Phosphoinositide regulation of endolysosomal membrane dynamics and nutrient signaling

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Date of defense: 25.11.2019

Affidavit

I declare that my PhD thesis at hand has been written independently and with no other sources and aids then quoted.

Berlin, August 20th , 2019

to Rosemarie Eva Christa Fortuna

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Contents

Publications

This cumulative dissertation is based on following publications:

1. Marat, A. L.*, **Wallroth, A.***, Lo, W. T., Müller, R., Norata, G. D., Falasca, M., Schultz, C., Haucke, V.: mTORC1 activity repression by late endosomal phosphatidylinositol 3,4-bisphosphate. *Science* **356,** 968 –972, 2017. Doi: 10.1126/science.aaf8310

   * these authors contributed equally to this work

2. **Wallroth, A.**, Koch, P.A., Marat, A.L., Krause, E., Haucke, V. : Protein kinase N controls a lysosomal lipid switch to facilitate nutrient signaling via mTORC1. *Nature Cell Biology* , 2019. Doi: 10.1038/s41556-019-0377-3

Other publications:

- **Wallroth, A.**, Haucke, V.: Phosphoinositide conversion in endocytosis and the endolysosomal system. *Journal of Biological Chemistry* **293**: 1526-1535, 2018

- **Wallroth, A.**, Haucke, V.: A lipid off-switch for mTORC1. *Molecular & Cellular Oncology*, **4**: e1356899, 2017

Abstract

The mammalian target of rapamycin complex 1 (mTORC1) on lysosomes and late endosomes (Ly/LEs) integrates intra- and extracellular nutrient signals and regulates metabolic pathways such as protein synthesis, lysosome biogenesis and autophagy. Many factors stimulate mTORC1 activity, including the production of phosphatidylinositol 3,4,5-trisphosphate [PI(3,4,5)P_3] by class I phosphatidylinositol 3-kinases (PI3Ks) at the plasma membrane and phosphatidylinositol 3-phosphate [PI(3)P] by class III phosphatidylinositol 3-kinase at endosomes. In contrast, within the course of this thesis phosphatidylinositol 3,4-bisphosphate [PI(3,4)P_2], synthesized by class II PI3K β was identified as a negative regulator of mTORC1. PI3KC2β is shown to repress mTORC1 activity locally on Ly/LEs, whereas loss of PI3KC2β hyperactivates mTORC1. Growth factor deprivation induces the association of PI3KC2β with the Raptor subunit of mTORC1 and local PI(3,4)P_2 synthesis triggers repression of mTORC1 activity through association of Raptor with inhibitory 14-3-3 proteins.

We used SILAC (stable isotope labeling with amino acids in cell culture) based quantitative mass spectrometry to identify growth factor dependent interaction partners and post-translational modifications of PI3KC2β. We could show that 14-3-3 protein binding to phosphorylated threonine 279 in the presence of growth factors competes with the recruitment of PI3KC2β to mTORC1 and to Ly/LEs. Furthermore, protein kinase N 2 (PKN2) was found to be the major kinase that phosphorylates PI3KC2β at T279, thereby triggering its complex formation and cytosolic sequestration with inhibitory 14-3-3 proteins to activate mTORC1. Conversely, loss of PKN2 or mutational inactivation of its target phosphorylation site in PI3KC2β repress nutrient signaling via mTORC1. Furthermore, *mammalian target of Rapamycin Complex 2* (mTORC2) was shown to activate PKN2, while mTORC2 inhibition mimicked the effects of PKN2-loss upon PI3KC2β.

Together, these results uncover a novel mechanism that couples mTORC2-dependent activation of PKN2 to the regulation of mTORC1-mediated nutrient signaling via PI3KC2β-mediated PI(3,4)P_2 synthesis.

Zusammenfassung

Der *mammalian target of Rapamycin Complex 1* (mTORC1) auf Lysosomen und späten Endosomen (Ly/LEs) registriert und integriert intra- und extrazelluläre Signale und reguliert zelluläre Stoffwechselwege wie die Proteinsynthese, Lysosomen Biogenese und Autophagie. mTORC1 Aktivität wird von vielen verschiedenen Faktoren stimuliert, vor allem über Produktion von Phosphatidylinositol 3,4,5-trisphosphat [PI(3,4,5)P_3] durch Klasse I Phosphatidylinsoitol 3-Kinasen (PI3Ks) an der Zellmembran und die lokale Produktion von Phosphatidylinositol 3-Phosphat [PI(3)P] durch die Klasse III PI3K. Wie im Verlauf dieser Arbeit gezeigt, fungiert Phosphatidylinositol 3,4-Bisphosphat [PI(3,4)P_2], welches von der Klasse II PI3K β produziert wird, als negativer Regulator von mTORC1. PI3KC2β inhibiert mTORC1 Aktivität lokal auf Ly/LEs, während die Deletion von PI3KC2β mTORC1 hyperaktiviert. Entzug von Wachstumsfaktoren induziert Komplexbildung von PI3KC2β mit der Raptor Untereinheit von mTORC1 und die lokale Synthese von PI(3,4)P_2 reprimiert die Aktivität von mTORC1 durch Assoziation von inhibierenden 14-3-3 Proteinen mit Raptor.

Durch SILAC basierte quantitative Massenspektrometrie zur Identifikation Wachstumsfaktor-abhängiger Interaktionspartner und posttranslationaler Modifikationen von PI3KC2β konnte gezeigt werden, dass 14-3-3 Proteine in Abhängigkeit von Wachstumsfaktorstimulation an phosphoryliertes Threonin-279 von PI3KC2β binden, mit der Interaktion von PI3KC2β mit mTORC1 konkurrieren und somit die Rekrutierung von PI3KC2β zu Ly/LEs verhindern. Im Folgenden konnte die Proteinkinase N 2 (PKN2) als hauptverantwortliche Kinase für die Phosphorylierung von T279 in PI3KC2β identifiziert werden, womit PKN2 deren Komplexbildung und zytosolische Sequestrierung mit inhibitorischen 14-3-3 Proteinen induziert und somit mTORC1 aktiviert. Im Gegenzug führt Deletion von PKN2 oder Inaktivierung der Phosphorylierungsstelle in PI3KC2β durch Mutagenese zu einer Repression der mTORC1 Aktivität. Darüber hinaus konnte gezeigt werden, dass *mammalian target of Rapamycin Complex 2* (mTORC2) für die Aktivierung von PKN2 verantwortlich ist. In der Tat hat mTORC2 Inhibierung ähnliche Effekte auf PI3KC2β wie die Deletion von PKN2.

Zusammenfassend enthüllen die Ergebnisse dieser Arbeit, dass die mTORC2-abhängige Aktivierung der PKN2 mTORC1-vermittelte Stoffwechselwege über PI3KC2β katalysierte PI(3,4)P_2-Synthese reguliert.

1 Introduction

1.1 Phosphatidylinositol-phosphates

Phosphoinositides are a minor class of comparably short-lived membrane phospholipids consisting of seven sub-species. These species are generated via phosphorylation or dephosphorylation of the inositol ring of phosphatidylinositol on its 3-, 4- and 5- OH group by phosphoinositide-kinases and phosphatases (Fig. 1).

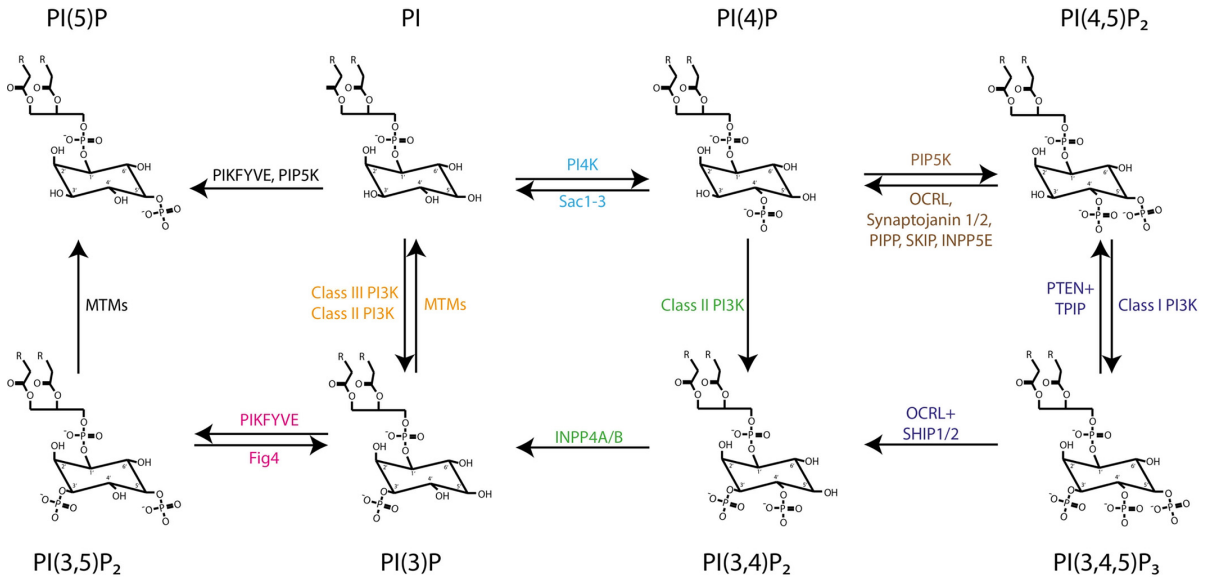

Figure 1.1 Interconversion of different PIs via PI-kinases and phosphatases. Taken from (Wallroth and Haucke, 2018)

Phosphoinositides mediate crucial cellular and organismal functions including signaling, gating of ion channels, cytoskeleton regulation and motility, development as well as the regulation of intracellular membrane traffic (Balla, 2013; Di Paolo and De Camilli, 2006). Different PI species display distinct cell biological functions and distinct subcellular distributions. PI 4-phosphates, like phosphatidylinositol 4-phosphate (PI(4)P) or phosphatidylinositol 4,5-bisphosphate, are localized the plasma membrane and exocytic pathways, the Golgi complex and the trans-Golgi network (TGN) and act as key regulators of membrane traffic (Balla and Balla, 2006; Balla, 2013). In contrast, PI 3-phosphates are on one hand produced upon growth factor stimulation at the plasma membrane and initiating signaling cascades and on the other hand mainly found as prominent components within the endo-lysosomal system (Cantley, 2002; Raiborg et al., 2013; Vanhaesebroeck et al., 2010a). PI 3-phosphates include phosphatidylinositol 3-phosphate (PI(3)P), phosphatidylinositol 3,5-bisphosphate,

phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3,4,5-trisphosphate, generated via phosphorylation of the 3- OH group of the inositol ring of phosphatidylinositol. Due to the rapidly reversible nature of phosphorylation and a very distinct subcellular localization of different PI 3-phosphate species, they serve as key regulators of signaling cascades as well as spatiotemporally controlled signposts of membrane identity (Fig. 2).

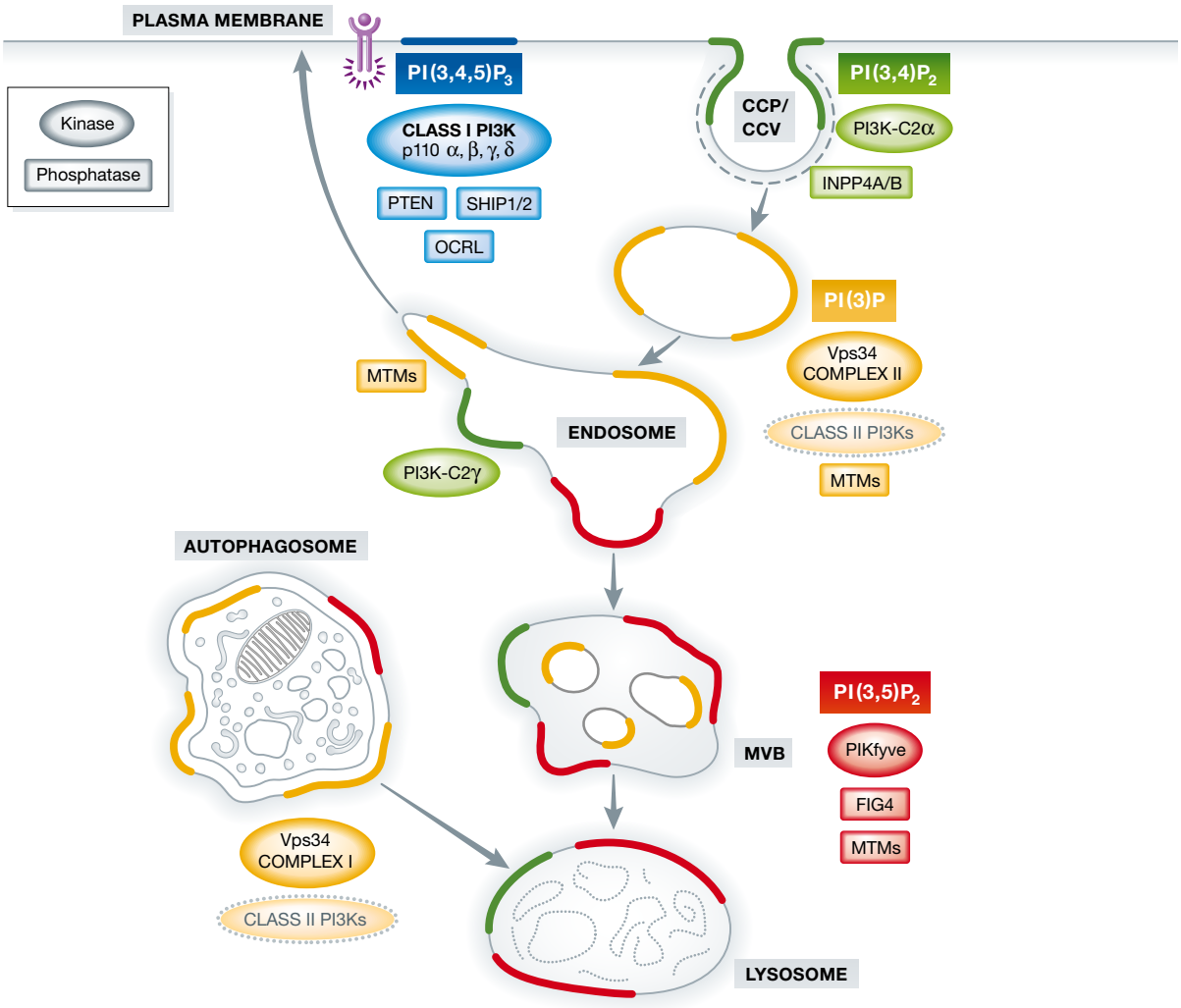

Figure 1.2 Localization of different PI 3-phosphates including kinases and phosphatases being responsible for their interconversion. Plasma membrane: receptor tyrosine kinase (purple) activate results in class I PI3Ks producing $PI(3,4,5)P_3$. $PI(3,4,5)P_3$ is a substrate for the PI 3-phosphatase PTEN and for the PI 5-phosphatases SHIP1/2 and OCRL. Furthermore class II PI3Kα $PI(3,4)P_2$ necessary for clathrin-coated pit (CCP) maturation during endocytosis. Early endosomes: PI(3)P is generated primarily by the class III PI3K Vps34 complex II with a possible contribution of class II PI3Ks, either by direct PI(3)P synthesis or indirectly via $PI(3,4)P_2$ hydrolysis by the PI 4-phosphatases INPP4A/B. Late endosomes/multivesicular bodies (MVBs)/Lysosomes: PI(3)P is converted into $PI(3,5)P_2$ via the PI(3)P 5-kinase PIKfyve. Endosomes and lysosomes also harbour a poorly understood pool of $PI(3,4)P_2$. In the liver, an endosomal $PI(3,4)P_2$ pool is synthesized by class II PI3Kγ. Autophagosomes: PI(3)P is produced by Vps34 complex I, and possibly by the class II PI3Ks (dashed line). $PI(3,5)P_2$ turnover at MVBs and/or lysosomes is mediated by MTMs together with the $PI(3,5)P_2$ 5-phosphatase Fig4. Taken from (Marat and Haucke, 2016)

$PI(3,4,5)P_3$ and $PI(3,4)P_2$ as a degradation product of $PI(3,4,5)P_3$ are generated at the plasma membrane upon extracellular growth factor stimulation (Cantley, 2002; Vanhaesebroeck et

al., 2010a) and PI(3,4)P_2 has been described as an essential mediator of late stages of clathrin mediated endocytosis (Posor et al., 2013). PI(3)P is found as the predominant PI-species of early endosomes and playing an important role in autophagosome biogenesis. During the maturation of endosomes from early to late stages, PI(3)P is converted into PI(3,5)P_2 , which is present on late endosomes/multivesicular bodies, lysosomal and autophagosomal compartments (Mayinger, 2012). Furthermore, an endosomal pool of PI(3,4)P_2 has been described with its function has yet to be established (Watt et al., 2004).

1.2 PI3-kinases

Phosphatidylinositol 3-kinases (PI3K) are phosphorylating the 3-OH group of the inositol ring. They act either on the plasma membrane, on endosomal or on autophagosomal membranes (Marat and Haucke, 2016). There are eight different PI3Ks grouped into three classes. The core of all PI3Ks, a so called 'signature motif' is fairly conserved through all PI3Ks, consisting of a catalytic active kinase domain, a helical domain and a membrane binding C2-domain (Balla, 2013; Vadas et al., 2011; Vanhaesebroeck et al., 2010a). The three different classes of PI3Ks are based on the conservation found outside of the kinase core, the appearance and type of regulatory domains as well as their preference of lipid-substrates.

1.2.1 Class I PI3Ks

Class I PI3Ks use PI(4,5)P_2 as a substrate and produce PI(3,4,5)P_3 . They are functioning as tightly regulated heterodimers, consisting of one of four possible catalytic subunits (p110α,β,γ,δ) and one of two possible regulatory subunits, consisting of two classes, the p85 class (splice variants: p85α, p55α, p50α, p85β, p55γ) and the p101/87 class (Hiles et al., 1992). The catalytic subunits p110α,β and δ (class IA subunits) interact with the regulatory p85 class while p110γ (class IB subunit) associates with the regulatory p110/87 class (Jean and Kiger, 2014; Vadas et al., 2011).

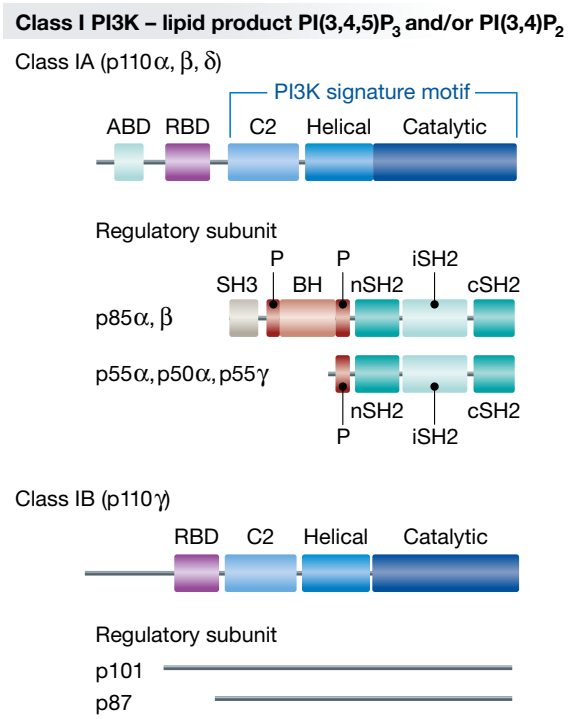

Figure 1.3 Domain structure of class I PI3Ks. ABD: Adaptor binding domain; RBD: Ras-binding domain; SH3: Src-homology 2 domain; SH2: Src-homology 3 domain; BH: Bar cluster region homology domain; iSH2: Intervening coiled-coil domain. C2: C2-domain. Adapted from (Marat and Haucke, 2016)

All assembled class I PI3Ks act downstream of receptor tyrosine kinases (RTKs) and guanine nucleotide-binding protein (G-protein)- coupled receptors and are activated upon growth factor or mitogen binding to these receptors, further controlling downstream signaling cascades regulating cell survival, - growth and proliferation, metabolism and autophagy. p110α and p110δ are mainly activated following RTK stimulation whereas p110β and p110γ are activated downstream of GPCR activation (Stephens et al., 1994; Stoyanov et al., 1995). Furthermore, small GTPases like active Ras and its family members (e.g. Rab5) can bind to the Ras binding domain (RBD) of p110 and lead to activation of class I PI3Ks (Vanhaesebroeck et al., 1997). Following activation they produce PI(3,4,5)P_3 mainly on the plasma membrane, but also on endosomes, leading to recruitment and activation of effectors binding PIP_3 , most notably Akt (or protein kinase B). Akt controls many downstream pathways such as the mammalian target of rapamycin complex-1 (mTORC1) pathway as well as FOXO transcription factors and the pro-apoptotic factor BAD (Dibble and Cantley, 2015; Vanhaesebroeck et al., 2010a). Due to their crucial role in early steps of signaling cascades controlling cell growth and proliferation, on one hand activating mutations of class I PI3Ks are associated with cancer and on the other hand they represent import drug targets (Vanhaesebroeck et al., 2010b).

1.2.2 Class II PI3Ks

Class II PI3Ks are the least studied class of PI3Ks with functions in signaling and membrane traffic. Class II PI3Ks are large monomeric enzymes lacking a regulatory subunit, but containing additional C-terminal PX and C2 domains compared to class I or class II PI3Ks. Three isoforms (PI3KC2α,β and γ) are present in mammals, whose main difference lies in their unstructured N-terminal extensions, which differ in length and amino acid sequence.

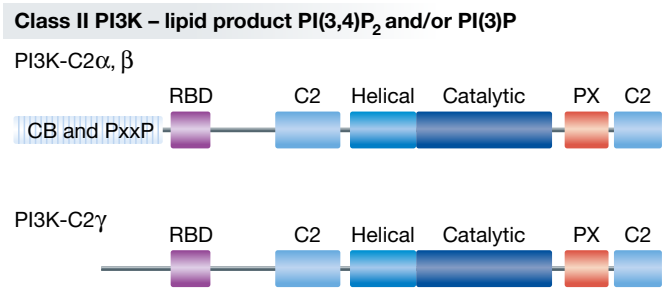

Figure 1.4 Domain structure of class II PI3Ks. RBD: Ras-binding domain; CB: clathrin binding region; PxxP: proline rich segment. PX: Phox homology domain. C2: C2-domain. Adapted from (Marat and Haucke, 2016)

These N-terminal extensions likely play regulatory roles by mediating isoform specific protein-protein interactions. For PI3KC2α and PI3KC2β clathrin has been shown to bind the N-terminal region and likely playing a regulating or recruiting role for these two kinases. Together with the N-terminal region, the Ras-binding domain (RBD) of class II PI3Ks likely play an important role in recruiting the kinases to the membranes they should act on, as the RBD PI3KC2γ has been shown to interact with Rab5 and PI3KC2γ acts on early endosomes (Braccini et al., 2015; Jean and Kiger, 2014; Sasaki et al., 2009; Vanhaesebroeck et al., 2010a). Beside the N-terminal region and the RBD, the class II specific PX- and C2 domains likely play important roles in regulation kinase activity depending on the localization of the kinase. It has recently been shown for PI3KC2α that the PX- and C2-domain fold back onto the kinase domain and keeping PI3KC2α in an autoinhibitory conformation, which is released when the PX-C2 module binds to $PI(4,5)P_2$ enriched membranes such as the plasma membrane, where PI3KC2α has previously shown to act (Wang et al., 2018). Similar mechanism with probably different PIP-specificities for the PX-C2-module are likely to be present in the regulation of the other two members of class II PI3Ks.

The lipid products of class II PI3Ks are PI(3)P and $PI(3,4)P_2$ and there is growing evidence that class II PI3Ks and their lipid products are coupling membrane traffic to cellular signaling events. The α isoform has been shown to synthesize $PI(3,4)P_2$, which is required for late stages

of CME, whereas for the γ isoform has been demonstrated that it produces an endosomal pool of PI(3,4)P_2 to sustain AKT2 activity upon insulin stimulation (Braccini et al., 2015; Posor et al., 2013). Class II PI3K α and β also have been linked to growth factor dependent signaling events, although their precise function as well as their lipid products in these events remain to be determined. In addition to their direct function in PI(3,4)P_2 synthesis, class II PI3K have also been implicated to regulate intracellular pools of PI(3)P important for endocytic traffic and autophagy, although it remains unclear whether these effects result from direct PI(3)P synthesis from class II PI3Ks or indirectly via further conversion of PI(3,4)P_2 (Arcaro et al., 2002; Brown et al., 1999; Devereaux et al., 2013; Franco et al., 2014; Jean et al., 2012; Leibiger et al., 2010; Yoshioka et al., 2012).

1.2.3 Class III PI3Ks

The class III of PI3Ks consists of only one kinase -Vps34- which was initially described in yeast as a gene regulating sorting from endosomes to lysosomes (Herman and Emr, 1990; Schu et al., 1993). Vps34 acts together with its regulatory subunit Vps15/p150, which its constitutively binds to. Vps15 is N-terminal myristoylated, targeting Vps34 to membranes. Vps34 occurs in two different complexes, which are present on different membranes and regulating different membrane trafficking events. Complex I consist of Vps15/p150, Beclin-1, ATG14L, and NRBF2 and is responsible for Vps34 producing PI(3)P during autophagosome formation. Complex II consist of Vps15/p150, Beclin-1 and UVRAG and acts on early endosomes (Cao et al., 2014; Funderburk et al., 2010; Kihara et al., 2001; Lu et al., 2014; Rostislavleva et al., 2015). Complex II is recruited to endosomal membranes via Rab5, which interacts with the WD40 domain of Vps15. The generation of PI(3)P via Vps34-complex II on Rab5 positives endosomes leads to the recruitment of various PI(3)P effectors regulating endosomal fusion, tubulation, maturation or intraluminal vesicle formation, e.g. early endosome autoantigen 1 (EEA1) or the ESCRT complex subunit hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) (Jean and Kiger, 2014; Ktistakis et al., 2012; Raiborg et al., 2013).

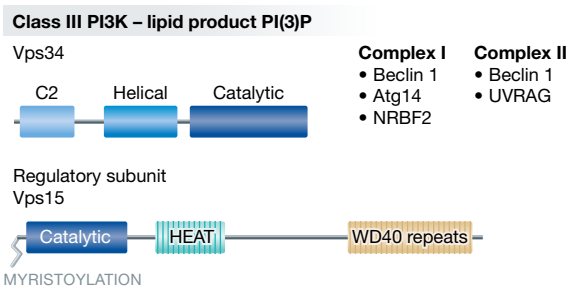

Figure 1.5 Domain structure of class III PI3Ks. HEAT: HEAT repeat domain; C2: C2-domain. Adapted from (Marat and Haucke, 2016)

In contrast to the positive regulation of autophagy via Vps34 in complex I, Vps34 derived PI(3)P has also been implicated in the activation of mTORC1, a negative regulator of autophagy. It has been reported that PI(3)P recruits phospholipase D1 (PLD1), resulting in formation of phosphatidic acid, which in turn triggers dissociation of the inhibitory mTORC1-subunit DEPTOR, finally resulting in mTORC1 activation (Bridges et al., 2012; Yoon et al., 2011; Yoon et al., 2015). Recently it has been shown that Vps34 derived PI(3)P on lysosomes leads to repositioning of the lysosomes towards the cell periphery, a process which has previously been shown to depend on the nutrient status and availability within the cell, also leading to mTORC1 activation (Hong et al., 2017; Korolchuk et al., 2011). Furthermore, in fashion of a feed forward loop, mTORC1 activity specifically inhibits formation of Vps34-complex I, the complex implicated in promotion of autophagy, while activating complex II via phosphorylation on UVRAG (Munson et al., 2015). Altogether, both class III PI3K complexes not only have opposing effects within the cell, but also negatively regulate each other via complex cellular mechanisms.

1.3 The mammalian Target Of Rapamycin (mTOR)

The mammalian or mechanistic Target Of Rapamycin (mTOR) is a serine/threonine kinase belonging to the family of PI3K-related kinases (PIKK). This kinase was identified as the direct target of Rapamycin, which acts in a complex with FKBP12, shortly after its homolog in yeast has been found using genetic screens for rapamycin resistance (Brown et al., 1994; Cafferkey et al., 1993; Heitman et al., 1991; Kunz et al., 1993; Sabatini et al., 1994). Rapamycin is antimicrobial compound first isolated from bacteria on the Easter Island, showing anti-fungal, anti-tumor and immunosuppressive properties (Eng et al., 1984; Martel et al., 1977; Vezina et al., 1975). In the cell it forms a complex with the protein FKBP12, which in turn directly inhibits kinase activity of mTOR via binding to its FRB-domain (Yang et al., 2013). mTOR kinase can

occur in two different complexes, mTORC1 and mTORC2, consisting of a slightly different subunit composition, have different localization, different downstream targets and differences in rapamycin-sensitivity. While FKBP12-rapamycin directly inhibits mTORC1, mTORC2 is insensitive to acute treatment (Jacinto et al., 2004; Sarbassov et al., 2004).

1.3.1 mTORC1

mTORC1 consists of three core-components: mTOR, mLST8 (mammalian lethal with Sec13 protein 8) and Raptor (regulatory protein associated with mTOR). Raptor defines the subcellular localization of mTORC1, is essential for the recruitment to the lysosomal membrane (the location of its activation) and facilitates substrate recruitment via binding to a TOR signaling motif (TOS) found in many mTORC1-substrates (Hara et al., 2002; Kim et al., 2002; Nojima et al., 2003; Schalm and Blenis, 2002). mLST8 has been described to bind the kinase domain of mTOR and stabilizes its activation loop, but more detailed functions within the complex remain to be described (Yang et al., 2013). In addition to these three core-subunits, mTORC1 contains the two inhibitory subunits PRAS40 (proline-rich Akt substrate of 40kDa) and DEPTOR (DEP domain containing mTOR interacting protein) (Peterson et al., 2009; Sancak et al., 2007; Vander Haar et al., 2007). In structural studies is has been shown that mTORC1 occurs in dimers with the monomers contacting each other via Raptor and the HEAT-repeats within mTOR (Aylett et al., 2016; Yip et al., 2010).

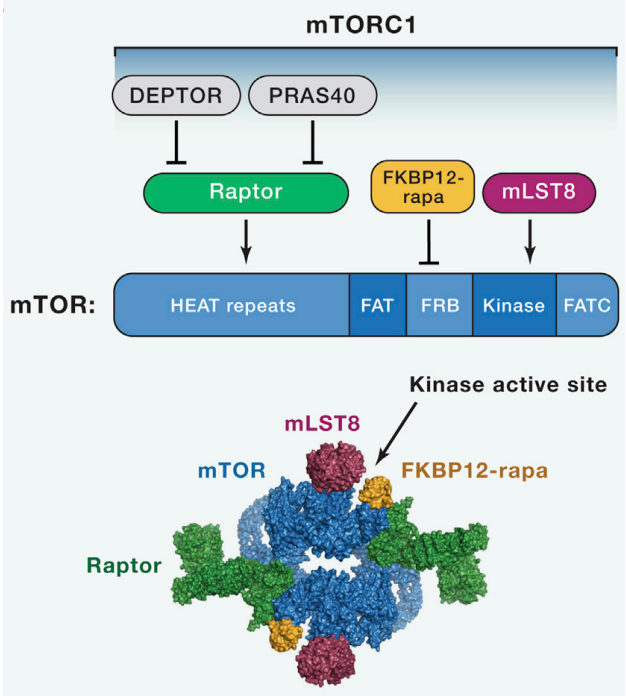

Figure 1.6 mTORC1 subunits, binding sites and structure of the core of mTORC1. A 5.9Å cryo-EM structure of the core of mTORC1 including FKBP12-rapamycin. Subunits are color coded. Adapted from (Saxton and Sabatini, 2017)

1.3.2 Regulation of mTORC1

mTORC1 is functioning as a central regulator of cell growth, survival and proliferation. A plethora of signals such as growth factor stimulation, nutrient status of the cell, cellular stress and energy availability feed into the regulation of mTOR kinase within the mTORC1 complex, which are then translated by mTORC1 into downstream signals. As mentioned earlier, growth factors binding to RTKs stimulate class I PI3Ks at the plasma membrane, which in turn generate PIP_3 . One of the major downstream effectors of PIP_3 is Akt, which is recruited via PIP_3 to the plasma membrane, where it gets fully activated by phosphorylation via mTORC2 and PDK1 (Phosphoinositide-dependent kinase-1) (Vanhaesebroeck et al., 2010a). Akt then plays direct and indirect roles in the activation of mTORC1. Akt directly phosphorylates the inhibitory mTORC1 subunit PRAS40, leading to binding of 14-3-3 proteins and dissociation from mTORC1 due to allosteric inhibition (Sancak et al., 2007). Furthermore, Akt acts as an indirect activator of mTORC1 via phosphorylation and inhibition of a key negative regulator of mTORC1, the Tuberous Sclerosis Complex (TSC). TSC is a heterotrimeric complex consisting of TSC1, TSC2 and TBC1D7 and acts as a GTPase activating protein (GAP) for the small GTPase Rheb, which is directly binding and activating mTORC1 (Dibble et al., 2012). Multisite phosphorylation of the TSC-complex by Akt leads to its dissociation from the lysosome, where Rheb and mTORC1 are localized (Dibble and Cantley, 2015; Inoki et al., 2003; Inoki et al., 2002; Manning et al., 2002). Similarly, downstream signaling of Ras can also activate mTORC1. Erk and its effector p90RSK, which act downstream of Ras, also phosphorylate and inhibit the TSC-complex (Ma et al., 2005; Roux et al., 2004). Additionally, growth factor pathways regulating mTORC1 also include Wnt signaling, which can also activate mTORC1 via inhibiting TSC (Feng et al., 2007; Inoki et al., 2006).

The regulation of mTORC1 activity upon cellular stresses like hypoxia or low ATP levels, e.g. due to glucose deprivation, mainly works via the central metabolic regulator AMP-activated protein kinase (AMPK), which is mainly sensing cellular ATP-levels and activated upon low cellular ATP-levels (Garcia and Shaw, 2017). AMPK inhibits mTORC1 directly and indirectly. AMPK directly phosphorylates Raptor, which leads to binding of 14-3-3 proteins to the phosphorylated motif of Raptor and inhibits substrate recruitment to mTORC1 (Gwinn et al., 2008). In an indirect mechanism, AMPK phosphorylates TSC2 leading to TSC activation and subsequent Rheb inhibition and mTORC1 inhibition (Feng et al., 2007; Inoki et al., 2006).

As amino acids are not only essential building blocks for protein synthesis, but also act as energy sources in metabolic pathways, mTORC1 activity is also tightly coupled to changes in amino acid concentrations. To be activated, mTORC1 needs to be recruited to the lysosome, the place where it can be fully activated by Rheb. mTORC1 localization at the lysosomal surface is tightly connected to its regulation via amino acids. mTORC1 can sense cytosolic amino acids as well as intra-lysosomal amino acids via distinct mechanisms. Cytosolic amino acids stimulate heterodimeric Rag GTPases, which are tethered to the lysosomal membrane via the pentameric Ragulator complex consisting of MP1, p14, p18, HBXIP and C7ORF59 (Bar-Peled et al., 2012; Kim et al., 2008; Sancak et al., 2010; Sancak et al., 2008). Amino acid stimulation converts the Rags to their active GTP-bound state, allowing them to bind Raptor and recruit mTORC1 to the lysosome, where it then can be activated by Rheb, implicating a tight interplay between amino acid levels within the cell and growth factor stimulation

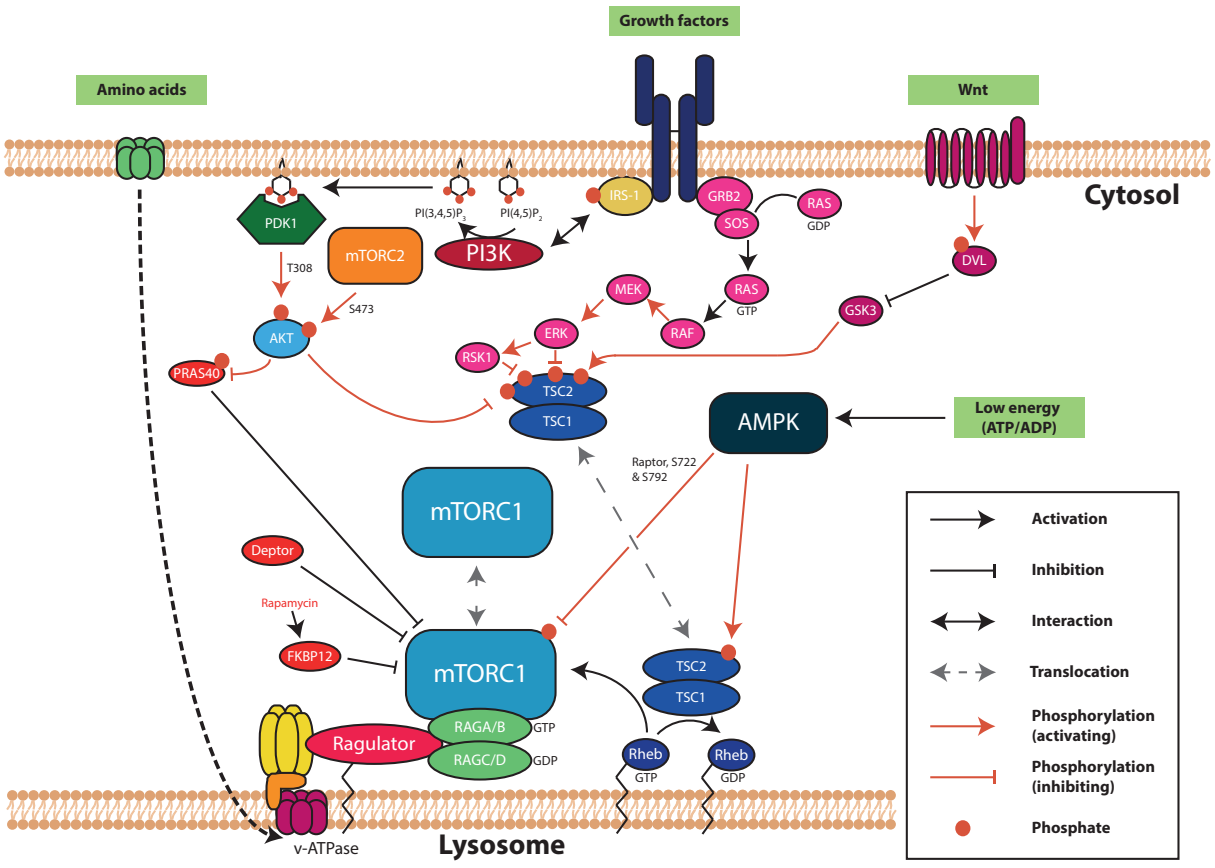

Figure 1.7 Regulation of mTORC1 localization and activity via amino acids, growth factors and energy. mTORC1 recruitment to the lysosomal surface is regulated via amino acid sensing within the cytosol and the lysosome. V-ATPase plays a critical role, promoting the GEF-activity of Ragulator towards the Rag-GTPases, which in turn rectuit mTORC1. Growth factor stimulation activates mTORC1 via signaling cascades involving the Akt- and the MAPK-signaling cascade and Wnt signaling, with the TSC-complex with its Gap-activity towards Rheb being the central target of all pathways. Energy levels regulate mTORC1 activity mainly via the ATP/ADP sensing kinase AMPK, which directly phosphorylates mTORC1 as well as activates TSC.

upstream of mTORC1 activation. In contrast, intra-lysosomal amino acids alter the nucleotide status of Rag GTPases via an interplay between the lysosomal amino acid transporter SLC38A9 and lysosomal v-ATPase. V-ATPase interacts with the Ragulator-Rag complex promoting the GEF (guanine-nucleotide exchange factor) of Ragulator towards RagA/Rag, leading to activation of RagA/B and subsequent recruitment of mTORC1 (Rebsamen et al., 2015; Wang et al., 2015; Zoncu et al., 2011).

1.3.3 Downstream targets of mTORC1

mTORC1 activity plays a critical role in balancing catabolic and anabolic processes within the cell in response to intra- and extracellular stimuli. Active mTORC1 promotes anabolic processes like lipid and protein synthesis and inhibits catabolic processes such as autophagy and lysosome biogenesis. The two key effectors of mTORC1 upregulating anabolic processes are eIF4E binding protein (4EBP) and the ribosomal p70S6 Kinase (S6K). 4EBP binds eIF4E and prevents formation of the eIF4F-complex. Direct phosphorylation of 4EBP by mTORC1 leads to its dissociation from eIF4E enabling 5'cap-dependent mRNA translation (Brunn et al., 1997; Gingras et al., 1999). Phosphorylation of the hydrophobic motif (Thr389) and probably the turn motif (S371) of S6K by mTORC1 allow phosphorylation by PDK1 and subsequent complete activation of S6K. Active S6K activates mRNA translation via phosphorylation and activation of various substrates, including the ribosomal S6 protein and eIF4B (Dorrello et al., 2006; Holz and Blenis, 2005; Pearce et al., 2010). Furthermore, S6K activation via mTORC1 also promotes lipid synthesis through the S6K target SREBP1 (sterol responsive element binding protein 1) (Duvel et al., 2010).

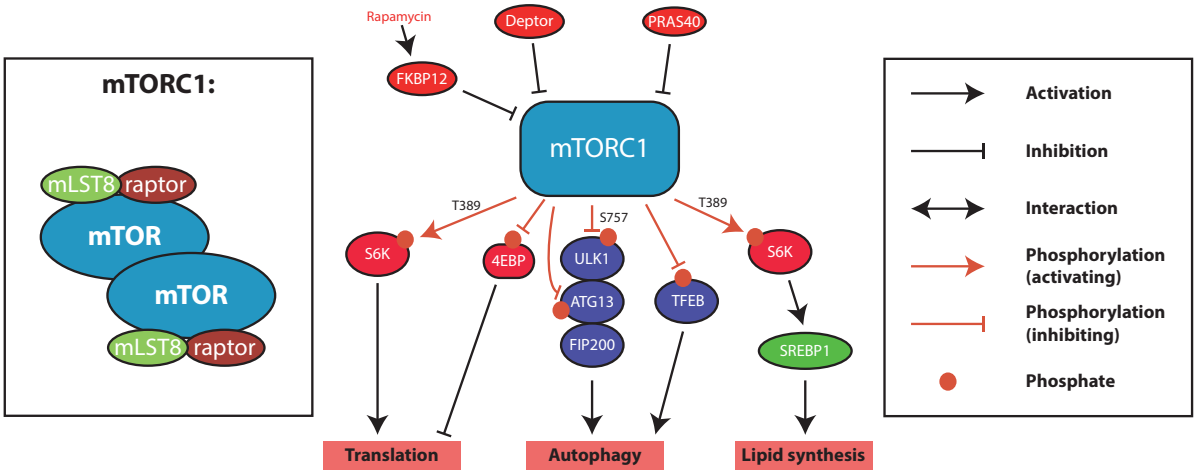

Figure 1.8 Major downstream pathways of mTORC1 signaling. The mTORC1 complex, acting as a dimer, directly phosphorylates S6K, 4EBP, ATG13, ULK1 as well as the transcription factor TFEB, leading to protein translation and lipid synthesis as well as inhibition of autophagy.

Key effectors in the downregulation of catabolic processes via mTORC1 are TFEB (Transcription Factor EB), ULK1 and UVRAG. TFEB is a transcription factor driving the expression of genes for lysosome biogenesis and the autophagy machinery. Phosphorylation of TFEB by mTORC1 leads to its binding to 14-3-3 proteins and subsequent inhibition of its translocation to the nucleus (Kim et al., 2011; Settembre et al., 2012). ULK1 is a kinase promoting autophagosome formation. Phosphorylation by mTORC1 prevents its activation by AMPK. The effects of phosphorylation of UVRAG by mTORC1 has been described previously (see 1.2.3).

1.3.4 mTORC2

Similar to mTORC1, mTORC2 consists of three core-components: mTOR, mLST8 (mammalian lethal with Sec13 protein 8) and Rictor (rapamycin insensitive companion of mTOR), which likely has analogous functions in mTORC2 as Raptor in mTORC1 (Jacinto et al., 2004; Sarbassov et al., 2004). In addition to these three core-subunits, mTORC2 contains the inhibitory subunit DEPTOR as well as the regulatory subunits mSin1 and Protor1/2 (Frias et al., 2006; Jacinto et al., 2006; Pearce et al., 2007; Peterson et al., 2009; Thedieck et al., 2007; Woo et al., 2007; Yang et al., 2006). In contrast to mTORC1, mTORC2 is insensitive to acute rapamycin treatment.

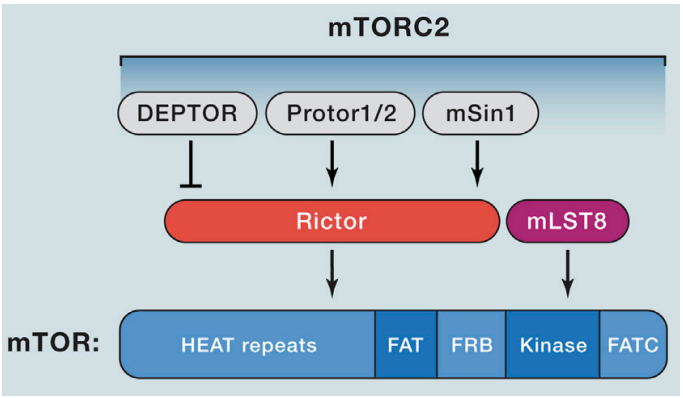

Figure 1.9 mTORC2 domain structure, subunits and binding sites. Adapted from (Saxton and Sabatini, 2017)

1.3.5 Regulation of mTORC2

In contrast to mTORC1, mTORC2 has been described as an effector of PI3K signaling only, thus functioning downstream of growth factor stimulation. The mSin1-subunit of mTORC2 contains a phosphoinositide-binding Pleckstrin-homology-domain (PH-domain). This PH-domain inhibits the catalytic activity of mTORC2. Upon generation of PIP_3 at the plasma membrane,

the autoinhibitory conformation is relieved and mTORC2 is activated (Liu et al., 2015; Yang et al., 2015). Furthermore, mTORC2 is also regulated via mTORC1 in negative feedback loops via Grb10, a negative regulator of insulin/IGF-1 signaling, and S6K, which phosphorylates IRS1 leading to its degradation. IRS1 is needed for class I activation upstream of mTORC2 (Harrington et al., 2004; Hsu et al., 2011; Shah et al., 2004; Yu et al., 2011).

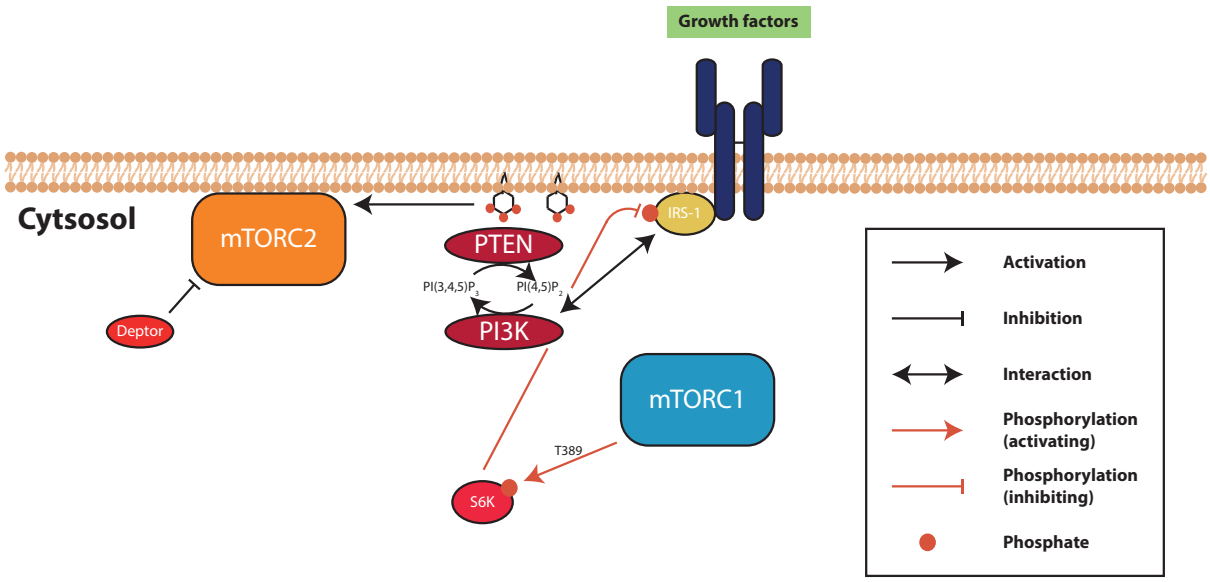

Figure 1.10 Regulation of mTORC2 activity. mTORC2 is activated downstream of growth factor stimulation. The mSin1 subunit binds PIP_3 causing a conformational change promoting mTORC2 activity. mTORC1 can act as a negative inhibitor of mTORC2 via S6K, which inhibits IRS-1, the activator of class I PI3Ks.

1.3.6 Downstream targets of mTORC2

Compared to mTORC1, which is regulates cell growth and metabolic pathways, mTORC2 regulates cell proliferation, cell migration, cell survival and cytoskeleton rearrangement, mainly by phosphorylating and activating most members of the AGC-kinase family. mTORC2 has been described to phosphorylate the turn-motif and hydrophobic motif within the C-terminus of the kinase domain of AGC-kinases, phosphorylations that are required for full activation of these kinases. mTORC2 has first been described to activate PKCα, a regulator of the actin cytoskeleton (Jacinto et al., 2004; Sarbassov et al., 2004). Later, mTORC2 has also been shown to phosphorylate and activate several other PKC-family members, namely PKCγ, PKCδ, PKCε and PKCζ , who all are involved in regulating cytoskeletal remodeling and cell migration (Gan et al., 2012; Ikenoue et al., 2008; Li and Gao, 2014; Thomanetz et al., 2013; Tobias et al., 2016). However, the most prominent target of mTORC2 is Akt, the key effector of class I PI3K signaling (Sarbassov et al., 2005). Akt, once activated by mTORC2 and PDK1,

promotes cell survival and cell proliferation, e.g. via phosphorylation of FOXO1, FOXO3a, BAD and the metabolic regulator GSK3β (Guertin et al., 2006; Jacinto et al., 2006). Furthermore Akt regulates cell growth via mTORC1, directly via phosphorylation of PRAS40 and indirectly via phosphorylation of TSC (Inoki et al., 2002; Sancak et al., 2007). Finally, mTORC2 has also been shown to phosphorylate SGK, which mediates ion transport as well as cell survival (Garcia-Martinez and Alessi, 2008).

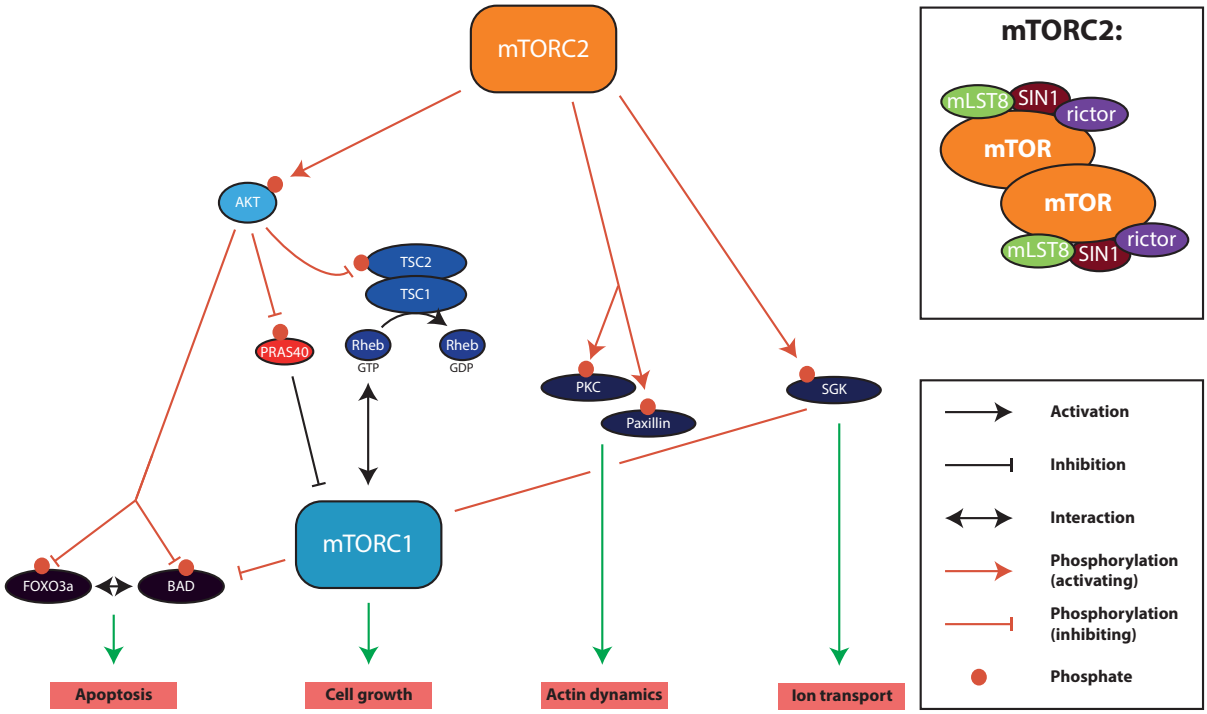

Figure 1.11 Major downstream effectors of mTORC2. The main targets of mTORC2 (also acting as a dimer) are kinases of the AGC-kinase family, such as PKC, Akt and SGK. Activation of these kinases by mTORC2 activates signaling cascades which are negatively regulating Apoptosis, promoting cell growth, as well as regulating actin dynamics and ion transport.

1.3.7 PI3Ks in mTOR-signaling

The regulation of nutrient signaling, in particular mTORC1 and mTORC2 signaling, is closely connected to phosphoinositides generated by PI3Ks. mTORC2 is activated by PIP_3 generated by class I PI3Ks at the plasma membrane (Liu et al., 2015; Yang et al., 2015). Full mTORC1 activation depends on the activity of Akt, which is activated by class I PI3Ks. Hence, mTORC1 activation is dependent on plasma membrane pools PIP_3 and $PI(3,4)P_2$, which is either generated as a degradation product of PIP3 or by class II PI3Ks (Vanhaesebroeck et al., 2010a; Braccini et al., 2015). Furthermore, PI3P generated by the class III PI3K VPS34 is implicated in mTORC1 activation indirectly via its effects on the positioning of lysosomes and the activation of PLD1 (Bridges et al., 2012; Yoon et al., 2011; Yoon et al., 2015; Hong et al., 2017; Korolchuk et al., 2011).

1.4 Aim of the study

The mammalian target of rapamycin complex 1 is a central regulator of cell growth and metabolism and autophagy. Therefore its activity needs to be tightly regulated. mTORC1 senses cellular energy, nutrient status and growth factor stimulation via various mechanisms. One of the major regulators of mTORC1 activity are PI3Ks and their lipid products. Class I PI3Ks, which produce PI(3,4,5)P_3 as well as class III PI3K, which produces PI(3)P, have been shown to play major roles in mTORC1 activation. Class II PI3Ks have also been suggested to play a role in growth factor signaling, but their specific function, potential implication in nutrient signaling via mTORC1 as well as their lipid products have not been fully understood yet.

This thesis aims to provide insight into the function of class II PI3Ks in nutrient signaling. Furthermore, it aims to answer the following questions: Which lipid product is produced by class II PI3Ks and on which membrane(s) are class II PI3Ks, especially PI3KC2β located? Furthermore: What are the downstream effects of PI3KC2β with respect to nutrient signaling and how is its activity PI3KC2β regulated?

2 Project I: mTORC1 activity repression by late endosomal phosphatidylinositol 3,4-bisphosphate

2.1 Overview of the project

Nutrient sensing and signaling by mTORC1 integrates internal and external signals to regulate cell metabolism, cell growth and autophagy while its dysfunction is implicated in diseases ranging from obesity and diabetes to cancer. mTORC1 activation is regulated by lipids, most notably by PIs, signaling lipids that localize to distinct compartments to execute a plethora of cell physiological functions. Mitogen-induced production of PI(3,4,5)P_3 by class I PI3Ks at the plasma membrane stimulates mTORC1 signaling via activation of Akt, a kinase that is co-activated by active mTORC2 (Dibble and Cantley, 2015; Vanhaesebroeck et al., 2010a). Furthermore, PI(3)P production on endosomal compartments by class III PI3K also plays a role in promoting mTORC1 activity, on one hand via inhibiting the inhibitory mTORC1 subunit DEPTOR and on the other hand via inducing the repositioning of lysosomes, the organelles where mTORC1 is activated, towards the cell periphery (Hong et al., 2017; Korolchuk et al., 2011; Yoon et al., 2011; Yoon et al., 2015).

To address a potential role of class II PI3Ks in nutrient signaling via mTORC1, we performed knockdown experiments of the two ubiquitously expressed class II PI3Ks -PI3KC2α and PI3KC2β- in HeLa cells. We did not study PI3KC2γ, an isoform specifically expressed in liver (Braccini et al., 2015). As a readout we examined the phosphorylation of S6K at Threonine-389 by mTORC1, the commonly used readout for mTORC1 activity. Surprisingly, only PI3KC2β had an effect on mTORC1 activity, and even more surprising, in contrast to any other PI3K investigated until that point, loss of PI3KC2β led to higher mTORC1 activity suggesting it to act as a negative regulator of mTORC1. We could confirm these results using CRISPR-Cas generated knockout-cell lines. Furthermore, loss of PI3KC2β led to an accumulation of p62, an increase in cell size and a more peripheral distribution of lysosomes, all indicators of higher mTORC1 activity. In an approach to detect the subcellular localization of PI3KC2β, we used CRISPR-Cas to endogenously tag PI3KC2β with an N-terminal eGFP. We also performed pulldown-assays using the unstructured N-terminus of PI3KC2β, the only region of this enzyme not conserved with PI3KC2α and thus likely to fulfill isoform specific functions. Via mass-

spectrometry, we found mTOR and the mTORC1 specific subunit Raptor as interaction partners of the N-terminus of PI3KC2β. We could identify the region within the N-terminus of PI3KC2β that binds to Raptor, confirmed the interaction by immunoprecipitation experiments using endogenously eGFP-tagged PI3KC2β, and found that PI3KC2β is localized to late endosomes/lysosomes (Ly/LEs). Interestingly, the recruitment of PI3KC2β to Ly/LEs as well as the interaction with mTORC1 were found to depend on growth factor starvation.

Moreover , we identified PI(4)P as the preferred substrate of PI3KC2β, suggesting that the main product of this class II PI3K is PI(3,4)P_2. We could further show that upon growth factor starvation, PI3KC2β is recruited to LY/LEs and produces PI(3,4)P_2. Analysis of potential effectors of PI(3,4)P_2 that might inhibit mTORC1 activity on LyLEs, revealed 14-3-3 proteins. Specifically, we found that loss of PI3KC2β reduces the interaction of Raptor with 14-3-3γ, an interaction that was previously shown to inhibit mTORC1 activity (Gwinn et al., 2008). Finally, we could establish 14-3-3γ as an effector of PI(3,4)P_2 via assays addressing direct binding and we saw that 14-3-3γ recruitment to Ly/LEs and mTORC1 depends on lysosomal PI(3,4)P_2.

Taken together our study identified the class II PI3K β as a novel inhibitor of mTORC1 activity locally at lysosomes. Surprisingly, PI(3,4)P_2, a phosphoinositide previously described to play an activating role for mTORC1 when produced at the plasma membrane (Vanhaesebroeck et al., 2010a), executes an opposing function, when produced locally on Ly/LEs under conditions of growth factor depletion.

2.2 Original publication

Marat, A. L.*, Wallroth, A.*, Lo, W. T., Müller, R., Norata, G. D., Falasca, M., Schultz, C., Haucke, V.: mTORC1 activity repression by late endosomal phosphatidylinositol 3,4-bisphosphate. *Science* **356,** 968 –972, 2017

https://doi.org/10.1126/science.aaf8310

* these authors contributed equally to this work

Personal contribution

First, I generated cell lines carrying a knock-out of PI3KC2β via CRISPR-Cas in order to confirm the effect of PI3KC2β on the activity on mTORC1, previously observed using siRNA mediated knockdown. Using this cell line not only the effect on mTORC1 activity using the phosphorylation of S6K could be confirmed, but I could also measure an increase in cell size of HEK293T cells carrying a knock-out of PI3KC2β in comparison to wild type HEK293T cells. Furthermore, I could see a change of LC3 levels within cells upon loss of PI3KC2β, suggesting a defect in autophagy, another sign of high mTORC1 activity. Using HEK293T cells as a background and re-expressing either wild type PI3KC2β or kinase-inactive PI3KC2β, I could show that the inhibiting effect of PI3KC2β towards mTORC1 depends on the kinase activity of PI3KC2β and hence on its lipid product, $PI(3,4)P_2$.

I then generated HEK293T cell lines expressing PI3KC2β N-terminal eGFP tagged from the endogenous locus, again using CRISPR-Cas technology. Using these cells, I could see PI3KC2β localizing to Ly/Les upon growth factor starvation. Furthermore I could also observe the interaction of PI3KC2β with mTORC1, which I found being growth factor starvation dependent as well. Interestingly, this interaction did not occur upon long term amino acid starvation, conditions where mTORC1 falls off Ly/LEs, suggesting that this interaction only occurs on Ly/LE-membranes.

Knowing that the inhibitory effects of PI3KC2β towards mTORC1 depend on its lipid product, I looked at potential mechanisms of this inhibition in more detail. While investigating potential changes of the subunit composition of mTORC1 depending on PI3KC2β/$PI(3,4)P_2$, I recognized a dramatic loss of interaction between Raptor and 14-3-3 upon loss of PI3KC2β, a previously described interaction that inhibits mTORC1 activity (Gwinn et al., 2008). I indeed was able to verify an interaction between 14-3-3γ and $PI(3,4)P_2$ and could show that the recruitment of 14-3-3γ to Ly/LEs depends on PI3KC2β and its lipid product. These data suggest that 14-3-3γ

is a direct effector of PI(3,4)P_2 and may contribute to the inactivation of mTORC1 on the Ly/LEs under conditions of growth factor deprivation.

3 Project II: Protein kinase N controls a lysosomal lipid switch to facilitate nutrient signaling via mTORC1

3.1 Overview of the project

We previously identified PI3KC2β as a repressor of mTORC1 activity, which acts locally at the lysosome. PI3KC2β recruitment and activation at the lysosome depend on growth factor depletion (Marat et al., 2017), similar to what previously has been described for other regulators of mTORC1 activity, e.g. the TSC-complex (Manning et al., 2002; Menon et al., 2014). The mechanism how growth factors regulate PI3KC2β localization as well as Ly/LEs $PI(3,4)P_2$ production remained unknown (Marat et al., 2017). In our previous work we could rule out a direct interplay between PI3KC2β and Akt as well as between PI3KC2β and AMPK. Hence, PI3KC2β regulation by growth factors must involve different, possibly novel components. To identify these factors and to further investigate the effects of growth factors on PI3KC2β I designed a SILAC-based mass-spectrometry screen using HEK293T cells endogenously expressing eGFP-PI3KC2β. I immunoprecipitated endogenous eGFP-PI3KC2β and quantitatively analyzed interacting proteins and the regulation of these interactions by growth factor starvation. As growth factor regulation often functions via signaling cascades involving protein phosphorylation, I determined phosphorylation sites within PI3KC2β via mass-spectrometry. 14-3-3 proteins were found as a prominent interaction partners of PI3KC2β under conditions of growth factor abundance. 14-3-3 proteins allosterically regulate target proteins by binding to specific phosphorylated motifs and are known to regulate a plethora of signaling pathways including mTORC1 signaling, with prominent examples of target proteins auch as TSC, PRAS40, Raptor and TFEB (Gwinn et al., 2008; Morrison, 2009; Sancak et al., 2007; Settembre et al., 2012). A motif surrounding phosphorylated threonine 279 (T279) of PI3KC2β was identified as the 14-3-3 binding motif. The 14-3-3 binding motif lies in the same region as the region that binds to Raptor, suggesting that the association of these proteins with PI3KC2β is mutually excluive. In agreement with that, 14-3-3 binding to PI3KC2β occurs in growth factor-rich conditions, i.e. conditions in which PI3KC2β does not interact with Raptor/mTORC1. Furthermore, 14-3-3 binding promoted the dimerization of PI3KC2β. In order to identify the kinase that phosphorylates PI3KC2β at T279 to promote 14-3-3 binding, I screened 245 serine/threonine kinases for their activity towards T279 of PI3KC2β. The only

kinase that could be confirmed to phosphorylate PI3KC2β at T279 in vitro and in HEK293T cells was PKN2. I could show that 14-3-3 binding to PI3KC2β depends on PKN2 and that recruitment of PI3KC2β to Ly/LEs and interaction with mTORC1 is inhibited by 14-3-3 and thus by PKN2. PI3KC2β recruitment to Ly/LEs was further shown to depend on Rab7, a small GTPase acting on late endosomes, and this recruitment only occurs with PI3KC2β that is not phosphorylated and 14-3-3 bound. These results suggest that PKN2 is a negative regulator of PI3KC2β and thereby might play a role in the activation of mTORC1. A loss of mTORC1 activity upon loss of PKN2 has been reported previously in a mouse model (Quétier et al., 2016). We could demonstrate that PKN2 loss indeed leads to reduced mTORC1 activity and increases the levels of $PI(3,4)P_2$ on Ly/LE-membranes. Both were rescued by the additional loss of PI3KC2β, suggesting that PKN2 regulates mTORC1 activity via regulating the recruitment of PI3KC2β to Ly/LEs. Finally, we were interested to determine how PKN2 itself is regulated by growth factor stimulation. mTORC2 is known as a central regulator of AGC-kinases, the kinase family to which PKN2 belongs. We therefore investigated whether PKN2 is a substrate of mTORC2 and whether loss of mTORC2 affects PKN2-kinase activity and thereby PI3KC2β-14-3-3 complex formation. We could confirm that mTORC2 phosphorylates PKN2 within its turn-motif, a phosphorylation which has been shown to be necessary for kinase activity of PKN2 (Lim et al., 2008). Furthermore, loss of mTORC2 led to loss of the interaction between PKN2 and PI3KC2β, loss of complex formation between PI3KC2β and 14-3-3 and loss of the phosphorylation of PI3KC2β.

Taken together, these results suggest a novel pathway of growth factor dependent regulation of mTORC1 signaling via an mTORC2-PKN2- PI3KC2β-axis.

3.2 Original publication

Wallroth, A., Koch, P.A., Marat, A.L., Krause, E., Haucke, V.: Protein kinase N controls a lysosomal lipid switch to facilitate nutrient signaling via mTORC1. *Nature Cell Biology*, 2019 https://doi.org/10.1038/s41556-019-0377-3

Personal Contribution

Since the mechanism of how growth factors regulate PI(3,4)P2 production of Ly/LEs was a major question remaining open after finding PI3KC2β as a novel inhibitor of mTORC1 activity, I initially designed screens to identify new interaction partners of PI3KC2β as well as potential post translational modifications. To be able to detect growth factor dependent quantitative differences concerning potential interaction partners of PI3KC2β as well as potential post-translational modifications, I performed SILAC-based mass-spectrometry screens. With these screens I Identified T279 of PI3KC2β as a phosphorylated residue in growth factor rich conditions. I could show that phosphorylation of this site is required for complex formation between PI3KC2β and 14-3-3 proteins. Following that, I designed and performed screens and experiments establishing PKN as the kinase phosphorylating PI3KC2β at T279 as well as showing that this phosphorylation and 14-3-3 binding results in dimerization of PI3KC2β. I then generated HEK293T cell lines carrying PKN1/2 knock-out, which I used to show that PKN loss leads to a loss of PI3KC2β-14-3-3-interaction, promotes PI3KC2β-Raptor interaction and leads to loss of mTORC1 activity. Generating HeLa cells expressing endogenously eGFP-tagged PI3KC2β I could show that loss of PKN2 not only promotes PI3KC2β-mTORC1 interaction, but also promotes recruitment of PI3KC2β to Ly/LEs. In addition, loss of PKN2 promotes PI(3,4)P_2 production on Ly/LEs. Furthermore, I could rescue mTORC1 activity and Ly/LE-PI(3,4)P_2 levels by additional depletion of PI3KC2β, suggesting that PKN2 promotes mTORC1 activity via inhibition of PI3KC2β. Furthermore, I could also rescue the mentioned effects appearing upon PKN2 loss by co-depletion of Rab7, a small GTPase which is necessary for Ly/LE-recruitment of PI3KC2β. Investigating, how PKN2 might be regulated by growth factor stimulation, I endogenously tagged PKN2 with mCherry in the HEK293T cells that already express endogenous eGFP-PI3KC2b. Trying to find a link between PKN2 and growth factor stimulation, I tested potential effects of mTORC2 manipulation on PKN2. Performing kinase assays I could show that mTORC2 can phosphorylate the turn motif of PKN2 at T958. With inhibition or

depletion of mTORC2 I could confirm these results in HEK293T cells. Furthermore, I could also show, that depletion of mTORC2 inhibits interaction between endogenous PKN2 and endogenous PI3KC2β, phosphorylation of T279 in PI3KC2β by PKN2 and finally leads to a loss of PI3KC2β-14-3-3 interaction. Collectively I could show that growth factor dependent regulation of PI(3,4)P_2 production on Ly/LEs and mTORC1-inhibtion functions via an mTORC2-PKN2-PI3KC2β axis.

4 Discussion

4.1 PI3KC2β in nutrient signaling

The class II of the PI3K-family has been the least studied class of these lipid kinases. Their role in cellular functions, their localization, their protein structure, even preferences for direct lipid products have not been fully described yet (Marat and Haucke, 2016). In the course of this thesis I identified the class II PI3K β as a novel player in nutrient signaling, giving first insights of the impact of this class II PI3K on cellular functions. I could show that PI3KC2β -and its main lipid product PI(3,4)P_2- act on late endosomal/lysosomal membranes and inactivate the central regulator of cell growth and cell metabolism, mTORC1. PI3KC2β and its lipid product are negatively regulated by growth factors via mTORC2 and PKN2 by sequestering the enzyme inactive in the cytosol preventing its recruitment and further activation. Upon growth factor depletion mTORC2 and following PKN2 are inactive. This leads to a release of the inactivating mechanism sequestering PI3KC2β and results in the recruitment to Ly/LEs and production of PI(3,4)P_2. Lysosomal/late-endosomal PI(3,4)P_2 then inhibits mTORC1 activity. The results of this thesis show that production of PI(3,4)P_2 has opposing effects on nutrient signaling to what has been seen before when produced on a different membrane (on Ly/LE-membranes instead on the plasma membrane). In addition to establishing PI3KC2β and late endosomal/lysosomal PI(3,4)P_2 as novel negative regulators of mTORC1, a new functional link between mTORC2 and mTORC1 was discovered. It could be shown that mTORC2 is not only promoting mTORC1 activity via activation of Akt, but also via activating PKN2 and thus inhibiting PI3KC2β and Ly/LE PI(3,4)P_2 production. These discoveries put the class II PI3K β in a central position acting as a downstream effector of mTORC2 and an upstream regulator of mTORC1, negatively regulating protein translation and cell growth and promoting autophagy and possibly being responsible for some physiological effects that have been described for mTORC2. While the functional relationship between PI3KC2β and mTORC1 have been investigated in detail in the course of this thesis, it remained open whether there are additional effects of mTORC2 or PKN manipulation that depend on PI3KC2β. In addition to regulating mTORC1, e.g. PKN as well as mTORC2 have been implicated in cytoskeleton remodeling and cell migration. It would be interesting to investigate whether PI3KC2β functions as a downstream effector of PKN and mTORC2 in these processes as well. A potential impact of PI3KC2β on cell migration in addition

to the discovered effects on cell growth would make PI3KC2β and its lipid product an interesting protein to investigate for example looking at cancer progression, a process where the regulation of cell growth and cell migration are tightly connected.

4.1.1 The lipid product of PI3KC2β

Class II PI3Ks and their lipid products have been the least studied among all PI3Ks. In previous studies, it has been shown that PI3KC2β is able to produce PI(3)P and PI(3,4)P_2 in vitro and in cell models such as hepatocyte-cultures (Alliouachene et al., 2015; Arcaro et al., 1998). In the course of this thesis, it was shown that recombinant expressed as well as immunoprecipitated human PI3KC2β had the highest affinity for PI(4)P as a substrate and very low affinities for other PI-species. In contrast to previous findings, immunofluorescence stainings using antibodies against PI(3,4)P_2 as well as a recombinant expressed FYVE-domain of Hrs to stain PI(3)P showed that endosomal PI(3,4)P_2 depends on the presence and activity of PI3KC2β whereas PI(3)P levels were completely unaffected upon PI3KC2β manipulation. The PI3KC2β dependent PI(3,4)P_2 pool is on LY/LEs, where a pool of PI(3,4)P_2 has not been described before. It was confirmed that PI3KC2β is only responsible for production of this pool of PI(3,4)P_2 at Ly/LEs and not on the plasma membrane. Manipulation of PI3KC2β did neither influence PI(3,4)P_2 at the plasma membrane nor phosphorylation of Akt, which is dependent on plasma membrane PI(3,4,5)P_3 or PI(3,4)P_2 (Ebner et al., 2017). Furthermore, we could not detect any effect of PI3KC2β on the late-endosomal/lysosomal pool of PI(3)P but only on PI(3,4)P_2. In agreement with these findings, it has recently been shown that VPS34 and its lipid product PI(3)P have opposing effects on mTORC1 activity and lysosome positioning compared to PI3KC2β and PI(3,4)P_2 production (Hong et al., 2017). However, an indirect implication of PI3KC2β in the regulation of late-endosomal/lysosomal PI(3)P levels under specific starvation, e.g. via PI-conversion of PI(3,4)P_2 to PI(3)P by the PI(3,4)P_2-4-phosphotases INPP4A/B (Hawkins and Stephens, 2016), might be possible. An involvement of PI3KC2β and PI(3,4)P_2 in the recruitment or regulation of other lipid-kinases and -phosphatases such as PIKFYVE or myotubularins (MTMs) -regulating endosomal and lysosomal PI(3,5)P_2 and PI(3)P levels- is also possible and an interesting point when addressing the role of PI3KC2β in PI-conversion on endosomal membranes. A functional relationship between MTMs and class II PI3K co-regulating a pool pf PI(3)P has previously been proposed in *drosophila* (Velichkova et al.,

2010). In an MTM1-deficient mouse model inhibition or depletion of PI3KC2β promoted rescue of myotubular myopathy symptoms (Sabha et al., 2016).

4.1.2 The localization of PI3KC2β

Class II PI3Ks have been the least studied class of PI3Ks and thus their subcellular localization and location of activity have been poorly understood. PI3KC2α has been implicated in signaling events downstream of the epidermal growth factor receptor (EGFR), in the maturation of clathrin coated pits in clathrin mediated endocytosis (CME) and in early endosomal trafficking and thus has been shown to act primarily at the plasma membrane and early endosomes (Arcaro et al., 2000; Campa et al., 2018; Franco et al., 2014; Posor et al., 2013). PI3KC2γ has been shown to be a Rab5 effector and regulates Akt2 signaling on early endosomes (Braccini et al., 2015). PI3KC2β has been the least studied kinase among the class II. PI3KC2β has been shown to interact with EGFR and Grb2 at the plasma membrane as well as clathrin in an undefined location. It also partly translocates to the cytoplasm and nucleus upon EGF stimulation (Arcaro et al., 2000; Banfic et al., 2009; Katso et al., 2006).

In the course of this thesis, the subcellular localization and location of activity of PI3KC2β was analyzed based on tracking endogenously expressed enzyme within the cell using CRISPR/Cas9-technology. Protein-protein-interactions were studied based on known sequence information and homologies between the class II PI3Ks. PI3KC2α and PI3KC2β have a very high sequence homology except in their unstructured N-termini. Since PI3KC2β was found to regulate mTORC1 activity but PI3KC2α does not, proteins that mediate this specific function of PI3KC2β were likely to bind within the non-homologous N-terminal region. Indeed, a specific interaction between PI3KC2β and mTORC1, which resides at late-endosomal/lysosomal membranes, could be described. This interaction depends on growth factor starvation. The growth-factor dependency is caused by a competing interaction between PI3KC2β and 14-3-3 proteins within the same sequence, which is dependent on phosphorylation by PKN2. 14-3-3 proteins bind to specific phosphorylated motifs and regulate the accessibility of binding surfaces, conformational changes or enzymatic activities and as scaffold proteins in a plethora of signaling pathways (Yaffe et al., 1997). This mechanism of regulation via phosphorylation and 14-3-3 interaction is specific for the β isoform of class II PI3Ks, since neither PI3KC2α nor PI3KC2γ contain 14-3-3 binding motifs within their N-termini. In agreement with this data, the endogenously GFP-tagged PI3KC2β in HEK293T and HeLa cells

showed a mainly cytosolic localization in conditions of high growth factors but the enzyme was recruited to Ly/LEs -the localization of mTORC1- upon growth factor depletion. Depletion of PKN2 had the same effect, suggesting that 14-3-3 proteins are keeping PI3KC2β cytosolic and prevent recruitment to Ly/LEs and mTORC1. Furthermore, Rab7, as small GTPase being active on LY/Les, was found as an additional factor in the mechanism of PI3KC2β recruitment to Ly/LEs. Whereas the phosphorylation and 14-3-3 binding to PI3KC2β generally inhibits the recruitment to Ly/LEs, once de-phosphorylated, Rab7 is needed to actively recruit the enzyme to its location of activity, probably via the RBD of PI3KC2β. A similar mechanism has been described for PI3KC2γ, which is recruited to early endosomes by the small GTPase Rab5 (Braccini et al., 2015). Since all three class II PI3Ks contain a RBD, recruitment of PI3KC2α to its location of activity via a small GTPase is also likely but has not been described yet.
In addition to the unstructured N-terminus and the RBD, the C-terminal PX-C2-modul of class II PI3Ks is also likely to play a role in their regulation or localization. It has been described that the PX and C2 domains are keeping PI3KC2α in a catalytically inactive conformation, which is resolved upon binding to PI(4,5)P_2, a lipid which is highly present at the plasma membrane (Wang et al., 2018). Similar mechanisms for PI3KC2β and PI3KC2γ remain unknown so far, but it is likely that not only recruitment via a small GTPase but also the PIP-identity of a membrane define to which membranes these kinases are recruited and activated.

4.1.3 Effects of lysosomal PI(3,4)P_2

It has been described that PI(3,4)P_2 is recognized by the PH-domain of Akt and thus can promote recruitment and activation of Akt (Ebner et al., 2017). These processes have been described for PI(3,4)P_2 which is either produced at the plasma membrane by class I PI3Ks or at early endosomes by PI3KC2γ (Braccini et al., 2015; Vanhaesebroeck et al., 2010a). Except from Akt, the PI3KC2α effector SNX9 and TAPP1/TAPP2 , downstream effectors of this PI have yet to be identified (Posor et al., 2013; Wullschleger et al., 2011). In mass spectrometry screens for the identification of proteins binding to different phosphoinositide species, 14-3-3γ was found (Jungmichel et al., 2014). In the course of this thesis, it could be confirmed that 14-3-3γ is able to bind to PI(3,4)P_2. However, a PIP-binding domain within the protein could not be identified. Functionally, manipulation of PI(3,4)P_2 levels via knockdown of PI3KC2β or adding exogenous PI(3,4)P_2-AM altered the recruitment of 14-3-3 to LY/LEs an the binding of 14-3-3γ to Raptor, a previously described interaction. Altogether, 14-3-3γ was found as a

$PI(3,4)P_2$ effector regulating mTORC1-activity. Interestingly, manipulation of $PI(3,4)P_2$ on LY/LEs not only influences mTORC1-activity but also the positioning of lysosomes within the cell. Whereas 14-3-3γ can work as a link coupling $PI(3,4)P_2$-generation to inhibition of mTORC1-activity, a functional relationship between 14-3-3γ recruitment to LY/LEs and changes of lysosome positioning could not be found, suggesting the presence of other effectors of lysosomal/late-endosomal $PI(3,4)P_2$ that regulate lysosomal positioning and maybe other effects of PI3KC2β which have not been described yet. Some proteins which have been shown to regulate the positioning of lysosomes are the small GTPases Arl8 and Rab7, the Ragulator complex as well as protruidin and FYCO1 (Filipek et al., 2017; Hong et al., 2017; Pu et al., 2017; Rosa-Ferreira and Munro, 2011; Wunderlich et al., 2001). Whereas loss of Ragulator has been shown to phenocopy the loss of PI3KC2β and suggests Ragulator as a potential downstream target of PI3KC2β, loss of Arl8 and FYCO1/protruidin leads to a perinuclear clustering of lysosomes, suggesting that PI3KC2β $PI(3,4)P_2$ production might activate GAPs of Arl8 or regulating factors of FYCO1/protruidin instead of these proteins directly. Furthermore, the positioning of lysosomes has been shown to be coupled to the pH gradient on the lysosomal membrane and thus might be coupled to v-ATPase activity (Johnson et al., 2016). Interestingly, lysosomal v-ATPase has already been suggested to be a direct effector of $PI(3,5)P_2$, so it likely is able to also bind $PI(3,4)P_2$, which might influence it activity as well (Li et al., 2014). Additionally, lysosomal $PI(3,4)P_2$ has been shown to activate the cholesterol transporter ORP1L, which is removing cholesterol from late-endosomal/lysosomal membranes (Dong et al., 2019). Cholesterol can activate mTORC1 via SCL38A9 (Castellano et al., 2017). ORP1L depletion hyperactivated mTORC1 suggesting ORP1L as another effector of $PI(3,4)P_2$ on Ly/LEs and link between PI3KC2β and mTORC1 (Dong et al., 2019).

In general, proteins recognize different PI-species via their PI-binding domains. For binding $PI(3,4)P_2$, PH-domains such as in Akt or TAPP1 as well as PX-BAR domains such as in SNX9 have been described. Unfortunately, 14-3-3γ , which was found in the course of this thesis as a $PI(3,4)P_2$ effector, lacks such a domain, similar to how v-ATPase has been described as a $PI(3,5)P_2$ effector. This raises the question whether the observed downstream effects are exclusively direct or whether yet unidentified PH- or PX/BAR-domain containing proteins are involved in these processes as well. Thus, looking for further $PI(3,4)P_2$ effectors on lysosomal membranes in future studies proteins containing PI-binding domains should be prioritized.

4.2 The regulation of PI3KC2β via PKN downstream of mTORC2

One characteristic of the class II PI3K β described in the course of this thesis is the growth factor starvation dependent recruitment and activation of this lipid kinase, which is a novel mechanism which has not been described for any other PI3K. The starvation dependent recruitment of PI3KC2β depends directly on association with active Rab7, but the key regulation occurs via phosphorylation dependent 14-3-3 binding of PI3KC2β, which shields the binding surface of PI3KC2β to raptor and keeps the kinase in an inactive cytosolic conformation. Screening 245 serine/threonine-kinases, the only kinases that could be identified carrying out this key phosphorylation were PKN1 and PKN2 (Protein kinase N 1 and 2). Using HEK293T and HeLa cells, only PKN2 but not PKN1 induced phosphorylation of PI3KC2β, suggesting PI3KC2β as an isoform specific target for PKN2. PKNs are PKC-related kinases within the family of AGC-kinases. Similar to how previously described for many other AGC-kinases (Pearce et al., 2010; Saxton and Sabatini, 2017), within this study mTORC2 was identified as a key activator of PKN2. mTORC2, which is activated in response to growth factor stimulation, phosphorylates and activates PKN2 which in turn phosphorylates PI3KC2β keeping it inactive in the cytosol. Altogether, this mTORC2-PKN2-PI3KC2β axis gives detailed insight into how the class II PI3K β is recruited to Ly/LEs and activated in response to growth factor starvation.

4.2.1 Regulation of mTORC1 by PKN

It has been previously suggested, that mTORC1 activity is affected by PKN2 and thus a downstream target. Induced PKN2-KO MEFs showed a reduction of phosphorylation at T389 of S6K, the mTORC1 target-site, whereas phosphorylation of Erk1/2 and Akt were unaffected (Quétier et al., 2016). These results could be confirmed depleting PKN2 via knock-out and knock-down in HEK293T cells in the course of this thesis. In addition, loss of PKN2 also decreased cell size, another indicator of lower mTORC1 activity. Interestingly, depletion of PKN1 did not affect mTORC1-activity, suggesting an isoform specific effect. The previously unknown mechanism of mTORC1 activation by PKN2 could be described as being indirect via PI3KC2β. PI3KC2β, a novel negative regulator of mTORC1 activity, is itself negatively regulated by PKN2, as described previously. Loss of PKN2 diminishes mTORC1 activity, which can be rescued by co-depletion of PI3KC2β. Interestingly, the co-loss of PI3KC2β did not completely rescue the PKN2 dependent loss of mTORC1 activity. This could suggest that other

unidentified targets of PKN2 also directly or indirectly affect mTORC1 activity and PI3KC2β is not the only effector of PKN2 influencing nutrient signaling via mTORC1. The results from this study indicate that in principle PKN1 and PKN2, two isoforms of PKN family sharing very high sequence homology (Mukai, 2003), are able to phosphorylate PI3KC2β and induce the inhibiting 14-3-3 interaction. However, in cell culture only PKN2 affected either mTORC1 activity or PI3KC2β–14-3-3 complex formation whereas loss of PKN1 had no effect on both and also co-depletion of PKN1 and PKN2 did not increase any effects. This strongly suggests that PI3KC2β and mTORC1 activity are PKN2 isoform specific targets and effectors. Still, similar effects of PKN1 on PI3KC2β and mTORC1 activity might occur in other cell types. Furthermore, it was not tested whether PKN1 can compensate for long-term PKN2 loss and takes over PKN2 functions. This is not unlikely since *in vitro* PKN1 and PKN2 phosphorylated PI3KC2β and forced 14-3-3-interaction.

4.2.2 The relationship between PKN and PI3KC2β

In the course of this thesis it has been shown that PKN2 is a key negative regulator of PI3KC2β localization and recruitment and thus an upstream regulator of mTORC1 activity. Phosphorylation of PI3KC2β at T279 induces 14-3-3 binding and dimerization and prevents binding of Raptor and Rab7, which are necessary for PI3KC2β to Ly/LEs. A remaining open question is where PKN2 and PI3KC2β come together,where the phosphorylation is happening and where PKN2 is localized in general. To address this question, live tracking of the HEK293T cell line carrying endogenous mCherry-tagged PKN2 might be helpful. One possibility would be that upon growth factor stimulation, PKN2 binds to active PI3KC2β at the Ly/LE-membrane, phosphorylating it and inducing PI3KC2β falling off Ly/LEs.

Furthermore, it would be interesting to obtain structural information, how dimers of PI3KC2β interact with dimers of 14-3-3 proteins and how this complex is preventing membrane recruitment of PI3KC2β. Both, 14-3-3 binding and dimerization of PI3KC2β have been shown to be dependent on growth factor starvation and phosphorylation at T279 of PI3KC2β. While it is clear that 14-3-3 binding directly prevents interaction with the mTORC1 complex because 14-3-3 proteins and Raptor are competing for the same binding surface, it remains unclear how 14-3-3 proteins prevent the binding of Rab7, which is probably occurring via the RBD of PI3KC2β. The RBD of PI3KC2β starts after the unstructured N-terminus approximately at

amino acid 320, whereas the binding surface for 14-3-3 proteins is a motif around amino acid 279, so the RBD is unlikely to be involved directly in PI3KC2β-14-3-3 complex formation.
Surprisingly, although the phosphorylation of T279 of PI3KC2β is inhibiting its recruitment to LY/LEs and thus inactivates the kinase, long term depletion of PKN2 and loss of this phosphorylation destabilizes PI3KC2β and decreases protein levels of PI3KC2β. This effect became obvious after depletion of PKN2 for more than 48h via siRNA mediated knockdown and was even more prominent while the attempt of generating a cell line carrying a T279A mutation within PI3KC2β via CRISPR/Cas. Successful mutation could be confirmed via genomic sequencing, but in clones with a homozygote T279A mutation PI3KC2β protein expression was not detectable anymore. These results suggest, that on one hand PKN2 phosphorylation of PI3KC2β functions inhibitory, but on the other hand stabilizes the inactive kinase. This suggests that the relationship between PI3KC2β and PKN2 is not straight forward. Long term inhibition or depletion of PKN2 might not permanently activate PI3KC2β and inhibit mTORC1 activity, but might phenocopy the loss of PI3KC2β and reverse the initially detectable cell biological phenotypes of PKN2 loss. If so, short term pharmacologic inhibition of PKN2 would inhibit cell growth via reducing mTORC1 activity, but long term the opposite might happen. Although data generated during this thesis and previous studies suggest that pharmacologic inhibition of PKN2 might be a novel way to inhibit mTORC1 activity and thus would be beneficial for treatment of diseases caused by mTORC1 miss- and upregulation, potential effects of long terms PKN2 inhibition on PI3KC2β as well as mTORC1 activity have to be investigated. Mechanistically, it remains elusive how PKN2 loss destabilizes PI3KC2β and this point would be interested to address in future studies. Since short term depletion of PKN2 leads to recruitment of PI3KC2β to LY/LEs, it is likely that PI3KC2β in the unphosphorylated and active conformation is e.g. prone to be degraded, maybe via Ubiquitination dependent pathways. Potential effects of degradative pathways on PI3KC2β stability, e.g. via proteasome inhibition, p62 depletion or inhibition of lysosomal degradation, should be addressed in future studies.

4.2.3 mTORC2 in PKN regulation

mTORC2 has been shown to be activated by growth factor stimulation via class I PI3Ks. The main mTORC2 downstream targets are kinases from the AGC-kinase family. It has been shown for many AGC kinases, that mTORC2 phosphorylates two sites within the C-terminus of the

kinase domain of AGC kinases, the turn motif and the hydrophobic motif with the most prominent kinases being phosphorylated by mTORC2 in both motifs being Akt and various PKC isoforms (Pearce et al., 2010; Saxton and Sabatini, 2017). These phosphorylations are necessary to allow further phosphorylation of the activation loop by PDK1. Thus, phosphorylation of the turn motif and the hydrophobic motif are necessary for substrate recognition and kinase activity of these kinases (Pearce et al., 2010). The turn motif of PKN1 and PKN2 are conserved with other PKCs whereas the hydrophobic motif -although it is mostly conserved- does not contain a phosphorylatable residue but a phospho-mimicking aspartate instead. This suggests that PKN1 and PKN2 could only be phosphorylated within the turn motif at threonine 958 and thus are only regulated via the turn motif phosphorylation, whereas the hydrophobic is in a permanently phosphorylated and active conformation due to the phospho-mimicking residue . It has been shown before that mutation of threonine 958 into alanine completely abolishes kinase activity of PKN2 (Lim et al., 2008). Furthermore T958 has been shown to be phosphorylated, although it has not been described before which kinase is phosphorylating this residue (Falk et al., 2014). Within this study, mTORC2 has been shown to be able to phosphorylate T958 of PKN2. Furthermore, mTOR inhibition and mTORC2 depletion diminished phosphorylation at T958 of PKN2 in HEK293T cells as well as inhibiting PKN2-PI3KC2β interaction, suggesting that this phosphorylation is performed by mTORC2 and is not only necessary for kinase activity but also for substrate recognition of PKN2. Furthermore, mTORC2 depletion also resulted in a dramatic reduction of PI3KC2β-14-3-3 complex formation strongly suggesting a mTORC2-PKN2 cascade that regulates PI3KC2β. Finally, confirming the previously described abolishment of PKN2 kinase activity, only wild type PKN2 but not PKN2-T958A could rescue PI3KC2β-14-3-3 complex formation in PKN1/PKN2 knockout-cells. Altogether, these results show a direct relationship between mTORC2 and PKN2 activity which in turn regulates localization and activity of PI3KC2β and finally mTORC1 activity. However, it is still possible, that mTORC2 is regulating PKN2 on additional ways, maybe even via direct phosphorylation at so far unidentified sites. Furthermore, Rho GTPases are implicated in PKN2 activation (Lim et al., 2008; Quétier et al., 2016). Indeed, within the course of this thesis it could be confirmed that the inhibition of Rho leads to a similar decrease of PI3KC2β-14-3-3 complex as depletion of mTORC2. Rho itself has also been suggested to be regulated by mTORC2, implicating that in addition to regulating PKN2 via direct phosphorylation, mTORC2

might also regulate PKN2 activity via Rho (Li and Gao, 2014). Open questions concerning the mTORC2-PKN2 relationship could be addressed in future studies.

4.3 Conclusion and outlook

Within the course of this thesis, the class II PI3K β could be identified as a novel negative regulator of mTORC1 activity. Additionally, mTORC1 regulation via PI3KC2β was shown to depend on upstream regulation via PKN2 and mTORC2. These results unveil a novel signaling pathway regulating mTORC1 activity upon growth factor stimulation independent of Akt. Furthermore, the discovered mTORC2-PKN2-PI3KC2β-mTORC1 signaling axis suggests a novel interplay between mTORC1 and mTORC2 activity in the regulation of cell growth and cellular metabolism.

These findings suggest that pharmacological targeting of PI3KC2β and PKN2 activity may open new possibilities for the treatment of diseases which are connected to deregulation of mTORC1 activity such as cancer, diabetes or obesity as well as X-linked myotubular myopathy. Going into that direction, it would be important to address the open question on how long term PKN2 loss destabilizes PI3KC2β.

From the cell biological perspective, it would be interesting to identify further effectors of lysosomal PI(3,4)P_2 to clarify the mechanism of how PI3KC2β regulates the positioning of lysosomes in addition to mTORC1 activity. In a bigger picture, PI3KC2β might the link in the relationship between mTORC1 activity and positioning of lysosomes.

Finally, investigating potential implications of PI3KC2β in other mechanisms regulated by mTORC2 and PKN2 could open new fields of research. Apart from regulating cell growth via mTORC1, mTORC2 as well as PKN2 are implicated in the regulation of cell adhesion and cell migration. Furthermore, loss of the Ragulator complex has not only been shown to phenocopy loss of PI3KC2β and thus is a potential downstream target of PI(3,4)P_2, but also implicated in focal adhesion dynamics and thus in cell adhesion and migration (Filipek et al., 2017; Pu et al., 2017; Schiefermeier et al., 2014; Wunderlich et al., 2001). Altogether, these findings suggest a potential involvement of PI3KC2β and its cell biological effects such as the repositioning of lysosomes not only in cell growth but also in cell adhesion and migration. PI3KC2β as an enzyme that effects and links both cell growth and cell adhesion/migration might be a powerful target in cancer therapy, where inhibition of both of these cellular functions is beneficial to inhibit on one hand tumor growth and on the other hand metastasis.

5 List of Figures

6 Bibliography

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