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PI3K isoforms in cell signalling and vesicle trafficking. — Source link

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PI 3-kinase isoforms at the crossroads of signalling and vesicular traffic

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Abstract | Phosphoinositide 3-kinases (PI3Ks) are a family of lipid kinases that phosphorylate intracellular inositol lipids to regulate signalling and intracellular vesicular traffic. Mammals have eight isoforms of PI3K, divided into three classes. The class I PI3Ks generate 3-phosphoinositide lipids which directly activate signal transduction pathways. In addition to being frequently genetically-activated in cancer, similar mutations in class I PI3Ks have now also been found in a human non-malignant overgrowth syndrome and a primary immune disorder which predisposes to lymphoma. The class II and III PI3Ks are regulators of membrane traffic along the endocytic route, in endosomal recycling and autophagy, with an indirect impact on cell signalling. Here we summarize current knowledge on the different PI3K classes and isoforms, focusing on recently uncovered biological functions and the mechanisms by which these kinases are stimulated. Areas covered include emerging evidence for isoform-specific regulation and function of Akt family members, potential non-cytotoxic actions of PI3K inhibitors in cancer and regulation of mTORC1 by class II and III PI3Ks. A deeper insight into the PI3K isoforms will undoubtedly continue to contribute to a better understanding of fundamental cell biological processes, and ultimately, in human disease.

The cDNA cloning of the first catalytic subunit of a PI3K (p110α, Ref.[1]) in 1992 revealed close sequence similarity to the *Saccharomyces cerevisiae* Vps34 gene product, which was soon thereafter documented to possess PI3K activity in that it could convert phosphatidylinositol (PI) to its 3-phosphorylated PI(3)P derivative[2]. Subsequent bioinformatic and molecular biology approaches based on sequence homology of the kinase domain of these enzymes allowed the isolation of multiple PI3K genes from a range of organisms, with the Waterfield group proposing the now generally-accepted classification of the isoforms of PI3K[3-6]. The main role of Vps34 in yeast in regulating the transport of proteins to the lysosome-like vacuole[7] indicates that regulation of vesicular traffic is the most ancient function of 3-phosphoinositides, with a role in signalling being a later addition in eukaryotic evolution[8].

Genetic and pharmacological approaches have now uncovered the broad functions of the different PI3K isoforms, some of which are targets of the first approved PI3K inhibitors for the treatment of human cancer. The challenges faced in effectively targeting PI3K in disease have illustrated that much remains to be learned about PI3K biology.

In this review, we summarize key recent insights into PI3K signalling and cell biology in mammals, for example, newly discovered human syndromes, resulting from constitutive activation of class I PI3Ks leading to tissue overgrowth[9] or immune deregulation[10, 11]. Despite having been discovered over two decades ago[12, 13], the class II PI3Ks remain the most enigmatic PI3K subfamily. Recent studies have started to uncover mechanisms by which these PI3Ks are regulated and how they contribute to $PI(3,4)P_2$ and PI(3)P lipid pools to regulate endocytosis and the endolysosomal system. Exciting progress has also been made in understanding the structural basis of the activation of Vps34, the sole class III PI3K member, and how its distinct endosomal and autophagy-related functions control the activity of several protein kinases.

Class I PI3Ks

Class I PI3Ks are heterodimers of a p110 catalytic subunit in complex with a regulatory subunit. They engage in signalling downstream of tyrosine kinases, G protein-coupled receptors (GPCRs) and monomeric small GTPases (FIG. 1). In contrast to *C. elegans* and *Drosophila,* which only have a single

Commented [BV1]: Phosphatidylinositol: A glycerophospholipid with the cyclohexanehexol *myo*-inositol as its headgroup. The 3'-, 4'- and 5'-OH groups can be reversibly phosphorylated, resulting in a total of 7 phosphoinositide species.

Commented [BV2]: G protein-coupled receptor: A receptor with seven trans-membrane helices that functions as a ligand-dependent GEF activating heterotrimeric G proteins for downstream signalling.

Commented [BV3]: Heterotrimeric G proteins: Guanine nucleotide-binding proteins composed of three subunits: Gα, Gβ and Gγ. Gα cycles between an inactive GDP-bound conformation and an active GTP-bound conformation, which is released from its Gβγ partners. Both Gα and Gβγ can then modulate the activity of downstream effector proteins.

Commented [BV4]: Monomeric small GTPases: GTP-binding proteins which are homologous to the Gα subunit of heterotrimeric G-proteins but occur as monomers. They bind to and hydrolyze GTP to GDP, and are mostly active in the GTP-bound state.

class I PI3K isoform (AGE-1 and Dp110, respectively), mammals have four class I PI3K catalytic subunits. These are p110α, β, γ and δ, encoded by the *PIK3CA*, *PIK3CB, PIK3CG* and *PIK3CD* genes, respectively.

Structural insights into class I PI3K activation have been reviewed recently[14-16], as have the roles of class I PI3K isoforms in normal physiology and disease[17-23]. Below we discuss emerging data on isoform-selective regulation of the class I PI3Ks and the finding that class I PI3K pathway components are not only mutationally-activated in cancer but also in human overgrowth and in a primary immune deficiency syndrome. We also discuss how class I PI3K phosphoinositide lipid products may act in early endosomes to regulate specific isoforms of the Akt protein kinase, resulting in isoform-selective phosphorylation of its substrates. Interesting emerging data suggest that clinical efficacy of class I PI3K inhibitors in cancer might be due to non-cytotoxic mechanisms, including through regulation of the responsiveness of cancer cells to hormone therapy and stimulation of the host anti-tumour immune response.

Commented [BV5]: Primary immune deficiency: An inherited disorder of the immune system that leads to immune dysregulation.

Commented [BV6]: Early endosomes: Membrane-bound intracellular vesicles marked by RAB5 and EEA1 and high levels of PI(3)P. Endocytic vesicles fuse with early endosomes and cargo is sorted for recycling or degradation. Early endosomes then mature into late endosomes.

Commented [BV7]: Hormone therapy (also known as endocrine therapy): Treatment that interferes with hormone signalling in hormone receptor-positive cancers, such as some type of breast or prostate cancer.

Class I PI3Ks: structure and signalling

Class I PI3K subunits and their binding partners. All PI3Ks contain a conserved so-called 'PI3K core', consisting of a C2 domain, helical domain and kinase domain (FIG. 1).

The class I PI3Ks are subdivided into class IA and IB enzymes, based on the differences in regulatory subunits (FIG. 1). Their catalytic subunits consist of the PI3K catalytic core, extended N-terminally with the so-called Ras binding domain (RBD) (which can bind Ras, Rac1 or Cdc42) and, in the case of the class IA PI3Ks, a binding domain for a regulatory subunit (FIG. 1).

The class IA catalytic subunits (p110α, β and δ) occur in complex with a p85 regulatory subunit, which keeps the p85/p110 heterodimer in an inactive cytosolic state. Mammals have three genes encoding p85 subunits (*PIK3R1*, *PIK3R2* and *PIK3R3*), which can give rise to five gene products (p85α/p55α/p50α, p85β and p55γ). Class IA PI3Ks become activated upon recruitment to the plasma membrane via binding of the SH2 domains of p85 to tyrosine-phosphorylated proteins such as receptor tyrosine kinases (RTKs) or other membrane-bound proteins such as the insulin receptor substrate (IRS) proteins. A study using mass spectrometry approaches and mouse models with endogenously-tagged class IA subunits has revealed that p85α and p85β bind equally to p110α and p110β, whereas p85α preferentially interacts with p110δ (Ref.[24]). The same study also provided evidence for the existence of p110-free p85 subunits[24].

p110γ is the sole class IB PI3K and occurs in complex with a p84 or p101 regulatory subunit. This PI3K is activated by Gβγ subunits released by activated GPCRs which engage the heterodimeric p110γ complex with the plasma membrane[25].

All class I catalytic subunits also interact, via their RBD, with membrane-bound small GTPase (G) proteins, namely Ras (p110α, p110δ, p110γ) or Rac1 or Cdc42 (p110β)[26, 27]. Amongst the class I PI3K isoforms, p110β is unique in its capacity to additionally interact with Gβγ subunits (through the C2-helical linker)[28] and with activated Rab5 in the helical domain[29] (FIG. 2). This makes the regulation of p110β amongst the most complex of the class I PI3Ks, with several inputs possibly required for full activation. This might also allow p110β to act as a coincidence detector and integrator of distinct types of upstream signals[30] (reviewed in Refs.[15, 31]).

Below, we use the terminology PI3Kα, PI3Kβ, PI3Kγ and PI3Kδ when referring to the heterodimeric class I PI3K complexes.

Phosphoinositides generated by class I PI3Ks. Activated class I PI3Ks phosphorylate $PI(4,5)P_2$ at the plasma membrane, to generate the lipid second messenger $PI(3,4,5)P_3$ (or PIP_3 in short), which is metabolised to $PI(3,4)P_2$ (FIG. 2) by 5-phosphatases such as SHIP. The PTEN 3-inositol lipid phosphatase is a key regulator of the cellular levels of PIP_3 and $PI(3,4)P_2$, converting these lipids to $PI(4,5)P_2$ and PI(4)P, respectively[32, 33].

Mass spectrometry approaches have revealed the existence of distinct $PI(4,5)P_2$ species, with a diversity of fatty-acyl profiles[34-36]. There are indications that the acylation pattern of

phosphoinositides can modulate their metabolism and the effector response[37] and early data in platelets show that some minor species of $PI(4,5)P_2$ become highly enriched upon stimulation[38]. While the functional implications of these observations remain to be determined, such analyses have the potential to uncover additional functional diversification of the cell's 3-phosphoinositide code.

There is emerging evidence that p85 subunits may also have functions independent of the catalytic p110 subunit, especially in cancer, where they may occur free of p110 or acquire mutations that convert them into neomorphic proteins with new functionalities, such as activation of JNK and ERK signalling pathways (reviewed in Refs.[39, 40]).

3-phosphoinositide protein effectors. PIP_3 and $PI(3,4)P_2$ interact with lipid-binding pleckstrin homology (PH) domains in a range of protein effectors, to modulate their localisation and/or activity. Well-established effectors include protein kinases (such as PDK1, Akt (also known as PKB) and Btk), regulators of monomeric GTPases (GTPase-activating proteins (GAPs) and GTP/GDP exchange factors (GEFs)) and scaffolding proteins (such as GAB proteins). These allow class I PI3Ks to regulate a broad range of biological activities, including nutrient sensing, cell cycle progression and migration[17, 19, 41] (FIG. 2).

While most class I PI3K protein effectors bind both PIP_3 and $PI(3,4)P_2$, some selectively bind $PI(3,4)P_2$ and could control $PI(3,4)P_2$-selective signalling pathways[42, 43]. It will be of interest to determine whether and how class I $PI(3,4)P_2$-driven biology integrates with that of $PI(3,4)P_2$ produced by the class II PI3Ks.

It is estimated that, in a typical mammalian cell, around 10-30 and possibly even more proteins can directly bind class I PI3K lipids[44-47]. Non-PH-domain-based interactions of proteins with PIP_3 and/or $PI(3,4)P_2$ have also been described, e.g. with specific isoforms of the myosin motor proteins[48, 49]. This indicates the existence of multiple potential additional class I PI3K-dependent effector pathways.

Akt is activated by PIP_3/$PI(3,4)P_2$-driven recruitment to the plasma membrane where it undergoes phosphorylation on the conserved Thr308 (by PDK1) and Ser473 (by mTORC2) sites, leading to its full activation[50]. The ease by which Akt activation can be monitored as a proxy for class I PI3K activation (using phospho-specific antibodies against the Ser473 and Thr308 activation sites), in part explains why a large focus thus far has been on this kinase. However, given the potential for the class I PI3K lipids to engage with a broader diversity of effectors, it is not surprising that Akt is not always absolutely required for PI3K-driven output (reviewed in Ref.[51]).

Over 100 Akt substrates have now been identified[50], including inhibitors of cell cycle progression (p21, p27), GAPs (TSC2, AS160), apoptosis inducers (BAD), protein kinases (GSK3β) and a subset of the forkhead box O (FOXO) transcription factors. The function of many Akt substrates has now been clarified, including that of phosphorylation of TSC2 in providing growth-factor input into mTORC1 activation (BOX1). This contrasts with the role of the FOXO transcription factors in PI3K signalling, whose functional role is most likely more context-dependent due to modulation by other cell type-specific proteins. FOXOs regulate a broad range of target genes, with Akt phosphorylation leading to exclusion of FOXOs from the nucleus, thus negatively regulating their transcription factor activity. Relieving a possible tumour suppressor role of FOXOs by PI3K/Akt signalling was a concept to rationalize this inverse correlation between PI3K activity and FOXO target gene expression[52]. However, recent data indicate that FOXOs are not classical tumour suppressors[53], with the overall importance of FOXO regulation in PI3K biology remaining unclear. In T-lymphocytes, however, a clear role for Akt-driven FOXO regulation in differentiation and function in response to nutrients and stress is emerging[54, 55].

Spatiotemporal signalling by Akt isoforms. Mammals have three isoforms of Akt (Akt 1, 2 and 3) for which no isoform-selective inhibitors are available[50]. Akt1 and Akt2 are both widely expressed, with an enrichment of Akt2 in insulin-responsive tissues, while Akt3 shows a more restricted tissue distribution in the brain[56]. While Akt isoforms have similar *in vitro* substrate specificity, emerging *in*

Commented [BV8]: Pleckstrin homology (PH) domain: A sequence of approximately 100 amino acids that can mediate specific binding to PI lipids and is present in many signalling molecules. Only a minority of PH domains actually bind lipids.

Commented [BV9]: GAP: A protein that inactivates small GTP-binding proteins, such as Ras and Rheb family members, by
increasing their rate of GTP hydrolysis..

Commented [BV10]: GEF: A protein that facilitates the exchange of GDP for GTP in the nucleotide-binding pocket of a GTP-binding protein.

vivo evidence indicates the potential for substrate specificity and isoform-specific biological functions[50, 57, 58]. The molecular basis for this specificity has remained unclear.

A recent study has provided evidence for isoform-selective Akt recruitment to specific subcellular compartments[59], potentially resulting in exposure of Akt2 to specific substrates residing in these compartments. Functional Akt isoform specificity might therefore be based on substrate accessibility, rather than due to differences in intrinsic catalytic substrate specificity. This conclusion is based on a report from Liu *et al*. who, using spatiotemporally-resolved quantification of PIP_3 and $PI(3,4)P_2$ in live fibroblasts stimulated with PDGF, showed that both lipids were generated at the plasma membrane, with only $PI(3,4)P_2$ being transferred via clathrin-mediated endocytosis to an early endosomal compartment where Akt2 is preferentially activated over Akt 1 and 3 (Ref.[59]).

Commented [BV11]: Clathrin-mediated endocytosis: The internalization of plasma membrane and receptors therein into small vesicles that is mediated by a protein coat containing clathrin, adaptors and accessory proteins.

These authors further showed that the short linker region between the PH and catalytic domains of the Akt isoforms determines their 3-phosphoinositide binding selectivity, with Akt1 and Akt3 showing strong selectivity for PIP_3 whereas Akt2 binds both PIP_3 and $PI(3,4)P_2$. This might help to explain why Akt1 and Akt3 are transiently activated only at the plasma membrane, whereas Akt2 is active for longer at the plasma membrane and also becomes recruited to early endosomes. Evidence is presented that this endosomal Akt2 is able to phosphorylate certain substrates such as GSK3β, but not FOXOs or TSC2. It is not clear if such Akt isoform substrate specificity also exists during the time they are active at the plasma membrane.

The notion that Akt2 can be selectively activated by $PI(3,4)P_2$ is also shown by inactivation of the INPP4B phosphatase, which converts $PI(3,4)P_2$ to PI(3)P, leading to selective activation of Akt2 but not Akt1 (Ref.[60]). WDFY2, a FYVE domain-containing protein found on early endosomes, has been shown to selectively interact and regulate the signalling of Akt2 but not of Akt1 (Ref.[61]), adding to the emerging concept that Akt2 is selectively recruited to endosomes where it can phosphorylate specific substrates, especially in metabolic tissues where it is the most abundant Akt isoform[56, 62]. Interestingly, $PI(3,4)P_2$ produced in an endosomal compartment by the class II PI3K-C2γ upon hepatocyte stimulation with insulin also results in a delayed and sustained activation of Akt2 as compared to Akt1, leading to phosphorylation of GSK3β but not of FOXO or the downstream substrate S6 (Ref.[63]).

Commented [BV12]: FYVE domain: A PI(3)P-binding domain of ~60–65 amino acids that is named after the four Cys-rich proteins — FAB1, YOTB (also known as ZK632.12), VAC1 and EEA1 — in which it has been found.

A recent study[64] has provided evidence that $PI(4,5)P_2$-generating kinases, class I PI3Ks, PDK1 and Akt can assemble on the IQGAP1 (IQ motif containing GTPase-activating protein 1) scaffold protein. It is clear that the existence of such higher order complexes of specific kinases, effectors and their substrates has the potential to compartmentalise and facilitate specific and efficient kinase-substrate signalling pathways. The recruitment of specific Akt isoforms to such IQGAP1 complexes has not been investigated.

Molecular mechanisms of class I PI3K isoform specificity. Mouse gene targeting studies have uncovered organismal roles of class I PI3Ks[65], including key roles in insulin signalling and organismal metabolism (PI3Kα[66, 67] but not PI3Kβ[68, 69]), angiogenesis (PI3Kα)[70] and immunity (PI3Kγ and PI3Kδ[71, 72]) (FIG. 2). Interestingly, no such overarching organismal role for the kinase activity of PI3Kβ has emerged to date. Pharmacological studies using isoform-selective PI3K inhibitors have confirmed these biological functions of class I PI3K isoforms in humans, apart from angiogenesis, which has not been addressed to date.

One element to explain the physiological functions of the class I PI3K isoforms may relate to their relative expression levels in different tissues. p110α and p110β show a broad tissue distribution, while p110γ and p110δ are highly expressed in white blood cells, but also found in some other tissues at lower levels than in leukocytes. This includes expression of p110γ in cardiomyocytes where it is upregulated under stress conditions, triggering adverse cardiac remodelling that contributes to heart failure[73, 74]. p110δ is also found in neurons[75, 76] and fibroblast-like cells[77, 78] with functions in axonal transport[79] and inflammatory responses[77], respectively. p110δ can also become highly expressed in some solid tumour types, such as breast[80, 81] and liver[82, 83], with emerging evidence that it could be a therapeutic target in these cancers[81-83].

Another parameter in isoform-selective function of PI3K isoforms is their differential capacity to respond to specific upstream signals (FIG. 2). For example, only PI3Kα regulates insulin signalling and organismal metabolism[66-69], despite PI3Kα and PI3Kβ both being expressed in metabolic tissues. This may be partially explained by the selective recruitment of PI3Kα over PI3Kβ to tyrosine-phosphorylated IRS proteins downstream of activated insulin receptors[66], possibly analogous to the higher recruitment of p110α/p85 over p110β/p85 to activated platelet-derived growth factor receptors (PDGF-Rs)[24]. Insulin may also not provide the plethora of upstream stimuli required for full activation of PI3Kβ (FIG. 2). However, in PDGF-stimulated mouse embryonic fibroblasts, despite being recruited about five-fold less efficiently to the stimulated PDGFR than PI3Kα, PI3Kβ still contributed 40% of the PIP_3 (Ref.[24]). This was explained by the fact that p110β also becomes activated by receptor-activated small GTPases (Rac/Cdc42)[24]. These observations illustrate how PI3Kβ can act as a coincidence detector[15, 30, 84] but also highlight the challenges in fully understanding the molecular mechanisms of isoform-selective functions of PI3Ks.

In this context, it is important to note that while class I PI3K isoforms often have dominant functional roles in normal physiology upon stimulation with specific stimuli, such isoform-selective function is often lost upon cell transformation[85, 86]. This might be due to aberrant expression of PI3K isoforms and/or co-incident ongoing activation of multiple distinct signalling pathways in cancer cells.

Class I PI3Ks in cancer signalling and therapy

Genetic PI3K pathway activation in cancer. Somatic genetic activation of the class IA PI3K pathway is very common in cancer, through multiple mechanisms[21, 22], including mutational activation of p110 catalytic subunits or inactivation of the PTEN tumour suppressor. Similar genetic lesions have now also been found in *PIK3CA*-related overgrowth spectrum (PROS), PTEN hamartoma tumour syndrome (PHTS) and the activated PI3Kδ syndrome (APDS), described in the paragraphs below.

Commented [BV13]: Somatic mutation: mutation that occurs after conception

Activating mutations in the p110 catalytic subunits in cancer, leading to single amino acid substitutions, are most frequent in *PIK3CA*, with a lower frequency of mutation in *PIK3CB* or *PIK3CD*. These mutations are found throughout the coding region of the p110 isoforms, but most are in 'hotspot regions' in the helical and kinase domains (FIG. 1), with the RBD conspicuously negative for such mutations. These mutations mimic and enhance dynamic events in the natural activation process of the auto-inhibited p85/p110 complex[87].

Evidence is emerging that *PIK3CA* mutant cancers show preferential sensitivity to PI3Kα inhibitors, but this correlation is not universal. In this context, the PTEN/PI3Kβ interdependency observed in some PTEN-deficient cancers is also not a general phenomenon[88, 89].

Genetic PI3K pathway activation can occur early or late in the development of cancer. Evidence has been presented that activating *PIK3CA* mutations are an early, clonal event in breast[90] and colon cancer[91] as is heterozygous PTEN loss in squamous lung carcinoma[92].

S*ignalling dynamics upon genetic PI3K pathway activation.* Recent studies have shown that Ras/Erk pathway-activating mutations in cancer cells can result in the loss of temporal resolution of transient signalling, resulting in so-called signal misinterpretation[93, 94]. How genetic PI3K pathway activation impacts on the mode of PI3K signalling and its biological output has not been investigated in detail. Whereas especially PI3K mutation is expected to lead to sustained signalling under basal, non-stimulated conditions, PI3K mutation or PTEN inactivation may also affect the amplitude and/or duration of transient PI3K signalling in response to growth factors[95, 96].

It has been known for a long time that transient and sustained tyrosine kinase signalling can results in distinct functional outputs[97]. This principle has also been documented for PI3K using PIP_3-stimulated exocytosis of the cytosolic vesicle-associated GLUT4 glucose transporter as a model system[98]. Indeed, GLUT4 exocytosis requires long-term, high amplitude PIP_3 signals, but is not induced by short-duration high-amplitude or persistent low amplitude PIP_3 signals. Deregulation of normally cyclical events in cells by constitutive PI3K activation may contribute to tumour-promotion, illustrated

by the deregulated centrosome amplification induced by mutated PI3K[90, 99], which can lead to chromosomal instability and possibly facilitate tumour evolution[100].

Non-cytotoxic anti-cancer effects of PI3K inhibitors. The frequent aberrant activation of the class I PI3K pathway in human cancer has led to intense efforts to develop PI3K pathway inhibitors for oncology. However, class I PI3K targeting has not been effective in solid tumours to date, in part due to effective non-genetic and genetic compensatory feedback loops upon high-dose treatment with PI3K inhibitors and poor drug tolerance (reviewed in Refs.[101, 102]), amongst other factors.

It is important to recognize that PI3K inhibitors are not cytotoxic for cancer cells and are cytostatic at best. This reflects the role of class I PI3K in growth factor/nutrient-sensing, with cells entering a dormant state resembling nutrient-deprivation upon class I PI3K pathway inhibition, without direct induction of cell death signalling pathways[103]. Moreover, transformed cells can survive with very low levels of PI3K activity[86].

Emerging evidence indicates that the clinical impact of PI3K inhibitors in cancer might derive from non-cytotoxic mechanisms such as enhancement of endocrine therapy, stimulation of an anti-cancer immune response, or interfering with tumour-stroma interactions, as detailed below.

PI3Kα inhibitors can enhance oestrogen pathway activity in breast cancer and increase the dependence of these cells on this hormone, as indicated by increased sensitivity to endocrine inhibition[104]. Reported mechanisms include an increase in oestrogen receptor expression through enhanced FOXO3A-mediated transcription[104] and an epigenetic mechanism involving Akt and its substrate KMT2D, a histone methyltransferase[105, 106]. Akt phosphorylation of KMT2D attenuates its methyltransferase activity, whereas PI3Kα inhibition enhances KMT2D activity, leading to a more open chromatin state which facilitates oestrogen receptor-dependent transcription[105]. PI3K/Akt inhibitors have also been shown to sensitize prostate cancer to androgen receptor blockade[107-110]. These observations help to explain the current clinical development focus on PI3Kα and Akt inhibitors, with several encouraging reports from phase II/III trials in breast (NCT02437318; NCT03310541)[111] and prostate cancer (NCT03072238; NCT03310541)[112], in combination with hormone therapy or paclitaxel, respectively.

An exciting recent discovery is that PI3K inhibition can stimulate anti-tumour immunity, either by providing immunostimulatory activity to tumour-associated macrophages (by PI3Kγ inhibitors)[113, 114] or by preferential inhibition of regulatory T-cells (by PI3Kδ inhibitors, which also dampen immunosuppressive myeloid-derived suppressor cells)[115-118]. This is the basis for the ongoing cancer immunotherapy trials of solid tumours using inhibitors of the leukocyte PI3K isoforms[101, 119].

Three inhibitors which differ in PI3K isoform-selectivity but share the ability to inhibit PI3Kδ are now approved for the treatment of specific B-cell malignancies[119]. Interestingly, the efficacy of these compounds does not depend on the PI3K/PTEN mutational status of the leukaemic cells and is unlikely to derive from direct cytotoxicity. Indeed, the main leukaemia-intrinsic impact of PI3Kδ inhibition appears to be a dampened response to intrinsic and extrinsic tumour-supporting signals, including B-cell receptor signalling. PI3Kδ inhibition also negatively impacts on specialised stromal cells that support the leukaemic cells, such as mesenchymal stromal cells and myeloid-derived nurse-like cells[101, 120]. This dual action of PI3Kδ inhibitors is expected to weaken the leukaemic cells and render them more vulnerable to combination therapy such as with B-cell-depleting antibodies (e.g. anti-CD20) or chemotherapy. This is especially the case in chronic lymphocytic leukaemia, where PI3Kδ inhibition often induces lymphocytosis[121], releasing the leukaemic cells from their protective niches in the bone marrow and lymph nodes into the peripheral blood. It is likely that the immunostimulatory activity of PI3Kδ inhibition also contributes to its overall clinical impact in B-cell malignancies.

Class I PI3Ks in human disease syndromes

PIK3CA-related overgrowth spectrum (PROS). Activating mutations in *PIK3CA* can give rise to the so-called *PIK3CA*-related overgrowth spectrum (PROS)[9]. In these rare, congenital disorders, *PIK3CA*

Commented [BV14]: Hormone therapy (also known as endocrine therapy): Treatment that interferes with hormone signalling in hormone receptor-positive cancers, such as some type of breast or prostate cancer.

Commented [BV15]: Placlitaxel: anticancer agent that acts by stabilizing microtubule through binding tubulin

Commented [BV16]: Congenital disease: a disease that is present from birth

mutations are acquired postzygotically, with the majority of cases likely arising during embryogenesis[9]. The resulting genetic mosaicism is associated with substantial phenotypic heterogeneity, reflecting differences in the exact timing and location of each mutation. Their mosaic nature, and the lack of germline transmission, suggest that heterozygous, activating *PIK3CA* alleles may be embryonic lethal[9], as observed in mouse models[90, 122].

The spectrum of *PIK3CA* mutations in PROS mirrors that of cancer, yet the overgrowth in PROS is benign and is typically not associated with cancer predisposition. Interestingly, *PIK3CA* mutations in PROS are mainly found in tissues derived from mesoderm (such as fat, muscle, bone and vasculature) and neuroectoderm (such as brain), possibly reflecting lineage-specific negative selection of mutant cells[9]. Conversely, somatic *PIK3CA* mutations are relatively rare in the sporadic cancers originating from these tissues, which might indicate only a weak tumour-promoting role of mutant *PIK3CA* in these tissue contexts. Another remarkable observation is an often low percentage of *PIK3CA*-mutant cells in the overgrowth tissue, indicative of potential paracrine effects of *PIK3CA*-mutant cells on non-mutant surrounding cells[9].

Vascular malformations are one of the most common clinical features in PROS. Somatic mosaic mutations in the PI3K pathway have been associated with a range of subtypes of vascular malformations (reviewed in Ref.[123]), with *PIK3CA* mutations driving so-called venous malformations[124-126] and lymphatic anomaly[127].

Treatment of PROS patients with a low dose of a PI3Kα-selective small molecule inhibitor has been found to effectively reduce PROS overgrowth, to be very well-tolerated and not to impact on the organismal growth of paediatric patients, thus offering great clinical promise for PROS[128]. This contrasts with the modest reduction in overgrowth by a low dose of the mTORC1 inhibitor sirolimus, which also led to frequent and considerable adverse events in this patient population[129].

PROS may hold important lessons for treatments of *PIK3CA*-associated cancers. Given the shared genetic defect, the clinical observations in PROS raise the important question whether a low-dose of PI3Kα inhibitor could also normalize aberrant PI3K signalling in cancer[130]. In cancer therapy, PI3K pathway inhibitors are currently used at maximum-tolerated doses, intended to block all cellular PI3K activity, but these drugs are poorly tolerated by the patients, and when used at high concentrations induce immediate signalling feedback loops that can neutralize the effect of PI3K inhibitors. Interestingly, such neutralising feedback loops do not seem to occur upon low-dose PI3K inhibition, possibly because of some remaining PI3K activity to support normal growth factor signalling under these conditions.

PTEN hamartoma tumour syndrome (PHTS). PHTS is a cancer predisposition syndrome caused by heterozygous *PTEN* dysfunction in all cells of the body, including germ cells[131, 132]. Thus, unlike *PIK3CA* mutations in PROS, heterozygous *PTEN* inactivation is compatible with human development, illustrating the distinct biological impact of these genetic defects. PHTS is also associated with macrocephaly and developmental disabilities[131, 132]. It remains to be determined whether these differences reflect the broader cellular function of *PTEN* and/or dose-dependent effects of class I PI3K signalling[9].

A limited number of case reports have been published on the treatment of PHTS patients with the mTOR inhibitors sirolimus (reviewed in Ref.[133]) or rapamycin[134, 135]. These treatments led to a variable clinical impact, with reappearance of symptoms upon drug discontinuation. It will be of interest to explore other PI3K pathway inhibitors in this clinical setting.

Activated PI3Kδ Syndrome (APDS). Dominant activating mutations in *PIK3CD* are the cause of a primary immunodeficiency disease, called Activated PI3Kδ Syndrome (APDS)[136] or PI3Kδ-activating mutation causing senescent T cell, lymphadenopathy and immunodeficiency (PASLI)[137] (reviewed in Refs.[10, 11]). *PIK3CD* mutations in APDS are of a similar nature and located in the equivalent hot-spot locations as in oncogenically-activated *PIK3CA*. These mutations are inherited from one of the parents who are heterozygous carriers of this mutation in all cells or only in the germline. An interesting observation is

that leukocytes with activated *PIK3CD* are very prone to cell death, indicating that high levels of PI3Kδ activity can be deleterious for cells[136, 137], possibly leading to cell senescence upon PI3Kδ overactivation[137].

A clinical phenocopy of this disease, sometimes called APDS2, is caused by specific mutations in *PIK3R1* which, for reasons that are unclear, preferentially activate p110δ and not p110α or p110β[10, 138], and therefore mainly act in the immune system. The recent finding that p85α preferentially interacts with p110δ over p110α and p110β (Ref.[24]) might help to explain this observation.

APDS predisposes to respiratory infections and airway damage, early death from infection-related causes and lymphoma[138]. Ongoing clinical trials with PI3Kδ inhibitors in APDS have provided promising evidence for immune normalisation and patient benefit, with no discernible adverse effects[139].

Class II PI3Ks

The class II PI3Ks remain the most enigmatic PI3K subfamily. Whereas a single class II PI3K is present in *C. elegans* and *Drosophila* (piki-1 and PI3K_68D, respectively), vertebrates have three class II PI3K isoforms. PI3K-C2α and -C2β are found ubiquitously, while PI3K-C2γ expression is mainly restricted to the liver[63, 140].

In contrast to the class I PI3Ks, class II PI3Ks are not classical signal transducers. Instead, they regulate intracellular membrane dynamics and membrane traffic and can thereby indirectly influence cell signalling. Only in a few cell type-specific settings has evidence been presented for signalling through agonist-dependent lipid pools generated by class II PI3Ks (Supplementary Table 1)[63, 141-144].

Class II PI3Ks are monomeric enzymes, consisting of the conserved PI3K catalytic core extended by a largely non-structured N-terminal region and C-terminal PX and C2 domains, the latter being the origin of the PI3K-C2 nomenclature (FIG. 1).

The lipid products of the class II PI3Ks have been a contentious issue, but are now accepted to include both PI(3,4)P_2 and PI(3)P. As 3'-phosphorylated phosphoinositides are key determinants of membrane identity throughout the endocytic system[145], it is of little surprise that these PI3Ks have been found to mainly operate in endocytosis, on early and recycling endosomes as well as on late endosomes/lysosomes (FIG. 3).

Generic as well as diverse and often cell-type-specific functions have now emerged for class II PI3Ks, some of which depend on the scaffolding function but not the catalytic activity of these kinases.

Activation of class II PI3Ks. As monomers lacking regulatory subunits, the class II PI3Ks have evolved a distinct mode of regulation that was recently uncovered for PI3K-C2α[146]. In solution, the carboxy-terminal PX-C2 module of PI3K-C2α folds back onto the kinase domain, thereby inhibiting membrane binding and catalytic activity. Only when recruited to the plasma membrane through its N-terminal interaction with clathrin[147, 148] will cooperative binding to PI(4,5)P_2 by the PX-[149] and C2-domain[150] unlock PI3K-C2α catalytic activity[146] (FIG. 4a). Of note, the critical residues in the loop of the kinase domain that contact the PX-domain are conserved in PI3K-C2β, indicating that PI3K-C2β may also be subject to this mode of auto-inhibition. Determining the lipid specificity of the PI3K-C2β PX-domain could be of key importance to molecular understanding of the highly isoform-specific activities of PI3K-C2α and PI3K-C2β, with different PX domain specificities potentially restricting activation to distinct subcellular locations. It remains unknown whether, in addition to this mechanism of auto-inhibition-release, class II PI3K activity is further regulated through post-translational modification or allosteric regulation, e.g. through interaction of small GTPases with the so-called Ras-binding domain.

Lipid products of class II PI3Ks. The substrate specificity of PI3Ks is largely determined by the activation loop in the kinase domain that contacts the inositol headgroup of the phosphoinositide lipid substrate[151]. In class I PI3Ks, this loop contains two clusters of basic residues that coordinate the 4'- and 5'-inositolphosphates of PI(4,5)P_2, whereas Vps34 has neither of these basic clusters. In both cases, this accurately reflects the *in vivo* substrate specificities of class I and class III PI3Ks, namely PI(4,5)P_2 and PI, respectively.

Commented [BV17]: Phox homology (PX) domain: A lipid- and protein-interaction domain that consists of 100–130 amino acids and is defined by sequences that are found in two components of the phagocyte NADPH oxidase (phox) complex.

Commented [BV18]: C2 domain: Membrane-binding domain homologous to the C2 domain of protein kinase C with mostly only moderate lipid specificity. Some C2-domains associate with membranes in a Ca^{2+}-dependent manner.

Commented [BV19]: Late endosome: Membrane-bound compartment late on the endocytic route just before fusion with the lysosome. Marked by the presence of Rab7 and other proteins also present on lysosomes, such as LAMPs.

The class II PI3Ks retain only the 4'-phosphate-binding basic residues in the PI3K activation loop, suggesting they use PI(4)P to produce PI(3,4)P_2. Experimental validation of the *in vivo* lipid products of class II PI3Ks, however, has been challenging. This is at least in part a consequence of the rapid conversion of PI(3,4)P_2 to PI(4)P or PI(3)P in many cell types[32], due to dephosphorylation by INPP4A/B 4'-phosphatases and the PTEN 3'-phosphatase[152], respectively. Changes in PI(3,4)P_2 levels are therefore difficult to detect, and fluctuations in PI(3)P may in fact derive from altered PI(3,4)P_2 synthesis. Indeed, functional and cell biological approaches addressing the question of the class II PI3K lipid products have provided more insight than lipid analyses. These include the use of cell-permeant phosphoinositide-derivates[148, 153, 154], visualization of local subcellular pools of phosphoinositides[63, 146, 148, 154, 155], and an activation loop mutation that limits PI3K-C2α to PI3P synthesis[148, 155, 156]. Those studies that unambiguously identified the lipid product revealed that all class II PI3K isoforms produce PI(3,4)P_2 in cells[63, 148, 154]. However, at least PI3K-C2α also makes PI(3)P (Refs.[155, 156]), and this appears to depend entirely on the inositol-lipid substrate species available to PI3K-C2α in different subcellular locations[157]. Indeed, as further detailed below, the lipid product of PI3K-C2α appears to largely depend on the relative local abundance of PI and PI(4)P: at the PI(4)P-rich[158, 159] but PI-de-enriched[160] plasma membrane, PI3K-C2α produces PI(3,4)P_2, whereas on endosomes the lack of PI(4)P may favour the formation of PI(3)P (FIG. 3).

Scaffolding roles of class II PI3Ks. In addition to their catalytic functions, class II PI3Ks can act as protein scaffolds, as highlighted by the role of PI3K-C2α in stabilizing the mitotic spindle during metaphase[161]. By simultaneously interacting with both clathrin and TACC3, PI3K-C2α stabilizes a protein complex required to crosslink microtubules within the kinetochore fibres[162]. Loss of PI3K-C2α protein expression leads to a delay in completion of mitosis and reduced proliferation, a role which does not depend on its catalytic activity[161]. This exemplifies the need to differentiate between kinase-dependent and scaffolding functions of class II PI3Ks, especially with respect to their potential as therapeutic targets for inhibitors of kinase-activity and the interpretation of data obtained using PI3K-C2α gene knockout mice.

Cell biological functions of class II PI3Ks conserved across cell types. In addition to a growing number of cell type-specific roles (discussed below), class II PI3Ks also perform generic functions across many cell types (FIG. 3). The sole *Drosophila* class II PI3K_68D is required for endosomal sorting and recycling, both in hemocytes[163] and postsynaptically at neuromuscular junctions[164], as well as for homeostasis of the endolysosomal compartment[165]. Interestingly, with the evolution to three class II isoforms in mammals, it appears that the roles in the early and late stages of the endocytic pathway have been largely attributed to PI3K-C2α and -C2β, respectively.

PI3K-C2α. During clathrin-mediated endocytosis, a complex interplay of phosphoinositide-metabolizing enzymes spatiotemporally organizes membrane internalization[166], and PI3K-C2α plays a part in this process. PI3K-C2α associates with clathrin through a clathrin-box motif in its amino-terminal region[147] and synthesizes a local pool of PI(3,4)P_2 that is required for the maturation of clathrin-coated pits[148] (FIG. 3). Formation of PI(3,4)P_2 mediates recruitment of the curvature-inducing PX-BAR domain proteins SNX9/18, leading to constriction of the membrane invagination at its neck and thus preparing a template for dynamin to catalyse scission of these newly-formed vesicles from the plasma membrane[148, 167]. This illustrates how phosphoinositides can couple vesicle formation to conversion of membrane identity – in this case, completion of clathrin-mediated endocytosis is coupled to formation of 3'-phosphoinositides, which are prevalent in the endosomal compartment[145].

Intriguingly, PI3K-C2α is also required for recycling cargo from endosomes to the plasma membrane, yet produces PI(3)P and not PI(3,4)P_2 in this context[155, 156] (FIG. 3). This function is thought to underlie the defective localization of the sonic hedgehog signal transducer Smoothened to primary cilia in PI3K-C2α$^{-/-}$ embryos[156]. A possible mechanism was revealed by studies that used a new FRET-based Rab11-activity sensor in combination with the PI(3)P-binding 2×FYVE probe[155]. These experiments indicate that PI3K-C2α synthesizes an early endosomal PI(3)P pool that activates Rab11,

Commented [BV20]: Kinetochore fibres: bundles of spindle microtubules that attach to the kinetochore, a protein complex at the centromere of each chromosome

Commented [BV21]: Endosomal sorting and recycling: Endocytosed cargo proteins are sorted in early endosomes either for degradation in lysosomes, recycling to the plasma membrane or retrograde trafficking to the *trans*-Golgi network.

Commented [BV22]: BAR domain: bin-amphiphysin-rvs, a family of membrane-curvature inducing and sensing protein domains.

Commented [BV23]: Dynamin: A large mechanochemical GTPase that assembles into a helical oligomer on highly curved membranes. GTP hydrolysis is coupled to conformational changes that catalyse constriction and eventually fission of the underlying membrane.

Commented [BV24]: FRET: Förster-resonance-energy-transfer, a biophysical method that uses resonance energy transfer between fluorophores as a measure of proximity, e.g. to differentiate between active and inactive conformations of small GTPases.

leading to budding of nascent Rab11-GTP-positive vesicles and recruitment of the 3-phosphatase MTM1 by Rab11-GTP. This results in removal of PI(3)P from vesicles bound for recycling[155].

PI3K-C2β. Whereas PI3K-C2β may play poorly understood roles in early stages of the endocytic pathway[168], a critical role for this PI3K has been found in the late endosomal/lysosomal compartment. Upon serum starvation, PI3K-C2β associates with the mTORC1 subunit Raptor on late endosomes/lysosomes and produces a local pool of $PI(3,4)P_2$ which suppresses mTORC1 activity in absence of growth factor signalling[154]. This suppression is mediated at least in part by $PI(3,4)P_2$ facilitating recruitment of 14-3-3 proteins that inhibit mTORC1 via Raptor. Indeed, in response to energy stress, AMPK phosphorylates Raptor, allowing phospho-Ser-binding 14-3-3 proteins to associate with Raptor and thereby inhibit mTORC1[169]. $PI(3,4)P_2$ thus appears to be required for lysosomal enrichment of 14-3-3 proteins and efficient inhibition of mTORC1.

Additionally, PI3K-C2β-derived $PI(3,4)P_2$ promotes perinuclear clustering of lysosomes[154], known to contribute to repression of mTORC1[170] (BOX 1). It remains unclear how cessation of growth factor signalling activates PI3K-C2β, and whether lysosomal positioning is cause or consequence of PI3K-C2β-mediated mTORC1 suppression.

Overall, these findings position $PI(3,4)P_2$ produced by PI3K-C2β in a functionally opposed context to class I PI3K-derived $PI(3,4)P_2$, which promotes mTORC1 activation. The correlation of $PI(3,4)P_2$ synthesis with perinuclear lysosomal clustering also contrasts with the role of Vps34-derived PI(3)P, which induces peripheral translocation of lysosomes and thereby enhances mTORC1 activity[170] (FIG. 6).

A seemingly unrelated process, cell migration, has also been reported to be regulated by PI3K-C2β, yet the molecular mechanism of this function remains obscure[143, 171, 172].

Cell-type specific roles of class II PI3K isoforms. Whereas the class I PI3Ks activate the same 'canonical' signalling pathways in different settings, no general modus operandi of the class II PI3Ks has emerged thus far. Their diverse roles in vesicular traffic translate to multiple biological functions, often in a cell type-specific manner (summarised in Supplementary Table 1). PI3K-C2α appears to be of particular importance in endothelial cells, where it is required for angiogenic signalling in response to vascular endothelial growth factor[173], sphingosine-1-phosphate[174], and transforming growth factor-β[175]. In all these cases, defective receptor internalization underlies the signalling defect[173-175]. This suggests that these endothelium-specific functions of PI3K-C2α may in fact be a consequence of its role in endocytosis[147, 148] and further illustrates how class II PI3Ks can indirectly regulate signalling through modulation of membrane traffic.

In hepatocytes, PI3K-C2β and -C2γ play opposing roles in the regulation of insulin signalling. Inactivation of PI3K-C2β by unknown mechanisms in hepatocytes causes expansion of the $APPL1^+$ early endosomal compartment, which in other cell systems is known to control signalling duration and output[176, 177]. This correlates with increased class I PI3K-dependent Akt-signalling upon insulin stimulation[168]. In contrast, PI3K-C2γ is a Rab5-effector that in response to insulin produces an endosomal $PI(3,4)P_2$ pool sustaining prolonged Akt2 activation. PI3K-C2γ thereby specifically enhances GSK3β activation, without affecting Akt1-dependent ribosomal S6-kinase or FOXO phosphorylation[63].

Moreover, PI3K-C2β functions specifically in the activation of T-cells[178, 179] and mast cells[180, 181] to regulate the Ca^{2+}-activated K^+ channel, KCa3.1. In response to immune receptor activation, PI3K-C2β promotes KCa3.1 opening to allow for compensatory K^+-efflux during Ca^{2+} signalling. The E3-ubiquitin ligase TRIM27 negatively regulates PI3K-C2β and KCa3.1 conductivity in this setting[178]. The physiological significance and potential therapeutic exploitation of these findings for example in auto-immunity or allergy remain to be explored.

Organismal roles of class II PI3Ks in mammals. Gene targeted mice have provided insight into the physiological functions of the class II PI3Ks (Supplementary Table 2). Of note, inhibitors with selectivity for class II PI3Ks are not available to date[182].

Homozygous *Pik3c2α* gene knockout[156, 173] or kinase inactivation by a point mutation in the ATP-binding site[183] result in lethality around embryonic day (E) 10.5 – E11.5 of development, with multiple

defects likely underlying this phenotype. Yoshioka *et al.* reported defective developmental angiogenesis in *Pik3c2a*$^{-/-}$ embryos, with conditional *Pik3c2a* knockout in vascular endothelial cells also causing embryonic lethality[173]. However, endothelial cell-specific deletion results in death only at day E16.5-18.5, implicating factors other than defective angiogenesis in the embryonic lethal phenotype of *Pik3c2a*$^{-/-}$ mice. Indeed, PI3K-C2α has also been found to be required for primary cilium elongation and ciliary sonic hedgehog signalling during development[156], potentially accounting for the retarded development and growth observed from E8.5 onwards. Of note, postnatal functions of PI3K-C2α have so far only been inferred from mice with heterozygous or incomplete loss-of-function (Supplementary Table 2). *Pik3c2a*$^{+/-}$ mice display increased vascular permeability[173] and impaired platelet function with a delay in thrombus formation[184], a phenotype that is also seen in heterozygous kinase-dead PI3K-C2α$^{D1268A/+}$ mice[185]. Furthermore, heterozygous loss of PI3K-C2α activity leads to leptin resistance and mild, late-onset insulin resistance and obesity in males[183]. Taken together, PI3K-C2α is clearly essential for embryonic development in mice yet its post-natal physiological functions remain poorly understood.

Surprisingly, homozygous PI3K-C2α deficiency has recently been reported in a small number of patients presenting with skeletal abnormalities and cataract formation, amongst other symptoms[186]. Whereas this may point to a functional divergence of PI3K-C2α in mice and humans, it should be noted that fibroblasts derived from these patients displayed strongly increased expression of PI3K-C2β. This raises the possibility of a compensatory mechanism not observed in mice[186].

Mice with homozygous null[187] or kinase-dead[168] alleles of the ubiquitously-expressed PI3K-C2β are viable and reach adulthood without overt phenotypes, and the same is true for mice that are homozygous null for the liver-enriched PI3K-C2γ[63]. Interestingly, systemic loss-of-function of these two PI3K isoforms causes strikingly different metabolic phenotypes, leading to improved (PI3K-C2β)[168] or reduced organismal insulin sensitivity (PI3K-C2γ)[63]. PI3K-C2β inactivation improves insulin sensitivity and protects against high-fat-diet-induced liver steatosis[168], correlating with augmented class I PI3K-dependent Akt-signalling in metabolic tissues in response to insulin. On the contrary, PI3K-C2γ in hepatocytes mediates insulin-dependent Akt2 activation required for GSK3β activation[63]. PI3K-C2γ knockout mice therefore display reduced glycogen storage in the liver, and by means of compensation generate more triglycerides and develop insulin resistance with age[63]. These distinct functions of PI3K-C2β and -C2γ in the liver illustrate the highly isoform-specific roles of the class II PI3Ks.

Another example of unexpected *in vivo* isoform-selectivity of PI3Ks was uncovered in the context of X-linked centronuclear myopathy (XLCNM). XLCNM is caused by loss-of-function mutations in the 3'-phosphatase MTM1 (Ref.[188]) that lead to impaired clearance of PI(3)P from recycling endosomes, resulting in defective exocytosis and intracellular accumulation of integrins[189, 190]. First shown in flies, muscle-specific depletion of the single *Drosophila* class II isoform *PI3K68D* rescues *mtm* deficiency[190]. In contrast, interfering with *Vps34*, which in most cell types synthesizes the bulk of PI(3)P, exacerbated the phenotype[190]. These findings were recently confirmed in an MTM1 knockout mouse model of XLCNM, where concomitant muscle-specific deletion of PI3K-C2β but not Vps34 fully restored muscle morphology, PI(3)P levels and viability[191]. The potential to rescue the phenotype by conditional deletion of PI3K-C2β even after disease-onset indicates the therapeutic potential of PI3K-C2β inhibition in this human condition.

Vps34 class III PI3K

Vps34, the primordial PI3K conserved from yeast to human, is the sole class III PI3K member. Through association with specific protein complexes and by producing PI(3)P in different subcellular compartments, Vps34 regulates autophagy, endosomal sorting, phagocytosis and macropinocytosis[192, 193].

Below we summarize recent developments in the understanding of Vps34 structure and regulation, the emerging evidence for the generation of distinct subcellular PI(3)P pools by Vps34 and control of signal transduction by protein kinases (such as SGK3, LKB1, AMPK and mTOR), and the organismal roles of Vps34 as derived from mouse gene targeting studies.

Commented [BV25]: Primary cilium: A solitary elongated protrusion of the plasma membrane supported by a centriole-based axoneme of 9 doublet microtubules. Primary cilia serve as sensory structures and specialized signalling platforms that are of particular importance in development.

Commented [BV26]: X-linked centronuclear myopathy: also called myotubular myopathy, a severe paediatric neuromuscular disorder causing muscle weakness.

Commented [BV27]: Autophagy: A cellular 'self-eating' process to break down protein and damaged organelles through lysosomal pathway in response to nutrient depletion or stress. It is characterized by membrane trafficking events that sequester cytoplasmic material in double-membrane structures, called autophagosomes, followed by degradation and recycling of cellular components by the lysosome.

Commented [BV28]: Phagocytosis: A largely actin-driven form of endocytosis to ingest large particles, such as cellular debris or whole microorganisms.

Commented [BV29]: Macropinocytosis: evolutionarily-conserved endocytic pathway allowing internalization of extracellular fluid via large endocytic vesicles called macropinosomes.

Structural insights: adaptation to membrane curvature and selective inhibitors. Vps34 consists of the minimal PI3K catalytic core with an N-terminal C2 domain, a helical domain and a C-terminal kinase domain (FIG. 1). Vps34 forms at least two tetrameric complexes, known as complex I and II. The core complex consists of Vps34, beclin1/Atg6 and the protein kinase-like Vps15/PIK3R4. In a mutually exclusive manner, this core associates with either ATG14 or UVRAG to form complex I or II, respectively[194]. Additional regulatory subunits can associate with these complexes, including NRBF2 or AMBRA in complex I and Rubicon in complex II (Ref.[192]; FIG. 5).

Complex I is activated and recruited to phagophore initiation sites mainly at the endoplasmic reticulum (ER), to produce a pool of PI(3)P critical for the formation and elongation of the nascent autophagosome, which encloses and isolates cytoplasmic components, whereas complex II controls endosome maturation[192].

Structural studies on human complex I and yeast complex II revealed a V-shaped architecture with membrane-binding domains located at the tip of each arm[195-197] (reviewed in Refs.[14, 198]) (FIG. 4b,c). One arm is formed by the Vps34-Vps15 dimer, whereas the other arm is formed via parallel arrangement of the coiled-coil domains of beclin1 associated with Atg14 (in complex I) or UVRAG (in complex II). The tips of both arms make contact with membranes, with one arm binding via the kinase domain of Vps34 and the other via the C-terminal BARA, BATS or BARA2 domains of beclin, ATG14 or UVRAG, respectively. This architecture allows the complexes to activate Vps34 and to adapt to membrane curvature.

Vps34 catalytic activity is regulated by the helical and kinase domains of Vps34 dynamically adopting a closed or opened conformation. Indeed, in solution when not bound to membranes, the C-terminal helix of Vps34 blocks its ATP-binding site to prevent ATP hydrolysis. Association of this helix with the membrane tethers Vps34 to the membrane and exposes the ATP-binding site[196].

The other arm is critical to adjust the complexes to different membrane curvatures. By virtue of the curvature-sensing amphipathic helix in the BATS domain located at the C-terminus of ATG14, complex I preferentially associates with highly-curved membranes such as the expanding autophagic isolation membrane[199]. In contrast, flexibility between the two arms of complex II allows it to open and act on low curvature membranes such as the relatively flat endosomal membranes[195].

The ATP-binding pocket in Vps34 is unusually-constricted[200]. This explains the relative Vps34 specificity of the widely-used 3-methyladenine compound, which fits in this small pocket, unlike most class I PI3K inhibitors which are too bulky[200]. However, 3-methyladenine is not selective for Vps34, and highly-selective Vps34 inhibitors have now been developed[201-206]. These have been used to confirm the role of Vps34 in autophagy and vesicular trafficking, to identify putative autophagy substrates and autophagy cargo receptors[202] and a possible involvement of Vps34 in the regulation of signalling[201, 207, 208].

Contribution of Vps34 to basal and stimulated PI(3)P pools. Vps34 and its binding partners ATG14, beclin1 or UVRAG are subject to extensive post-translational modifications (reviewed in Refs.[192, 198]). These include phosphorylation by mTORC1, AMPK and ULK1 (in response to nutrient availability and energy depletion) and by growth factors via Akt, CDK1/5 and mTORC1 (to inhibit autophagy and allow proliferation)[192]. Vps34 and members of the Vps34 complexes can also be modulated by ubiquitination, SUMOylation and acetylation under certain conditions[192]. The functional relevance of these post-translational modifications remains to be determined.

The contribution of Vps34 to basal cellular PI(3)P varies considerably between cell types. For instance, Vps34 is believed to be responsible for 65% of basal cellular PI(3)P in mouse embryonic fibroblasts[209], 30-40% in megakaryocytes and ~10% in platelets[210]. These observations indicate that Vps34 is not the universal main source of cellular PI(3)P, as had long been thought. Other enzymes, which directly or indirectly affect PI(3)P-production, including the class II PI3Ks and lipid phosphatases, likely contribute to the maintenance of the basal PI(3)P pool.

In addition to regulating basal PI(3)P production, Vps34 may also contribute to dynamic PI(3)P pools in the context of nutrient sensing, to modulate endocytosis and autophagy, in a Vps34 complex-

Commented [BV30]: Phagophore: also called isolation membrane is double membrane cupshaped structure that engulfs cytoplasmic material. It is the precursor of the autophagosome.

Commented [BV31]: BARA domain: β-α-repeated, autophagy-specific domain

Commented [BV32]: BATS domain: Barkor/Atg14 autophagosome targeting domain which binds to the autophagosome membrane via the hydrophobic surface of an intrinsic amphipathic alpha helix.

Commented [BV33]: Amphipathic helix: α-helical sequence in which polar and charged amino acids are oriented to one side and hydrophobic ones to the other side. The lipophilic side dips into bent membranes displaying packing defects in the outer leaflet, thus sensing membrane curvature.

specific manner. For instance, glucose starvation increases the autophagic PI(3)P pool while overall cellular PI(3)P levels are decreased[211]. Glucose and amino-acid starvation have been shown to specifically stimulate Vps34 complex I without affecting total Vps34 activity[211, 212]. Likewise, amino-acid starvation enhances Vps34 activity in complex I to induce autophagy whereas ATG14-free Vps34 complexes are inhibited[211]. In contrast, insulin stimulation selectively induces complex II activity, thereby promoting endocytic trafficking during growth factor stimulation[212, 213].

Vps34 also contributes to agonist-stimulated PI(3)P under conditions other than nutrient sensing. This is the case in platelets in which Vps34 inhibition barely affects basal PI(3)P levels but significantly reduces stimulation-dependent PI(3)P production[210]. Similarly, Vps34 seems to be the main producer of light-induced PI(3)P production in retinal rods[214].

These findings highlight the complex regulation of Vps34, especially in the context of nutrient signalling: nutrient starvation can both inhibit and activate Vps34 kinase activity, in a Vps34-complex-dependent manner. A similar paradox was reported in macrophages with loss of Rubicon, a regulator of Vps34, which displayed increased overall PI(3)P but a significant decrease in PI(3)P on LAPosomes (LC3-associated phagocytosis-engaged phagosomes)[215]. This suggests that Vps34 activity is fine-tuned in different complexes in response to specific environmental stimuli.

Regulation of protein kinase signalling by Vps34. Like the class II PI3Ks, Vps34 is not a direct signal transducer. However, emerging evidence indicates that Vps34, mainly through its endosomal function, can regulate signalling by several protein kinases. This may involve regulation of PTEN, which has been shown to bind via its C2 domain, to a Vps34-dependent endosomal PI(3)P pool, recruiting this phosphatase to the endosomes[216] where it could dephosphorylate PIP_3 to terminate class I PI3K signalling.

SGK3 (Serum- and Glucocorticoid-regulated Kinase-3) is a Ser/Thr kinase structurally and functionally closely related to Akt, including its substrate specificity. However, SGK3 contains a PI(3)P-binding PX domain instead of the PIP_3/$PI(3,4)P_2$-binding PH domain in Akt, and is in fact the only Ser/Thr protein kinase with a PI(3)P-binding domain. Upon growth factor stimulation (such as by IGF1), SGK3 is recruited via its PX domain to the endosomal membrane by PI(3)P generated by two sources, namely by Vps34 complex II (Refs.[201, 207]) and by sequential dephosphorylation of PIP_3 by the SHIP/INPP4 lipid phosphatases[217]. Growth factor-activated PDK1 and mTORC2, which have been found on endosomal membranes[218, 219] then activate SGK3 kinase activity in an analogous way to activation of Akt, by phosphorylation of the activation loop in the kinase-domain (PDK1) and of the C-terminus (mTORC2)[201, 207]. These findings are relevant in the context of class I PI3K in cancer. Firstly, many breast tumours cell lines with *PIK3CA*-activating mutations show low levels of Akt activity and instead rely on SGK3 (Refs.[208, 220]). Second, prolonged treatment of breast cancer lines with class I PI3K or Akt inhibitors upregulates Vps34-dependent SGK3 signalling as an adaptive resistance response, allowing to phosphorylate a subset of Akt substrates, including mTORC1, independently of class I PI3Ks[208].

Another kinase regulated by Vps34 is the endosomally-localized pool of LKB1 kinase, an activator of AMPK and a regulator of cell polarity[221]. This polarity function of LKB1 depends on its binding to the endosomally-localised WDFY2, a FYVE domain-containing protein, and the transit of LKB1 from Rab5- to Rab7-positive compartments during endosomal maturation. Inhibition of Vps34 leading to impaired endosomal maturation and mislocalization of WDFY2 has been shown to dysregulate the localization of LKB1 in the endosomes, with aberrantly-localized increased LKB1 signalling giving rise to a disruption of epithelial organization[221].

Vps34 also controls the AMPK pathway, a critical sensor of low cellular energy levels. Indeed, a key impact of systemic Vps34 inactivation is an organismal activation of AMPK signalling. This most likely results from reduced autophagic proteolysis upon Vps34 inhibition, leading to reduced availability of key metabolites important for mitochondrial respiration. This leads to a lowering of cellular ATP levels, thereby activating AMPK[204] (see under 'Organismal functions of Vps34' for more details).

Vps34 also has a complex interrelationship with mTORC1 signalling, as described in the next paragraph.

Interplay between Vps34 and mTORC1 in nutrient signalling. In the presence of nutrients and growth factors, active mTORC1 prevents autophagosome formation by phosphorylating the Ser/Thr kinase ULK1 in the autophagy initiator complex, and by phosphorylation of Atg14 in the Vps34 complex I, resulting in inhibition of Vps34 activity[222]. Conversely, upon nutrient starvation or treatment with rapamycin, mTORC1 inactivation and ensuing lack of inhibition of these pro-autophagic complexes allows autophagy to proceed.

Whereas Vps34 does not seem to control the basal activity of mTORC1[223], it has been implicated in the acute activation of mTORC1 by amino acids in mammalian cells[224-227]. This is somewhat counterintuitive, given that Vps34 on the one hand stimulates autophagy, and on the other activates mTORC1 which downregulates autophagy. This may be related to the involvement of distinct Vps34 complexes and potentially part of a feedback loop to switch off autophagy when nutrients become available. Two mutually non-exclusive mechanisms by which Vps34 could contribute to mTORC1 activation in response to amino acid-stimulation have recently been reported, both based on PI(3)P production by Vps34 at the lysosome.

A first mechanism is based on the recruitment of phospholipase D1 (PLD1) via its PI(3)P-binding PX-domain to the lysosomal surface[224]. Phosphatidic acid produced by PLD1 is then thought to dissociate mTORC1 from its inhibitory DEPTOR subunit, thereby triggering mTORC1 activation[228] (FIG. 6b,c).

Commented [BV34]: Phospholipase D1: A phosphodiesterase that hydrolyses phosphatidylcholine and other glycerophospholipids to generate phosphatidic acid and the free headgroup of the substrate lipid.

A second mechanism is based on the lysosomal recruitment of the PI(3)P-binding proteins protrudin and FYCO1 (Refs.[229, 230]). Protrudin is an integral ER protein which occurs in complex with kinesin-1, a microtubule motor protein. Under nutrient starvation, lysosomes localize perinuclearly and mTORC1 is predominantly found in the cytosol. Upon amino acid stimulation, mTORC1 is recruited to lysosomes where Vps34 also produces PI(3)P. This PI(3)P allows protrudin to make contacts with the lysosome and to transfer kinesin-1 to FYCO1, a so-called motor adaptor protein involved in the transport of intracellular vesicles along microtubules, which has also become recruited to the PI(3)P at the lysosomes. Lysosomes loaded with kinesin-1 then translocate along microtubules to the cell periphery, bringing the lysosomal mTORC1 complex in close proximity to growth factor and nutrient signalling complexes at the plasma membrane, leading to an increase in mTORC1 activity (FIG. 6c).

Organismal roles of Vps34 in mammals: scaffold versus kinase activity. Several Vps34-deficient mouse models have been reported, mostly using gene-targeting approaches aimed at ablation of Vps34 protein expression (Supplementary Table 2). Because the stability of the Vps34 complexes relies on the presence of the Vps34 protein, these models have invariably resulted in loss-of-expression of Vps34 binding partners. This is likely to be further confounded by the fact that different studies targeted different exons of the *Vps34* gene, resulting in loss-of-expression of full length Vps34 or the possible generation of truncated Vps34 proteins. These factors complicate the interpretation of the observed phenotypes which may indeed not depend on Vps34 only, and also fail to discriminate between its catalytic and scaffolding functions[209, 213, 231, 232].

Global *Vps34* KO revealed that this PI3K is critical for embryonic development, with homozygous Vps34 KO mice dying at early stages of embryogenesis i.e. between E6.5 and E8.5 (Refs.[204, 233]). Notably, autophagy-deficient mice (i.e. mice deficient in ATG3, ATG5, ATG7, ATG12 or ATG16L1) characteristically die much later, shortly after birth[234]. This suggests that non-autophagic functions of Vps34 also contribute to embryonic development.

Tissue-specific *Vps34* KO results in distinct phenotypes, ranging from mild to severe, mostly leading to lethality (Supplementary Table 2). The majority of these phenotypes have been associated with impaired endocytic trafficking or autophagy, or both.

A Vps34 kinase-dead knock-in strategy in mice was aimed at assessing the organismal importance of Vps34 catalytic activity and to model the impact of pharmacological intervention by Vps34 kinase-inhibitors[204]. Interestingly, whereas homozygosity for kinase-dead Vps34 mice also led to lethality

between E6.5 and 8.5, heterozygous Vps34 mice were healthy and fertile, but displayed increased insulin sensitivity and partial protection against high-fat-diet induced liver steatosis[204]. This effect was mimicked by a small molecule inhibitor of Vps34, in both preventative and therapeutic settings of insulin sensitivity. These observations suggest that a therapeutic window exists for organismal dampening of Vps34, with potential exploitation in overcoming insulin resistance. Possibly due to the partial inactivation of Vps34, only very mild defects in autophagy and endosomal trafficking were observed. Indeed, studies using Vps34 inhibitors showed that in order to fully block autophagy in cell-based models, >90% of Vps34 activity needs to be inhibited, indicating that very low levels of Vps34 activity are sufficient for autophagy, at least under non-stressed conditions[202]. A key impact of partial systemic Vps34 inactivation is an organismal activation of the energy sensing AMPK pathway. This most likely results from a mild autophagy defect, leading to reduced availability of key amino acid substrates for mitochondrial respiration and an ensuing reduced ATP production which results in AMPK activation. As a consequence of AMPK-stimulated processes such as reduction in hepatic gluconeogenesis and increased glucose uptake in the muscle, overall levels of glucose in the blood are reduced[204].

Liver-specific *Vps15* gene knock-out has also been shown to lead to increased organismal insulin sensitivity, through a mechanism different from that reported for Vps34 inactivation[213]. Indeed, Vps15-null hepatocytes show an increase in insulin-mediated Akt signalling, which was attributed to prolonged residence of the insulin receptor in endosomes[213]. This contrasts with genetic or pharmacological inactivation of Vps34 which does not affect insulin-stimulated Akt[201, 204]. A possible explanation is that this effect of Vps15 deletion is due to the reduced expression of beclin in these cells, as a consequence of the loss of the scaffolding function of Vps15 (Ref.[213]). This is analogous to observations in breast cancer cells, where loss of beclin expression prolongs the residence of the EGFR in early endosomes, resulting in sustained EGF-stimulated Akt/ERK signalling[235].

Conclusions and perspectives

Much progress has been made in understanding the roles and mechanisms of action of the different PI3K isoforms. An emerging theme is that pools of the same lipid derived from different PI3K isoforms are not necessarily functionally equivalent. This is illustrated by $PI(3,4)P_2$ which, when produced by PI3K-C2β, does not activate Akt[154], unlike the $PI(3,4)P_2$ produced by the class I PI3Ks[32, 152] and by PI3K-C2γ in the liver[63]. Likewise, several independent and functionally distinct subcellular pools of PI(3)P exist, such as in endosomal maturation, endocytic recycling and initiation of autophagy. It has also become clear that Vps34 is not always the main producer of cellular PI(3)P, with contributions from class II and possibly class I PI3Ks, although the latter remains to be firmly established. It will be a technical challenge to determine the contributions of individual PI3K isoforms to the specific subcellular pools of phosphoinositides.

With regards to class IA PI3Ks, it has become clear that timing and tissue context determine the impact of mutational activation, with the presence of *PIK3CA* mutation not necessarily predisposing to cancer development, as is the case in PROS[9]. Much still has to be learned on how the different class IA PI3K subunits engage with each other and with upstream activators[24], the compartmentalisation of their downstream effectors such as Akt and their substrates[59], and the ensuing biological functions.

Essential cellular and organismal roles of the class II PI3Ks have now been uncovered, with the three class II isoforms apparently carrying out entirely distinct functions. The upstream regulatory inputs that activate these PI3Ks remain largely unknown. With a structural concept for the regulation of PI3K-C2α activity now in place[146], it will be exciting to understand how this applies to the other class II isoforms and how this integrates with other regulatory inputs such as phosphorylation or other posttranslational modifications. While PI3K-C2α is clearly essential for embryonic development[156, 173], we still know very little about its organismal role in post-natal stages. The first clear indication for therapeutic potential in targeting class II PI3Ks, as seen in the context of X-linked centronuclear myopathy[190, 191], has the potential to spur efforts to develop isoform-selective inhibitors for these enigmatic PI3Ks.

Structural studies have provided insight into how Vps34 lipid kinase activity is regulated in complex I and II. It remains to be determined how the multiple posttranslational modifications of the distinct subunits[198] may affect the composition and function of the Vps34 complexes. Highly-selective Vps34 inhibitors have helped define the various cellular functions and uncovered new biological roles for Vps34 in cancer[208, 236, 237] and metabolic sensitization[204, 208]. Further studies are required to define the biological roles of the distinct Vps34 complexes, and development of complex-specific modulators would certainly help to address this fundamental question but also open opportunities for more refined interference with Vps34 function in disease, such as to selectively interfere with autophagy in cancer[238].

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BOX 1 - Regulation of mTORC1 by class I PI3Ks

The Ser/Thr kinase mTORC1 is a central regulator of cell growth. Under nutrient-rich conditions (growth factors and amino acids), mTORC1 potentiates anabolic pathways (such as protein synthesis) and inhibits catabolic processes (such as autophagy). When nutrients are scarce, mTORC1 becomes inactive, resulting in reduced cell growth and induction of autophagy. Apart from growth factors, many other inputs including oxygen, energy, and inflammation involving Ras-ERK, RSK, or AMPK pathways have also been identified as regulators of mTORC1.

Activation of mTORC1 occurs at multiple levels (shown in panels A and B). The first level of activation of mTORC1 occurs on the lysosome, through input from amino acids and growth factors[239]. The amino acid branch causes translocation of mTORC1 from the cytosol to lysosomes, its obligate platform of activation, whereas the growth factor axis activates lysosomally-bound mTORC1. The second level is through positioning of the lysosome within the cell[170]. Under conditions of starvation, lysosomes cluster perinuclearly. Under nutrient-rich conditions, lysosomes become positioned near the plasma membrane, bringing mTORC1 close to input from cell surface receptors.

Amino acid sensing occurs through cytoplasmic sensors and amino acid transporters which, through regulation of a GEF and GAP (RAGULATOR and GATOR, respectively) for the RagC/RagA GTPase heterodimer, results in a switch of the GDP/GTP-loading on these small GTPases. The lysosome-bound active Rag heterodimer then recruits mTORC1 from the cytoplasm to the lysosomal surface where its activator, the small GTPase Rheb, resides.

Once localized to the lysosome, mTORC1 becomes fully activated if growth factors are also present. This occurs through a class I PI3K/Akt-driven process that removes a lysosomally-bound Rheb inactivator to the cytosol, allowing Rheb to become activated through GTP-loading and to stimulate mTORC1 activity. This Rheb inactivator is TSC2, a GAP for Rheb. TSC2 is bound to TSC1, forming the so-called tuberous sclerosis complex. Upon growth factor stimulation, Akt phosphorylates TSC2, inducing the dissociation of the TSC complex from the lysosomal membrane. This relieves the inhibition of Rheb, allowing it to stimulate mTORC1 activity. Class II/III PI3Ks can also regulate mTORC1 (see text).

FIGURE LEGENDS

Fig. 1. | **Substrate specificity and domain organisation of the PI3K isoforms and subcellular distribution of their lipid products. a** | Left panels: PI lipid substrate specificity. SHIP is a lipid phosphatase which converts $PI(3,4,5)P_3$ to $PI(3,4)P_2$. Right panels: Domain structure of PI3Ks. For highly homologous proteins, a single overall domain structure is shown. No specific domain structures are present in the class IB regulatory PI3K subunits. Three specific single amino acid substitutions, due to hotspot mutations found in human *PIK3CA*, are shown. Indicated are the name of the protein and (between brackets) the name of the gene encoding the PI3K family subunits. The class IA regulatory subunits are encoded by three distinct genes: *PIK3R1* (which encodes p85α, p55α and p50α, through differential promotor usage), *PIK3R2* (which encodes p85β) and *PIK3R3* (which encodes p55γ). The genes encoding Beclin, Atg14 and UVRAG have not been classified as *PIK3R* genes and are referred to as accessory subunits rather than regulatory subunits. Vps34 occurs in two main multiprotein complexes, referred to as complex I and II, which are made up of Vps34, Vps15, beclin and Atg14 (complex I) or Vps34, Vps15, beclin and UVRAG (complex II). **b** | Subcellular distribution of the 3-PIs directly generated by PI3Ks. Not shown is $PI(3,5)P_2$, which derives from phosphorylation of PI(3)P by the PI5K PIKfyve. The three classes of PI3Ks produce 3'-PI pools in distinct subcellular locations. Whereas $PI(3,4,5)P_3$ is only produced by class I PI3Ks, distinct pools of $PI(3,4)P_2$ and PI(3)P are generated by more than one class of PI3Ks. ABD, p85 adaptor binding site; BH, BCR-homology domain; P, proline-rich region; RBD, ras-binding domain; SH2, Src-homology 2 domain; iSH2, inter-SH2 domain. Myr, N-terminal myristoylation of Vps15, which helps to anchor Vps34 complexes to the membrane. HEAT, Huntingtin, EF3, α subunit of PP2A, TOR1 domain; WD-40 repeat, Trp-Asp (W-D) (which is also

called β-transducin repeat); BH3, Bcl-2 homology 3 domain; CC1/2, coiled-coil domain 1/2; C2, protein kinase C conserved region 2; BATS, Barkor/Atg14(L) autophagosome targeting sequence; BARA, β/α-repeated, autophagy-related domain.

Fig. 2. | **Isoform-selective regulation of class I PI3K and Akt family members**. Shown are the activating inputs into the distinct class I PI3K isoforms, resulting in the generation of PIP_3 from $PI(4,5)P_2$ at the plasma membrane. Conversion of PIP_3 to $PI(3,4)P_2$ by 5-phosphatases such as SHIP (not shown) leads to the formation of $PI(3,4)P_2$ which is also found on the plasma membrane but which, in contrast to PIP_3, is also being transferred via clathrin-mediated endocytosis to an early endosomal compartment[59]. Evidence is emerging[59-61] that, in this location, Akt2 is preferentially activated over Akt 1 and 3 with endosomal Akt2 being able to phosphorylate certain substrates (such as GSK3β, but not FOXOs or TSC2) to which other isoforms have less access. It is not clear if such Akt isoform substrate specificity also exists during the time they are active at the plasma membrane. PIP_3 production enables the recruitment of cytosolic inactive Akt to the membrane through its PH domain binding. This leads to phosphorylation and full activation of Akt by the PH-domain containing kinase PDK1 (which phosphorylates the T-loop on T308) and the mTORC2 complex (in which the SIN1 component contains a PH domain), which phosphorylates the hydrophobic motif on S473. Phosphorylation by Akt mainly leads to functional inactivation of its substrates (illustrated by the blunt-ended line), with activation (indicated by an arrow) of other substrates, such as eNOS (endothelial nitric oxide synthase).

Fig. 3. | **Cell biological functions of class II PI3K isoforms**. The class II PI3K isoforms have distinct and non-redundant functions. (a) PI3K-C2α is recruited to clathrin-coated pits through association with clathrin and synthesizes a local pool of $PI(3,4)P_2$ that is required for maturation of the pit and constriction of the membrane invagination at its neck. This effect is mediated at least in part by $PI(3,4)P_2$-dependent recruitment of the PX-BAR-domain proteins SNX9/18. (b) PI3K-C2α is also required for recycling of endosomal cargo back to the plasma membrane. On early endosomes, PI3K-C2α produces a pool of PI(3)P required for activation of Rab11. Rab11-GTP recruits the 3'-phosphatase MTM1 leading to depletion of PI(3)P, which is required for the formation of vesicles bound for the endocytic recycling compartment and effectively restricts PI(3)P to the early endosomal compartment. (c) In the pericentriolar recycling compartment, PI3K-C2α produces a pool of PI(3)P for Rab11 activation required for transport of cargo towards the primary cilium, such as the sonic hedgehog effector smoothened. (d) During mitosis, PI3K-C2α interacts with clathrin and TACC3 to stabilize the microtubule-crosslinking complex of CHTOG, TACC3 and clathrin on kinetochore fibres. This function is entirely independent of the kinase activity of PI3K-C2α. (e) In the absence of growth factor signalling, PI3K-C2β associates with the mTORC1 subunit raptor on lysosomes and produces $PI(3,4)P_2$ to facilitate lysosomal recruitment of 14-3-3γ. Under low-energy conditions, AMPK phosphorylates raptor and ensuing association of phospho-serine-binding 14-3-3 proteins with raptor inhibits mTORC1. Lysosomal $PI(3,4)P_2$ thus facilitates AMPK-driven inhibition of mTORC1 by local enrichment of inhibitory 14-3-3 proteins. (f) In hepatocytes, PI3K-C2β and PI3K-C2γ have opposing roles in insulin signalling. Whereas PI3K-C2γ is directly involved in insulin signal transduction by producing an insulin-stimulation dependent endosomal pool of $PI(3,4)P_2$ that sustains prolonged activation of Akt2, PI3K-C2β negatively regulates insulin receptor signalling. This effect correlates with an expansion of the $APPL1^+$ endosomal compartment observed upon inactivation of PI3K-C2β in hepatocytes; APPL1 endosomes can serve as signalling platforms for activated receptors.

Fig. 4. | **Activation mechanisms of PI3K-C2α and Vps34**. **a** | The kinase activity of PI3K-C2α is regulated by its C-terminal PX- and C2-domains. In the cytoplasmic, inactive state, PI3K-C2α is auto-inhibited by the PX-C2 module that folds back onto the PI3K core. This closed conformation is stabilized by intramolecular contacts (indicated by red double-sided arrows) of the PX-C2 module with a loop in the C-lobe of the catalytic domain and with the Ras-binding domain (RBD). PI3K-C2α is

recruited to sites of clathrin-mediated endocytosis by interaction of its unstructured N-terminal region with the clathrin terminal domain. Release of auto-inhibition requires the presence of PI(4,5)P_2 in the plasma membrane, which binds to both the PX- and C2-domains to dislodge them from the PI3K core. This stabilizes the membrane-associated open conformation of PI3K-C2α and enables unhindered access of PI(4)P lipid substrate to the catalytic site. This figure is based on Ref.[146]. **b** | Vps34 complex assembly and adaptation to membrane curvature. Both Vps34 complexes adopt a V-shaped conformation[195-197]. Vps15 is positioned at the base of the assembly and shapes the complex. The tip of each arm interacts with membranes via membrane binding-domains. These domains include the C-terminal kinase domain of Vps34, the BARA domain of beclin1, and the BATS domain of Atg14 for complex I or the BARA2 domain of UVRAG for complex II. The activity of complex I and II is regulated by membrane curvature. Complex I prefers highly curved membranes such as omegasomes and autophagosomes through the curvature-sensing Atg14-BATS domain[199]. In contrast, complex II is adapted to relatively flat membranes such as endosomes, possibly due to the flexibility of the hinge region. Figure adapted from Ref[195]. **c** | The C-terminal helical and kinase domains (HelCat) of Vps34 adopt different conformations. In the inactive state, the Vps34 kinase domain is tightly associated with the pseudokinase domain of Vps15, likely preventing access to the lipid substrate and blocking the ATP binding site. Upon membrane binding, the Vps34 kinase domain dissociates from Vps15 and via a long C2-helical linker dislodges from the rest of the complex to access its substrate and become catalytically active[196]. Figure adapted from Ref.[197].

Fig. 5. | **Vps34 in endosomal trafficking and autophagy**. Vps34 produces PI(3)P in intracellular compartments and regulates autophagy, endosomal sorting and phagocytosis. These pathways converge at the lysosome for degradation of the vesicular contents. Vps34 functions in two main complexes consisting of Vps34, Vps15, Beclin1 either with Atg14 (complex I) or UVRAG (complex II). **a** | Complex II is recruited to early endosomes through binding of Vps15 to Rab5-GTP and generates PI(3)P to recruit PX- or FYVE-domain containing effectors such as EEA1 that drive endosomal fusion. It also regulates endocytic sorting by controlling the maturation of Rab5-positive early endosomes into Rab7-positive late endosomes. The complex II subunit UVRAG promotes late endosome–lysosome fusion through its interaction with the HOPS (homotypic fusion and vacuole protein sorting) complex, a Rab7 effector in late endosomal membrane fusion events[240]. **b** | It is not clear which Vps34 complexes generate PI(3)P on lysosomes to regulate lysosomal positioning and activation of mTORC1 (FIG. 6)[230]. **c** | During phagocytosis, phagosomes acquire traits characteristic of early endosomes and Vps34 complex II is recruited to the nascent phagosome through interaction with Rab5. Phagosomes then mature into late endosomes and fuse with lysosomes where the sequestered particle is degraded. The negative regulator of Vps34 complex II, Rubicon, displaces UVRAG from the HOPS complex, thereby altering endosome maturation and Rab7 GTPase activation[241, 242]. **d** | Complex I is required for PI(3)P production on autophagosome precursor membranes. Initiation of autophagy is regulated by the class I PI3K/mTORC1 pathway (see BOX1 for details) and Vps34. Upon nutrient depletion, ULK1 is no longer repressed by mTORC1 and translocates to the ER where it phosphorylates many proteins including Atg14 and Vps34, leading to the recruitment of Vps34 complex I to the ER. Local PI(3)P production then leads to the formation of an isolation membrane emerging from an Ω (omega)-shaped subdomain membrane from the ER. This local PI(3)P pool recruits WIPI2 and DFCP1 proteins required for the recruitment of the ATG12-ATG5-ATG16L1 conjugation system which functions as an E3-like ligase to mediate the lipidation of LC3 protein, enabling it to associate with the nascent autophagosome. LC3 plays an important role in cargo recognition, autophagosome expansion and closure. After maturation, autophagosomes fuse with either late endosomes or lysosomes to form autolysosomes, in which the sequestered cytoplasmic materials are digested. Although this maturation/fusion process involves complex II, the precise mechanism of autophagosome–lysosome fusion remains poorly understood.

Fig. 6. | **Regulation of mTORC1 signalling by PI3K-C2β and Vps34.** Activation of mTORC1 is regulated by different PI3K isoforms at multiple levels. **a** | In absence of growth factor signalling and under low-

nutrient conditions, lysosomes are clustered in the perinuclear area, mTORC1 is inhibited and autophagy is taking place. PI3K-C2β contributes to suppression of mTORC1 activity by producing a lysosomal pool of $PI(3,4)P_2$ that promotes association of 14-3-3γ with lysosomes. Low energy levels cause AMPK to phosphorylate raptor on Ser^{722} and Ser^{792}, and binding of 14-3-3 proteins to these sites inhibits mTORC1. $PI(3,4)P_2$ produced by PI3K-C2β is required for efficient lysosomal recruitment of 14-3-3γ and suppression of mTORC1. It is at present unclear how $PI(3,4)P_2$ regulates lysosomal positioning, yet lysosomal $PI(3,4)P_2$ at least indirectly promotes perinuclear clustering of lysosomes. **b** | Availability of amino acids leads to activation of mTORC1 on the lysosomal surface (BOX 1). In response to amino acid stimulation, Vps34 synthesizes PI(3)P on lysosomes and thereby enhances activation of mTORC1 by at least two different mechanisms. PI(3)P-driven recruitment of phospholipase D1 (PLD1) leads to generation of phosphatidic acid in the lysosomal membrane, which then causes the inhibitory mTORC1 subunit Deptor to dissociate from the complex, relieving inhibition. Vps34-derived PI(3)P also promotes peripheral translocation of lysosomes by providing docking sites for the PI(3)P-binding proteins FYCO1 and protrudin. Protrudin is an integral membrane protein of the ER that associates with the anterograde microtubule motor protein kinesin-1, whereas FYCO1 serves as an adaptor between cargo and motor protein. Increased lysosomal PI(3)P levels promote ER-lysosome contacts and allow kinesin-1 to be transferred from protrudin to the lysosome-bound FYCO1. This results in kinesin-1-driven translocation of lysosomes to the cell periphery. **c** | In presence of amino acids and growth factors, lysosomes are located in the cell periphery, mTORC1 is active and autophagy is being inhibited. Growth factors activate class I PI3Ks and, through Akt, relieve inhibition of Rheb, resulting in activation of mTORC1. Positioning of lysosomes in proximity to signalling receptor tyrosine kinases at the plasma membrane potentiates growth-factor-mediated activation of mTORC1. This figure is based on Ref.[230].

Supplementary Table 1: ***Cell type-specific functions of the class II PI3K isoforms***

	Cell type	Function	References
PI3K-C2α	neuroendocrine cells	ATP-dependent priming of neurosecretory granules	243, 244
	L6 myoblasts	Insulin-induced GLUT4 translocation	141
	pancreatic β-cells	Glucose-stimulated insulin secretion	142, 245
	3T3-L1 adipocytes	Insulin- and amino acid-stimulated mTORC1 activation	246
	rat insulinoma cells	Insulin granule exocytosis	247
	endothelial cells	VE-cadherin trafficking and VEGFR signalling	173
	endothelial cells	Signalling from sphingosine-1-phosphate and TGFβ receptors	174, 175
	endothelial cells	Cell survival	248
	megakaryocytes/platelets	Structure of the internal membrane reserve system, platelet formation and function	184, 185
	hippocampal neurons	Neurite initiation and dendrite morphogenesis	249
	Drosophila neuromuscular junction postsynapse	Presynaptic homeostatic potentiation	164
	Madin-Darby canine kidney cells	Contribution to $PI(3,4)P_2$ pool as a marker of the apical membrane domain in a 3D-cyst polarization model	250
PI3K-C2β	hepatocytes	Dampening of insulin- and class I PI3K-dependent Akt activation	168
	T-cells, mast cells	Regulation of KCa3.1 K^+-channel activity	178-181
	acute myeloid, brain and neuroendocrine tumour cells	Proliferation and survival	251
	endothelial cells	Migration	248
	Madin-Darby canine kidney cells	Contribution to $PI(3,4)P_2$ pool as a marker of the apical membrane domain in a 3D-cyst polarization model	250
PI3K-C2γ	hepatocytes	Sustained endosomal insulin-stimulated Akt2-activation	63

Supplementary Table 2: Major phenotypes of mice with targeted class II or class III PI3K genes

	Target tissue	Mouse model	Viability	Major phenotypes	Reference
PI3K-C2α	**global**	constitutive KO	Lethal between E10.5 and E11.5, delayed development from E8.5	Defective vascular formation. Heterozygous mice are viable and fertile with increased vascular permeability.	173
		constitutive KO	Lethal between E10.5 and E11.5, delayed development from E8.5	Defective turning and left-right patterning during development, impaired ciliary Hedgehog signalling.	156
		constitutive kinase-dead KI	Lethal between E10.5 and E11.5	Heterozygous male mice display early-onset leptin-resistance and mild age-dependent obesity.	183
		constitutive KO / inducible RNAi	Lethal between E10.5 and E11.5 (constitutive KO)	Heterozygous mice have defects in the internal membrane reserve system in megakaryocytes and platelets, impaired platelet adhesion and thrombus formation.	184
		constitutive kinase-dead KI	Heterozygous KIs are viable	Heterozygous mice show defects in megakaryocyte / platelet membrane structure and α-granules and impaired platelet function.	185
		hypomorphic gene trap allele	Viable, reduced growth, 30% lethality at 6 months of age	Renal failure associated with glomerulonephropathy.	252
	vascular endothelium	Tie2-Cre	Lethal between E16.5 and E18.5	Defective vascular formation.	173
PI3K-C2β	**global**	constitutive KO	Viable and fertile	No overt phenotype observed.	187
		constitutive kinase-dead KI	Viable and fertile	Enhanced insulin sensitivity and glucose tolerance, resistance to high-fat-diet-induced liver steatosis.	168
PI3K-C2γ	**global**	constitutive KO	Viable and fertile	Reduced liver glycogen storage, and compensatory adiposity and insulin resistance with age.	63

	Target tissue	Mouse model	Viability	Major phenotypes	Reference
Vps34	**global**	Meox-Cre (epiblast)	Lethal between E7.5 and E8.5	Early embryonic lethality with severely reduced cell proliferation. Heterozygous mice show no overt phenotypes.	233
		Vps34 kinase-dead knock-in	Lethal between E6.5 and E8.5	Heterozygous mice are viable, healthy and display a robustly enhanced insulin sensitivity and glucose tolerance.	204
	heart	mck-Cre	Post-natal lethality between 5 and 13 weeks	Cardiomegaly	231
		mck-Cre	Post-natal lethality between 11 and 15 weeks	Hypertrophic cardiomyopathy. Accumulation of $CryAB^+/desmin^+/p62^+/K63pUb^+$ aggregates.	253
	liver	Alb-Cre	Post-natal lethality at 1 year	Hepatomegaly and hepatic steatosis, increased liver protein content, lack of glycogen deposition.	231
	skeletal muscle	Ckmm-Cre (creatine kinase)	Post-natal lethality between 4 and 9 weeks	Muscular dystrophy and severe dilated cardiomyopathy.	254
		Acta1 -Cre	Post-natal lethality at 14 weeks	Mild myopathic abnormalities. Exacerbates the Mtm1 KO phenotype.	191
	kidney	Nphs2-Cre (podocyte)	Post-natal lethality between 3–9 weeks	Rapid podocyte degeneration and early-onset glomerulosclerosis, proteinuria.	255
		Pax8-Cre (proximal tubular cells)	Post-natal lethality at 4 weeks	proximal tubular cell vacuolation and intracellular sequestration of megalin.	256
		Podocin-Cre (podocyte)	Post-natal lethality at 9 weeks	Severe kidney lesions, with severe glomerulosclerosis. Renal tubular dilation, severe proteinuria, and mild to moderate interstitial inflammation and fibrosis	257
	white adipose tissue	Fabp4-Cre	Viable	Improved glucose tolerance and reduced adiposity in middle and old age mice. Adipose tissue browning.	258

	Target tissue	Mouse model	Viability	Major phenotypes	Reference
	retina	Rhodopsin-Cre and iCRE-75 (retinal rod cells)	Viable	Rapid rod cell degeneration. Retinas have an increased number of apoptotic cells.	214
		MLR10-Cre	Viable	Congenital cataract and microphthalmia.	259
	megakaryocyte / platelet	PF4-Cre	Viable	Impaired thrombus formation, aggregation, granule secretion.	260
		PF4-Cre	Viable	Mild microthrombocytopenia and platelet granule abnormalities.	210
	Schwann cell	myelin protein zero (P0)-Cre	Viable	Severe hypomyelination in peripheral nerves. Unsteady gait, hind limb weakness, and tremor. Defective myelination in Vps34 mutant nerves.	261
	immune system	Lck-Cre (T cell)	Viable	T lymphocytes exhibit increased apoptosis and reduced IL-7Rα surface expression.	262
		CD4-Cre (T cell)	Viable	Inflammatory wasting syndrome characterized by weight loss, intestinal inflammation, and anemia at 18 to 24 weeks.	263
		CD4-Cre (T cell)	Viable	T cell lymphopenia. Reduced number of T cells but normal T cell development. Impaired autophagy in T cells.	232
	nervous system	CaMKII-Cre (hippocampus, pyramidal neurons)	Viable	Loss of dendritic spines, neurodegeneration, reactive gliosis.	264
		Advillin-Cre (sensory neurons)	Post-natal lethality at 2 weeks	Neurodegeneration, increased lysosomes in small diameter neurons, vacuolization in large diameter sensory neurons.	265

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Box 1

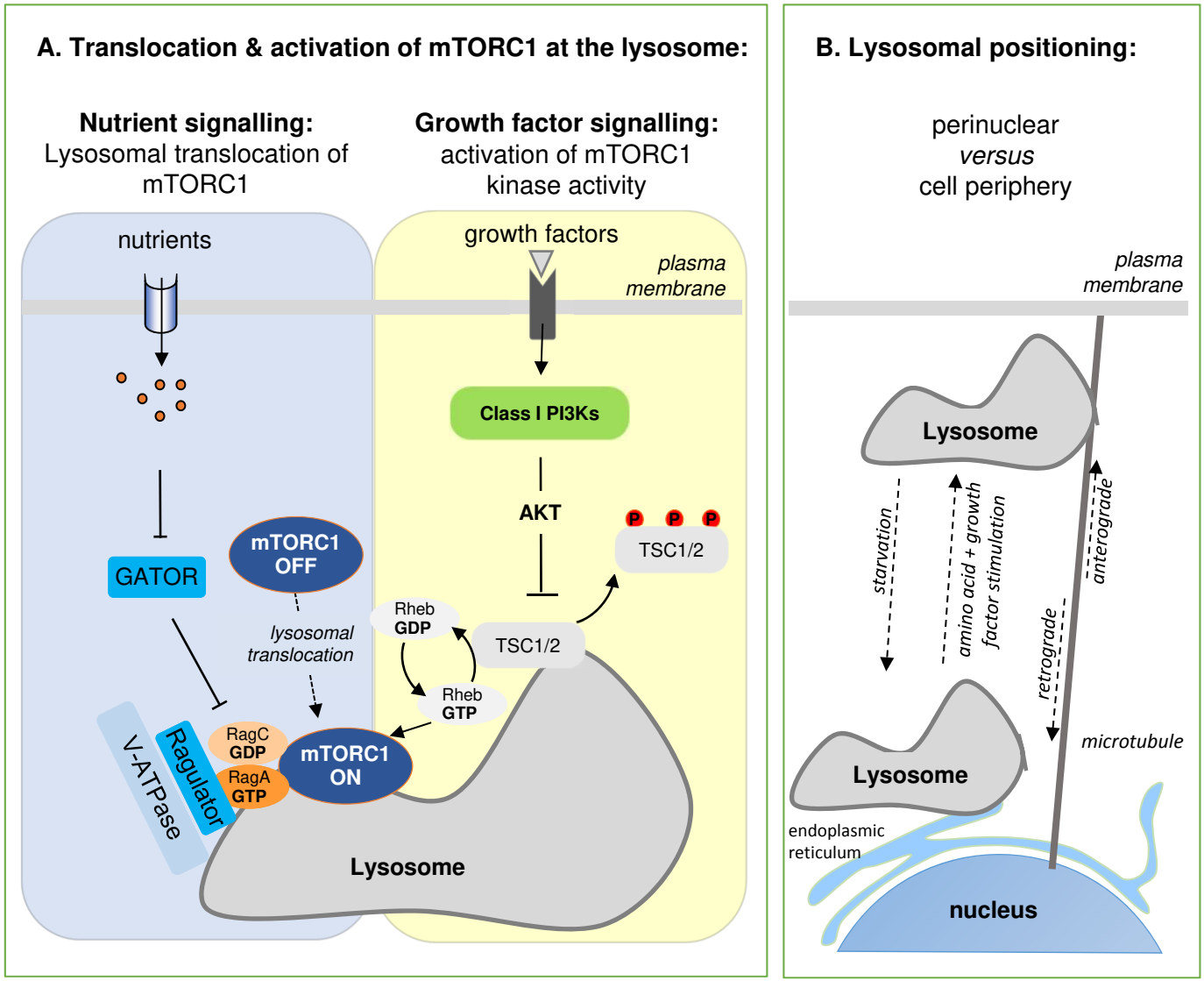

A. Translocation & activation of mTORC1 at the lysosome:
Nutrient signalling:
Lysosomal translocation of mTORC1
Growth factor signalling:
activation of mTORC1 kinase activity
nutrients
growth factors
plasma membrane
Class I PI3Ks
AKT
GATOR
mTORC1 OFF
lysosomal translocation
TSC1/2
Rheb GDP
Rheb GTP
TSC1/2
V-ATPase
Ragulator
RagC GDP
RagA GTP
mTORC1 ON
Lysosome
B. Lysosomal positioning:
perinuclear versus cell periphery
plasma membrane
Lysosome
starvation
amino acid + growth factor stimulation
anterograde
retrograde
microtubule
Lysosome
endoplasmic reticulum
nucleus

Figure 1

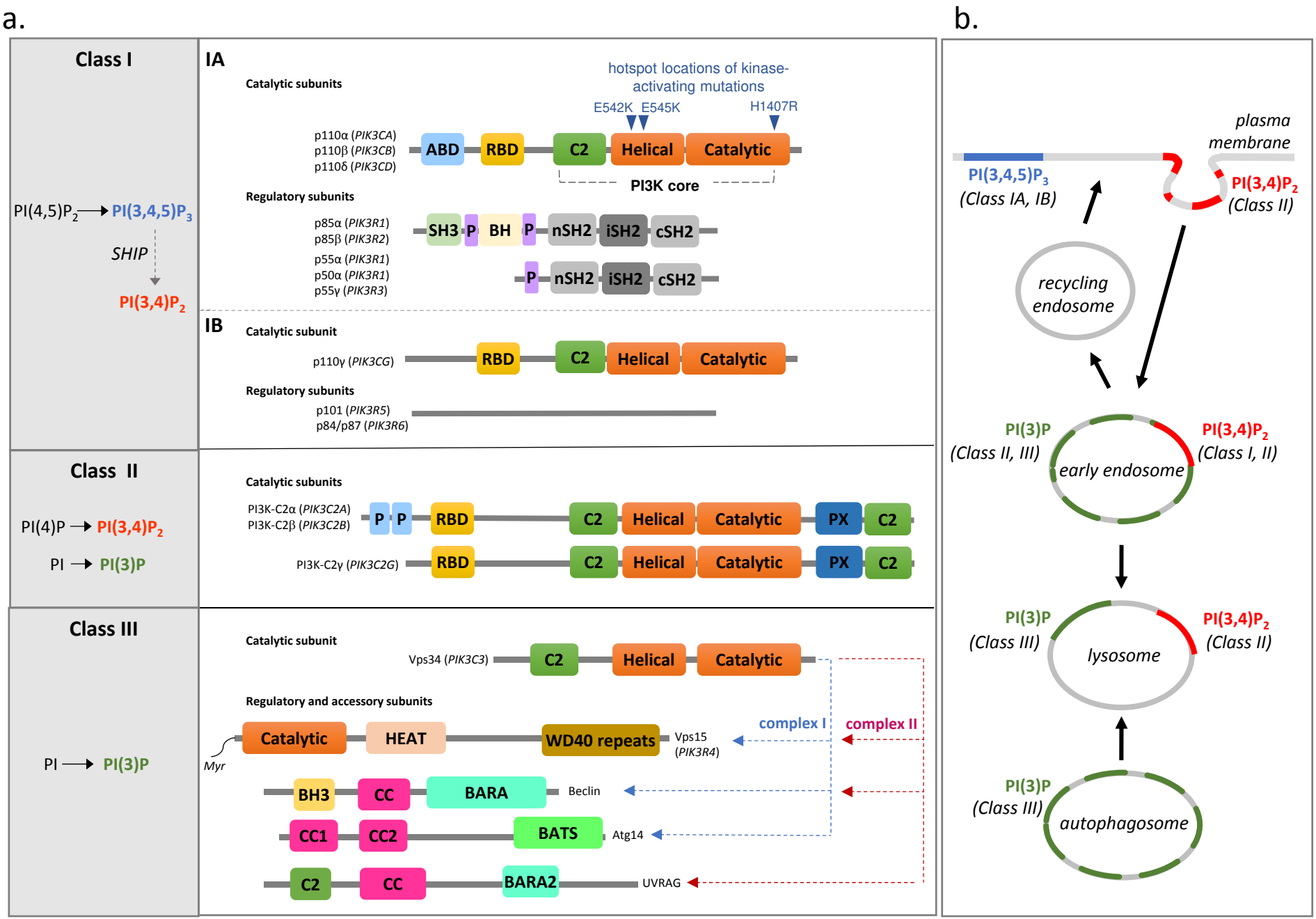

a.
b.
Class I
PI(4,5)P2 → PI(3,4,5)P3
SHIP
PI(3,4)P2
IA
Catalytic subunits
hotspot locations of kinase-activating mutations
E542K E545K
H1407R
p110α (PIK3CA)
p110β (PIK3CB)
p110δ (PIK3CD)
ABD
RBD
C2
Helical
Catalytic
PI3K core
Regulatory subunits
p85α (PIK3R1)
p85β (PIK3R2)
SH3
P
BH
P
nSH2
iSH2
cSH2
p55α (PIK3R1)
p50α (PIK3R1)
p55γ (PIK3R3)
P
nSH2
iSH2
cSH2
IB
Catalytic subunit
p110γ (PIK3CG)
RBD
C2
Helical
Catalytic
Regulatory subunits
p101 (PIK3R5)
p84/p87 (PIK3R6)
Class II
PI(4)P → PI(3,4)P2
PI → PI(3)P
Catalytic subunits
PI3K-C2α (PIK3C2A)
PI3K-C2β (PIK3C2B)
P
P
RBD
C2
Helical
Catalytic
PX
C2
PI3K-C2γ (PIK3C2G)
RBD
C2
Helical
Catalytic
PX
C2
Class III
PI → PI(3)P
Catalytic subunit
Vps34 (PIK3C3)
C2
Helical
Catalytic
Regulatory and accessory subunits
Myr
Catalytic
HEAT
WD40 repeats
Vps15 (PIK3R4)
complex I
complex II
BH3
CC
BARA
Beclin
CC1
CC2
BATS
Atg14
C2
CC
BARA2
UVRAG
plasma membrane
PI(3,4,5)P3 (Class IA, IB)
PI(3,4)P2 (Class II)
recycling endosome
PI(3)P (Class II, III)
early endosome
PI(3,4)P2 (Class I, II)
PI(3)P (Class III)
lysosome
PI(3,4)P2 (Class II)
PI(3)P (Class III)
autophagosome

Figure 2

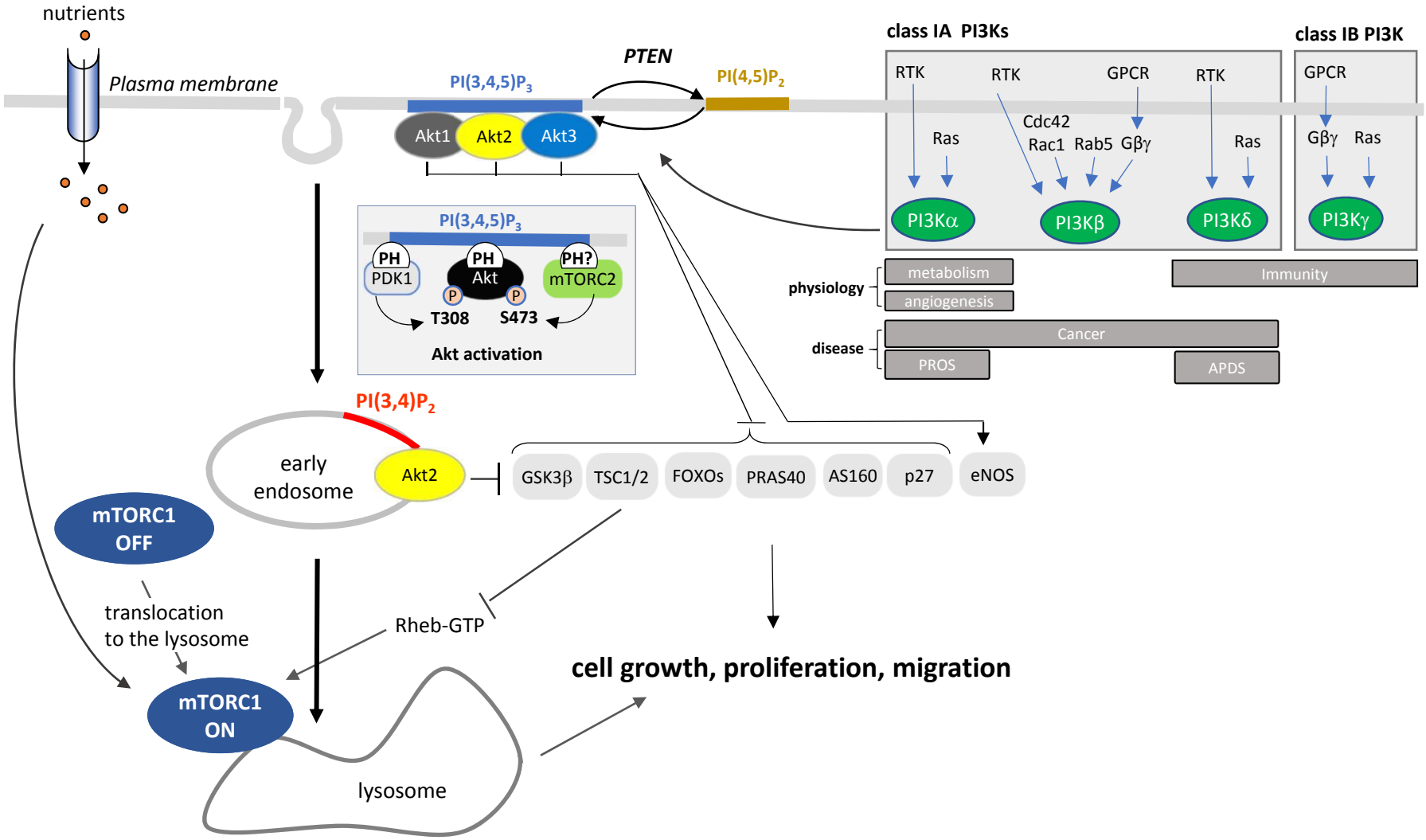

nutrients
Plasma membrane
PI(3,4,5)P3
PTEN
PI(4,5)P2
Akt1
Akt2
Akt3
class IA PI3Ks
class IB PI3K
RTK
RTK
GPCR
RTK
GPCR
Ras
Cdc42
Rac1
Rab5
Gβγ
Ras
Gβγ
Ras
PI3Kα
PI3Kβ
PI3Kδ
PI3Kγ
PI(3,4,5)P3
PH
PDK1
PH
Akt
PH?
mTORC2
P
P
T308
S473
Akt activation
physiology
metabolism
angiogenesis
Immunity
disease
Cancer
PROS
APDS
PI(3,4)P2
early
endosome
Akt2
GSK3β
TSC1/2
FOXOs
PRAS40
AS160
p27
eNOS
mTORC1
OFF
translocation
to the lysosome
Rheb-GTP
cell growth, proliferation, migration
mTORC1
ON
lysosome

Figure 3

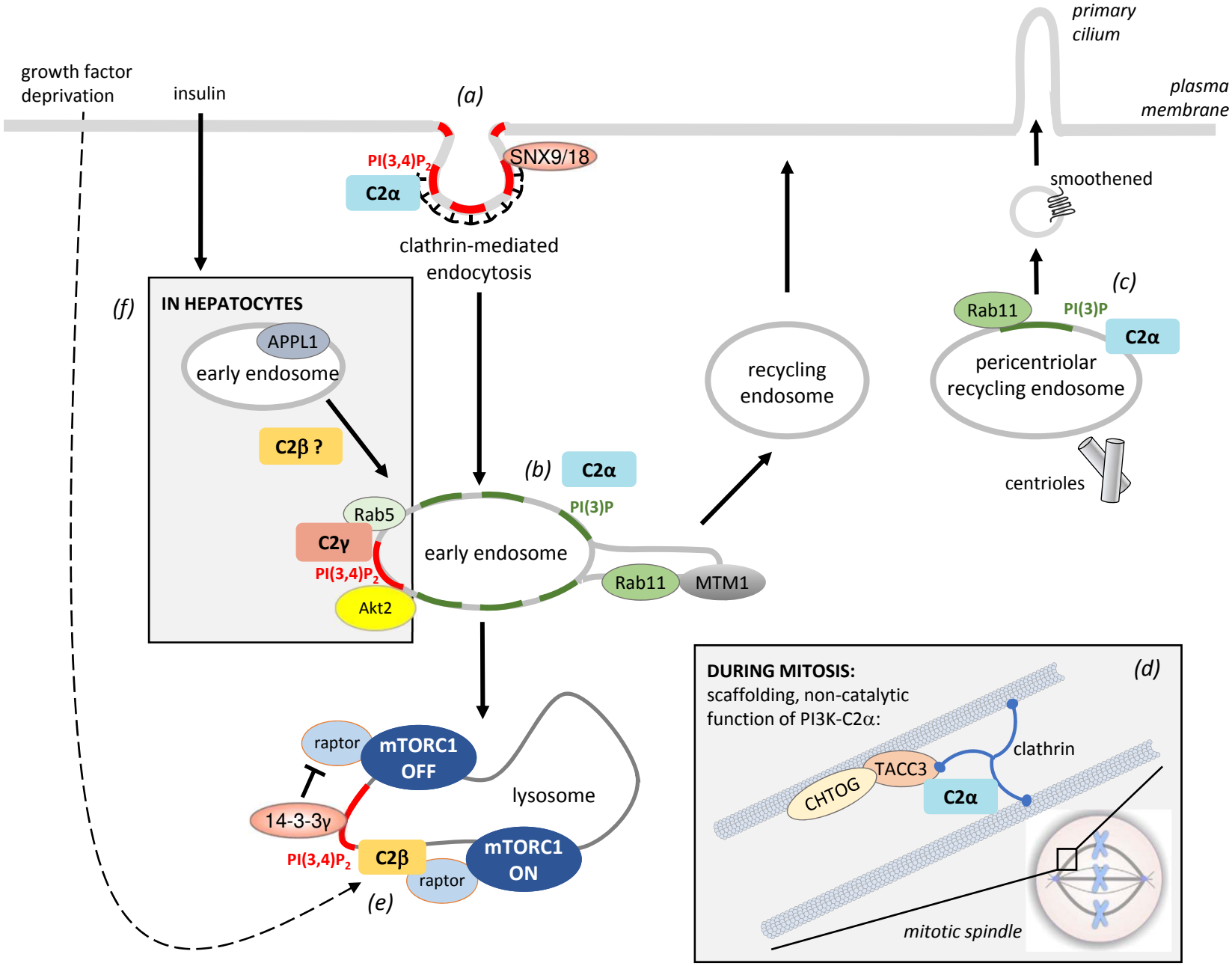

primary cilium
plasma membrane
growth factor deprivation
insulin
(a)
PI(3,4)P2
SNX9/18
C2α
clathrin-mediated endocytosis
smoothened
(c)
Rab11
PI(3)P
C2α
pericentriolar recycling endosome
centrioles
recycling endosome
(f)
IN HEPATOCYTES
APPL1
early endosome
C2β ?
(b)
C2α
PI(3)P
Rab5
C2γ
PI(3,4)P2
Akt2
early endosome
Rab11
MTM1
raptor
mTORC1 OFF
14-3-3γ
lysosome
PI(3,4)P2
C2β
mTORC1 ON
raptor
(e)
DURING MITOSIS:
scaffolding, non-catalytic function of PI3K-C2α:
(d)
clathrin
TACC3
CHTOG
C2α
mitotic spindle

Figure 4

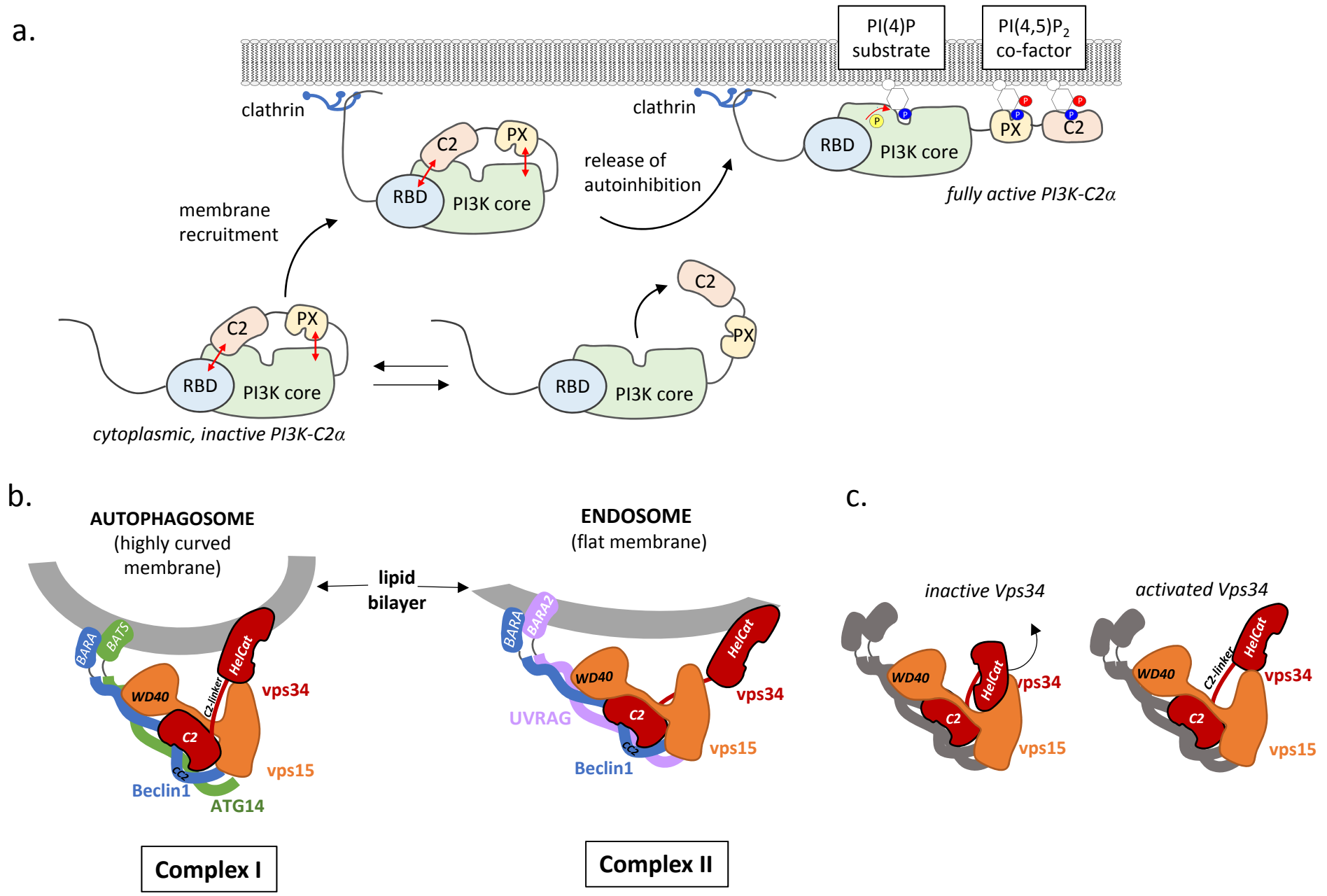

a.
PI(4)P substrate
PI(4,5)P2 co-factor
clathrin
clathrin
C2
PX
RBD
PI3K core
release of autoinhibition
fully active PI3K-C2α
membrane recruitment
cytoplasmic, inactive PI3K-C2α
b.
AUTOPHAGOSOME
(highly curved membrane)
lipid bilayer
ENDOSOME
(flat membrane)
BARA
BATS
BARA2
HelCat
WD40
C2-linker
vps34
vps15
C2
Beclin1
ATG14
UVRAG
Complex I
Complex II
c.
inactive Vps34
activated Vps34

Figure 5

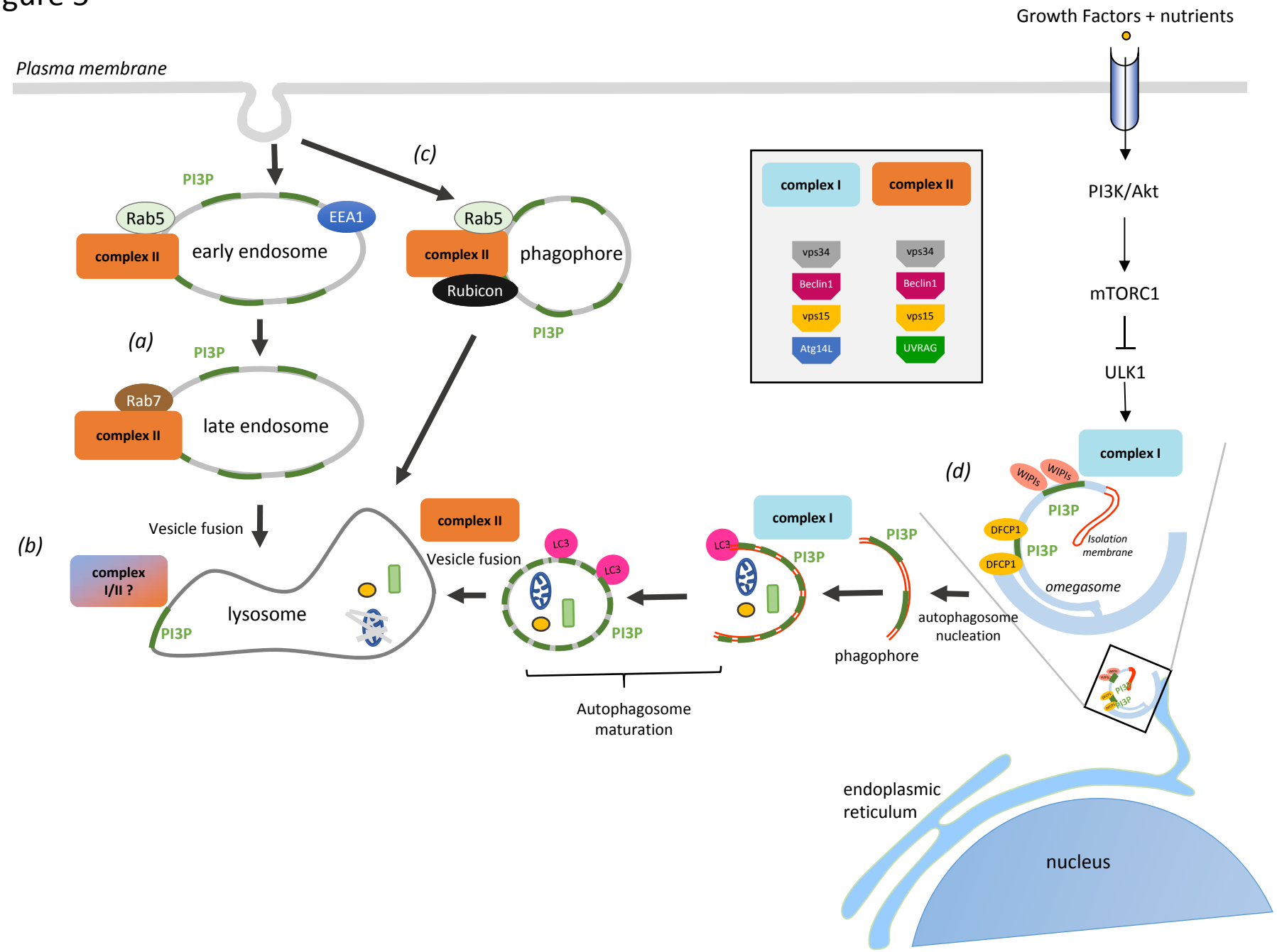
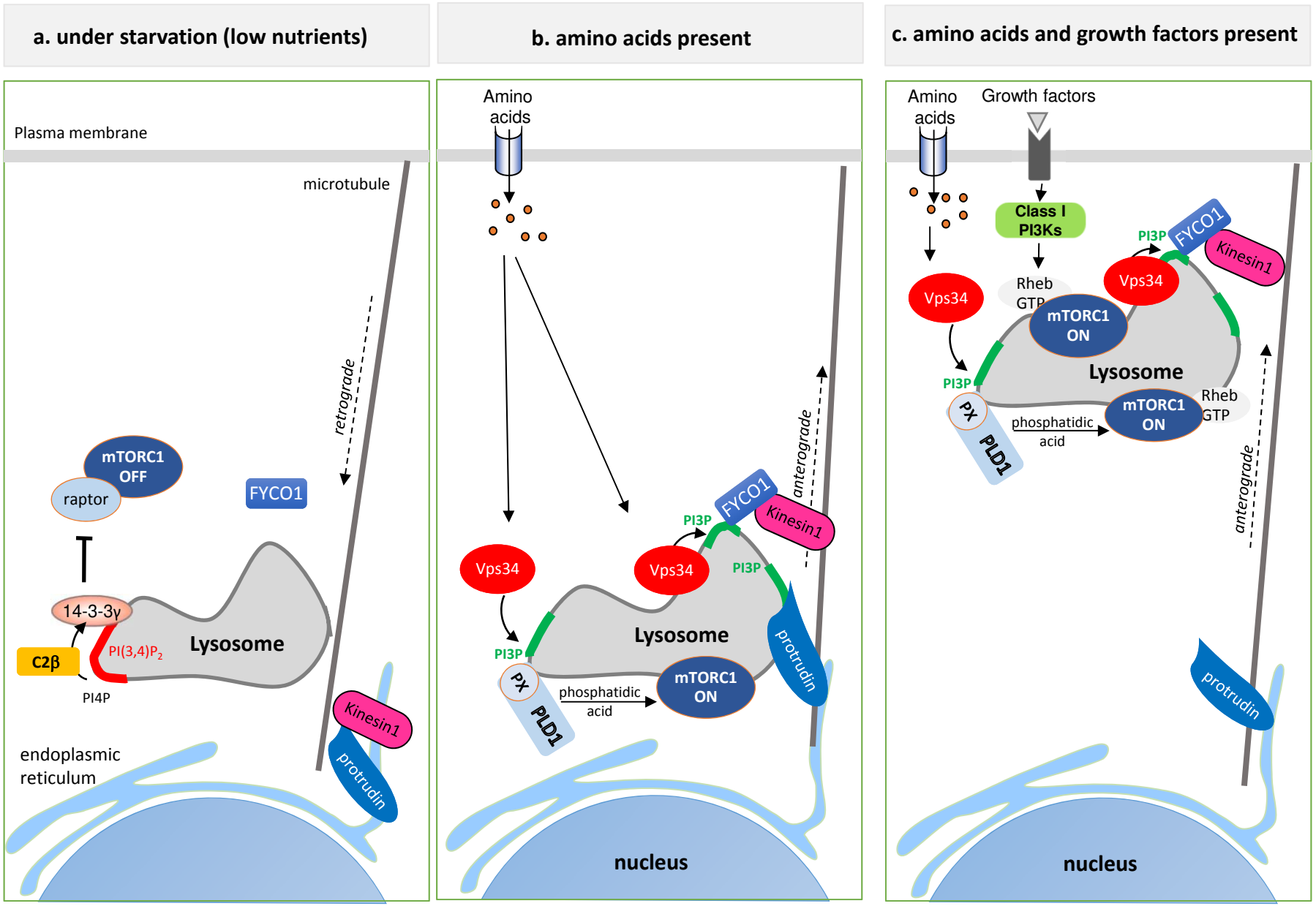

Growth Factors + nutrients
Plasma membrane
PI3K/Akt
mTORC1
ULK1
complex I
complex II
vps34
Beclin1
vps15
Atg14L
UVRAG
(c)
PI3P
Rab5
EEA1
complex II
early endosome
phagophore
Rubicon
(a)
Rab7
late endosome
(b)
Vesicle fusion
complex I/II ?
lysosome
LC3
Autophagosome maturation
autophagosome nucleation
(d)
WIPIs
DFCP1
Isolation membrane
omegasome
endoplasmic reticulum
nucleus

Figure 6


a. under starvation (low nutrients)
b. amino acids present
c. amino acids and growth factors present
Plasma membrane
microtubule
retrograde
mTORC1
OFF
raptor
FYCO1
14-3-3γ
C2β
PI(3,4)P2
Lysosome
PI4P
Kinesin1
protrudin
endoplasmic
reticulum
Amino
acids
Vps34
PI3P
FYCO1
Kinesin1
anterograde
Lysosome
PI3P
PX
PLD1
phosphatidic
acid
mTORC1
ON
protrudin
nucleus
Amino
acids
Growth factors
Class I
PI3Ks
Vps34
Rheb
GTP
mTORC1
ON
PI3P
FYCO1
Kinesin1
Lysosome
PI3P
PX
PLD1
phosphatidic
acid
mTORC1
ON
Rheb
GTP
anterograde
protrudin
nucleus