid for 5 min to visualise the protein bands and then rinsed with 5% (v/v) acetic acid to remove the excess of the unbound dye. Nitrocellulose filters were then cut with a razor blade into strips. Strips containing the proteins of interest were incubated for 30 min at RT in blocking buffer (TTBS (0.05% (w/v) Tween 20, 150 mM NaCl, 20mM Tris-HCl pH 7.5) with 1% (w/v) BSA). The strips were subsequently incubated with fresh blocking buffer containing the primary antibody at its working concentration (see Table 2.4 for the antibody dilution used).

After 2-3 h of incubation at RT, or overnight at 4 °C, the primary antibodies were removed and the strips washed 3 times with TTBS (10 min each wash). Strips were incubated for 1 h with the appropriate HRP-conjugated secondary antibody (diluted 1:10,000 for ECL development) and washed 3 times (10 min each wash) with TTBS and one time with PBS. After washing, strips were incubated with the developing solution:
strips were incubated with the ECL (Euroclone, UK) developing solution for 1 min at RT and visualised by chemiluminescence.

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<th>Company or other source</th>
<th>Animal source</th>
<th>Dilution</th>
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Table 2.4 List of antibodies used for Western blot in this study

2.8 Preparation of total cell lysates and immunoprecipitation

2.8.1 Total cell lysates

Total cell lysates were obtained by scraping cells into Lysis Buffer (20mM Tris pH 7.5, 150 mM NaCl, 1mM EDTAm 0,1 % Triton) with protease and phosphatase inhibitors cocktail (Roche). The suspension was centrifuged at 14000 rpm for 10 min at 4°C. The supernatant obtained from the centrifugation was recovered. Lysates from mouse livers were prepared as follows: Livers put in Lysis buffer (25mM TRIS-HcL pH 7.4,
150mM NaCl, 1mM EDTA, 1% NP40) supplemented with protease and phosphatase inhibitors cocktail (Roche) were homogenized with a 5mm bead in the Tissue lyser for 2 min at 50Hz. Samples were centrifuged at 13000 rpm at 4°C, the supernatant was collected and underwent a second centrifugation. The supernatant was recovered and analysed by SDS PAGE.

2.8.2 Immunoprecipitation for mass spectrometry analysis

All the following steps were performed on ice or at 4°C, using ice-cold solutions. 15*10⁶ HeLa cells were seeded in 15 cm dishes. Transient transfection of GFP-FAPP1 or empty GFP vector as a control was performed using 30 μg and 90 μL of TransIT-LT1 DNA as indicated in session 2.3.1. After 16 hrs of transfection, cells were washed with cold PBS and lysed with 1 mL of lysis buffer (25mM TRIS pH 7.4, 100 mM NaCl, 1 mM EDTA, 0.5% lauryl maltoside, supplemented with protease and phosphatase inhibitors cocktail (Roche)). Cells were harvested 15 minutes at 14,000 rpm at 4°C. The clarified lysates were incubated with anti-GFP (Consorzio Mario Negri Sud; 1μg ab/ 500μg lysate) overnight at 4°C. Immune-complexes were collected by incubation for 1 h at 4°C with protein A Sepharose (Sigma- Aldrich, Germany), previously equilibrated with lysis buffer. The immunoprecipitates were washed for 6 times in lysis buffer and 2 times in the same buffer without detergent by 2 min centrifugation at 500g. Proteins were eluted adding 2X sample buffer (SB) and denaturating at 95°C for 5 min. Eluates were sent to the Central Proteomics facility (South Parks Rd., Oxford) for mass spectrometry analysis.
2.8.3 Co-immunoprecipitation analysis

All the following steps were performed on ice or at 4°C, using ice-cold solutions. Total lysates (0.5-1.0 mg) from HeLa cells or mouse livers were prepared as in 2.8.1 and incubated with anti FAPP1 and anti SAC1 (1μg ab/ 500μg lysate) overnight at 4°C. Immune-complexes were collected and eluted as described in 2.8.2. Input, flow through and immunoprecipitate (eluate) were analysed by SDS PAGE (10 or 12 %) and Western blotting with anti VAP-A, anti VAP-B, anti-FAPP1 and anti-SAC1 antibodies.

2.9 Cytosol-membrane fractionation

Cytosol-membrane fractionation was performed as previously described (Tenorio et al., 2016). All the following steps were performed on ice or at 4°C, using ice-cold solutions. Total lysates from HeLa cells Mock or FAPP1 KD were obtained by scraping cells into homogenization buffer (0.1 M KH2PO4, 0.1 M K2HPO4, 5 mM MgCl2, 0.25 M sucrose, pH 7.4) supplemented with protease and phosphatase inhibitors cocktail (Roche).

Homogenization was done by passing the suspension of scraped cells 30 times through a ball-bearing cell-cracker and the homogenate was spun at 1,000 x g for 10 min. The post-nuclear supernatant (700μl) was ultra-centrifuged at 100,000 x g for 30 min. The supernatant, designated as cytosol (C), was collected and analysed immediately. The pellet was washed once with homogenization buffer and centrifuged as before. The second supernatant was discarded and the pellet (P) was resuspended in 300μl of homogenization buffer, and analysed immediately. After protein concentration estimation, equal amounts of each fraction was analysed by SDS PAGE (10%) and
western blotting with antibodies anti-GOLPH3, anti-VAP-A (used as a control of transmembrane proteins) and anti-βTubulin (used as a control of cytosolic proteins).

2.10 Electron microscopy techniques

The following experiments were performed by Elena Polishchuck (Tigem, Pozzuoli, Italy).

For the analysis of Golgi ultrastructure in control and FAPP1 KD HeLa cells, the cells were fixed by adding to the culture medium the same volume of a mixture of PHEM (10 mM EGTA, 2 mM MgCl\textsubscript{2}, 60 mM PIPES, 25 mM HEPES, pH 6.9) buffer, 4% paraformaldehyde, 2% glutaraldehyde for 2 h, and finally stored in storage solution (PHEM buffer, 0.5 % paraformaldehyde) overnight. After washing with 0.15 M glycine buffer in PBS, the cells were scraped and pelleted by centrifugation, embedded in 10% gelatin, cooled on ice, and cut into 0.5-mm blocks in the cold room. The blocks were infused with 2.3 M sucrose, which acts as a cryo-protectant, and then placed onto small specimen pins. The pins were frozen by immersion in liquid nitrogen, quickly transferred to a pre-cooled (−60 °C) cryo-chamber fitted onto an ultramicrotome (Leica Ultracut R) and trimmed to a suitable shape. The sections were cut at −120 °C using a dry diamond knife and collected on the knife surface. Sections were retrieved from the knife by picking them up on a small drop of a 1:1 mixture of 2.3 M sucrose and 2% methyl cellulose and transferred onto formvar- and carbon- coated specimen grids.

2.10.1 Pre-embedding immunogold labeling

FAPP1 sub-Golgi localization through electron microscopy was performed by pre-embedding immunogold labelling techniques. The name of these techniques derives from the fact that the labelling is performed before the sample is embedded. Briefly,
the sample is chemically fixed and the antigen is labelled by means of a specific primary antibody that is recognized by a secondary antibody conjugated with ultrasmall gold particles.

The cells were fixed with a mixture of 4% paraformaldehyde and 0.05% glutaraldehyde in 0.15 M HEPES for 10 min at RT and then put in a 4% paraformaldehyde in 0.15 M HEPES for 30 min at RT. Afterwards, the cells were incubated in blocking solution for 30 min at RT and then with a rabbit anti-GFP antibody overnight at 4°C. The following day cells were incubated with anti-mouse Fab’ fragments of anti-rabbit IgGs coupled to 10 nm gold particles for 2h and washed with PBS. GoldEnhance™ EM kit (Nanoprobes) was used to enhance ultrasmall gold particles. Then the cells were scraped, pelleted, post-fixed in OsO4 and uranyl acetate and embedded in Epon.

2.11 Real Time PCR

RNAs were obtained from HeLa cells by using RNeasy mini kit (Quiagen, UK). The cDNAs were generated by the Quantitect kit for the qRT-PCR analysis. The qRT-PCR reactions were performed with listed primers and carried out with the Roche Light Cycler 480 system. The PCR reaction was performed using cDNA (200-500 ng), 10 μl of the SYBR Green Master Mix (ROCHE) and 400 nM primer, in a total volume of 20 μl. The PCR conditions for all the genes were as follows: preheating, 95°C for 5 min; cycling, 40 cycles of 95°C for 15 s, 60°C for 15 s and 72°C for 25 s. Quantification results were expressed in terms of cycle threshold (Ct). The Ct values were averaged for each triplicate. The \textit{Hprt} was used as the endogenous control for the experiments. Differences between the mean Ct values of the tested genes and those of the reference gene were calculated as $\Delta Ct_{\text{gene}} = Ct_{\text{gene}} - Ct_{\text{reference}}$. Relative
expression of the genes of interest in siRNAs compared to mock treated cells was analysed as $2^{-\Delta Ct}$. Relative fold changes in expression levels were determined as $2^{-\Delta\Delta Ct}$.

The sequences of oligonucleotide primers are summarized in Table 2.5.

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Table 2.5. List of the sequences of the primers used in qRT-PCR experiments.

2.12 High content screening

The High content screening was performed in collaboration with the High Content Screening facility (Telethon institute of genetics and medicine TIGEM, Italy).

HeLa cells were reverse-transfected using Lipofectamine RNAiMAX (Thermo Fisher Scientific) according to the manufacturer’s protocol. Briefly, siRNA duplexes were spotted into 384-well culture plates using a liquid-handler system (Hamilton) to give a final concentration of 40 nM. A mixture containing the transfectant agent and OPTI-MEM (Thermo Fisher Scientific) was added to each well and incubated for 20-30 minutes at room temperature after which 2,500 cells were added to each well. After 72 hours the cells were fixed for 15 min at room temperature by addition of 1 volume of 4% PFA (paraformaldehyde in PBS) to the growth medium, and stained with FAPP1 or GOLPH3 antibodies. Cells were co-stained also with a Golgi marker, GM130
and the DRAQ-5 dye that fluoresces brightly in the nucleus and dimly in the cytoplasm, allowing segmentation of the cytoplasmic and nuclear compartment. For image acquisition at least 25 images per field were acquired per well of the 384-well plate using confocal automated microscopy (Opera high content system; Perkin-Elmer). A dedicated script was developed to perform the analysis of FAPP1 and GOLPH3 intensity at the Golgi complex (Harmony and Acapella software; Perkin-Elmer). In particular, the script recognizes the Golgi area thanks to the staining with the Golgi marker GM130 and the cytosol thanks to the DRAQ-5 dye, and calculates the ratio between the intensity of FAPP1 or GOLPH3 in the Golgi area and in the cytosol. The results were normalized using negative (mock cells) and positive (FAPP1- or GOLPH3-KD cells) control samples in the same plate. P-values were calculated on the basis of mean values from independent wells.

2.13 SAC1 phosphatase in vitro assay

The following experiments were performed by Giuseppe Di Tullio (Tigem, Pozzuoli, Italy).

PI4P-containing liposomes were prepared starting from lipid stock solutions in chloroform using the following molar ratios of lipids: DOPC 45%; DOPE (20%; DOPS 5%; porcine brain PI4P 30%). Lipid mixtures in chloroform were dried under a nitrogen stream in a glass tube and hydrated in 50 mM Hepes pH 7.4, 120 mM KAc, 1 mM MgCl2, to produce a 2 mM lipid suspension. Lipid suspensions were transferred to LoBind tubes (Eppendorf), subjected to six freeze-thaw cycles (frozen in liquid nitrogen and thawed at 37°C) and extruded using a polycarbonate membrane with a pore diameter of 100 nm.
To measure the in cis activity of Sac1, 2 mol% DOGS-Ni-NTA was used in the preparation of the PI4P-containing liposomes at the expense of DOPC. The DOGS-Ni-NTA-containing liposomes were preincubated for 30 min at room temperature with 6XHis-hSac1\textsuperscript{1-522} (0.5 µM) to allow binding of the protein to the lipids. 20 µl of the liposomes were transferred to a well of 384-well plate and brought to 100 µl with the addition of reaction buffer (50 mM HEPES pH 7.2, 120 mM KAc, 1 mM MgCl\textsubscript{2}).

Aliquots (12.5 µl) of the mixture were withdrawn at different time points and added to 10 µl of 200 mM NEM to stop the reaction. Sac1 phosphatase activity was measured based on the amount of phosphate released in solution by adding 50 µl of malachite green reagent and reading the absorbance at 620 nm after 20 min.

To measure the in trans activity of Sac1, 6XHis-hSac1\textsuperscript{1-522} (0.5µM) was preincubated for 30 min at room temperature with 2 mM DOPC liposomes containing 2 mol% DOGS-NiNTA. 20 µl of these liposomes were mixed with 20 µl of PI4P-containing liposomes and 60 µl of DTT-containing reaction buffer and assayed as above. To measure the effects of FAPP1, GST or GST-FAPP1 was added to the reaction mixture at the indicated final concentrations.

2.14 Wound healing assay

These experiments were performed in collaboration with the High Content Screening facility (Telethon institute of genetics and medicine TIGEM, Italy).

A549 cells were treated with the indicated siRNAs in 6-well plates. After 48 h cells are trypsinized and plated in a 96 well to grow a confluent monolayer. After additional 20-22 h, the cell layer was scratched with a 10µl tip and this represents the starting point of the experiment (Time 0). Images were automatically acquired with the Operetta
high content imaging system with a 10x objective at time intervals of 2h for 24h. For the entire experiment duration, the cells are kept in a controlled atmosphere in the presence of 5% CO₂ at 37°C. Given the low magnification of the acquisition, the stitching of 9 fields at the centre of the well is able to capture the entire length of the wound. Images were analysed with the MRI Wound Healing Tool, a macro of the ImageJ software that uses a variance filter to separate, in a field, the zone occupied by tissue from the empty zone. In this way the MRI macro is able to calculate the area of the wound on a stack of images representing a time-series. The area at different time-points has been expressed, for each well as a % of their own time 0 with Microsoft Office Excel.

2.15 Matrigel invasion assay

A549 cells were treated with the indicated siRNAs in 6-well plates. After 72h the cells were trypsinized, counted, resuspended in low-serum medium (0.5% FBS) and plated in the Matrigel coated invasion chamber (Biocoat), previously placed in a 24 well plate. 500 µl of complete medium (10% FBS) were added in the bottom of the 24 well. In this way, the cells placed in the upper chamber tend to migrate for chemotaxis in the bottom well and, according to their invasion ability, will reach the lower side of the chamber by degrading the matrigel layer. After 24h the cells on the lower part of the chamber were washed twice in PBS, fixed with cold methanol for 2 min and stained with a 1:1 solution of Toludine Blue/ Sodium Borate for 2 min at RT. The excess of dye was removed by two rinses in distilled water. 12 random field/well were imaged with a phase contrast microscope (Zeiss Apotome)t and the number of cells that reached the lower membrane of the chamber were counted. Three wells were analysed for each condition and the mean number of invading cells was calculated with Microsoft Office Excel.
2.16 Generation of FAPP1-KO MEFs

The protocol used to generate FAPP1 KO mice was that described for FAPP2 KO mice in D’Angelo et al., 2013. Briefly, the FAPP1 gene was isolated from a mouse genomic BAC library derived from the 129Sv/J mouse strain (RPCI-22: Children’s Hospital Oakland Research Institute). An FRT-flanked SA-IRES-beta-geo-polyA cassette with an upstream loxP site was inserted into intron 5 and a loxP site was introduced into intron 7 in the FAPP1 targeting vector. This construct was used for the generation of the heterozygous FAPP1geo/+ mice. Homozygous FAPP1 geo/geo mice (hereon indicated as FAPP1 KO mice) were obtained by mating. The absence of FAPP1 was verified by Western blot and by PCR analysis. The PCR primers used were:

5’-AAAAACACCCAGTGTCAGGCTAGC-3’ (Primer1),

5’-TAACTGAGCACCAGGAGGAGAAGG-3’ (Primer2),

5’-CCGTACAGTCCACAAAGGCATCCT -3’ (Primer 3),

where primer pair 1 and 2 detected the wild-type allele and primers 1 and 3 the geo allele of FAPP1. MEFs were isolated from E13.5 embryos. Three embryos were pooled together to generate each single MEF clone. Briefly, after isolation and removal of internal organs, the embryos were trypsinized (0.125% trypsin-EDTA) at 37°C for minutes and put in culture in DMEM with 20% FCS plus amphotericin and penicillin/streptomycin for the first week and kept for several passages.
2.17 ApoB100 secretion

HepG2 mock treated or FAPP1-siRNA treated cells were incubated in lipid-free medium (containing 5% delipidated serum). After 24 or 48 h the medium was collected and the cells were lysed as described in par.2.8.1. ApoB-100 concentration in cells and media was determined with an ELISA assay kit (Mabtech, USA). Briefly, a 96well plate is pre-coated with the capture antibody, that binds ApoB-100 in the cellular or medium solution and immobilizes it. The sequential addition of a biotynilated monoclonal antibody against ApoB-100 (detection antibody), streptavidin conjugated with the reporter horseradish peroxidase (SA-HRP) and the enzyme substrate determines the formation of a colored substrate product with an intensity that is directly proportional to the concentration of ApoB-100 in the sample. The absorbance developed by the reaction product is read at 450nm and it is directly proportional to the concentration of ApoB-100.
Aims

Phosphatidyl-Inositol-4-Phosphate, PI4P, is a Golgi phosphoinositide with pleiotropic roles in the cell, coordinated via recruitment and activation of its multiple effectors. The importance of PI4P functions implicates that its levels must be tightly regulated in time and space. It is very well understood that PI4P homeostasis at the Golgi is maintained by a balance in its production by PI4-Kinases and its consumption by the PI4-phosphatase SAC1, however the upstream control orchestrating this system is not known. Over the last few years, a growing body of evidence describe that some PI4P binding proteins act as PI4P sensors: they not only bind PI4P but are also able to control its levels, via stimulation of PI4P enzymes or counter-exchange it with other lipids. FAPP1 has been one of the first PI4P effectors described, but, despite its early identification, its function has remained elusive so far. Thus, the specific aims of my project were to:

- Investigate the role of FAPP1 as a PI4P sensor at the Golgi complex;
- Identify the biological role of FAPP1 in the cell.
Chapter 3

Results

3.1 FAPP1 is localized at the TGN

FAPP1 has been previously shown to be localized at the TGN in COS7 and HeLa cells (Godi et al., 2004). In this study, however, FAPP1 and FAPP2 antibodies were used indistinctly and the difference in localization and function of FAPP1 compared to FAPP2 was not clearly determined. To analyze the function of the FAPP1 protein, I assessed first the sub-cellular localization of both endogenous and GFP-tagged FAPP1 in HeLa cells. As shown in Fig. 3.1 FAPP1 is mainly localized in a peri-nuclear area that corresponds to the Golgi apparatus, as demonstrated by the colocalisation with Golgi markers. In particular, FAPP1 (both endogenous and over-expressed) exhibits a preferential trans- Golgi localization, as it shows a major overlap with the trans-Golgi marker TGN46, rather than the cis-Golgi marker GM130 (Fig. 3.1).
Figure 3.1 FAPP1 localizes at the Golgi complex and is enriched at the TGN. A) HeLa cells were stained for FAPP1 and a cis- or trans-Golgi marker (GM130 and TGN46, respectively). B) HeLa cells were transfected with GFP-FAPP1 and stained with GM130 and TGN46. In both conditions FAPP1 shows a strongest overlap with TGN46 rather than with GM130.

To further investigate the intra-Golgi FAPP1 localization, I performed the same experiment in HeLa cells treated with the fungal metabolite Nocodazole, that results in
Golgi mini-stack formation due to microtubules disassembly, allowing for better visualization of Golgi subdomains (cis and trans). Analysing the Golgi ministacks, FAPP1 signal overlaps mostly with TGN46, and much less with GM130, as confirmed by the colocalization coefficient and the intensity plot (Fig. 3.2).

**Figure 3.2** FAPP1 localizes at the Golgi complex and is enriched at the TGN. A) HeLa cells were treated with Nocodazole 33μM for 3 hours and then stained with FAPP1 together with a cis- or trans-Golgi marker (GM130 and TGN46, respectively). Representative stacks shown in the insets evidence an overlap of FAPP1 (green) with TGN46 (red) compared to GM130 (blue). B) Co-localization of FAPP1 with GM130 and TGN46 was quantified in each ministack with the ZEN software (Zeiss). The box-plot represents mean ± st.dev of ~30 stacks. Unpaired t-test was calculated with the GraphPad software. ****P-value < 0.0001 C) A representative region is shown (white line in Fig. A). Intensity plot of signal intensity (y-axis) against distance in μm (x-
axis) shows that FAPP1 and TGN46 channels (green and red curves, respectively) overlap, and are separated from GM130 (blue curve).

Finally, the intra-Golgi distribution of FAPP1 was assessed also by immuno-electron microscopy. To this aim, GFP-FAPP1 overexpressing HeLa cells were fixed and immuno-gold labeled to reveal the GFP signal. FAPP1, although present on cis-medial Golgi cisternae, is clearly enriched at the TGN, as demonstrated by counting the number of GFP-FAPP1 gold particles localized on TGN or cis/medial Golgi cisternae (Fig 3.3).

**Figure 3.3 Immuno-gold electron microscopy analysis of FAPP1 intra-Golgi distribution.** A) HeLa cells expressing GFP–FAPP1 were processed for immuno-electron microscopy and labeled for GFP (10nm gold particles). GFP-FAPP1 particles are enriched in the last trans-cisterna/TGN. The arrowhead indicates a clathrin-coated vesicles (CCV) profile that identifies the trans pole of the Golgi. B) Box-plot showing the quantification of GFP-FAPP1 gold particles in the last trans-cisterna/TGN and in the cis-medial cisternae. Unpaired t-test was calculated with the GraphPad software. ****P-value < 0.0001.
The FAPP1-PH domain is a clear example of coincident binding to PI4P and the small GTPase ARF1 (Godi et al., 2004). Thus, if one of the two determinants of FAPP1 Golgi localization is perturbed, FAPP1 should redistribute in the cytosol. To inhibit the production of PI4P or the activation of ARF1, I treated HeLa cells with a selective inhibitor of PI4KIIIβ, PIK93, or with the fungal toxin Brefeldin A, respectively. As shown in Fig. 3.4, in both conditions FAPP1 detaches from the Golgi complex and re-localizes to the cytosol.

**Figure 3.4** FAPP1 localizes at the TGN via coincident binding with PI4P and ARF1. HeLa cells were treated with 250nM PIK93 for 30 min or with 2.5 μg/mL of Brefeldin-A for 3’ (a timeframe in which the Golgi is still compact) and then co-stained with FAPP1 and GM130. Under both treatments FAPP1 loses its Golgi localization and diffuses in the cytosol.
All together these experiments indicate that FAPP1, both endogenous and over-expressed, localizes at the Golgi complex showing a significant enrichment in the TGN, due to its binding with PI4P and ARF1.

3.2 FAPP1 depletion causes an increase in Golgi PI4P levels

FAPP1 is very different compared to all the other Golgi PI4P effectors. In fact, it is not a lipid transfer protein, and its apparent function identified so far is to bind PI4P (see par. 1.7). This observation prompted me to investigate a possible role of FAPP1 in the regulation of PI4P levels at the Golgi Complex (GC).

I followed a siRNA-based approach to down-regulate FAPP1 in HeLa cells and I measured the PI4P levels on the Golgi complex by using a monoclonal specific PI4P antibody (Hammond, Schiavo and Irvine, 2009). Fig 3.5 shows that, compared to the control, FAPP1 KD cells display an increase in PI4P signal at the GC, identified by the trans-Golgi marker Golgin-97 (G-97). The quantification of the ratio between PI4P and G-97 fluorescence intensity in the Golgi area (delimited by G-97) confirms that FAPP1 depletion causes an increase in PI4P levels at the GC (Fig. 3.5 B).
Figure 3.5 FAPP1 depletion increases PI4P levels at the GC. A) HeLa cells were Mock- or FAPP1 siRNA-treated for 72h and stained with antibodies against PI4P and the trans-Golgi marker Golgin 97 (G-97). The images show a clear increase of PI4P signal in FAPP1 KD cells compared to the control. B) The ratio between PI4P and G-97 fluorescence intensity in the Golgi area (delimited by G-97) was quantified in each cell with ImageJ. The mean value of control (Mock) cells was determined and considered as 100%. Histogram bars represent mean ± SEM of ~200 cells. Unpaired t-test was calculated with the GraphPad software. ****P-value < 0.0001. C) Western blot analysis shows the reduction of FAPP1 protein level after siRNA treatment. Total lysates (40μg) from each sample were separated by SDS-PAGE (12%) gel. The asterisk indicates a non-specific band recognized by FAPP1 antibody.

To formally exclude the possibility of a non-specific effect due to siRNAs off-targeting for FAPP1, I re-expressed a siRNA-resistant GFP-tagged FAPP1 in FAPP1 KD cells via infection with an adeno-associate virus (AAV) carrying a GFP- tagged FAPP1. Notably,
the re-expression of FAPP1 is able to rescue the increase of PI4P levels in FAPP1 KD cells (Fig 3.6).

Figure 3.6  FAPP1 KD-induced PI4P increase is rescued by FAPP1 over-expression. A) HeLa cells were Mock- or FAPP1 siRNA-treated for 48h and infected with an adenovirus carrying siRNA resistant FAPP1 (AAV2/2-GFP-FAPP1). After additional 24h, cells were stained with anti-PI4P and anti- Golgin 97 (G-97) antibodies. In cells overexpressing FAPP1 (white asterisks) the ratio between PI4P and G-97 signals is lower than in surrounding, non-infected cells, both in control and in FAPP1 KD conditions. B) The ratio between PI4P and G-97 fluorescence intensity in the Golgi area (delimited by G-97) was quantified with ImageJ and expressed as percentage of non-infected Mock cells. Mean ± SEM are shown. ANOVA was calculated with the GraphPad
Moreover, I isolated and immortalized murine embryonic fibroblasts (MEFs) from WT and FAPP1 KO mice and I analyzed the levels of PI4P at the Golgi. As shown in Fig. 3.7, PI4P levels are significantly increased in FAPP1 KO MEFs, compared to the WT.

**Figure 3.7 PI4P levels increase in MEFs from FAPP1 KO animals.** A) Immortalized MEF isolated from WT or FAPP1 KO mice were stained with antibodies against PI4P and the trans-Golgi marker Golgin 97 (G-97). B) The ratio between PI4P and G-97 fluorescence intensity in the Golgi area (delimited by G-97) was quantified in each cell with ImageJ. The mean value of control (WT) cells was determined and considered as 100%. Histogram bars represent mean ± SEM of samples. Unpaired t-test was calculated with the GraphPad software. ****P-value < 0.0001. C) Western blot analysis shows the absence of FAPP1 band in KO cells. 40μg of total
lysate from each sample were separated by SDS-PAGE (12%) gel. The asterisk indicates a non-specific band recognized by FAPP1 antibody.

These results indicate that FAPP1 depletion causes an increase in PI4P levels at the Golgi Complex.

3.3 FAPP1 regulates PI4P homeostasis at the level of ER-Golgi Contact Sites (ERTGoCS) via binding and stimulation of the 4-Phosphatase SAC1

To understand the mechanism by which FAPP1 controls PI4P at the Golgi, a mass spectrometry approach was used to identify FAPP1 putative interactors. HeLa cells overexpressing GFP-FAPP1 or GFP-empty vector were immunoprecipitated with a GFP antibody. Samples were sent to the Central Proteomics facility (South Parks Rd, Oxford) for mass spectrometry analysis. The result (Fig. 3.8) evidenced two important findings:

- FAPP1 interacts with SAC1, the only known 4-phosphatase in the cell

- FAPP1 is able to interact with ER proteins
Figure 3.8 Mass spectrometry analysis of FAPP1 interactors.  

A) HeLa cells overexpressing GFP-FAPP1 were immunoprecipitated with an anti-GFP antibody, analyzed by MS/MS and the interactors grouped according to cellular component. Interactors belonging to the secretory pathway were taken in consideration and further classified (right pie graph). Most of the interactors belonging to the secretory pathway are ER proteins. B) FAPP1 partners were ranked based on their score. The 4-Phosphatase SAC1 represents one of the strongest FAPP1 interactors. The presence of nuclear interactors is justified by the evidence that FAPP1 displays also a nuclear signal (data not shown). So far we never had evidence of FAPP1 localizing on...
mitochondria, and the presence of mitochondrial interactors can be ascribed to the cytosolic pool of FAPP1. Further analysis will be performed to assess the functional relevance of these interactions.

To corroborate the mass spectrometry data, endogenous FAPP1 was immunoprecipitated from HeLa cell lysate and the interaction with SAC1 was confirmed. The other way round, FAPP1 was detected in HeLa cells lysate immunoprecipitated with SAC1 antibody, enforcing the evidence that the two proteins interact (Fig. 3.9).

**Figure 3.9 FAPP1 interacts with the 4-Phosphatase SAC1.** HeLa cells were immunoprecipitated with control IgG or antibody anti-FAPP1 (left) or anti-SAC1 (right). Western blot analysis shows that a band at 66 KDa, the molecular weight of SAC1, is recognized by anti-SAC1 antibody in FAPP1 IP and not in IgG pre-immune. Similarly, a band of 33KDa, the molecular weight of FAPP1, is detected by anti-FAPP1 antibody in SAC1 IP, while it is absent in IgG sample. Immunoprecipitations are indicated as IP, flow through samples are indicated as FT.
Upon steady state conditions, SAC1 is mainly localized in the ER, while FAPP1 is, as previously shown, a TGN protein (Fig. 3.1-3.3 and par. 1.7) suggesting that their interaction could occur at level of ER-Golgi Membrane Contact Sites (ERTGoCS) (see par. 1.5). (Fig. 3.10)

Figure 3.10 FAPP1 and SAC1 localize in different sub-cellular compartments. HeLa cells were transfected with GFP-FAPP1 and stained with anti-SAC1 antibody. The two proteins, as highlighted in the inset, are localized in different compartments.

Intriguingly, a more careful analysis of FAPP1 subcellular distribution by immuno-electron microscopy allowed me to appreciate a nice FAPP1 localization at the TGN-ER interface (Fig 3.11).
Figure 3.11 FAPP1 is able to localize at ER-Golgi membrane contact sites (ERTGoCS). HeLa cells expressing GFP–FAPP1 were processed for immuno-electron microscopy and labelled for GFP (10nm gold particles). GFP-FAPP1 particles are enriched at the interface between the ER (colored in red) and the TGN (colored in green).

To confirm that FAPP1 exerts its function at the level of ERTGoCS, FAPP1 interaction with the VAP proteins was verified. VAPs are known to target many ER-organelles membrane contact sites, because they represent the ER determinant for all those protein (containing FFAT or FFAT-like motif) that simultaneously binds the ER and another organelle (e.g. Golgi, endosomes, PM; Murphy and Levine, 2016; see par. 1.5.2). Moreover, VAPs act as tethers in many contact sites (see par. 1.5.2), and, specifically, studies in our lab evidenced a role for VAPs in the maintenance and formation of ER-Golgi contact sites (Venditti et.al under revision). Fig 3.12 shows that
VAPA is co-immunoprecipitated by the antibody against endogenous FAPP1. I was also able to detect VAPB in endogenous FAPP1 immunoprecipitates from mouse liver lysates. The evidence that the VAPB band is absent in the IP from FAPP1 KO lysates corroborates the specificity of the interaction (Fig. 3.12).

Figure 3.12 FAPP1 interacts with the VAP proteins. **A)** HeLa cells were immunoprecipitated with control IgG or anti-FAPP1 antibody. VAPA antibody recognizes a band at 28 KDa, the molecular weight of VAPA, in FAPP1 IP and not in IgG pre-immune sample. **B)** Liver lysates from WT or FAPP1 KO mice were immunoprecipitated with control IgG or anti-FAPP1 antibody. FAPP1 was efficiently immunoprecipitated in WT cells (right) while is absent in FAPP1 KO samples (left). A band corresponding to the molecular weight of VAPB is recognized by an anti-
VAPB specific antibody in FAPP1 IP from WT mice, while is absent in FAPP1 IP from KO samples and in control IgG. Immunoprecipitations are indicated as IP, flow through samples are indicated as FT.

The mechanism by which FAPP1, via binding SAC1, regulates PI4P levels, was clarified by in vitro malachite assay studies performed to measure the 4-Phosphate activity of a recombinant human SAC1, in presence or absence of FAPP1. The activity of SAC1 was measured in conditions in which the phosphatase SAC1 and its substrate PI4P were put on the same membrane (cis conformation) or on different membranes (trans conformation), and the effect of adding a recombinant FAPP1 was assessed in both systems. Fig. 3.13 shows that, while SAC1 activity in trans per se is lower in steady state conditions, the addition of recombinant human FAPP1 has a strong stimulatory effect on SAC1 4-phosphatase activity in trans, and much less on the SAC1 activity in cis (Fig. 3.13).
Figure 3.13 FAPP1 preferentially stimulates SAC1 4-phosphatase activity in trans. PI4P containing liposomes were prepared as described in Methods. His-tagged recombinant SAC1 (His6-Sac1(1-522)) was bound to PI4P containing liposomes (cis conformation) or on different liposomes (trans conformation) for the indicated times, in presence of recombinant GST or GST-FAPP1. Sac1 phosphatase activity was measured based on the amount of phosphate released in solution by adding malachite green. The graph shows the in vitro phosphatase activity of SAC1 (expressed in pmol) in presence of GST-FAPP1 (+FAPP1) or GST alone (w/o FAPP1) in the cis (full line) or trans (dashed line) conformation (schematized in the above panel). The effect of FAPP1 on the stimulation of SAC1 phosphatase activity was much higher when SAC1 and PI4P are in the in trans conformation.
These data demonstrate that FAPP1 stabilizes SAC1 on PI4P-rich membranes, thus promoting its in trans activity to dephosphorylate PI4P at ERTGoCS.

ERTGoCS have been extensively studied in Antonella De Matteis’ laboratory in the last years. In particular a strategy for the visualization and the stabilization of ERTGoCS has been set up (described in Rega L.R., 2012, “The role of endoplasmic reticulum-trans Golgi network membrane contact sites”, Open University PhD Thesis, Life Science). This is based on a construct encoding for a TGN reporter (TGN46) and an ER reporter (the C-tail of cytochrome b5, Cb5), fused to two tags, HA and mCherry, respectively. With the aim of introducing a chemical inducible bridge between the two proteins, TGN-HA and mCherry-Cb5 were also fused with two domains, FRB and FKBP respectively, that dimerize under Rapamycin addition. A short treatment with rapamycin (200nM for 2’) allows the stabilization of existing ERTGoCS, that in this way can be also visualized by immunofluorescence, as Cb5 gets recruited in the TGN area (Fig. 3.14 A and 3.14 B (red channel)).

I took advantage of this powerful tool to analyze the ability of FAPP1 to stimulate SAC1 activity in vivo. A stable cell line overexpressing the TGN46-FRB-HA-mCherry-FKBP-Cb5 construct was interfered with siRNAs targeting FAPP1, treated with Rapamycin for 2, 4 and 15 minutes, fixed and stained for PI4P. In control cells (Mock), even a short time of ERTGoCS stabilization with Rapamycin is sufficient to allow PI4P consumption by SAC1. Interestingly, in FAPP1 KD cells, although ERTGoCS are equally formed compared to control (Fig. 3.14 B, red channel), PI4P needs more time to be reduced, and it reaches a reduction percentage comparable to the control only after 15’ of treatment (Fig. 3.14 C).
Figure 3.14 FAPP1 is required for PI4P efficient consumption at ER-Golgi Contact Sites (ERTGoCS). A) Schematic representation of the ERTGoCS stabilization system. TGN46-HA and Cb5-mCherry were used as reporters of TGN and ER and were fused with the FRB and the FKBP domain, respectively. The addition of Rapamycin induces the dimerization of FRB and FKBP and, in turn, the stabilization of ERTGoCS. B) HeLa cells stably expressing the TGN46-FRB-HA-mCherry-FKBP-Cb5 construct were left untreated (time 0) or treated with 200nM Rapamycin for the indicated times. ERTGOCs stabilization, visualized by Cb5 recruitment in the Golgi area, induces PI4P consumption at the Golgi in Mock treated cells (left panel). In FAPP1 KD cells (right panel), though Cb5 is equally recruited in the Golgi area, PI4P reduction is slower. C) Quantification of PI4P levels calculated as a ratio between the fluorescence intensity of PI4P and G-97 (not shown in Fig. B). PI4P values are expressed as % of values in cells at time 0 (untreated). ****P-value < 0.0001. While in control cells (black bars), PI4P is efficiently consumed already at early times of Rapamycin treatment, FAPP1 KD cells (grey bars) are slower in reducing PI4P, and reach the control levels (red dashed line) only after 15’. In order to demonstrate that FAPP1 regulates PI4P homeostasis stimulating SAC1 activity in trans also in vivo, FAPP1 silencing was performed in a context of VAPs depletion, where the OSBP1 mediated PI4P consumption in cis (Mesmin et al., 2013; see par. 1.5.3.3) is prevented, as VAPs represent the ER anchor for OSBP1 (Murphy and Levine, 2016). In VAPs KD cells a short time of Rapamycin tratement (2 and 4min), is not sufficient to induce ERTGoCS stabilization (Fig. 3.15, red dashed lines) and the subsequent PI4P consumption (Fig. 3.15, red full lines), which can be observed only under a longer rapamycin stimulation (15 min). In conditions of simultaneous FAPP1 and VAPs depletion, although the formation of ERTGoCS is partially restored after 15 minutes of treatment (Fig. 3.15, green dashed line), PI4P levels are not reduced (Fig.
3.15, green full line), confirming that FAPP1 is necessary to stimulate the 4-phosphatase activity of SAC1 in trans also in vivo.

Figure 3.15 FAPP1 is required to sustain SAC1 in trans dephosphorylation of PI4P at the level of ERTGoCS. Quantification of the Golgi PI4P levels (full lines) and number of cells forming ERTGoCS (dashed lines) in control, VAPs KD and VAPs + FAPP1 KD cells treated with 200 nM rapamycin for the indicated times. PI4P levels were calculated as the ratio between PI4P and Golgin97 fluorescence intensity at the Golgi complex and expressed as % of values in cells at time 0 (not exposed to rapamycin). ERTGoCS were calculated by counting the number of cells showing co-localization of Cb5 with TGN46 (i.e. red channel in Fig. 3.14 B) and expressed as % of total cells number. After a long rapamycin treatment (15 min) VAPs KD cells show a partial stabilization of ERTGoCS (red dashed line) and PI4P reduction (red line), but only in presence of FAPP1. In cells simultaneously depleted for VAPs and FAPP1, indeed, although ERTGoCS are partially formed under the long rapamycin treatment (green dashed line), PI4P levels are no
longer reduced (green line). *P-value < 0.005, **P-value < 0.001, ***P-value < 0.0001, ****P-value < 0.0001. ns= not significant.

All together these data demonstrate that FAPP1 regulates PI4P homeostasis via binding and stimulation of SAC1 activity at the level of ERTGoCS.

3.4 FAPP1 regulates anterograde trafficking of specific cargoes.

Since the Golgi PI4P pool has a very well established role in the control of Golgi to plasma membrane trafficking (see Chapter 1), I decided to investigate if the increase of PI4P in FAPP1 KD cells could affect anterograde membrane trafficking. To this aim, I analyzed the trafficking of the cargo reporter VSVtsO45-G, a modified form of a glycoprotein of vesicular stomatitis virus (VSV). VSV-G is widely used as a cargo prototype to study membrane secretion, because of its easy synchronization and manipulation. The modification in a hydrophobic pocket of the protein (Gallione and Rose, 1985) results in a higher sensitivity to the temperature and therefore can be used to synchronize this cargo. At 40°C, VSV-G is retained in the ER due to mis-folding that is rescued by a temperature shift at 20°C, which allows VSV-G ER-exiting and accumulation at the TGN. After a further increase of temperature at 32°C, VSV-G is trafficked from the TGN to the plasma membrane. Mock and FAPP1 KD cells were infected with VSV-G, incubated at 40°C for 3 hours and at 20°C for additional 2 hours (to synchronize the cargo in the ER and in the TGN, respectively), and finally shifted at 32°C. The ratio between luminal versus total VSV-G was analyzed by immunofluorescence at different time-points (15, 30 and 60 minutes) after the 32°C shift. In control cells, after 15 min at the permissive temperature, VSV-G is distributed between the TGN and the plasma membrane, while at 30 and 60 min, VSV-G localization at the TGN becomes weaker and disappears, respectively, in favor of
plasma membrane localization. The depletion of FAPP1 does not alter VSV-G trafficking, which proceeds at a similar rate of control cells (Fig. 3.16).
**Figure 3.16 VSV-G trafficking is not altered in FAPP1 KD cells.** A) HeLa cells treated with mock or FAPP1 targeting siRNAs were infected with VSVGts045 for 45 minutes at 32°C and then shifted at 40°C for 3 hours to accumulate VSV-G into the ER. Subsequently, the cells were shifted at 20°C for 2 hours, to synchronize the viral cargo at the TGN. Finally, the cells were incubated at 32°C and analyzed by immunofluorescence. To measure the arrival of VSV-G to the plasma membrane, a specific antibody recognizing the luminal domain of VSV-G was used before permeabilizing cells (upper panel). Subsequently, cells were permeabilized with saponin 0.2% and immunostained with an antibody recognizing a cytosolic portion of VSV-G (P5D4, lower panel). B) VSV-G arrival to the plasma membrane was calculated measuring the ratio between the intensity fluorescence of VSV-G at the plasma membrane and the total intracellular protein. Data represents mean ± st.dev. expressed as % of Mock time 0’. An absence of effect in VSV-G trafficking does not exclude that a defective secretion of other, more specific endogenous cargoes, can occur, since it has been proved that some cargo rely on a more specific machinery to be trafficked (see below).

Thus, I decided to analyze the transport of collagen, an endogenous cargo that, because of its big size, requires a special machinery to be trafficked. Some proteins, indeed, are important for procollagen secretion, but dispensable for small cargo trafficking (Venditti *et al.*, 2012).

To understand if it could be the case for FAPP1, I performed a collagen transport assay in control and FAPP1 KD human fibroblast (HF) which express procollagen type I (PC-I). Procollagen secretion can be monitored by immunofluorescence, as the cargo gets unfolded, and thus retained in the endoplasmic reticulum, at 40°C, similarly to the above described VSV-G synchronization protocol. A shift to the permissive temperature of 32°C allows collagen proper folding and its exit from the ER. In control
cells, after 15 min at 32°C, the majority of the cell population shows a clear PC-I Golgi localization, which starts to decrease at 30 min and is almost completely lost after 60 min, since PCI is secreted. PCI trafficking is not impaired in FAPP1 KD cells, in which the collagen localization is indistinguishable from control cells at each analysed time-point (Fig. 3.17).

**Figure 3.17** FAPP1 depletion does not affect procollagen trafficking. **A**) Human fibroblasts mock or FAPP1 si-RNA treated for 72h were incubated for 3h at 40°C (time 0’) and then shifted at 32°C and processed by immunofluorescence at the indicated time-points. PCI trafficking through the Golgi is not affected by FAPP1 depletion. **B**) Western blot analysis shows the efficient reduction of FAPP1 protein level also in this cellular model.
These results indicate that FAPP1 does not affect secretion of PCI nor VSV-G transport from the Golgi to the plasma membrane.

On the other hand, one indication of a possible cargo that could be affected by FAPP1 depletion came from important studies performed in our laboratory about the lipid transfer protein ORP10 (Venditti et al., under revision). ORP10 belongs to the OSBP-like (OSBPL) gene family, can bind PI4P via a PH domain and localizes at the Golgi complex (Nissilä et al., 2012). Experiments performed in Antonella De Matteis laboratory unraveled a role for ORP10 in the regulation of PI4P at the level of ER-TGN membrane contact sites (ERTGoCS) (Venditti et al., under revision). In particular ORP10 depletion impairs the formation of ERTGoCS and, as a consequence, displays a strong accumulation of PI4P at the Golgi (Venditti et al., under revision). Importantly, ORP10 had been previously demonstrated to be a negative regulator of β-lipoprotein secretion, since ORP10 silencing in hepatic cell line increases the secretion of ApoB-100, without affecting the general secretion (Nissilä et al., 2012).

Since FAPP1 and ORP10, although through different mechanisms, share a similar role in the regulation of the Golgi pool of PI4P at the level of ERTGoCS, I decided to investigate the role of FAPP1 in ApoB-100 secretion in hepatic cells (HepG2).

To examine β-lipoprotein secretion, HepG2 mock- or FAPP1-siRNA treated cells were incubated in lipid-free medium (containing 5% delipidated serum) for 24 hours, after which the medium content of ApoB-100 was determined by an ELISA assay. This kind of assay is defined as “sandwich”, because the molecule to be measured is bound between two primary antibodies: the capture antibody and the detection antibody (Fig. 3.18). A 96well plate is pre-coated with the capture antibody, that binds ApoB-100 in the cellular or medium solution and immobilizes it. The sequential addition of a biotynilated monoclonal antibody against ApoB-100 (detection antibody), streptavidin
conjugated with the reporter horseradish peroxidase (SA-HRP) and the enzyme substrate determines the formation of a colored substrate product with an intensity that is directly proportional to the concentration of ApoB-100 in the sample.

Figure 3.18 Schematic representation of the ELISA assay.

For each sample, the amount (ng) of ApoB-100 measured in the medium was calculated and normalized for the cellular protein content. Surprisingly, FAPP1 depletion caused a significant increase in ApoB-100 secretion (Fig. 3.19).
Figure 3.19 FAPP1 depletion results in increased ApoB-100 secretion. HepG2 cells were mock or FAPP1 siRNA-treated for 72 hours. For the last 24 of interference, cells were incubated in lipid free medium (containing 5% delipidated serum). ApoB-100 concentration in the medium was determined with a specific sandwich enzyme-linked immunosorbent assay (ELISA; Mabtech). The absorbance developed by the reaction product (see text) is read at 450nm and it is directly proportional to the concentration of ApoB-100 (ng). The histogram shows the amount of ApoB-100 in the medium expressed in ng per mg of cellular proteins. Values are mean ± st.dev of two independent experiments.

This important finding, together with the knowledge on ORP10 function, strongly supports the hypothesis that the pool of PI4P directly controlled by FAPP1 is needed to regulate the secretion of this specific cargo.
3.5 *FAPP1 and GOLPH3 are co-expressed in a variety of tissues*

In order to identify a more general role for the pool of PI4P controlled by FAPP1 at the level of ER-Golgi contact sites, we used a wide bioninformatic approach aimed to identify a co-expression network for FAPP1 (Mario Failli). In particular, analyzing RNAseq data from 2921 samples divided in 25 tissues, it was possible to identify 36 consensus modules of co-expressed genes, meant as genes whose expression level oscillates in the same fashion in most of the samples. Focusing my attention on the module containing the FAPP1 gene (PLEKHA3) I found that, interestingly, one of the genes co-expressing with FAPP1 is another PI4P effector, GOLPH3 (Fig. 3.20 A). Since GOLPH3 is an oncogene up-regulated in a variety of tumors (see par. 1.6.4.1), the same co-expression analysis was performed also in tumor conditions. Importantly, the strong co-expression between FAPP1 and GOLPH3, observed in a variety of normal tissues, is lost in tumor samples (Fig. 3.20 B).
**Figure 3.20** FAPP1 and GOLPH3 are co-expressed in samples from normal, but not tumoral tissues. Co-expression module containing FAPP1 and GOLPH3 (highlighted in red). The graph shows a co-expression network, purple lines indicate connections among genes. Rectangles size indicate the “module membership value” of the correspondent gene. White and pink rectangles represent genes showing a positive or negative co-expression correlation, respectively, compared to the principal component of the module. 

**B)** Correlation values between FAPP1 and GOLPH3 expression in normal (pink bars) and tumoral tissues (grey bars). The inset represents an example of GOLPH3 and FAPP1 expression levels in different normal and tumor samples. While in control condition (pink) FAPP1 and GOLPH3 expression levels oscillate in the same manner in the different samples, this correlation is completely lost in tumor conditions (grey).

These observations suggest that FAPP1 and GOLPH3 could cooperate in some cellular processes, and, in particular, the functional interaction between the two proteins might be important in cancer progression. In order to understand if the co-expression of FAPP1 and GOLPH3 translates in a functional cooperation of the two proteins in cells, I silenced FAPP1 via siRNA interference and looked at GOLPH3-related functions, starting from the analysis of the Golgi complex architecture.

### 3.6 FAPP1 KD induces Golgi fragmentation

Since GOLPH3 is important for the maintenance of Golgi morphology (Dippold et al., 2009), I analyzed the structure of the Golgi Complex (GC) in FAPP1 KD cells looking at the distribution of two different Golgi markers (Fig. 3.21 and 3.25) using super resolution-Structured illumination microscopy (SR-SIM) technique. FAPP1 depletion in HeLa cells leads to a strong alteration of the GC architecture, as highlighted from the staining of GM130 in Fig. 3.21 A, and measured as an increase in the Golgi particles per...
cell analyzed with the ImageJ software (Fig. 3.21 B). The super-resolution analysis coupled with the 3D reconstruction (Fig 3.21 A inset) clearly show that the GC was not simply expanded or dilated, but fragmented, as several particles are distinguishable and separated one from the other.

Figure 3.21 FAPP1 KD induces Golgi fragmentation. A) HeLa cells were Mock- or FAPP1 siRNA-treated for 72h and stained with the Golgi marker GM130. Samples were analyzed by Super Resolution- Structured Illumination Microscopy (SR-SIM). The insets show single cell 3D reconstruction. B) Quantification of Golgi particles by ImageJ software. Data represent the number of Golgi spots per cell in Mock and FAPP1 KD, respectively. Unpaired t-test was calculated with the GraphPad software. ****P-value < 0.0001. C) Western blot analysis show the reduction of FAPP1 protein level after siRNA treatment. 40μg of total lysate from each sample were separated by SDS-PAGE (12%) gel.
The Golgi complex is composed of flats cisterna grouped in several stacks, interconnected via a tubulo-membrane system known as “non-compact zone” to form the “Golgi ribbon”. This architecture can be broken at different levels, going from the unlinking of the stacks, without affecting their structure and polarity, to the unstacking and disassembly of cisternae in small vesicles, a process known as vesiculation (Fig 3.22 A). To better characterize the Golgi fragmentation observed in FAPP1 KD cells, Golgi ultrastructure was analyzed by Electron Microscopy. As shown in Fig 3.22 B, in FAPP1 KD cells Golgi stacks are present, and the cis-trans polarity is preserved, indicating that FAPP1 depletion does not results in unstacking and/or vesiculation of the GC.
Figure 3.22 FAPP1 KD does not cause Golgi vesiculation or loss of cisternae polarity. A) Scheme illustrating different kind of Golgi fragmentation. Adapted from Valente and Colanzi, Front.Cell Dev. Biol. 2015. B) HeLa cells were Mock- and FAPP1 siRNA- treated for 72h and samples were processed for transmission electron microscopy analysis (TEM). The Golgi Complex in FAPP1 KD cells is more fragmented than the control, but does not exhibit any sign of vesiculation, nor loss of ci-trans polarity.

3.7 Golgi fragmentation in FAPP1 KD cells is a consequence of PI4P homeostasis unbalance

To understand whether the process underlying the Golgi fragmentation observed upon FAPP1 depletion is a direct consequence to the increase of PI4P level at the Golgi or not, I simultaneously down-regulated FAPP1 and the Golgi most abundant PI4-Kinase, PI4KIIIβ, which is the major responsible kinase for PI4P production at the GC. As shown in Fig. 3.23, the effect of FAPP1 depletion on GC fragmentation is rescued by the double KD of FAPP1 and PI4KIIIβ.
Figure 3.23 Golgi fragmentation in FAPP1 KD cells depends on PI4P increase at the GC. A) HeLa cells were treated with Mock or the indicated siRNAs for 72h and stained with the Golgi marker GM130. The images show that the Golgi fragmentation induced by FAPP1 KD is rescued by the simultaneous depletion of PI4KIIIβ. B) Quantification of Golgi particles measured by ImageJ software. ANOVA was calculated with GraphPad software. ****P-value < 0.0001. ns = not significant. C) Quantification of the ratio between PI4P and Golgin-97 fluorescence intensity at the GC (mean ± std.dev), expressed as percentage of Mock treated cells. The graph shows that PI4KIIIβ KD was efficient in reducing PI4P levels at the GC. D) Western blot analysis shows the reduction of interfered proteins level after siRNA treatment. 40μg of total lysate from each sample were separated by SDS-PAGE (10%) gel. The asterisk indicates a non-specific band recognized by FAPP1 antibody.
As a parallel approach, I over-expressed the 4-phosphatase SAC1 version that cannot be retrieved in the ER because of a mutation in its COP-I binding site and is constitutively localized at the Golgi (SAC1-K2A) (Blagoveshchenskaya et al., 2008). GFP-SAC1-K2A, as expected, was very efficient in lowering PI4P levels at the GC (Fig. 3.24 B). The reduction in PI4P levels caused by SAC1 overexpression, as observed with PI4KIIIß depletion, reverted the Golgi fragmentation induced by the knock down of FAPP1 (Fig. 3.24).
Figure 3.24 Golgi fragmentation in FAPP1 KD cells depends on PI4P increase at the GC. A) HeLa cells Mock or FAPP1 KD were transfected with the GFP-SAC1-K2A mutant and, after 22-24h were stained with the Golgi marker GM130. SAC1 over-expressing cells (white asterisks) clearly show a more compact Golgi structure, compared to the surrounding, non-transfected cells. B) Quantification of Golgi particles with ImageJ software. Data represent the number of Golgi spots per cell in Mock and FAPP1 KD at steady state (grey distributions) and under GFP-SAC1-K2 overexpression (red distributions). ANOVA was calculated with GraphPad software. ****P-value < 0.0001 ns= not significant. C) PI4P levels quantification shows that GFP-SAC1-K2 was effective in reducing PI4P levels at the GC.

All together these observations indicate that the GC fragmentation observed in FAPP1 KD cells is dependent on the increase in PI4P level.

3.8 Golgi fragmentation induced by FAPP1 KD is mediated by GOLPH3

GOLPH3 regulates Golgi morphology by simultaneously binding the GC (via PI4P) and the F-actin cytoskeleton (via the unconventional myosin MYO18A) (Dippold et al., 2009). Perturbing GOLPH3 levels, indeed, disrupts Golgi architecture, with GOLPH3 over-expression resulting in Golgi fragmentation, and GOLPH3 silencing leading to Golgi collapse (Dippold et al., 2009; Ng et al., 2013). The evidence that FAPP1 depletion induces Golgi fragmentation as a consequence of PI4P increase (par. 3.7) suggests the hypothesis that GOLPH3 may be “hyper-activated” in this system. To test whether FAPP1 KD-dependent Golgi fragmentation was mediated by GOLPH3 activity, I performed a double KD of FAPP1 and GOLPH3 in HeLa cells and I analysed the Golgi morphology by immunofluorescence. Surprisingly, GOLPH3 depletion counteracts the Golgi fragmentation caused by FAPP1 depletion alone, resulting in a more compact GC morphology compared to FAPP1 KD cells (Fig 3.25 A and B).
This effect is better visualised by SR-SIM microscopy and its 3D reconstruction (3.25 C). This important result points out that elevated levels of PI4P at the GC hyper-activate the PI4P effector GOLPH3 in FAPP1-KD cells, leading, in turn, to Golgi fragmentation.
Figure 3.25 FAPP1 KD induced Golgi fragmentation is mediated by GOLPH3. A) HeLa cells were Mock-treated or treated with the indicated siRNAs for 72h and stained with the Golgi marker Golgin 97 (G-97). The Golgi fragmentation induced by FAPP1 KD is rescued by the simultaneous depletion of GOLPH3 B) Quantification of Golgi particles measured by Imagej software. ANOVA was calculated with GraphPad software. ****P-value < 0.0001. ns= not significant. C) Super-resolution images with 3D reconstruction of the Golgi structure in Mock and the indicated siRNAs- treated cells. D) Western blot analysis show the reduction of interfered protein level after siRNA treatment. The asterisk indicates a non-specific band recognized by FAPP1 antibody.

Interestingly, the effect of FAPP1 KD on Golgi complex morphology is similar to the effect of GOLPH3 over-expression in the same system (Fig. 3.26), confirming the hypothesis that FAPP1 KD induced Golgi fragmentation is caused by the enhancement of GOLPH3 function.
Figure 3.26 Golgi fragmentation induced by FAPP1 depletion and GOLPH3 over-expression are comparable. A) HeLa cells Mock or FAPP1 KD were transfected with a GFP-GOLPH3 construct and, after 22-24h were stained with the Golgi marker TGN46. GOLPH3 over-expression induces fragmentation of the Golgi, compared to the surrounding, non-transfected cells in control (Mock) but not in FAPP1 KD cells. B) Quantification of Golgi particles with
ImageJ software. Data represent the number of Golgi spots per cell in Mock and FAPP1 KD at steady state (grey distributions) and under GFP-GOLPH3 overexpression (red distributions). ANOVA was calculated with GraphPad software. ****P-value < 0.0001 ns= not significant.

3.9 FAPP1 KD does not affect GOLPH3 localization at the GC

In FAPP1-KD cells, the higher PI4P amount in the Golgi may lead to a strongest association of GOLPH3 to the GC. To test this hypothesis, I analysed the Golgi localization of GOLPH3 by immunofluorescence in both control and FAPP1 KD cells, using a specific antibody against the GOLPH3 protein. I decided to use the antibody at two different dilutions: the regular concentration (1:400) and at its 1:10 dilution (1:4000), in order to trite the antibody concentration to catch any difference in the protein localisation. Unfortunately, in the two tested conditions I did not observe any difference in GOLPH3 localization at the GC in FAPP1 KD compared to control cells (Fig. 3.27).
Figure 3.27 FAPP1 KD does not influence GOLPH3 localization at the GC. HeLa cells were Mock or FAPP1 siRNA treated for 72h and co-stained with anti GM130 and anti-GOLPH3 antibody at two different dilutions (indicated in the figure). No difference in GOLPH3 signal at the Golgi were observed in FAPP1 KD cells compared to the control.

GOLPH3 association to the Golgi is highly dynamic, with the protein rapidly moving between membrane and cytosolic pools (Tenorio et al., 2016). To monitor this incredibly dynamic association, I moved to a more quantitative approach. I performed a cytosol-membrane fractionation in control and FAPP1 KD conditions and I measured the GOLPH3 distribution in the two fractions. Fig. 3.28 shows that GOLPH3 partitioning between cytosol and membrane does not change in absence of FAPP1, confirming that FAPP1 depletion does not alter GOLPH3 association to the GC.
Figure 3.28 FAPP1 KD does not influence GOLPH3 localization at the GC. A) HeLa cells lysates (L) obtained from Mock and FAPP1 KD cells were used to prepare cytosolic (C) and membrane (M) fractions, with a procedure described in Methods. Equivalent amount of each fraction (30μg of proteins) was separated by SDS-PAGE (10%) and immuno-blotted with anti-GOLPH3 antibody. GOLPH3 is present in both cytosolic and membrane fractions as already described (Tenorio et al., 2016) and the distribution does not change in absence of FAPP1. Tubulin and VAPB were used as a control of a cytosolic and a membrane protein, respectively. B) Densiometric quantification of the immunoblot signal of GOLPH3.

From these experiments emerge that FAPP1 regulates GOLPH3 pathway, not influencing its Golgi localization, but just enhancing its activity at the GC.

3.10 GOLPH3 regulation mediated by FAPP1 and PI4P is important for its oncogenic activity

The GC fragmentation in FAPP1 KD cells is a proof that FAPP1 KD does hyper-activate GOLPH3. Thus, I tried to understand if FAPP1 could play a role in GOLPH3-driven oncogenesis.

3.10.1 FAPP1 KD increases cell migration hyper-activating the GOLPH3 pathway through PI4P.

The mechanism through which GOLPH3 promotes oncogenesis is not unequivocal but it has been demonstrated that the ability of GOLPH3 to drive cell migration and invasion depends on PI4P binding (Tokuda et al., 2014; Xing et al., 2016; Halberg et al., 2016). Thus, to test the hypothesis that FAPP1, regulating PI4P amount at GC, controls GOLPH3 oncogenic activity, I analyzed the role of FAPP1 in cell migration. To this aim, I
performed a wound-healing assay following the pipeline shown in Fig. 3.29 and I measured the extent of wound closure under depletion of FAPP1, GOLPH3 or both.

Interestingly, in a context in which GOLPH3 KD causes a reduction in cell migration (Fig. 3.30), FAPP1 depletion has an opposite effect, showing a significant increase in the extent of wound closure compared to control cells. This effect, however, is blunted by
the simultaneous depletion of GOLPH3, indicating that the increase in cell migration driven by FAPP1 KD is mediated by GOLPH3.
Figure 3.30 FAPP1 KD cells accelerate cell migration hyper-activating the GOLPH3 pathway.

A) A-549 cells were treated with Mock or the indicated siRNAs and grown to a confluent monolayer in 96 well (procedure illustrated in Methods and in Fig.3.29). After 72h, a wound was scratched with a 10μL plastic tip and cells were placed in the Operetta (Perkin Elmer) chamber at 37°C with 5% CO2. Phase contrast images were automatically acquired at two hours intervals for 24h and the migration area was quantified with ImageJ at every time-point. The figure shows images of 5 representative time-points for every condition as they appear after the acquisition (upper panels) and post processed with an ImageJ macro that identifies and calculates the area of the wound, here shown in white (lower panels). FAPP1 KD cells migrate faster than the control and this effect is abolished by simultaneously knocking down GOLPH3.

B) Quantification of the Wound Area. Values are mean ± st.dev of 6 replicates per condition, and are expressed as % of time 0 values.

A further proof that FAPP1 KD indirectly hyper-activates GOLPH3 pathway through the regulation of PI4P levels, inducing cell migration, comes from the evidence that FAPP1 acceleration in cell migration is rescued by the simultaneous depletion of the other component of GOLPH3 pathway, i.e. MYO18A, which has been shown to have a role in driving cell migration (Xing et al., 2016) (Fig 3.31).
Figure 3.31 FAPP1 KD cells accelerate cell migration hyper-activating the GOLPH3 pathway.

Quantification of the Wound Area in cells Mock treated or treated with the indicated siRNAs and processed as in Fig. 3.30. Values are mean ± st.dev of 6 replicates per condition, and are expressed as % of time 0 values. FAPP1-KD cells appear to be faster than the control in promoting the wound closure, but the simultaneous depletion with one member of the GOLPH3 pathway (GOLPH3 (Fig. 3.30) or MYO18A (Fig. 3.31)), counteracts this effect.

If the effect of FAPP1 depletion on cell migration is dependent on GOLPH3 hyper-activation, the ablation of the Golgi PI4P pool, necessary for GOLPH3 function, should decrease per se the cell migration phenotype in FAPP1 KD cells, mimicking the GOLPH3 down regulation. To test this hypothesis, I performed the wound healing assay in cells where I depleted the Golgi PI4P pool inhibiting the PI4KIIIβ activity both by siRNA treatment and functionally, using the inhibitor PIK93. Notably, FAPP1 acceleration in cell migration was abolished by PI4P reduction (Fig. 3.32).
Figure 3.32 FAPP1 KD-dependent increase in cell migration rate depends on PI4P regulation.

A) A-549 cells were treated with Mock or the indicated siRNAs and the wound healing assay was performed as in Fig. 3.30. Contextually to the wound scratching, Mock and FAPP1 KD cells were untreated or exposed to 50nM PIK93 until the end of the experiment. Phase contrast images were automatically acquired at two hours intervals for 24h and the migration area was quantified with ImageJ at every timepoint. The figure shows images of 2 representative timepoints for every condition as they appear after the acquisition (upper panels) and post processed with ImageJ (lower panels). B) Quantification of the Wound Area. Values are mean ± st.dev of 6 replicates per condition, and are expressed as % of time 0 values. C) Quantification of PI4P levels shows that both PI4KIIIβ KD and PIK-93 treatment were effective in reducing PI4P at the GC. Values of fluorescence intensity ratio between PI4P and the Golgi marker G-97 are calculated with ImageJ and expressed as % of Mock. ****P-value < 0.0001.

Importantly, immortalized MEFs isolated from FAPP1 KO mice are also faster in closing the wound compared to cells coming from WT mice (Fig. 3.33), confirming that the effect on cell migration can be ascribed to FAPP1 depletion and is not due to a non-specific effect of the siRNAs.
Figure 3.33 MEF from FAPP1 KO mice recapitulate the increase in cell migration observed with FAPP1 siRNAs. Quantification of the Wound Area in WT or FAPP1 KO MEFs processed as in Fig. 3.30-3.32. Values are mean ± st.dev of 10 replicates per condition, and are expressed as % of time 0 values. FAPP1 KO MEFs migrate faster than the WT.

3.10.2 FAPP1 KD increases cell invasion hyper-activating the GOLPH3 pathway through PI4P.

GOLPH3 oncogenic potential has been also linked to its ability to promote cell invasion, a key step for the metastatic cascade. To assess the role of FAPP1 in GOLPH3-driven cell invasion, I performed a transwell invasion assay, using Matrigel coated chambers (Biocoat), as illustrated in Fig. 3.34.

Figure 3.34 Schematic representation of the Matrigel coated chamber (Biocoat) used for the transwell invasion assay. The matrigel chamber is placed in a sterile well. Cells are seeded in the upper part of the chamber in serum free medium while the well is filled with serum completed medium. After 24h the cells that were able to invade the matrigel layer and reached the lower part of the chamber were fixed, stained and counted under a phase contrast microscope as described in methods.
GOLPH3 depletion resulted in a reduction of invading cells through the Matrigel compared to control cells (Tokuda et al., 2014 and Fig 3.35). Interestingly, FAPP1 depletion increases the number of invading cells, and this effect is abolished by the simultaneous KD of GOLPH3 or PI4KIIIβ (Fig. 3.35).

Figure 3.35 FAPP1 KD promotes cell invasion by hyper-activating the GOLPH3 pathway through PI4P. A) Mock treated or treated with the indicated siRNAs A-549 cells after 72h were trypsinized and plated in equal number in the Biocoat Matrigel chambers as illustrated in Fig. 3.34. After 24h cells that invaded the matrigel layer were fixed and stained with Toluidine Blue.
procedure illustrated in Methods) and imaged with Zeiss Apotome microscope. The images show one representative field for each condition. FAPP1 KD cells display an increase in the number of invading cells compared to control, but this effect is rescued by the simultaneous KD of both PI4KIIIβ and GOLPH3. B) Quantification of the invading cells. Data represent the number of cells that invaded the Matrigel layer in each field. 3 wells per condition were considered and 12 random fields were acquired for each well. ANOVA was calculated with the Graph Pad software. ****P-value < 0.0001. ***P-value < 0.001. C) Quantification of PI4P levels shows that PI4KIIIβ KD was effective in reducing PI4P at the GC.

Collectively, these results (par. 3.10.1 and 3.10.2) indicate that FAPP1 controls GOLPH3 activity in promoting cell migration and invasion by regulating PI4P at the Golgi Complex.

3.11 High content screening approach to identify pathways responsible for FAPP1 and GOLPH3 co-regulation

Since FAPP1 and GOLPH3 are co-expressed genes (see par. 3.5), I tested the hypothesis that the two genes share one or more common transcription factors. Thus, I decided to run a high content screening approach aimed to identify eventual common regulators of FAPP1 and GOLPH3 expression.

3.11.1 Experimental workflow and primary screening

To identify common transcriptional regulators of FAPP1 and GOLPH3 proteins, I transfected HeLa cells with a siRNA library of 971 transcription factors extrapolated from a whole drugable genome siRNA library to see if one or more of them were able to cause a reduction in both FAPP1 and GOLPH3 expression. To run the high content screening I followed the workflow illustrated in Fig. 3.36. As primary assay, I choose to
monitor FAPP1 and GOLPH3 levels by immunofluorescence analysis using a specific antibody for FAPP1 and GOLPH3 and a Golgi marker (GM130). A dedicated script was developed to quantify FAPP1 and GOLPH3 fluorescence on the Golgi. To check the specificity of FAPP1 and GOLPH3 detection on the Golgi, I used cells transfected with siRNAs targeting FAPP1 and GOLPH3 respectively, which show very reduced Golgi staining of the two target proteins. In addition, to validate the assay sensitivity I performed a dose response of both siRNA concentration and antibody dilutions, for the two proteins, FAPP1 and GOLPH3, and I choose the optimal siRNA and antibody concentration to use in the screening (Fig 3.36). Images were acquired using confocal automated microscopy Opera system (high content system; Perkin-Elmer).
Figure 3.36 High Content Screening workflow.
For each transcription factor corresponding to specific siRNA, I obtained as output a value comprised between 0 and 1, where 0 is the mean of positive control values (FAPP1 or GOLPH3 siRNA treated cells), and 1 is the mean of negative control values (Mock treated cells), represented in Fig. 3.37 as a black or a yellow dashed line, respectively. All the values from 0 to 0.6, which resulted to be statistically significant were considered positive hits and are listed in Fig. 3.38. SiRNAs that selectively down-regulated FAPP1 or GOLPH3 had, as expected, the highest scores, while few other siRNAs (red dots in Fig. 3.37) were able to simultaneously decrease FAPP1 and GOLPH3 signal, although with a lower score.

I decided to extend the validation step also to other hits, since the threshold might be too stringent and arbitrary. First of all I choose the 10 best hits exclusive for FAPP1 or GOLPH3 down-regulation. In addition to these hits, I included also those siRNAs that, from the gene ontology analysis, resulted to be part of the same pathways, prompted by the rationale that FAPP1 and GOLPH3 may be co-regulated in response to a common biological condition, rather than transcribed by the same TF. With these criteria, I re-tested 80 siRNAs from the first screening, divided as shown in Fig. 3.37
Figure 3.37 High content screening output. A) HeLa cells were reverse-transfected in 384-well as described in methods. After 72h, cells were immunostained with FAPP1 or GOLPH3 antibodies. A dedicated script was used to calculate the Golgi intensity values of FAPP1 (left plot) and GOLPH3 (right plot) in cells treated with siRNAs from the transcription factors library. A score (y-axis) is assigned to each value, considering the positive control (FAPP1 or GOLPH3 siRNA) as 0 (black dashed line), and the negative control (Mock treated cells) as 1 (yellow dashed line). siRNA with a score lower than 0.6 were considered hits and are shown in the plots. Values represent mean ± standard deviation of values in triplicates. The hits with the
strongest score (dots close to the black dashed line) were exclusive for FAPP1 or GOLPH3, while the siRNA that simultaneously downregulated FAPP1 and GOLPH3 exhibit a lower score (red dots). B) Scheme showing hits selected for the validation.

3.11.2 Best hits high-throughput validation

The results of the first high-throughput siRNA validation confirmed that the strongest hits for FAPP1 did not have the same effect on GOLPH3 and viceversa (i.e. full and dashed green circles in Fig. 3.38) while the siRNAs that showed a common effect on FAPP1 and GOLPH3 down-regulation had a much lower score (black circles in Fig. 3.38). The evidence that the best hits are exclusive for FAPP1 or GOLPH3 indicates that, with this approach, it was not possible to identify a strong, common regulator for the two proteins.
Figure 3.38 High Content Screening Hits validation. 80 Hits from the High content screening were selected as indicated in Fig. 3.37 and underwent a second screening for the validation. HeLa cells were reverse-transfected in 384-well as described in methods. After 72h, cells were immunostained with FAPP1 or GOLPH3 antibody. The plot shows the Golgi intensity values of FAPP1 (blue dots) and GOLPH3 (red dots) in cells treated with each of the 80 siRNAs (listed on x-axis). A score (y-axis) is assigned to each value, considering the positive control (FAPP1 or GOLPH3 siRNA) as 0, and the negative control (Mock treated cells) as 1. Values are in triplicate and mean ± standard deviation are shown. The strongest hits for FAPP1 (green circles) did not show a simultaneous effect on GOLPH3 level (green dashed circles) and viceversa. 10 siRNAs that reduced the levels of both FAPP1 and GOLPH3 (black circles) were considered positive hits (listed in the box) and chosen for the secondary assay validation.

I choose 10 genes showing a partial reduction of both FAPP1 and GOLPH3 staining at the Golgi for the low-throughput validation (secondary assay).

3.11.3 Best hits low-throughput validation: secondary assay
To validate the results obtained from the siRNA-based screening, Western blot and Real-time analyses were performed on cells treated with the specific siRNA hits listed in fig 3.38. Although some of the siRNAs used were able to down-regulate FAPP1, they failed to have any effect on GOLPH3 levels (Fig. 3.39). In conclusion, these validation studies confirmed the results obtained by immunofluorescence, as none of the identified hits show a convincing simultaneous depletion of FAPP1 and GOLPH3.
Figure 3.39 High Content Screening common hits between FAPP1 and GOLPH3: low-throughput validation. A) Lysates from HeLa cells transfected with the indicated siRNAs for 72h were separated by SDS-PAGE (12%) and immunoblotted with FAPP1 and GOLPH3 antibodies. B) FAPP1 and GOLPH3 levels after the treatment with the indicated siRNAs were monitored by Real time PCR and expressed as percentage of Mock treated cells (black dashed line). None of the siRNA tested was able to simultaneously reduce FAPP1 and GOLPH3 levels.
SiRNAs that came out from the HCS as exclusive regulators of FAPP1 or GOLPH3 (e.g. green circles in Fig. 3.38) were effective in reducing their protein levels, as a control of the reliability of the screening (Fig. 3.40).

**Figure 3.40 High Content Screening exclusive hits for FAPP1 and GOLPH3: low-throughput validation.** Lysates from HeLa cells transfected with the indicated siRNAs for 72h were separated by SDS-PAGE (12%) and immunoblotted with FAPP1 and GOLPH3 antibodies. siRNAs that in the primary screening specifically down-regulated FAPP1 or GOLPH3 are indicated with blue and red asterisks, respectively.

### 3.11.4 Best hits effect on PI4P levels

Despite the lack of a common regulator of FAPP1 and GOLPH3 expression, almost all the siRNAs from the validation analysis confirmed to reduce FAPP1 and GOLPH3 localization at the Golgi also on coverslips (Fig 3.41).
Figure 3.41 Hits from HCS efficiently reduce FAPP1 and GOLPH3 fluorescence signal at the GC. A-B) HeLa cells were interfered with the indicated siRNAs for 72h and immunostained with FAPP1 or GOLPH3 antibodies and a Golgi marker (GM130). The fluorescence intensity in the Golgi area of FAPP1 (A) or GOLPH3 (B) was quantified with the ImageJ software. Values are
shown as mean ± range (min to max) of ~150 cells. C-D) Examples of FAPP1(C) and GOLPH3 (D) Golgi signal reduction under the treatment with the indicated siRNAs.

This finding prompted me to investigate if FAPP1 and GOLPH3 reduction at the GC was mediated by changes in PI4P levels. As shown in Fig. 3.42, some of the tested siRNA did not alter PI4P levels at the Golgi complex, and, so, they probably induce FAPP1 and GOLPH3 displacement by siRNA off-targeting and/or by unknown mechanisms (e.g. alteration of Golgi morphology or impairment of ARF1 functions, also responsible at least for FAPP1 localization at the Golgi (Godi et. al, 2004)). Interestingly, 5 out of the 10 siRNAs causes a reduction in PI4P levels, which is compatible with a detachment of FAPP1 and GOLPH3 from the GC (Fig. 3.42).
Figure 3.42 High Content Screening common hits between FAPP1 and GOLPH3: effect on PI4P levels. A) PI4P and G9-97 immunostaining in cells interfered with siRNAs coming from the HCS. B) The fluorescence intensity ratio between PI4P and G-97 was calculated with ImageJ and expressed as percentage of Mock. Five out of ten siRNA tested induce a reduction in PI4P levels at the GC compared to the mean of Mock treated cells (black dashed line).

Two of these proteins are correlated with the regulation of Golgi function: XBP1, which is a master regulator of the Unfolded Protein Response (UPR) and SREBP1, a membrane bound TF, that, in response to sterol levels activates a transcriptional programme aimed to increase cholesterogenesis and lipogenesis (Glimcher, 2010; Eberlé et al., 2004). Interestingly, the two proteins converge in a common pathway, which is the Unfolded Protein Response (UPR). Many reports indicate that XBP1, besides its role in transcribing UPR gene targets, also controls lipid synthesis, via both direct transcription of lipogenic genes (Lee et al., 2008) or by binding SREBP promoter and inducing lipogenesis under insulin treatment (Ning et al., 2011). On the other hand, SREBP has been found to be activated by ER-stress (Kammoun et al., 2009), thus confirming a crosstalk between the two pathways.

To verify if UPR could control PI4P levels, I induced ER-stress in HeLa cells both with Thapsigargin and MG132 and I measured the PI4P levels at the Golgi complex. As shown in Fig. 3.43, the two treatments both induced a significant increase of the PI4P levels.
Figure 3.43 ER-stress modulate PI4P levels at the Golgi. A) HeLa cells were stimulated with MG132 or Thapsigargin to induce ER-stress and PI4P levels at the GC were monitored by immunofluorescence. B) PI4P/G-G97 fluorescence intensity ratio expressed as percentage of untreated cells. PI4P levels increase under ER-stress conditions.

This evidence is coherent with an UPR dependent modulation of PI4P. As a matter of fact, PI4P levels increase under UPR induction (MG132 and Thapsigargin), while
decrease when the UPR is blunted or non-functional (XBP1- SREBP1 KD, see Fig. 3.42). Thus, starting from 900 transcription factors, this HCS approach allowed me to identify an unexpected and novel connection between UPR and PI4P homeostasis.

3.12 Other functions of FAPP1

To explore other functions of FAPP1, I analyzed the co-expression module containing FAPP1 (see par. 3.5), prompted by the rationale that the co-expression of a group of genes could imply their involvement in a common pathway. To this aim I assigned functional categories (five in total) to each gene of the module and I calculated the gene enrichment in each category expressed as a percentage of total module genes number (Fig. 3.44).

![Functional classification of the genes belonging to the FAPP1 co-expression module](image)

Figure 3.44 Functional classification of the genes belonging to the FAPP1 co-expression module. One or more functions were assigned to each gene of the co-expression module.
The number of genes enriched for each function was determined and expressed as a percentage of total genes number of the module.

The results highlight important evidences:

- In the module there are some Golgi genes, as expected (FAPP1 and GOLPH3 contribute to enrich this category).

- FAPP1 is co-expressed with phosphoinositide metabolizing genes such as the 5-phosphatase MTMR2 and the PI3-Kinase PIP4K2A, enforcing its role as a PI4P regulator. Moreover, specific PI4P related genes are present, such as GOLPH3 but also PITPNB, shown to regulate PI4P at the Golgi via PI transport (Carvou et al., 2010).

- The module contains genes important for migration events and cancer progression, confirming the possible role of FAPP1 in this pathway. Interestingly, some of them are genes involved in membrane trafficking of integrin and other pro-migrating factors, suggestive of a possible mechanism of action that is worthy to be further investigated.

- Surprisingly, FAPP1 is co-expressed with many genes enriched in endosome/endocytosis and autophagy/lysosomes pathways, which is quite unexpected for a Golgi protein.

In the light of these evidences I decided to test a possible role of FAPP1 in endocytosis and autophagy.
3.12.1 FAPP1 does not show colocalization with endosomal markers and is not involved in endosome to Golgi trafficking.

Prompted by the co-expression of FAPP1 with endo-lysosomal genes, I analyzed the FAPP1 localization in the endo-lysosomal compartments. This is plausible since a pool of PI4P is also present on endosomes (Dong et al., 2016). In steady state conditions, FAPP1 is localized at the TGN (see par.3.1, Fig. 3.1-3.3) and no colocalization with early (EEA1) or late (Rab-7/LAMP1) endosomal markers was observed (Fig. 3.45).
Figure 3.45 FAPP1 is not localized to the endo-lysosomal compartment.  

A) HeLa cells were stained with antibodies against FAPP1 and early or late endosomes markers.  

B) The colocalization of FAPP1 with EEA1 and TGN was assessed with ImageJ . Mean ± standard deviation values of ~ 100 cells were calculated and plotted as percentage of the total FAPP1.
If a protein cycles between multiple compartments it is possible that its colocalization with a subcellular compartment is transient and, then, difficult to catch at steady state conditions. Thus, to visualize an eventual FAPP1 transient association to these organelles, I exposed the cells to a 18°C temperature block or treatment with NH4Cl2 to delay or block endosomes acidification, respectively, and expand the endo-lysosomal compartment (Snyder et al., 2006). FAPP1 does not change its Golgi localization upon 18°C temperature block, nor after NH4Cl2 treatment, a condition in which other proteins (i.e. TGN46) are visualized on LAMP1 positive structures (Fig. 3.46).
Figure 3.46 FAPP1 is not localized to the endo-lysosomal compartment. A) FAPP1 colocalization with the endosomal marker EEA1 was assessed in HeLa cells left untreated or exposed to 18°C for two hours. B) HeLa cells were treated with 50mM of NH4Cl and immunostained with the indicated antibodies. While TGN46 shows a relocalization to LAMP1 positive structures, FAPP1 does not show any overlap with the endo-lysosomal compartment (insets).
Although FAPP1 does not localizes to the endosomes, it could still regulate endosome-to-Golgi trafficking staying at the TGN. Indeed, PI4P at the Golgi has been demonstrated to be required for efficient retromer-mediated trafficking, in particular in the phase of cargo release at the TGN (Niu et al., 2013). Thus, I followed the internalization and trafficking of the bacterial toxin Shiga-toxin, which is internalized via multiple endocytic pathways and then retrogradely transported to the Golgi and ER (Mallard et al., 1998). I used it to monitor the retrograde endosome-to-Golgi trafficking in FAPP1 KD cells compared to control cells. Cells were incubated with Shiga-Txn at 4°C, to allow the binding (Time 0') and then shifted to the temperature of 37°C to allow internalization and to follow its arrival to the TGN. As shown in Fig.3.47, FAPP1 depletion does not alter Shiga Toxin arrive at the TGN, measured as colocalization value between Shiga-Txn and the TGN46 marker, that is comparable between FAPP1 and Mock treated cells at different time-points.
Figure 3.47 Shiga Toxin trafficking is not altered in FAPP1 KD cells. A) Mock and FAPP1 KD cells were loaded with Alexa-568-conjugated Shiga Toxin for 30’ at 4°C (Time 0’), shifted at...
37°C and stained with the indicated antibodies after different times of internalization. 30' is shown as a representative timepoint. B) Manders’ colocalization coefficient of Shiga toxin with TGN46 was measured for each timepoint with ImageJ and plotted as mean ± standard deviations of ~100 cells per condition. Shiga-Txn arrival at the TGN is not altered by FAPP1 depletion.

These data indicate that FAPP1 does not seem to have a role in endocytosis or in the retromer-mediated trafficking and that, probably, the group of genes enriched the endocytosis inside the co-expression module does not involve a direct co-expression with FAPP1.

3.12.2 FAPP1 KD cells show increased autophagosomes number

Intrigued by the hypothesis that a Golgi protein may have a role in autophagy, I silenced FAPP1 in HeLa cells and monitored LC3 levels. Notably, FAPP1 depletion caused a marked increase in LC3 spots followed with a specific antibody by immunofluorescence and in LC3II levels observed by western blot (Fig. 3.48). The increase in LC3 spots caused by FAPP1 depletion was also confirmed in HK2 cells, a model of proximal tubule renal cells (Fig. 3.48 A, lower panel).
Figure 3.48 LC3 is increased in FAPP1 KD cells. A) HeLa (upper panel) or HK2 (lower panel) cells were treated with Mock or the FAPP1 siRNAs for 72h and stained with the indicated antibodies. B) Boxplot shows mean and distribution of the number of LC3 spots per cell quantified with ImageJ. Unpaired t-test was calculated with the GraphPad software. ****p-value < 0.0001 C) Western blot analysis shows the reduction of FAPP1 protein level after siRNA treatment.

The increase in LC3 positive structures is not due to a siRNA off-targeting effect, because it is rescued by the re-expression of FAPP1 (Fig. 3.49).
Figure 3.49 Autophagy increase in FAPP1 KD cells is not due to siRNAs off-targeting effect. A) HeLa cells were Mock of FAPP1 siRNA-treated for 48h and transfected with GFP-FAPP1. After additional 24h cells were immunostained with antibodies against LC3 and LAMP1 (not shown). FAPP1 re-expression is able to reduce LC3 spots in FAPP1 KD cells (white asterisks). B) LC3 spots per cell (dots) were quantified with ImageJ and shown as mean and distribution (boxplots). ANOVA was calculated with the GraphPad software. ****P-value < 0.0001 ns= not significant.
To discriminate whether the high number of autophagosomes in FAPP1 KD derives from increased biogenesis or decreased degradation, I treated cells with the vacuolar H+ATPase inhibitor Bafilomycin-A1 (Baf-A1). In FAPP1 KD cells, the number of LC3 structures, already high in untreated cells, further increases upon Baf-A1 treatment, although to a lesser extent compared to the effect of Baf-A1 treatment in control cells (Fig. 3.50).

**Figure 3.50** FAPP1 KD cells show both increased production and defective degradation of autophagosomes. A) HK2 cells Mock or FAPP1 siRNAs treated for 72h were left untreated (-) or exposed to 100 nM Bafilomycin for 3h at 37°C (+) and immunostained with the indicated
antibodies. B) Mean ± SEM of LC3 spots per cell quantified with ImageJ. ANOVA was calculated with the GraphPad software. **P-value < 0.01. In FAPP1 KD cells (red bars) LC3 spots further increase in presence of Bafilomycin, although the difference with the untreated cells is lower than in Mock (blue bars).

These results indicate that FAPP1 KD cells display increased number of autophagosomes as a result of both increased biogenesis and defective degradation.

3.12.3 LC3 increase in FAPP1 KD cells is not due to mTOR signaling inhibition, nor to FAPP1 re-localization on autophagosomes.

To further investigate on the possibility that FAPP1 depletion may increase LC3 levels by inhibiting mTOR signaling, I interfered HeLa cells for FAPP1 and I analysed the phosphorylation status of an mTOR target (ULK1) and of mTOR itself. Western blot analyses show that mTOR signaling is not inhibited upon FAPP1 depletion (Fig 3.51).
**Figure 3.51 mTOR signalling is not altered by FAPP1 depletion.** HeLa Mock or FAPP1 KD cells were left untreated or starved in HBSS medium for 3 hours. Cells were lysed and 40μg of each sample were separated by SDS PAGE (10%) and immunoblotted with the indicated antibodies. Phosphorylation of mTOR targets monitored with specific antibodies is reduced under starvation as expected, but no significant differences between FAPP1 KD and control cells are present.

Another possibility is that FAPP1 may re-localize to the autophagosomes under autophagy induction. Fig. 3.52 A shows that, after 3h of starvation in HBSS medium, FAPP1 is still localized at the Golgi. Moreover, although HBSS treatment was effective in inducing LC3 spots, FAPP1 is not present on those structures (Fig. 3.52 B).
Figure 3.52 **FAPP1 Golgi localization in unchanged under autophagy induction.**

A) HeLa cells were left untreated or starved with HBSS medium for 3 hours and immunostained with the indicated antibodies. B) HeLa cells were transfected with GFP-FAPP1 for 22H, starved in HBSS medium for 3H and stained with an LC3 antibody. Under starvation FAPP1 (both endogenous and overexpressed) preserves its Golgi localization and does not relocalize to LC3 positive structures.
3.12.4 Autophagosomes increase in FAPP1 KD cells depends on the Golgi pool of PI4P

The evidence that FAPP1 does not change its localization under autophagy induction suggests that FAPP1 regulates autophagy exerting its function at the Golgi. To see if LC3 accumulation in FAPP1 KD cells was secondary to its function in PI4P regulation at the GC, I looked at LC3 structures in HeLa cells depleted for both FAPP1 and PI4KIIIβ (Fig 3.53).

Interestingly, the simultaneous depletion of PI4KIIIβ rescued the autophagy defect observed in FAPP1 KD cells, indicating that FAPP1 regulates autophagy as a consequence of its role as a PI4P sensor at the Golgi (Fig 3.53). This was particularly interesting in the light of the known role of Golgi complex ion autophagy (see below).
Figure 3.53 Increased LC3 in FAPP1 KD cells is rescued by the simultaneous depletion of PI4KIIIβ. HeLa cells were treated with Mock or the indicated siRNAs for 72h and stained with anti-LC3 and anti-LAMP1 antibodies. The depletion of PI4KIIIβ counteracts the increase in LC3 structures due to FAPP1 depletion.

3.12.5 ATG9 trafficking is unbalanced in FAPP1 KD cells

Searching for a link between Golgi and autophagy, I decided to follow the distribution and trafficking of ATG9, the only trans-membrane autophagy protein described, which is mainly localized at the TGN and, under autophagy induction, relocalizes to
peripheral structures that dynamically interact with phagophores and autophagosomes and contribute to autophagosomes biogenesis (Young et al., 2006). As expected, in control cells ATG9 is localized at the TGN while, under autophagy induction, ATG9 signal at the Golgi is reduced in favor of an extra-Golgi component (Fig. 3.54). Surprisingly, in FAPP1 KD cells ATG9 is already redistributed in steady state conditions at the same extent of Torin treated cells, and the double KD of FAPP1 with PI4KIIIβ reverts this phenomenon (Fig. 3.54). Notably, PI4KIIIβ KD cells show an opposite effect on ATG9 localization, that is retained in the TGN also under autophagy induction, similarly to what happens by depleting ULK1, extensively demonstrated to be required for ATG9 trafficking (Fig. 3.54).
Figure 3.54 ATG9 trafficking is increased in FAPP1 KD cells. A) HeLa cells interfered with Mock or FAPP1 siRNAs for 72h were left untreated or exposed to 1μM Torin for 2 hours and stained
with anti-ATG9 and anti-TGN46 antibodies. FAPP1 KD cells show an extra-Golgi component of ATG9 already in steady state conditions that disappears under simultaneous KD of PI4KIIIβ. ULK1 is known to be required for ATG9 dissociation from the Golgi under autophagy induction and was used as a control of the assay. **B)** The ratio between ATG9 fluorescence intensity in the Golgi and in the cytosol area was quantified with ImageJ and expressed as a percentage of Mock cells at steady state (red dashed line). ANOVA was calculated with the GraphPad software. ****P-value < 0.0001 ns=not significant.

These data suggest that FAPP1 depletion, by increasing PI4P levels at the Golgi, boosts ATG9 trafficking, leading, in turn, to increased autophagosomes production.
FAPP1 is a PI4P sensor acting at ER-TGN membrane contact sites (ERToGoCS)

PI4P, together with PI(4,5)P2 is the most abundant phosphoinositide in the cell. Although it is present on the plasma membrane and has been detected on many endomembrane compartments, PI4P is enriched at the Golgi complex, particularly at the TGN, where its steady state levels result from the precise activity of two kinases (PI4KIIIβ and PI4KIλα) and of one 4-phosphatase, Sac1. Initially considered as a mere precursor for highly phosphorylated PI species, it is now clear that PI4P is active in its own right, and it has been shown to coordinate a large variety of functions, including membrane trafficking, autophagy, sphingolipid metabolism, cell migration and many others (see Chapter 1). Given its pivotal role in regulating cellular functions, PI4P levels need to be tightly controlled in time and space. Multiple mechanisms co-exist in the cell to control the localization and/or the activity of the PI4P regulatory enzymes. However, during the last years, a growing number of reports indicate that the control of PI4P homeostasis also relies on sensors, identified as those PI4P effector proteins that not only bind PI4P but are also able to control its levels by modulating the activity of PI4P metabolizing enzymes (See par. 1.5.3.4.3). One of the first PI4P effectors identified was the four-phosphate adaptor protein 1 (FAPP1), which, by virtue of its high degree of homology with FAPP2, is included in the lipid transfer protein class of PI4P effectors (see par. 1.7). Intriguingly, and in spite of its classification, FAPP1 does not possess any canonical lipid transfer domain and its function has remained elusive so far.
In this thesis work I showed that FAPP1 acts as a PI4P sensor at the Golgi complex. FAPP1 depletion causes a marked increase in Golgi PI4P amount, both in cellular and animal models (section 3.2). FAPP1 regulates PI4P homeostasis at the level of ER-TGN membrane contact sites (ERTGoCS), where it binds both VAP proteins, which are required to physically establish the contact site, and the 4-phosphatase Sac1. In vitro malachite assays demonstrated that FAPP1 enhances Sac1 activity, and this occurs to a much higher extent when Sac1 and PI4P are placed on distinct liposomes (in the trans conformation) compared to a condition in which Sac1 and PI4P are on the same liposomes (in the cis conformation) (section 3.3). It seems plausible that this occurs also in vivo, since ERTGoCS are close enough (on average 12nm, data not shown) to allow the TGN-localized FAPP1 to bind Sac1 in the ER and stimulate its activity in trans to dephosphorylate PI4P across the TGN membranes. Indeed, I observed that the stabilization of ERTGoCS in HeLa cells with a rapamycin-inducible system resulted in a massive depletion of the Golgi PI4P pool, already occurring after 2 minutes of treatment (section 3.3). However, when FAPP1 is absent, the reduction of PI4P under ERTGoCS stabilization is significantly slower, demonstrating that FAPP1 is required for Sac1 to efficiently dephosphorylate its substrate PI4P at the level of ERTGoCS. Another PI4P effector, OSBP1, had been previously shown to regulate PI4P homeostasis at the level of ERTGoCS, where PI4P is exchanged for cholesterol and transported to the ER to be dephosphorylated in cis by Sac1 (Mesmin et al., 2013). Interestingly, also in condition of VAP depletion, where OSBP1 loses its ER anchor and cannot stimulate Sac1 activity in cis at the level of ER-TGN contact sites, FAPP1 is required for PI4P consumption under ERTGoCS stabilization, demonstrating that FAPP1 stimulates Sac1 activity in trans also in vivo. From these observations, at least two distinct mechanisms to control PI4P levels at the level of ERTGoCS coexist: one relies on OSBP1 that, via
exchanging PI4P for cholesterol, regulates PI4P hydrolysis in cis, and this pool does not impact on total PI4P levels at least under steady state conditions; the other mechanism is based on FAPP1, which directs and stabilizes Sac1 activity in trans towards those sites showing high levels of PI4P. Given its low affinity for PI4P, FAPP1 acts as a pure sensor controlling a pool of PI4P that significantly contributes to the total PI4P levels at the TGN.

**FAPP1 controls the activity of the oncogene GOLPH3 by regulating PI4P at the Golgi**

While the impact of PI4P depletion on cell physiology has been extensively exploited, much less is known about the consequences of increasing the PI4P levels in the Golgi complex. This is mainly due to the lack of negative PI4P regulators in mammals, so that all knowledge on the effects of an increase in PI4P levels relies on Sac1 KD studies. However, Sac1 is the only PI4-phosphatase and its depletion impacts on PI4P homeostasis in every sub-cellular compartment, making it difficult to discriminate the effects of the Golgi PI4P pool on the global cellular phenotype, which is, however, severely compromised (Liu et al., 2008).

FAPP1, acting as a negative regulator of PI4P at the TGN, represents a powerful tool to understand the global consequences of increasing PI4P levels at the Golgi and, thus, why it would be important for the cell to keep the Golgi PI4P levels low.

I found that FAPP1 depletion, by increasing PI4P levels, hyper-activates the PI4P binding protein GOLPH3 at the Golgi complex. GOLPH3 is required for the maintenance of Golgi architecture since it bridges the Golgi (via PI4P binding) with the actin cytoskeleton (via binding of the unconventional myosin MYO18A) (Dippold et al., 2009). More importantly, GOLPH3 is an oncogene, hyper-activated in a variety of solid tumors (see par. 1.6.4.1). In cellular models, GOLPH3 overexpression increases cell
migration and invasion rate, which are, conversely, decreased by GOLPH3 down-regulation (Xing et al., 2016; Halberg et al., 2016; Tokuda et al., 2014). I found that FAPP1 depletion in a cellular model of non-small cell lung cancer (A549) increases the cell migration rate compared to control cells (Section 3.10.1). The effect of FAPP1 on cell migration is secondary to its role in the regulation of PI4P, since it is abolished by inhibiting the expression or the activity of the main Golgi PI4-kinase (PI4KIIIβ). Similarly, the FAPP1 KD-induced acceleration in cell migration is rescued upon the simultaneous depletion of GOLPH3. A comparable effect was observed looking at cell invasion, monitored as the ability of the cells to degrade a matrigel layer. Also in this case, FAPP1-KD cells showed an increased invasive potential compared to the control (Section 3.10.2), and this effect is rescued by the simultaneous inhibition of PI4P or GOLPH3. Together, these results indicate that the pool of PI4P regulated by FAPP1 is important to control the activity of the oncogene GOLPH3 at the Golgi complex. Interestingly, FAPP1 depletion induces a PI4P- and GOLPH3-dependent Golgi fragmentation defect that is comparable to the one observed under GOLPH3 overexpression, thus confirming the hyper-activation of GOLPH3 in this system (sections 3.7 and 3.8). GOLPH3 overexpression has been proposed to drive cancer by multiple mechanisms, but the one that best fits with its role at the Golgi complex is the ability to stimulate the secretion of pro-migration and pro-invasion factors (see par. 1.6.4.1). It will be important to assess if FAPP1 acts through a similar mechanism.

In addition, bioinformatics co-expression analysis performed on deposited RNAseq data highlighted a surprisingly high degree of co-expression between FAPP1 and GOLPH3 in a variety of normal, but not tumor tissues. The loss of co-expression in tumor conditions suggests that FAPP1 can act as an oncosuppressor, whose expression levels change concomitantly with those of the oncogene GOLPH3, to tightly control its
activity. If FAPP1 levels are lowered and the correlation is lost, GOLPH3 gets hyper-activated and starts the tumor cascade.

Future studies will be aimed at verifying this hypothesis by monitoring FAPP1 expression levels on patient biopsies, searching for an inverse correlation with tumor malignancy.

To dissect the regulation between FAPP1 and GOLPH3 co-expression, I performed an unbiased approach aimed at identifying the common transcription factors driving the expression of the two genes. Using a siRNA library of transcription factors to run a high content screen, I evaluated whether the absence of any transcription factor affects the levels of FAPP1 or GOLPH3, or both. This approach did not result in the identification of one unequivocal transcriptional regulator for FAPP1 and GOLPH3, even though several transcription factors exclusive for each of the two proteins were identified (Sections 3.11.2 and 3.11.3, Fig. 3.40). These latter will be equally important to analyze, following the hypothesis that the transcriptional regulation of FAPP1 may control GOLPH3 levels via a feedback mechanism, and vice versa. Moreover, this powerful approach allowed me to establish a novel and unpredicted connection between the Unfolded Protein Response (UPR) pathway and PI4P levels, since PI4P levels at the Golgi increased in response to ER-stress stimuli, and were reduced upon depletion of the UPR master regulator XBP1 (Section 3.11.4). Further studies will be needed to assess the biological significance of this interesting finding.
**The Golgi PI4P pool regulated by FAPP1 controls post-Golgi trafficking of specific cargoes**

The ablation of the Golgi PI4P pool, both via PI4KIIIβ depletion or the acute recruitment of Sac1 to the Golgi, has been shown to block anterograde membrane trafficking from the TGN (Hausser et al., 2005; Blagoveshchenskaya et al., 2008). My thesis work shows that the increase in PI4P levels (obtained by FAPP1 depletion) causes an opposite effect, resulting in the increased membrane trafficking of specific cargoes. In particular, FAPP1 depletion increases the secretion rate of the β-lipoprotein ApoB-100 in liver cells, without affecting the trafficking of other cargoes, i.e. the general cargo reporter VSV-G (Section 3.4). Some cargoes appear, thus, to be more sensitive to the PI4P content of the TGN than others. ApoB-100 is an essential component of very low density lipoproteins (VLDL) and its metabolites intermediate-density (IDL) and low-density (LDL) lipoproteins. Increased VLDL and reduced high-density-lipoprotein (HDL) levels are main features of dyslipidemia, a crucial determinant for accelerated atherosclerosis in diabetes and metabolic syndrome. The evidence that FAPP1 is needed for efficient ApoB-100 secretion sheds light on a possible physiological role of the PI4P pool controlled at the level of ER-TGN membrane contact sites. A tight control of PI4P levels is important to modulate cellular secretion, and highly secretory tissues, such as the liver, may be particularly sensitive to this modulation. FAPP1-KO mice are viable and show no overt phenotype (our unpublished observations). It will be interesting to challenge these animals with a high fat diet in order to understand if the increase of β-lipoprotein secretion observed in vitro translates to increased levels of circulating lipids and in the onset of a hyperlipidemic phenotype in vivo.
The hypothesis that an imbalance in PI4P homeostasis results in mis-trafficking of specific cargoes is confirmed by a previously unidentified role of PI4P in regulating the membrane trafficking of the autophagy protein ATG9, the only trans-membrane member of the ATG family identified so far. Under steady state conditions, ATG9 resides at the TGN even though it is also present on cytoplasmic punctae positive for endosomal markers (Young et al., 2006). Following the induction of autophagy, ATG9 is redistributed from its juxtanuclear position in the TGN to peripheral endosomal membranes contributing to autophagosome formation (Young et al., 2006). Studies in yeast have found that Pik1, the ortholog of PI4KIIIβ, regulates autophagosome biogenesis by controlling the trafficking of Atg9 through the Golgi (Wang et al., 2012). In my work, I found that mammalian PI4KIIIβ is also required for efficient trafficking of ATG9 that, upon autophagy induction, fails to dissociate from the Golgi in PI4KIIIβ-KD cells. Importantly, FAPP1 depletion elicits an opposite effect, with a strong redistribution of ATG9 to extra-Golgi structures already in non-stimulated conditions, in a PI4P-dependent manner. ATG9 mis-trafficking in FAPP1-KD cells translates into increased autophagosome biogenesis, monitored by LC3 lipidation (Section 3.12.2). Consistent with the ATG9 mis-trafficking, the increase in autophagosome number observed in FAPP1-KD cells is rescued upon the simultaneous depletion of PI4KIIIβ. Together, these results suggest that the PI4P pool modulated by FAPP1 at the TGN regulates the post-Golgi trafficking of ATG9, in turn promoting autophagosome formation (section 3.12.5). Takahashi and colleagues demonstrated that the modulation of post-Golgi carrier formation (acting on fission) regulates ATG9 trafficking (Takahashi et al., 2011), suggesting that ATG9 might exit the Golgi via a PI4P-mediated mechanism. The PI4P effector that is involved in PI4P-mediated secretion in this context remains to be assessed. Given its role in post-Golgi carrier
formation, GOLPH3 could be an interesting target to test, especially for a putative role in PI4P-mediated trafficking of ATG9. In fact, the evidence that the co-expression module containing FAPP1 and GOLPH3 also includes a lot of genes involved in autophagy (Fig. 3.44) may be taken as an indication that the two proteins are acting together in this pathway.

In conclusion, my thesis work unravels a novel mechanism of PI4P regulation occurring at the level of ER-Golgi membrane contact sites, where FAPP1 binds and stabilizes the ER-localized 4-phosphatase Sac1 towards PI4P-rich sites, in order to promote Sac1 activity in trans to dephosphorylate PI4P at the TGN (Fig 4.1, upper panel). In this context, FAPP1 is a negative PI4P regulator that, by avoiding the aberrant consequences of an increase of PI4P at the Golgi, acts as a gatekeeper of Golgi functions, among which I found (Fig 4.1, lower panel):

1. Hyper-activation of the Golgi oncogene GOLPH3 leading to:
   - Golgi fragmentation
   - Increased cell migration and invasion

2. Aberrant post-Golgi trafficking of specific cargoes, in particular:
   - ApoB-100, secreted in the extracellular medium
   - ATG9, trafficked from the TGN to induce autophagosome formation
Figure 4.1 Working model. Abbreviations are: ER = endoplasmic reticulum; TGN = Trans Golgi Network; MCS = membrane contact sites.
### List of Abbreviation

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ApoB</td>
<td>Apolipoprotein B</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
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<tr>
<td>CCV</td>
<td>clathrin coated vesicles</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
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<tr>
<td>COPI/COPII</td>
<td>coat protein I/I or coatamer</td>
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<tr>
<td>CPY</td>
<td>carboxypeptidase Y</td>
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<tr>
<td>DAG</td>
<td>diacylglycerol</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified minimal Essential Medium</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleasic acid</td>
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<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
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<td>EM</td>
<td>Electron Microscopy</td>
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<td>ER</td>
<td>Endoplasmic Reticulum</td>
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<td>ERES</td>
<td>Endoplasmic Reticulum Exit Sites</td>
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<tr>
<td>ERTGoCS</td>
<td>Endoplasmic Reticulum-Trans Golgi network contact sites</td>
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<tr>
<td>E-syt</td>
<td>extended synaptotagmin</td>
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<tr>
<td>FAPP</td>
<td>phosphatidylinositol four phosphate adaptor protein</td>
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<td>FCS</td>
<td>Fetal Calf Serum</td>
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<tr>
<td>FFAT</td>
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<td>mTOR</td>
<td>Mechanistic Target Of Rapamycin Kinase</td>
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<tr>
<td>Abbreviation</td>
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<td>PAGE</td>
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<td>revolution per minute</td>
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<td>weight/volume</td>
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<td>XBP</td>
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