

Phosphatidylinositol 4-kinase III beta promotes oncogenic signaling in breast cancer by controlling endocytosis

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Abstract

Endosomes are now recognized as important sites for regulating signal transduction. Here we show that the lipid kinase phosphatidylinositol 4-kinase III beta (PI4KIII β) regulates both endocytic kinetics and receptor signaling in breast cancer cells. PI4KIII β generates phosphatidylinositol 4-phosphate from phosphatidylinositol and is highly expressed in a subset of breast cancers. However, the molecular mechanism by which PI4KIII β promotes breast cancer is unclear. We demonstrate that ectopic PI4KIII β expression increases the rates of both endocytic internalization and recycling. Furthermore, PI4KIII β deletion reduces endocytic kinetics. Regulation of endocytic function by PI4KIII β is independent of its kinase activity but requires interaction with the Rab11a GTPase. Additionally, we find that PI4KIII β activates IGF-IR β signaling, dependent on endosome function. Finally, we observe that PI4KIII β deletion decreases the growth rate of mammary tumours in mice. Our work suggests a novel regulatory role for PI4KIII β in endosome function and plasma membrane receptor signaling, providing a mechanism by which increased PI4KIII β expression could promote breast cancer oncogenesis.

Keywords

Breast cancer, Rab11a, phosphatidylinositol 4-phosphate, recycling, endocytosis, signaling

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Table of Contents

Abstract	ii
Acknowledgements	iii
Table of Contents	iv
List of abbreviations	vi
List of figures.....	ix
Chapter 1: Introduction	1
1.1 Phosphoinositides and lipid signaling.....	1
1.2 Phosphatidylinositol 4-kinases.....	15
1.3 Phosphatidylinositol 4-kinase III beta.....	19
1.4 PI4KIII β and breast cancer	19
1.5 Rab GTPases	20
1.6 PI4KIII β -Rab11a interaction	27
1.7 The endocytic system	27
1.8 Plasma membrane receptor signaling	33
1.9 Endocytic regulation of plasma membrane receptor signaling	38
1.10 Signaling endosomes	39
1.11 Research hypothesis and objectives.....	40
Chapter 2: Materials and Methods	42
2.1 Cell culture	42
2.2 Plasmids	43
2.3 Western blot	44
2.4 Growth factor stimulation assays.....	45
2.5 Transferrin pulse-chase and uptake assays.....	45
2.6 Immunofluorescence	46
2.7 Proximity ligation assay	48
2.8 In vivo tumour studies	49
Chapter 3: Results	50
3.1 PI4KIII β enhances the intensity and/or duration of IGF-IR β , A-Raf, and Shc activation	50

3.2	PI4KIII β expression alters endocytic kinetics	55
3.3	Lipid kinase-independent control of endocytic function	66
3.4	PI4KIII β interacts with Rab5, Rab7, and EEA1	75
3.5	PI4KIII β mediated activation of IGF-IR β is dependent on endosome function ...	80
3.6	PI4KIII β deletion decreases IGF-IR β activation.....	85
3.7	PI4KIII β regulates mammary tumourigenesis	90
Chapter 4: Discussion		93
	Signaling endosomes and PI4KIII β	101
	PI4KIII β and cancer	110
Conclusion		112
	Future directions	112
References		115
Contributions of collaborators.....		139

List of abbreviations

A

ADE	Arf6-dependent endocytosis
ANTH	AP180 amino-terminal homology
AP	adaptor protein
APPL	adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper
AR	acidic region
Arf	ADP-ribosylation factors

C

C2	conserved region 2 of protein kinase C
cAMP	cyclic adenosine monophosphate
CDE	caveolar-dependent endocytosis
CDP-DAG	cytidine diphosphate diacylglycerol
CERT	ceramide transfer protein
CLIC/GEEC	clathrin-independent carrier/GPI-AP-enriched endosomal compartment
CME	clathrin-mediated endocytosis
CPZ	chlorpromazine
CR	cysteine-rich
CTCF	corrected total cellular fluorescence

D

DAG	diacylglycerol
DLIC	dynein light intermediate chain

E

EEA1	early endosome antigen 1
eEF1A2	elongation factor 1 α 2
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EMT	epithelial-mesenchymal transition
ENTH	epsin amino-terminal homology
ER	endoplasmic reticulum
ERC	endocytic recycling compartment
ESCRT	endosomal sorting complexes required for transport

F

FACS	fluorescence-activated cell sorting
FAPP	four-phosphate adaptor protein
FDE	flotillin-dependent endocytosis
Fe ³⁺	ferric ion
FERM	4.1, Ezrin, Radixin, Moesin
Fwd	four-wheel drive
FYVE	Fab1, YOTB, Vac1, and EEA1

G

GAP	GTPase-activating protein
GDF	GDI displacement factor
GDI	GDP dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GGA2	Golgi-localized, γ adaptin ear-containing, ADP-ribosylation factors-binding 2
GLUT4	glucose transporter type 4
GOLPH3	Golgi phosphoprotein 3
GPCR	G-protein coupled receptor
GTP	guanosine triphosphate

H

HOPS	homotypic fusion and protein sorting
Hrs	hepatocyte growth factor-regulated tyrosine kinase substrate

I

IGF-I	insulin-like growth factor type I
IGF-IR	insulin-like growth factor type I receptor
IGF-IR β	insulin-like growth factor type I receptor beta domain
Inpp	inositol polyphosphate-4-phosphatase
IP ₃	inositol 1,4,5-trisphosphate

L

LKU	lipid kinase unique
LPA	lysophosphatidic acid
Lpd	lamellipodin

M

MAPK/Erk	mitogen-activated protein kinases/extracellular signal-regulated kinases
MTOC	microtubule-organizing centre
mTORC	mammalian target of rapamycin complex
MVB	multivesicular body
MYO18A	unconventional myosin-XVIIIa
MyoV	myosin V

N

NCS-1	neuronal calcium sensor-1
NLS	nuclear localization signal

O

ORPL9	oxysterol-binding protein-related protein 9
OSPB	oxysterol-binding protein 1

P

PDGF	platelet-derived growth factor
PDK	pyruvate dehydrogenase kinase
PH	pleckstrin homology
PI	phosphoinositides

PI(3,4)P ₂	phosphatidylinositol 3,4-bisphosphate
PI(3,4,5)P ₃	phosphatidylinositol 3,4,5-trisphosphate
PI(4,5)P ₂	phosphatidylinositol 4,5-bisphosphate
PI3K	phosphatidylinositol 3-kinase
PI3P	phosphatidylinositol 3-phosphate
PI4K	phosphatidylinositol 4-kinase
PI4KIII α	phosphatidylinositol 4-kinase III α
PI4KIII β	phosphatidylinositol 4-kinase III β
PI4KII α	phosphatidylinositol 4-kinase II α
PI4KII β	phosphatidylinositol 4-kinase II β
PI4P	phosphatidylinositol 4-phosphate
PI5K	phosphatidylinositol 5-kinase
PI5P	phosphatidylinositol 5-phosphate
PIKfyve	phosphoinositide kinase, FYVE-type zinc finger containing
PIP5K	phosphatidylinositol 4-phosphate 5-kinase
PIS	phosphatidylinositol synthase
PKB	protein kinase B (aka Akt)
PKC	protein kinase C
PLA	proximity ligation assay
PLC	phospholipase C
PR	proline-rich
PROPPIN	β -propellers that binds phosphoinositides
PTB	phosphotyrosine binding
PtdIns	phosphatidylinositol
PTEN	phosphatase and tensin homolog
PX	Phox homology
R	
REP	Rab escort protein
RTK	receptor tyrosine kinase
S	
SFUM	serum free uptake media
SH2	Src homology 2
SHIP	SH2 domain-containing inositol 5-phosphatase
SNARE	soluble NSF attachment protein receptor
SNX	sorting nexin
T	
TAPP	tandem PH-domain containing protein
TGN	<i>trans</i> -Golgi network
W	
WASP	Wiskott-Aldrich syndrome protein

List of figures

Figure 1.1 Phosphoinositide structure and metabolism.....	3
Figure 1.2 Intracellular distribution of phosphoinositides.....	6
Figure 1.3 Phosphoinositide effector domains.	8
Figure 1.4 Structural characteristics of the PI4K isoforms.	18
Figure 1.5 Subcellular distribution of Rab GTPases.	22
Figure 1.6 Endocytic internalization and recycling.	31
Figure 3.1 PI4KIII β increases the intensity of IGF-IR β and A-Raf activation.	52
Figure 3.2 PI4KIII β increases the intensity and duration of Shc activation.	54
Figure 3.3 High expression of PI4KIII β increases endocytic recycling.....	57
Figure 3.4 High PI4KIII β expression increases the rate of endocytic internalization.....	59
Figure 3.5 Deletion of PI4KIII β does not affect Golgi structure in BT549 cells.	61
Figure 3.6 Deletion of PI4KIII β does not affect Golgi structure in NIH3T3 cells.	63
Figure 3.7 PI4KIII β deletion decreases endocytic recycling.	65
Figure 3.8 Deletion of PI4KIII β decreases endocytic internalization.	68
Figure 3.9 PI4KIII β rescue cell lines.	70
Figure 3.10 PI4KIII β -Rab11a interaction rescues endocytic recycling.	72
Figure 3.11 Catalytically activate PI4KIII β rescues endocytic internalization.....	74
Figure 3.12 Rab11a interacts with ectopic PI4KIII β	77
Figure 3.13 PI4KIII β colocalizes with compartments of the endocytic system.....	79
Figure 3.14 PI4KIII β does not affect Rab11a recycling endosome number, shape or size.	82

Figure 3.15 Ectopic PI4KIII β regulates IGF-IR β signaling dependent on endosome function.	
.....	84
Figure 3.16 IGF-IR β signaling does not appear to be affected by PI4KIII β knockout.....	87
Figure 3.17 PI4KIII β deletion causes increased total IGF-IR β	89
Figure 3.18 <i>Pi4kb</i> deletion inhibits mammary tumorigenesis in mice.	92
Figure 4.1 PI4KIII β -mediated regulation of endocytic recycling.	99
Figure 4.2 PI4KIII β -mediated receptor signaling via regulation of endocytosis.	106

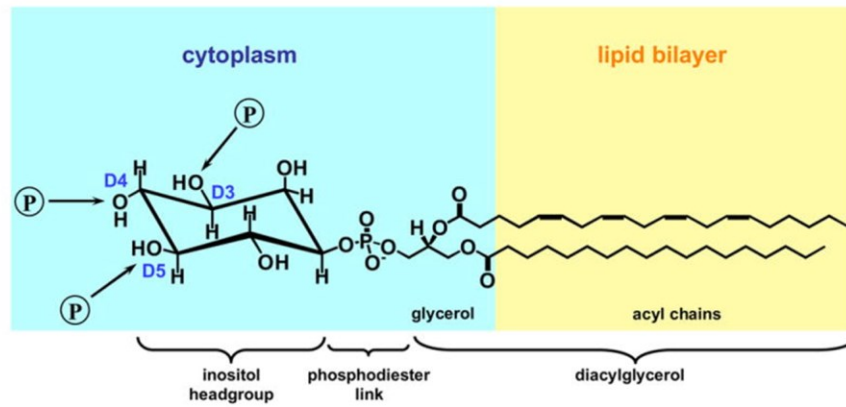
Chapter 1: Introduction

1.1 Phosphoinositides and lipid signaling

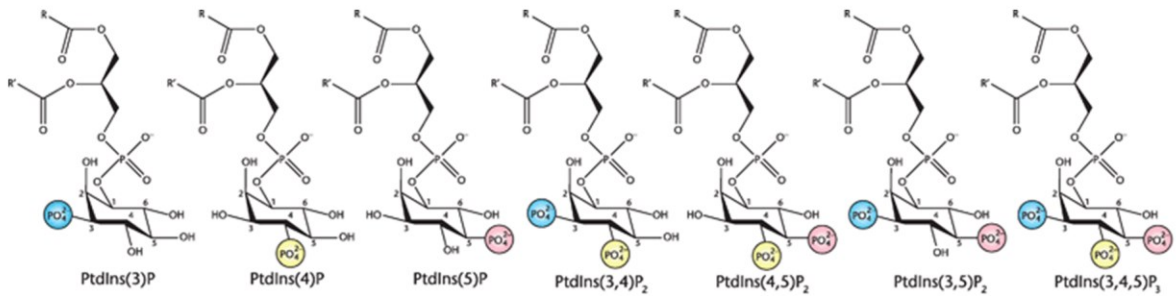
Phosphoinositides (PIs) are essential regulators of the function and survival of cells. They are involved in most cellular processes, including, membrane identity, intracellular trafficking, signaling, and metabolism (sections 1.1.1-1.1.7) ¹. Phosphatidylinositol (PtdIns), the base form of PIs, is synthesized by the addition of a *myo*-inositol head group to cytidine diphosphate diacylglycerol (CDP-DAG), mediated by the phosphatidylinositol synthase (PIS) enzyme ². Traditionally, PtdIns synthesis was thought to take place in the endoplasmic reticulum (ER) alone, but mounting evidence suggests there may also be plasma membrane localized production of PtdIns ^{2,3,4,5}. The diacylglycerol (DAG) backbone, made of 1-stearoyl-2-arachanonyl-3-phosphoglycerol, of PtdIns, is connected to the D1 position of the *myo*-inositol head group and anchors the PtdIns to membranes (Figure 1.1a) ^{1,6}. This leaves the *myo*-inositol head group in the cytosol and five exposed hydroxyls free for phosphorylation; although only three are known to be phosphorylated naturally (Figure 1.1a) ^{1,6}. From PtdIns, seven different PI isoforms can be generated, through the reversible mono-, di-, or tri-phosphorylation at the D3, D4, and D5 positions of the inositol ring (Figure 1.1b) ⁷. The PI isoforms can be interconverted by phosphatidylinositol kinases and phosphatases (Figure 1.1c) ⁷.

PIs make up less than 15% of cellular phospholipids, however, due to their widespread cellular locations and interactions, have critical roles in normal cellular function ^{8,9}. The spatiotemporal regulation of phosphatidylinositol kinases and phosphatases allow

a



b



c

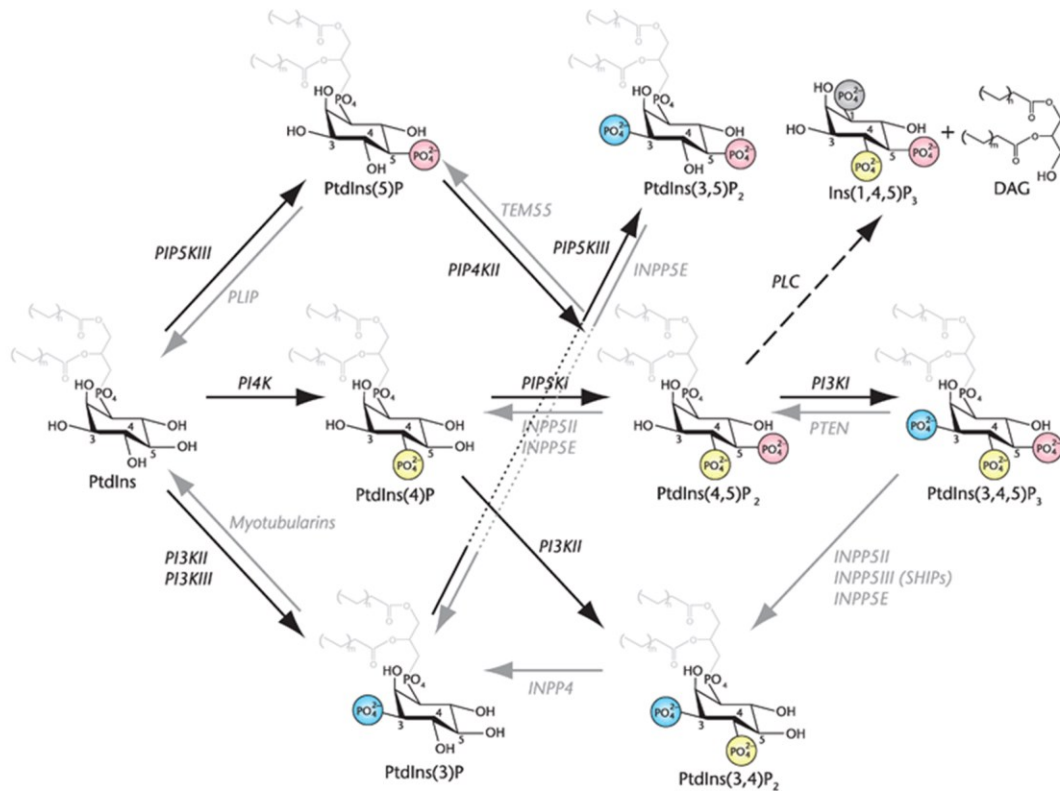


Figure 1.1 Phosphoinositide structure and metabolism.

(a) The chemical structure of phosphatidylinositol. The inositol headgroup is linked to diacylglycerol via the phosphorylation of its D1 position. The diacylglycerol chains anchor the phospholipid into membranes, leaving the inositol head group exposed in the cytoplasm to be modified through kinase and/or phosphatase activity. (b) Stereochemical representation of the seven *d-myo*-phosphatidylinositol phosphate species with the three modifiable phosphates at the D3, D4 and D5 positions shown as blue, yellow and red spheres, respectively. (c) The seven phosphoinositides and the converting enzyme reactions are depicted. Kinase reactions are indicated by solid black arrows, phosphatase reactions as solid grey arrows, and the PLC reaction is indicated by the dashed black arrow. The enzymes catalyzing the reactions are indicated in black (kinases and PLC) and grey (phosphatases). Figure adapted from Strahl and Thorner, 2007, and Leitha, 2011^{6,7}.

for the generation of distinctive pools of PIs located throughout the cell (Figure 1.2)¹⁰. The diversity in subcellular location, or time in the lifecycle of a cell that a PI is generated enables even the same isoform of PIs to perform various functions within the cell^{1,11}. Moreover, distinct functions for PIs come from which combination of their hydroxyl groups have been phosphorylated, and how this affects their interaction network¹².

Proteins have evolved different modules to bind PIs, termed phosphoinositide binding or recognition domains, that are used to regulate both their function and localization¹². These domains differ in affinity and specificity for PIs depending on the PIs cytosolic composition and include: pleckstrin homology (PH), AP180 amino-terminal homology (ANTH), conserved region 2 of protein kinase C (C2), epsin amino-terminal homology (ENTH), 4.1, Ezrin, Radixin, Moesin (FERM), Fab1, YOTB, Vac1, and early endosome antigen 1 (EEA1) (FYVE), Golgi phosphoprotein 3 (GOLPH3), β -propellers that bind PIs (PROPPINs), phosphotyrosine binding (PTB), Phox homology (PX), and Tubby domains (Figure 1.3)¹⁰. PI species are recognized by different combinations of these recognition domains, and while some recognition domains are able to bind several different PIs, such as the PH and PX domains, others interact with only one PI, for example, the FYVE domain and PI3P (Figure 1.3)^{10,12}. Although a recognition domain like PH can recognize many PIs, different proteins containing PH domains will interact preferentially with different PI species^{10,12}. For example, the PH domain of Akt has a high affinity for PI(3,4)P₂ and PI(3,4,5)P₃, whereas the PH domain of dynamin preferentially targets PI(4,5)P₂ (Figure 1.3)^{10,12}. The selective interactions between different proteins and PIs ensures the precise targeting of proteins throughout the cell¹². The specific generation, localization,

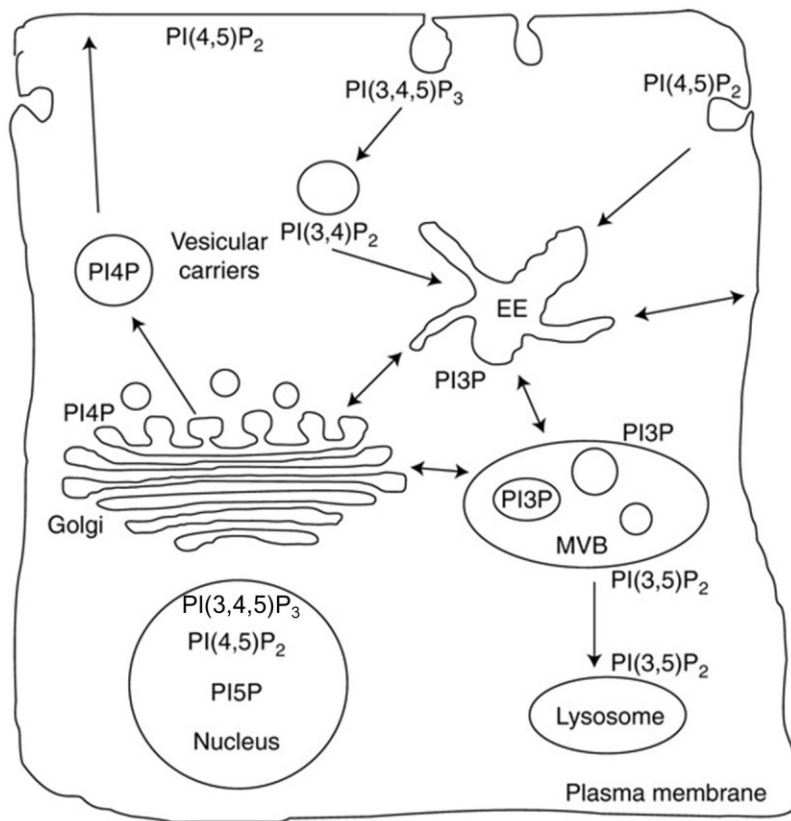


Figure 1.2 Intracellular distribution of phosphoinositides.

The predominant localization of PI species in subcellular compartments is depicted. There is some overlap of PI signature between membrane compartments, and heterogeneity of PI distribution on membrane compartments also occurs. PI(4,5)P₂ and PI(3,4,5)P₃ are enriched at the plasma membrane, possibly in raft-like domains. PI(3,4)P₂ dominates in early endocytic membranes and at the plasma membrane. PI(3)P is concentrated on early endosomal (EE) membranes and the multivesicular body (MVB) compartment. PI(4)P is enriched at the Golgi complex and in Golgi-derived carriers. PI(3,5)P₂ concentrates on late compartments of the endocytic pathway, the MVB, and lysosome. PI(5)P and PI(3,4,5)P₃ are localized in the nucleus, and generation of nuclear PI(4,5)P₂ is key to regulating some aspects of gene expression. Not all cellular compartments are illustrated and arrows are not intended to represent the entire cohort of known endocytic trafficking routes. Figure adapted from Shewan *et al*, 2011¹⁰.

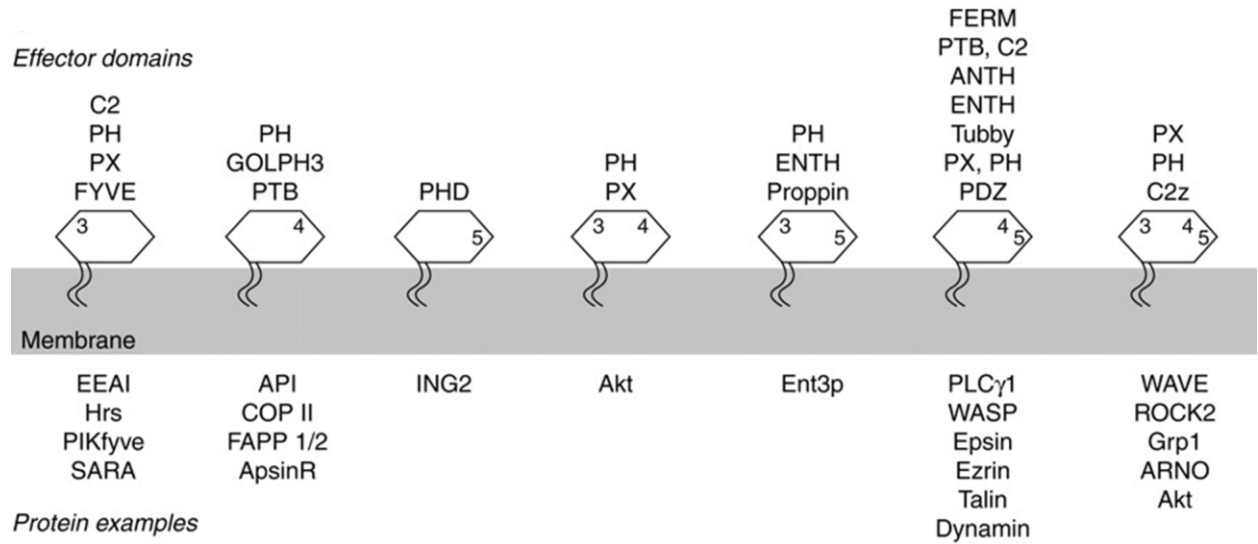


Figure 1.3 Phosphoinositide effector domains.

A depiction of the protein binding domains associated with each of the different PI isoforms. Examples of protein interactions with different PIs are provided. Abbreviations: PH (pleckstrin homology), ANTH (AP180 amino-terminal homology), C2 (conserved region 2 of protein kinase C), ENTH (epsin amino-terminal homology), FERM (4.1, Ezrin, Radixin, Moesin), FYVE (Fab1, YOTB, Vac1, and EEA1), GOLPH3 (Golgi phosphoprotein 3), PROPPINS (β -propellers that bind PIs), PTB (phosphotyrosine binding), PX (Phox homology), and Tubby domains. Figure adapted from Shewan *et al*, 2011¹⁰.

and roles of the seven different PIs will be discussed further in the next sections (sections 1.1.1-1.1.7).

1.1.1 Phosphatidylinositol 3-phosphate (PI3P)

PI3P is predominately generated by class III, and to a lesser extent class II, phosphatidylinositol 3-kinase (PI3K) phosphorylation of PtdIns, and is catabolized by myotubularin 3-phosphatases^{13,14}. Localized to early endosomes and autophagosomes, PI3P has numerous roles due to its large number of effector binding domains, especially the FYVE and PX binding domains (Figure 1.3)^{15,16}. Through its interactions with Rab5 and EEA1, PI3P is indispensable for the formation and trafficking of early endosomes^{17,18}. Additionally, in the early endosomes, PI3P effectors, sorting nexin (SNX) proteins are involved in the sorting of cargo for recycling, or hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs) and endosomal sorting complex required for transport (ESCRT) to multivesicular bodies (MVBs) for degradation^{19,20,21}. PI3P and its effector's aid in the formation of autophagosomes and subsequent steps through autophagy²². Additionally, PI3P is involved in exocytosis, cytokinesis and signaling^{23,24,25}.

1.1.2 Phosphatidylinositol 4-phosphate (PI4P)

The second most abundant PI in the cell, PI4P, accounts for 30% of PIs and is produced by the phosphorylation of PtdIns by phosphatidylinositol 4-kinases (PI4Ks)^{26,27}. PI4P abundance is regulated via dephosphorylation by Sac1 4-phosphatase to PtdIns or further phosphorylation by PI3Ks or phosphatidylinositol 5-kinases (PI5Ks) to generate PI(3,4)P₂ or PI(4,5)P₂, respectively²⁸.

Predominantly found in the Golgi and associated membranes, PI4P is crucial for normal Golgi function^{9,29}. PI4P recruits and anchors GOLPH3 to the *trans*-Golgi membrane, allowing it to interact with unconventional myosin-XVIIIa (MYO18A)³⁰. The linking of the Golgi to F-actin via the PI4P-GOLPH3-MYO18A complex provides the tensile force necessary to maintain the normal shape and trafficking of the Golgi³⁰. Furthermore, PI4P recruits Golgi-localized, γ adaptin ear-containing, ADP-ribosylation factors (Arf)-binding 2 (GGA2) and adaptor protein (AP)-1 to the Golgi membrane, promoting the formation of clathrin-coated vesicles, leaving the *trans*-Golgi network (TGN), destined for the plasma membrane^{31,32}.

PI4P has a role in the regulation of sphingolipid metabolism³³. PI4P recruits ceramide transfer protein (CERT), oxysterol-binding protein 1 (OSPB), oxysterol-binding protein-related protein 9 (ORPL9) and four-phosphate adaptor protein (FAPP) 1 and 2^{34,35,36}. These lipid-transfer proteins are all involved in the formation of sphingolipids, which when coupled with the previously mentioned TGN-to-plasma membrane transport allow the maintenance of the plasma membrane³⁷.

1.1.3 Phosphatidylinositol 5-phosphate (PI5P)

PI5P is the least well-studied of all PIs and, like PI3P, is estimated to be 100-fold less abundant than PI4P in mammalian cells^{38,39}. Although the route for the biogenesis of PI5P is not completely known, evidence suggests that the phosphoinositide kinase, FYVE-type zinc finger containing (PIKfyve) enzyme may have a role either by directly phosphorylating PtdIns generating PI5P or by phosphorylating PI3P to generate PI(3,5)P₂ which is then

dephosphorylated by the myotubularin family of 3-phosphatases^{40,41,42,43}. Additionally, type I and II PI(4,5)P₂ 4-phosphatases can generate PI5P through dephosphorylation of PI(4,5)P₂^{44,45}. In the nucleus, PI5P levels are upregulated by cellular stress such as UV irradiation, free radicals or DNA damage⁴⁶. As such, PI5P regulates apoptosis and gene expression in response to this stress and DNA damage^{47,48}. Furthermore, PI5P interacts with the Cul3-SPOP ubiquitin ligase to modulate nuclear protein function⁴⁹. Cytoplasmic PI5P is involved with actin remodelling, through the breakdown of F-actin⁵⁰. It is believed that PI5P regulates actin dynamics in order to facilitate endosome movement throughout the cell^{39,50,51}. The remodeling of F-actin for the trafficking of glucose transporter type 4 (GLUT4), for example, has a well-characterized dependence on insulin but also appears to be dependent on PI5P^{50,52,53}. This control of the actin cytoskeleton by PI5P contributes to cell motility^{54,55}.

1.1.4 Phosphatidylinositol 3,4-bisphosphate (PI(3,4)P₂)

Emerging evidence suggests roles for PI(3,4)P₂ as a regulator of endocytosis and an important signaling molecule⁵⁶. The generation of PI(3,4)P₂ can occur through two distinct pathways. The majority is produced by 5-phosphatase-mediated dephosphorylation of PI(3,4,5)P₃ and also through class II PI3K-mediated phosphorylation of PI4P^{57,58,59}. Due to the different pathways of generation, PI(3,4)P₂ is produced at different locations or steps throughout the endocytic and/or signaling pathways^{56,57,58,59}. The spatiotemporal differences in PI(3,4)P₂ production, allow it to perform unique functions depending on its origin, whether it is endocytosis at the plasma membrane, migration via cytoskeletal restructuring or signaling on the surface of an endosome^{56,57,58,59}.

The generation of PI(3,4)P₂ from PI(3,4,5)P₃ dephosphorylation occurs when plasma membrane receptor activation of class I PI3Ks causes the phosphorylation of PI(4,5)P₂ to produce PI(3,4,5)P₃⁵⁷. The PI(3,4,5)P₃ is subsequently dephosphorylated by a 5-phosphatase, such as Src homology 2 (SH2) domain-containing inositol 5-phosphatase (SHIP) 1 or 2, generating PI(3,4)P₂⁵⁸. Accumulation of class I PI3K-generated PI(3,4,5)P₂ to PI(3,4)P₂ on the surface of macropinosomes recruit either SNX proteins to promote cleavage of macropinosomes or tandem PH-domain containing protein (TAPP) 1 to promote dorsal ruffle formation, ultimately both leading to the scission of the macropinosome from the plasma membrane^{60,61,62}. Furthermore, SHIP1/2-mediated production of PI(3,4)P₂ has been implicated in a newly documented clathrin-independent endocytosis pathway, fast endophilin-mediated endocytosis (FEME)^{63,64}. PI(3,4)P₂ recruits lamellipodin (Lpd) and its effectors, which promote the cleavage of the growing endosome from the plasma membrane^{63,64}. Finally, the recruitment of Lpd and its effectors by PI(3,4,5)P₃ generated PI(3,4)P₂, has been shown to have a role in the restructuring and growth of actin filaments, to promote the formation of lamellipodia^{65,66}. Concomitantly, PI(3,4)P₂-mediated actin remodeling can promote the recruitment of effectors, such as Tks5, to form extracellular matrix degrading podosomes, which in cancer can develop into invadopodia^{67,68}.

Whereas, PI(3,4)P₂ generation from PI4P occurs when a class II PI3K, such as PI3K2Cα, is recruited to a clathrin-coated pit or early endosome and promotes the phosphorylation of PI4P at the D3 position of the inositol ring^{59,69}. PI(3,4)P₂ accumulation in clathrin-coated pits causes recruitment of downstream effectors such as SNX9 and Wiskott-Aldrich syndrome protein (WASP) which promote the cleavage of the early endosome from

the plasma membrane^{59,69}. Additionally, PI(3,4)P₂ generated by PI3KC2γ has been observed to promote the adaptor protein, phosphotyrosine interacting with PH domain and leucine zipper (APPL) 1/2-mediated activation of Akt2 on the surface of signaling endosomes⁷⁰. Regardless of how it was generated, PI(3,4)P₂ is regulated through its metabolism to PI3P by inositol polyphosphate-4-phosphatase (Inpp) A and B or to a lesser extent PI4P by phosphatase and tensin homolog (PTEN)^{71,72}.

1.1.5 Phosphatidylinositol 4,5-bisphosphate (PI(4,5)P₂)

The best characterized and most abundant of PIs, PI(4,5)P₂ accounts for 60% of all PIs⁷³. PI(4,5)P₂ is synthesized by the phosphorylation of PI4P by class I or II PI4P 5-kinases (PIP5Ks)⁷⁴. Like other PIP₂ species, PI(4,5)P₂ can be dephosphorylated by PI(4,5)P₂ 4- or 5-phosphatases to generate PI5P or PI4P respectively or further phosphorylated by PI3K to produce PI(3,4,5)P₃^{75,76}. Unique to PIs, PI(4,5)P₂ is hydrolyzed by phospholipase C (PLC) to produce inositol 1,4,5-trisphosphate (IP₃) and DAG^{77,78}. IP₃ translocates to the smooth ER to facilitate the release of Ca²⁺ ions, while DAG remains at the plasma membrane where it recruits protein kinase C (PKC); ultimately, cooperating to activate PKC at the plasma membrane^{79,80}.

Beyond metabolism, interactions between intact PI(4,5)P₂ and its effectors have important roles in cytoskeletal dynamics, the endocytic system, and ion channel activation⁷³. PI(4,5)P₂ is extensively involved with actin cytoskeletal dynamics and along with Cdc42 and N-WASP promotes the polymerization of the actin cytoskeleton via ARP2/3 activation⁸¹. PI(4,5)P₂ also recruits talin, via its FERM recognition domain, to promote the interaction

of the cytoskeleton with the plasma membrane⁹. Additionally, PI(4,5)P₂ recruits AP2 complex, AP180/CALM, epsin, Dab2 and HIP1/1R in order to generate the formation and internalization of clathrin-coated vesicles^{82,83,84}. Conversely, in neuronal cells PI(4,5)P₂ in cooperation with synaptotagmin-1 on synaptic vesicles facilitates the exocytosis of neurotransmitters and hormones^{85,86,87}. Finally, PI(4,5)P₂ regulates the activation of calcium, potassium and sodium ion channels and transporters^{88,89}.

1.1.6 Phosphatidylinositol 3,5-bisphosphate (PI(3,5)P₂)

PI(3,5)P₂ is the least abundant of the PIs, and as such until recently, very little was known about its functions^{90,91,92}. It is synthesized through PIKfyve phosphorylation of PI3P and returned to PI3P by Sac3 5-phosphatases^{93,94}. As mentioned earlier, it has been shown to be a large source of cellular PI5P by the myotubularin 3-phosphatases^{41,42,43}. PI(3,5)P₂ is highly involved with the endocytic system including the fusion and subsequent fission of endolysosomes, formation of and sorting of cargo to multivesicular bodies and recycling of endosomes back to the Golgi^{95,96,97,98,99}. PI(3,5)P₂ is also involved in exocytosis, stress response, autophagy, and endolysosome acidification^{96,100,101,102,103,104}. However, the exact role of PI(3,5)P₂ in these functions remains unclear^{96,100,101,102,103,104}.

1.1.7 Phosphatidylinositol 3,4,5-trisphosphate (PI(3,4,5)P₃)

The product of PI3K-mediated phosphorylation of PI(4,5)P₂, PI(3,4,5)P₃ is a very important PI, in part, due to its metabolites PI(3,4)P₂ and PI(4,5)P₂ (sections 1.1.4 and 1.1.5)^{62,63,66,67}. However, PI(3,4,5)P₃ itself has crucial roles in cellular growth, survival, and differentiation^{105,106}. In the plasma membrane PI(3,4,5)P₃ recruits numerous different

proteins from the cytoplasm to bind with its PH domain¹⁰⁷. Upon binding to PI(3,4,5)P₃ the proteins undergo conformational changes that result in the initiation of signaling¹⁰⁷. The best-characterized example of this plasma membrane recruitment and activation is the serine/threonine kinase, Akt^{108,109,110}. The conformational change exposes the catalytic loop of Akt, allowing for phosphorylation by pyruvate dehydrogenase kinase (PDK) 1 at the Thr308 residue and mammalian target of rapamycin complex (mTORC) 2 at the S473 residue^{110,111}. This activation of Akt and that of other proteins, such as PDK1, Btk, GRP1 and ARNO promote cell survival, proliferation and differentiation^{107,108}. Although this effector recruitment is well-documented at the plasma membrane of the cell, nuclear PI(3,4,5)P₃ can promote similar recruitment and effects inside the nucleus¹⁰⁵. Finally, PI(3,4,5)P₃ interacts with guanine nucleotide exchange factors (GEFs) involved in the activation of the Rho GTPase family, promoting rearrangement of the cytoskeleton and the formation of lamellipodia for cell migration¹¹².

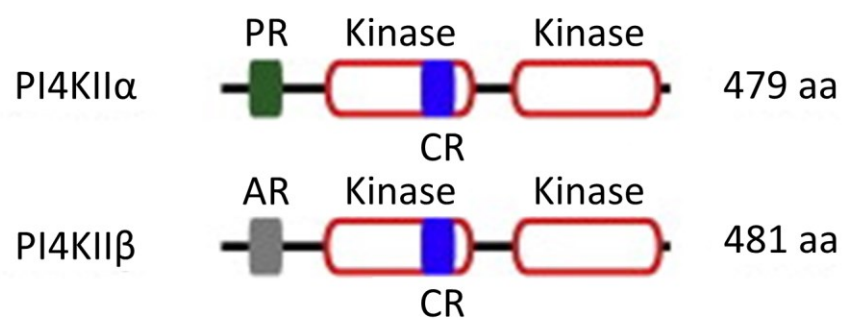
1.2 Phosphatidylinositol 4-kinases

PI4Ks are responsible for the generation of PI4P by phosphorylating PtdIns at the D4 position of the inositol headgroup²⁷. The importance of PI4P was highlighted previously (section 1.1.2), and it is the precursor molecule for PI(4,5)P₂ and PI(3,4,5)P₃, as well as maintaining the structure and function of the Golgi^{9,29}. There are four different mammalian isoforms of PI4K; PI4KIIα, PI4KIIβ, PI4KIIIα, and PI4KIIIβ²⁷. Each isoform performs that same catalytic function by generating PI4P, however, due to their different localizations throughout the cell, they create unique pools of PI4P that go on to perform various functions (see section 1.1.2)^{76,113}.

The type II PI4Ks are composed of a kinase domain, divided in two by an insert region (Figure 1.4a)⁷⁶. The N-terminal region of the kinase domain contains a cysteine-rich region that is palmitoylated for membrane tethering (Figure 1.4a)^{76,114}. The major difference between PI4KII α and PI4KII β is found upstream of the kinase domain, in the N-terminal proline-rich or acid regions, respectively (Figure 1.4a)¹¹⁵. It is believed that this difference allows the differential membrane targeting of the two isoforms¹¹⁶. PI4KII α is mainly localized to the TGN, but has also been seen in other endosomal vesicles, including late endosomes¹¹⁷. The best known function of PI4KII α is in endosome trafficking, and it has been shown to be involved in both the trafficking from the TGN to the plasma membrane, and trafficking of epidermal growth factor (EGFR) to late endosomes for degradation^{118,119}. On the other hand, PI4KII β is found in the cytosol and trafficking endosomes^{115,120}. The exact function of PI4KII β is not fully understood, but it appears to regulate the activation of signaling pathways, such as the platelet-derived growth factor (PDGF) pathway¹²¹.

PI4KIII α is composed of a proline-rich region, followed by a nuclear localization signal, lipid kinase unique domain and pleckstrin homology domain, and one continuous kinase domain (Figure 1.4b)⁷⁶. PI4KIII α is responsible for the main pool of plasma membrane PI4P and is found at the plasma membrane, *cis*-Golgi network, and nucleolus^{122,123,124}. Finally, PI4KIII β , which will be discussed further in the next section (section 1.3), is found mainly in the TGN¹²⁵.

a



b

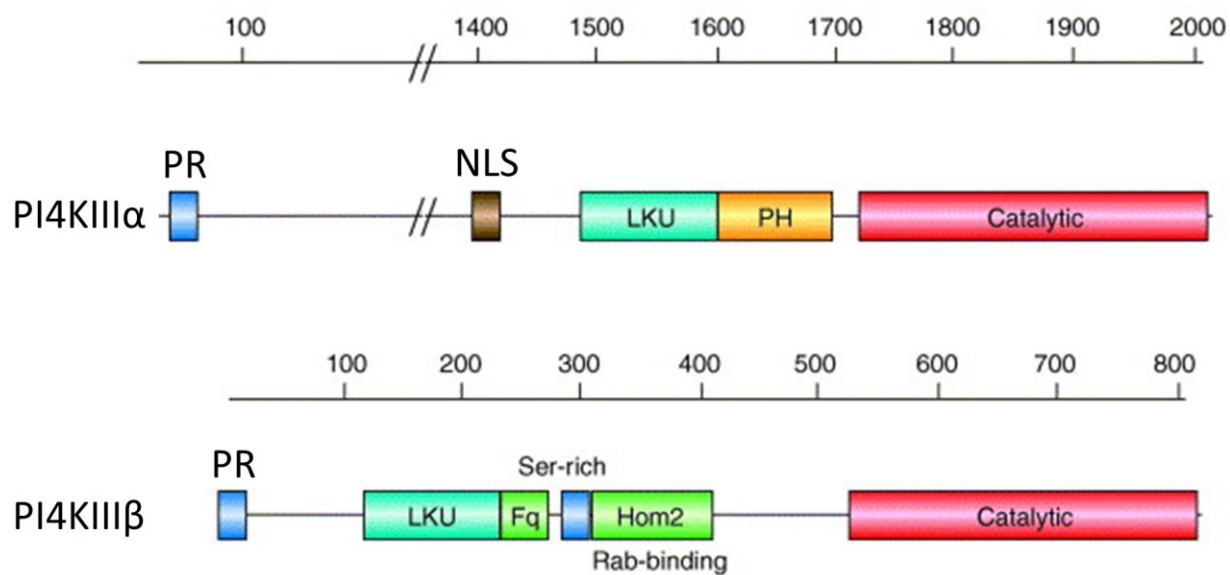


Figure 1.4 Structural characteristics of the PI4K isoforms.

(a) The type II PI4Ks comprise two large kinase domains that are divided by an insert region. The N-terminal lipid kinase domain contains a cysteine-rich (CR) region. This CR region is post-translationally palmitoylated for membrane docking. PI4KII α contains an N-terminal proline-rich (PR) region and PI4KII β contains an N-terminal acidic region (AR). The differences in the PR region and AR target the kinases to their unique cellular locations. (b) The type III PI4Ks contain one lipid kinase (catalytic) domain, which lacks a CR region. Both type III PI4Ks have N-terminal PR regions followed by lipid kinase unique (LKU) domains, which acts to scaffold protein-protein interactions during its catalytic activity. Finally, PI4KIII α contains a nuclear localization signal (NLS) and pleckstrin homology (PH) domain, which promote its association with membranes, whereas PI4KIII β contains a Rab-binding domain, where it binds Rab11a. Figure adapted from Balla and Balla, 2006, and Sasaki *et al*, 2009^{27,76}.

1.3 Phosphatidylinositol 4-kinase III beta

Through the generation of TGN localized pools of PI4P, PI4KIII β has a well-characterized role in the maintenance of the structure and function of the Golgi^{1,27,126}. The differences in the composition of PI4KIII α and PI4KIII β are that PI4KIII β lacks nuclear localization signal and PH domains but has a Rab-binding domain (Figure 1.4b)⁷⁶. PI4KIII β is recruited and binds to the *trans*-Golgi membrane via the small GTPase Arf1¹²⁷. At the Golgi, PI4KIII β is phosphorylated at Ser294 by protein kinase D1 (PKD1), which increases lipid kinase activity¹²⁸. The increased production of PI4P at the TGN promotes Golgi to plasma membrane transport via the PI4P-GGA2-FAPP complex¹²⁹. Additional roles for PI4KIII β through the production of TGN pools of PI4P were previously discussed (section 1.1.2), including maintaining the structure of the TGN and production of sphingolipids^{30,33}. Another protein responsible for PI4KIII β activation is neuronal calcium sensor-1 (NCS-1)¹³⁰. Because, NCS-1 is not found at the Golgi, where exactly it effects PI4KIII β is not known, but NCS-1-mediated activation of PI4KIII β promotes the exocytosis of insulin in pancreatic β cells^{130,131}. Additionally, one of the main focuses of this thesis is the interaction between PI4KIII β and Rab11a, which is important for normal Rab11a localization and function (further discussed in section 1.6)¹³².

1.4 PI4KIII β and breast cancer

Although other phosphatidylinositol kinases, such as PI3K, are well studied for their roles in cancer, emerging evidence implicates PI4KIII β in the progression and development of cancer¹³³. For example, transcriptional analysis of 1992 primary human breast tumours

by Curtis *et al.* identified *PI4KB*, the human gene encoding PI4KIII β , as a tumour driver due to its frequent amplification in primary tumour tissue¹³⁴. Additionally, we have reported that the PI4KIII β protein is expressed in low amounts in normal tissue, but highly expressed in ~20% of primary human breast tumours¹³⁵. Ectopic PI4KIII β expression also disrupts three-dimensional epithelial morphogenesis of breast cells and promotes cell motility and actin remodeling^{136,137}. In MDA-MB-231, triple-negative breast cancer cells, PI4KIII β has an anti-apoptotic effect dependent on endosomal signaling function¹³⁸. Finally, our lab has reported that PI4KIII β is able to directly activate Akt signaling¹³⁵.

1.5 Rab GTPases

The Rab family consists of over 70 different proteins that are all part of the Ras GTPase superfamily¹³⁹. Due to the sheer number of proteins in the Rab family, they regulate multiple cellular functions and are located throughout the cell (Figure 1.5)¹⁴⁰. However, most Rab proteins are primarily involved in vesicle trafficking¹⁴¹. Beginning with endocytic internalization, the Rab proteins are involved in the regulation of most aspects of the endocytic system, including recycling, degradation, and exocytosis¹⁴¹.

The Rab proteins are structurally highly conserved with a GTPase fold common to all Ras proteins^{142,143}. One source of variation between the Rab proteins is in the switch I and II regions; which controls the conformational change when guanosine triphosphate (GTP) or guanosine diphosphate (GDP) are bound¹⁴⁴. The largest difference between the Rab proteins, however, is found in their hypervariable C-terminus¹⁴⁵. These differences allow the different Rab proteins to be targeted for specific membranes and perform their unique

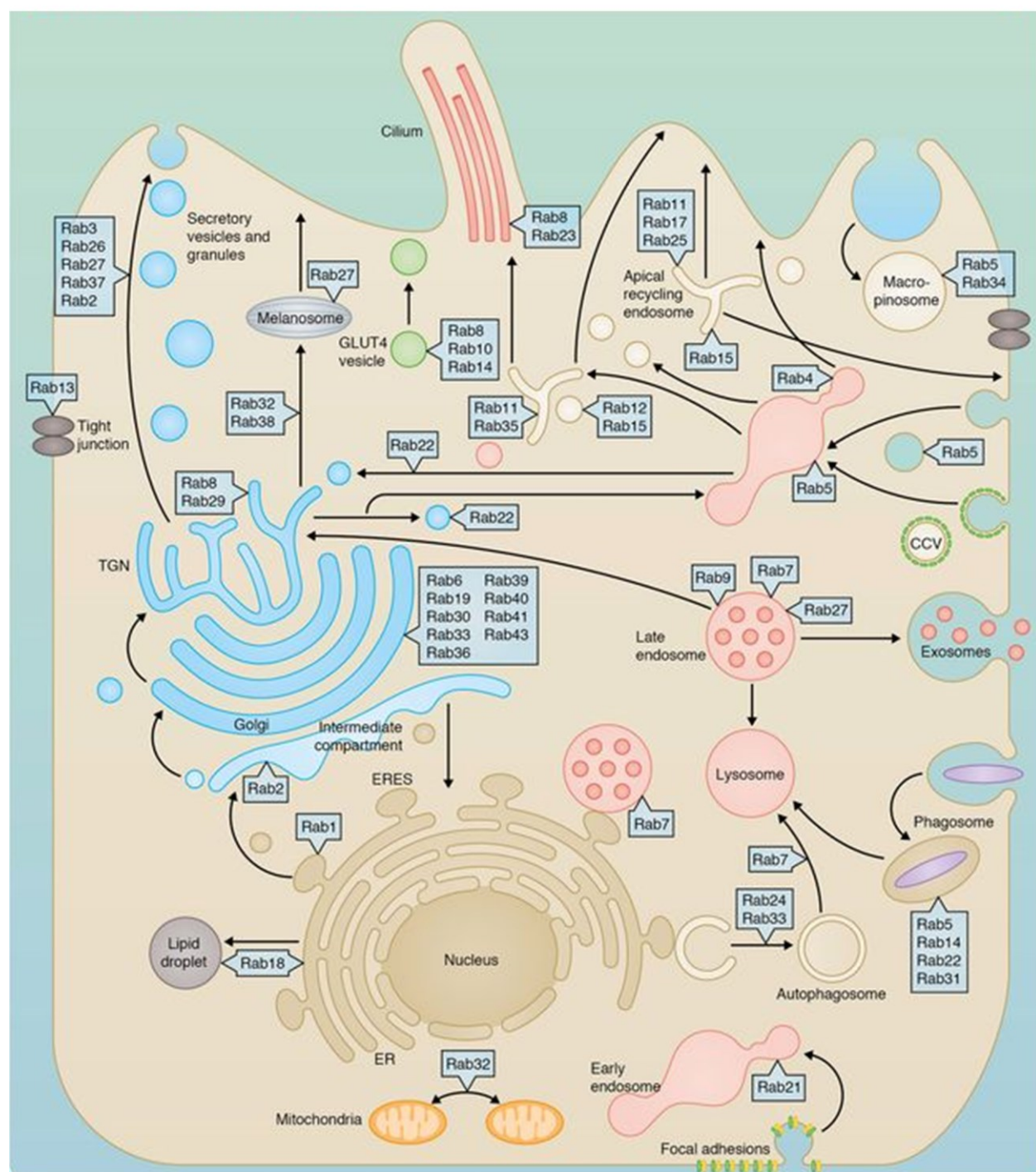


Figure 1.5 Subcellular distribution of Rab GTPases.

The predominant subcellular locations and involved pathways of the specific Rab proteins are shown schematically. The Rab proteins provide both unique and overlapping critical roles in numerous aspects of intracellular membrane trafficking. Abbreviations: ER (endoplasmic reticulum), ERES (endoplasmic reticulum exit sites), CCV (clathrin-coated vesicle), GLUT4 (glucose transporter type 4), and TGN (*trans*-Golgi network). Figure adapted from Zhen and Stenmark, 2015¹⁴⁰.

functions^{144,145}. Immediately after being synthesized the Rab proteins are post-translationally geranylgeranylated, allowing them to be incorporated into lipid membranes¹⁴⁶. Rab escort proteins (REPs) bind to the geranylgeranylated C-terminus of newly generated Rab proteins and escort them to their intended membranes¹⁴⁷. Rab proteins are activated through traditional GTP switch¹⁴⁸. GEFs catalyze the exchange of bound GDP for GTP, causing a conformational change in the Rab protein^{148,149}. The active Rab is able to interact with its downstream effectors and carry out any of the numerous functions associated with the different Rab proteins¹⁵⁰. Once the Rab protein has performed its function, GTPase-activating proteins (GAPs) will promote the conversion of GTP to GDP, returning the Rab to its inactive conformation¹⁵¹. GDP dissociation inhibitors (GDIs) interact with inactive Rab proteins and return them to their original membranes via interaction with GDI displacement factors (GDFs)¹⁵².

1.5.1 Rab5

Rab5 is activated by numerous GEFs, including Rabex-5, Rin1-3, ALS2, and GAPVD1, all of which contain a Vps9 domain that catalyzes the nucleotide exchange on Rab5^{153,154,155,156}. Conversely, Rab5 activity can be terminated by the GAP, RabGAP-5, or through a Rab5/Rab7 switch that occurs with endosome maturation^{157,158}. Rab5 is mainly found at the plasma membrane on newly forming endosomes and the surface of early endosomes¹⁴⁰. Upon activation at the plasma membrane, Rab5 interacts with PI3K to stimulate the production of PI3P^{159,160}. PI3P and Rab5-rich, developing endosomes recruit Rab5 effector proteins rabenosyn5 and EEA1^{161,162}. The rabenosyn5-EEA1 complex interacts with Rab5 GEFs to create a positive feedback loop on the developing endosome, recruiting and

activating more Rab5^{161,162}. Eventually the feedback loop is stopped and the growing vesicle pinches off of the plasma membrane to form an early endosome¹⁶³. After the early endosome is created Rab5 regulates the association and movement of these endosomes along microtubules¹⁶⁴. Additionally, Rab5 and its effectors EEA1 and Rabaptin-5 are involved in endosome fusion through the production of soluble NSF attachment protein receptor (SNARE) complexes^{165,166}. This allows the growth and maturation of endosomes through the fusion of early endosomes with each other or the endocytic recycling compartment (ERC)^{165,166}. Beyond endosome formation, Rab5 and its effectors are involved in regulation of EGFR signaling, APPL-mediated Akt signaling on endosomes, and formation of circular ruffles connecting endosomes to the actin cytoskeleton^{167,168,169}. Finally, as the endosome matures a Rab5/Rab7 switch occurs allowing the endosome to develop into a late endosome¹⁵⁸. As the early endosome matures, Mon1-Ccz1 complexes are recruited from the cytosol and by removing Rabex-5 from the membrane, terminate the previously mentioned Rab5 activation feedback loop^{158,170}. This in turn promotes the dissociation of Rab5 from and recruitment of Rab7 to the endosome^{158,170}. The Mon1-Ccz1 complex also interacts with the homotypic fusion and protein sorting (HOPS) complex, which is the GEF responsible for Rab7 activation^{158,170}.

1.5.2 Rab7

As mentioned, Rab7 is activated by the GEF, HOPS, and, conversely, its activity is terminated by the GAP, TBC1D15^{171,172}. Rab7 is often associated with degradative pathways of vesicle trafficking, including late endosomes, lysosomes, and even has roles in autophagy^{173,174,175}. Rab7 is primarily responsible for the maturation of early to late

endosomes¹⁷³. The previously discussed Rab5/Rab7 switch on early endosomes initiates the activation of Rab7, which traffics maturing endosomes toward the microtubule-organizing centre (MTOC), where it promotes the fusion of late endosomes and lysosomes^{158,173}. The exact mechanism of late endosome-lysosome fusion is unknown, however, it is hypothesized that there is continuous fusion and fission between the organelles, forming an intermediate hybrid organelle called an endolysosome^{176,170}. Analogous to its roles in the endocytic system, in autophagy, Rab7, is thought to be responsible for the localization of autophagosomes around the perinuclear region and subsequent fusion with lysosomes^{177,178}.

By regulating degradative pathways, Rab7 is also responsible for the regulation of membrane receptor activation¹⁷⁹. For instance, EGFR, upon binding epidermal growth factor (EGF), is activated and goes through a typical receptor tyrosine kinase (RTK) activation pathway (discussed in detail in section 1.8) and is ultimately internalized into the cell via endocytosis¹⁸⁰. Once internalized, signaling can persist on the surface of endosomes, but the fate of the activated EGFR-EGF complex is, ultimately, to be recycled back to the plasma membrane for further activation, or termination of signaling by lysosome-mediated degradation^{181,182}. As discussed, Rab7 promotes the degradative pathways and as such will lead to the degradation and termination of EGFR signaling¹⁸³.

1.5.3 Rab11a

The Rab11 family is comprised of 3 isoforms; Rab11a, Rab11b, and Rab11c (or Rab25)^{184,185}. Rab11a is ubiquitously expressed throughout the body whereas Rab11b is

expressed in the brain, testis, and heart, and Rab25 is expressed in the lungs, kidney and GI tract^{186,187,188}. My thesis focuses on the Rab11a isoform. At the nexus of the endocytic and exocytic pathways, Rab11a, localizes to the TGN, post-Golgi vesicles, and recycling endosomes¹⁸⁹. The exact GEFs regulating Rab11a in mammals are unclear, however, evidence from *Drosophila* suggest DENND4 is a promising candidate^{190,191}. Whereas, the GAPs, TBC1D15, TBC1D11, and Evi5 have more well-defined roles in Rab11a regulation^{172,192}.

Rab11a has a substantial network of possible interactions, both upstream and downstream of its activation; as such, it can perform many roles in the cell, depending on which interactions it makes^{184,185}. PI4KIII β recruits Rab11a to the TGN, where it promotes the activation of Rab11a, to regulate Golgi to plasma membrane trafficking¹³². Once activated, Rab11a interacts with one of the five members of the Rab11 family-interacting proteins (Rab11FIPs), which act as adaptors to motor proteins such as myosin V (MyoV) or dynein light intermediate chain (DLIC) to mediate the transport of the Rab11a-positive vesicle to its destination¹⁸⁴. The Rab11-Rab11FIP-motor protein complexes are essential for normal mitosis and meiosis, and vesicle trafficking, such as endocytic recycling^{193,194,195}.

Rab11a is primarily involved in the intracellular trafficking of cargo, including the movement of endosomes from the plasma membrane to the TGN, and the reverse, the slow recycling of endosomes back to the plasma membrane and exocytosis in the secretory pathway^{196,197,198,199}. In my thesis, I primarily examine the role of Rab11a in the slow recycling route of endocytosis. In the slow recycling pathway, early endosomes mature into sorting endosomes, where cargo is sorted for degradation or slow recycling²⁰⁰. Cargo

destined for slow recycling, or degradation, is shuttled to the perinuclear Rab11a-positive ERC^{193,200,201}. Smaller Rab11a-positive vesicles containing cargo break off of the maturing ERC to form recycling endosomes, which translocate to the plasma membrane to release any recycled cargo^{199,200}.

1.6 PI4KIII β -Rab11a interaction

The PI4KIII β -Rab11a interaction is the only documented binding between PI4KIII β and a Rab GTPase and is well characterized for its role in normal endosome function^{125,132}. For example, PI4KIII β , independent of PI4P, cooperates with active Rab11a to facilitate transport from the Golgi to the plasma membrane¹²⁵. Likewise, in *Drosophila melanogaster*, the PI4KIII β homolog four-wheel drive (*Fwd*) regulates Rab11 localization and endocytic function, independent of PI4P¹⁹⁵. *Fwd*-Rab11 interaction is necessary for normal cytokinesis in *Drosophila* spermatocytes and loss of *Fwd* leads to sterility in males¹⁹⁵. Similarly, in yeast and mammalian cells PI4KIII β , independent of PI4P, is necessary for the binding and localization to the Golgi of Rab11a and its effectors^{125,132}. Finally, PI4KIII β -mediated activation of Akt is independent of PI4P generation but requires Rab11a¹³⁵. Taken together PI4KIII β appears to have an important functional relationship with Rab11a and endosome function independent of its kinase activity.

1.7 The endocytic system

The endocytic system consists of distinct membrane compartments; best known for bringing molecules into, and their subsequent movement through, the cell^{200,202}. The principal features of the endocytic system are the internalization of cargo that is unable to

diffuse through the plasma membrane, the recycling of cargo back to the plasma membrane for further use, and the degradation of cargo for energy or defense^{203,204,205}. Each aspect of the system is important, and contributes to the overall health and survival of the cell, through the uptake of nutrients, plasma membrane homeostasis, destruction of pathogens, and regulation of signaling events, among other cellular processes^{203,204,205}. The reverse counterpart of the endocytic system, the exocytic system, occurs simultaneously and often in concert with the endocytic system, to provide comprehensive movement of valuable molecules within and between cells²⁰⁶. Where endocytosis allows a cell to react to its environment, exocytosis allows a cell to influence its environment²⁰⁶. Dysregulation of the endocytic system has been associated with numerous diseases, including neurodegeneration, diabetes, improper organ and tissue development, and cancer^{207,208}.

1.7.1 Endocytic internalization

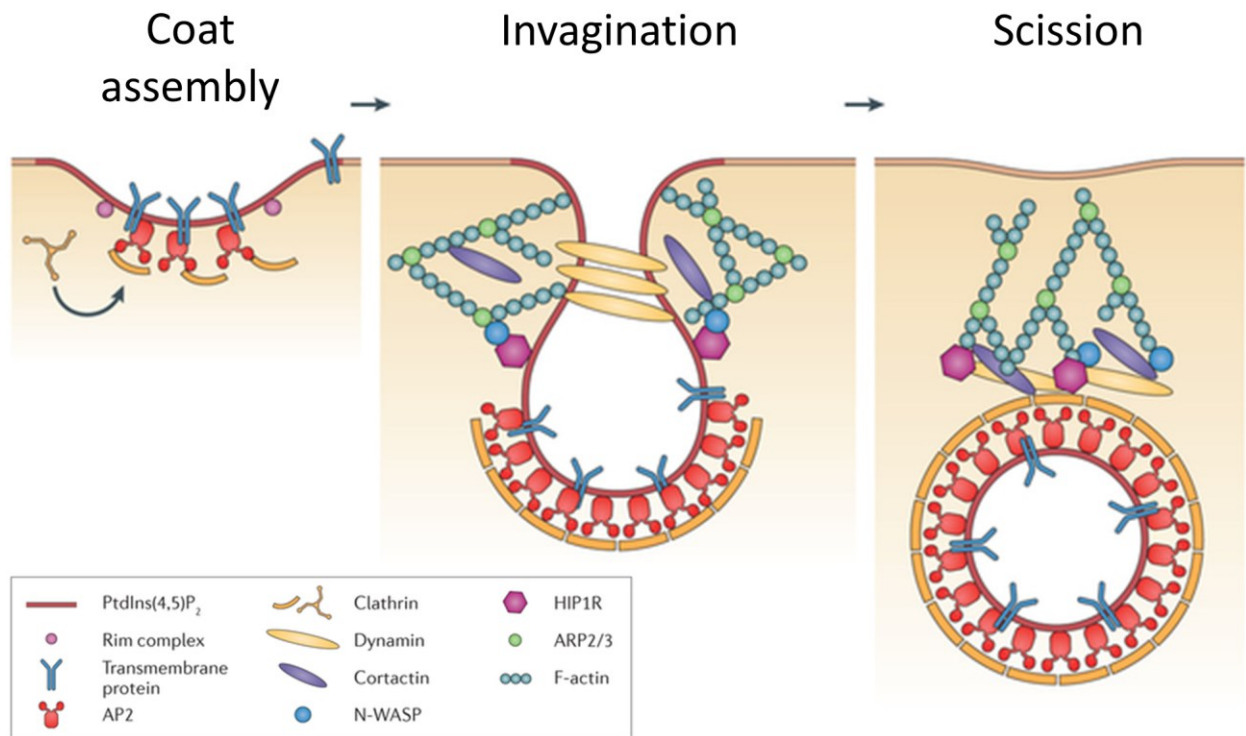
Endocytosis is important for normal cellular function such as nutrient uptake, cell signaling, cell morphology, and uptake and destruction of pathogens²⁰³. The best-defined internalization pathways include clathrin-mediated endocytosis (CME), caveolar-dependent endocytosis (CDE), clathrin-independent carrier/GPI-AP-enriched early endosomal compartment (CLIC/GEEC) pathway, IL2R β pathway, Arf6-dependent endocytosis (ADE), flotillin-dependent endocytosis (FDE), phagocytosis, macropinocytosis, circular dorsal ruffles, and entosis²⁰⁹. The pathways are generally defined by the major proteins or complexes required to perform their specific internalization mechanisms²⁰⁹. CME, for example, requires clathrin-coated pits on the intracellular side of the plasma membrane²¹⁰. Extracellular ligand-binding of transmembrane receptors promotes selective intracellular

binding to AP-2 (Figure 1.6a)^{211,212}. AP-2 sequesters activated transmembrane receptors and recruits clathrin and other adaptor proteins to promote clathrin-mediated invagination of the membrane (Figure 1.6a)¹⁶³. Dynamin regulated scission from the plasma membrane allows the movement of the clathrin-coated vesicle and its cargo into the cell (Figure 1.6a)¹⁶³. Although the majority of cargo can be internalized through many of the possible pathways, some can only enter through one specific route; thyroxine and CDE for example^{209,213}. Clathrin-mediated endocytosis is primarily responsible for the internalization of both RTKs and transferrin^{214,215}. As such, this thesis will focus primarily on the CME route of endocytic internalization.

1.7.2 Endocytic recycling

As a cell internalizes vesicles, through any of the previously mentioned internalization pathways (section 1.7.1), the plasma membrane loses both membrane proteins and lipids²⁰⁴. Therefore, the reciprocal process of endocytic recycling, is required in order to maintain plasma membrane composition¹⁹⁹. In addition to membrane homeostasis, endocytic recycling is involved in cell migration, signaling, morphogenesis, adhesion, and division^{216,217,218,219,220}. Two major recycling pathways exist; the fast and slow recycling pathways (Figure 1.6b)^{199,200}. The fast recycling route is dependent on Rab4 and Rab35, although their exact mechanisms of regulation are unknown^{221,222,223,224}. It is hypothesized that Rab4 prevents early endosome to ERC transport, forcing the endosome and cargo to be rapidly recycled out of the cell²²⁵. The role of Rab35 is even more uncertain, however, it is required for rapid recycling of endosomes internalized through both CME and clathrin-independent endocytosis^{224,226}. On the other hand, the

a



b

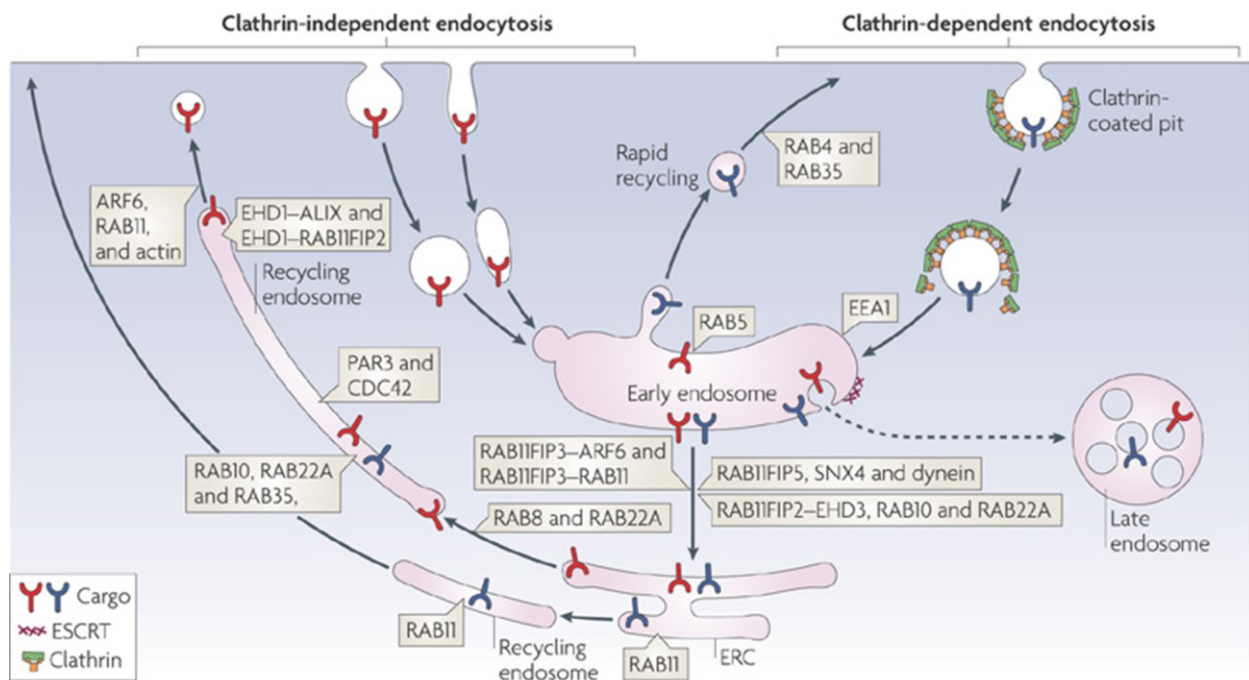


Figure 1.6 Endocytic internalization and recycling.

(a) A schematic figure of the internalization of transmembrane proteins through clathrin-mediated endocytosis. When the extracellular domain of the transmembrane protein binds to ligand, the protein undergoes a conformational change, which promotes the binding of adaptor protein (AP) 2 to its intracellular domain. AP2 recruits clathrin, which coats the intracellular side of the membrane and causes it to invaginate into the cell. Dynamin-dependent scission terminates the formation of a complete vesicle inside the cell. (b) A schematic diagram of endocytic internalization and recycling. Once a vesicle has been internalized, it is shuttled toward Rab5 containing early endosomes, where it fuses and releases its cargo. The cargo can then be quickly recycled back to the surface via the Rab4 and Rab35-mediated rapid recycling pathway, or continue onto the late endosome for destruction or the endocytic recycling compartment for further sorting (ERC). In the ERC, cargo is sorted for further use, or recycled back to the surface via Rab11-mediated recycling endosomes. Figure adapted from Grant and Donaldson, 2009, and Humphries and Way, 2013^{199,227}.

slow recycling pathway requires Rab11²²⁸. Discussed previously (section 1.5.3), the slow recycling route is often measured experimentally by assays such as transferrin pulse-chase assays^{229,230}.

1.7.3 Endocytic degradation

The degradative pathway of endocytosis is important for the destruction of internalized pathogens, termination of signaling events, and catabolism of macromolecules for energy and repurposing of their constituents^{231,232}. After being internalized, like in the slow recycling pathway, cargo destined for degradation will be sent to the perinuclear ERC^{201,233}. The ERC sorts the cargo, assigning it to one of two pathways; the previously discussed slow recycling pathway (section 1.7.2) or the degradative pathway²³³. While recycling endosomes split off, ERCs develop into MVBs with decreasing pH and travel toward the late endosomes²³⁴. The late endosome acts as another sorting station and can deliver cargo back to the TGN for further use or to the lysosome for degradation^{205,235}. As discussed earlier (section 1.5.2), the transition from late endosome to lysosome is believed to be achieved through continuous fusion and fission of late endosomes and lysosomes, making the hybrid endolysosome, before fully fusing with the lysosome^{176,170}. Unlike in recycling, where the membrane lipids and proteins are eventually restored to the plasma membrane, late endosome fusion with lysosomes results in the degradation of both the cargo and late endosome components¹⁷⁰. The degradative waste is then either removed from the cell via exocytosis or re-used, such as in the formation of lysosomes²³⁶. The trafficking and maturation of endosomes to late endosomes and endolysosomes is regulated primarily by, the previously discussed, Rab7 (section 1.5.2)^{158,170,173}.

1.7.4 Transferrin and the transferrin receptor

The majority of iron is brought into cells via the transferrin pathway^{215,237,238}. Transferrin is a protein with two iron, ferric ion (Fe^{3+}), binding domains^{215,237}. Loaded with iron, transferrin binds with transferrin receptor on the surface of a cell and is internalized via CME^{239,240}. As the endosome matures the pH decreases, releasing the bound iron but not dissociating transferrin from its receptor²⁴¹. The transferrin ligand-receptor complex is then recycled back to the surface of the cell to allow further uptake of iron^{242,243}. The transferrin complex can go through a quick recycling pathway ($t_{1/2}$: 4 minutes) but the majority goes through the slow recycling pathway ($t_{1/2}$: 10 minutes)^{200,244}. As such the transferrin protein and/or receptor are frequently used to track endocytic internalization and recycling^{217,229,245,246}.

1.8 Plasma membrane receptor signaling

Activation of transmembrane receptors through ligand binding allows a cell to react to environment and neighbouring cells²⁴⁷. There are three main classes of plasma membrane receptors: ligand-gated ion channels, enzyme-linked receptors and G-protein coupled receptors (GPCRs)²⁴⁷. Ligand-gated ion channels are generally found in the central nervous system and involved in neurotransmission²⁴⁸. The channels are made up of, usually, five transmembrane receptor subunits with α -helices, that together form a central pore²⁴⁹. In the unactivated state the central pore is closed²⁴⁹. When a ligand binds to one or more of the receptor subunits, it causes a conformation change that opens the central pore²⁴⁸. Ions flood into the cell, changing the cellular ion concentration²⁴⁸. The channel

then closes and is desensitized, until the ion concentration returns to normal, where the receptor will be activated again ²⁴⁸.

Enzyme-linked receptors can be broken further to RTKs, receptor serine/threonine kinases, tyrosine kinase associated receptors, receptor-like tyrosine phosphatases, histidine kinase associated receptors, and receptor guanylyl cyclases ²⁵⁰. Enzyme-linked receptors are made up of an extracellular binding domain, a transmembrane helix and an intracellular catalytic domain ²⁴⁷. The general mechanism of action for these receptors is when a ligand binds to the receptor, it causes dimerization and transautophosphorylation of the intracellular catalytic domains, which go on to catalyze further intracellular signaling ²⁴⁷. RTKs include the mitogen-activated protein kinases/extracellular signal-regulated kinases (MAPK/Erk) and Akt pathways, which are associated with cell survival, proliferation and motility, and often dysregulated in cancers ^{251,252}. Previously, our lab has explored the role of PI4KIII β in Akt activation and this thesis investigates its role in the activation of insulin-like growth factor type I receptor beta domain (IGF-IR β), A-Raf and Shc (sections 1.8.1-1.8.4) ¹³⁵.

Finally, GPCRs can be further divided into six different classes: rhodopsin-like, secretin receptor family, metabotropic glutamate, fungal mating pheromone receptors, cyclic adenosine monophosphate (cAMP) receptors and Frizzled/Smoothed ²⁵³. The different classes all share similar structures and mechanisms of action ²⁴⁷. GPCRs have an extracellular N-terminal domain followed by seven transmembrane helices separated by loop regions, which serves as a ligand binding domain, and finally an intracellular C-terminal domain ²⁴⁷. Extracellular ligand binding, causes a conformational change in GPCRs, which

allows them to act as a GEF for G-proteins²⁴⁷. G-proteins, are made of three subunits (α , β , and γ), and when activated and bound to GTP the α -subunit dissociates from the others and goes on to catalyze further intracellular signaling²⁵⁴. Specific GPCRs subgroups are involved in numerous pathways and can be stimulated by Wnt, Hedgehog, hormones, and chemokines, to name a few^{255,256}. Activated G-proteins are involved in cAMP-mediated activation of protein kinase A (PKA), PLC-mediated catabolism of PI(4,5)P₂ to IP₃ and DAG, and involvement in RTK pathways, like MAPK/Erk and Akt^{78,257,258}. An alternative, but interesting, consequence of GPCR activation was observed with Rab11a-mediated activation of Akt²⁵⁹. Activation of GPCRs by lysophosphatidic acid (LPA), caused the typical G-protein α subunit dissociation leading to further intracellular signaling, but also to the G-protein β and γ subunits interacting with Rab11a of recycling endosomes and promoting PI3K γ recruitment and Akt activation on the surface of the endosome²⁵⁹.

1.8.1 Akt

Akt, also known as protein kinase B (PKB), is a serine/threonine-specific protein kinase, with three isoforms (Akt1, Akt2 and Akt3), that are involved in cellular survival, proliferation and cell cycle progression²⁶⁰. In addition to the overlapping functions of the Akt isoforms, each isoform appears to have unique roles^{261,262,263}. For example, Akt1 is involved in apoptosis, Akt2 regulates insulin activity and Akt3 is involved in the development of the brain^{261,262,263}. Activated RTKs stimulate the activation of class I PI3K to produce PI3P, PI(3,4)P₂ or PI(3,4,5)P₃^{264,265}. As previously discussed (sections 1.1.4 and 1.1.7), PI(3,4)P₂ and PI(3,4,5)P₃ both differentially regulate and are responsible for the

activation of Akt^{70,110}. Aberrant Akt activation is frequently associated with oncogenesis^{266,267}.

1.8.2 IGF-IR

Insulin-like growth factor type I receptor (IGF-IR) is a receptor tyrosine kinase made up of an extracellular alpha chain, a transmembrane domain, and an intracellular beta chain²⁶⁸. The ligand, insulin-like growth factor I (IGF-I), binds to the extracellular alpha chain inducing a conformational change in IGF-IR structure, allowing for dimerization with another activated IGF-IR and transautophosphorylation of their intracellular tyrosine kinase domains of the beta chains²⁶⁹. Activated IGF-IR then acts downstream to activate the MAPK/Erk and Akt pathways²⁶⁹. As IGF-IR acts as a nexus for many signaling pathways associated with cell survival and growth it is strongly implicated in the development and progression of breast cancer, among others^{270,271}.

1.8.3 A-Raf

A member of the Raf serine/threonine-specific protein kinase family, A-Raf is the least characterized of this family^{272,273}. Although A-Raf and the other members of the Raf kinase family, B-Raf and C-Raf (or Raf-1), share similar structures, they are involved in numerous functions throughout the cell^{272,273}. Each of the Raf proteins has three conserved regions; CR1, CR2 and CR3^{274,275}. CR1 and CR2 make up the regulatory region and function for Ras binding and auto-inhibition of the Raf protein, respectively, while CR3 is the kinase domain and is required for Raf activation^{274,275}. Immediately upstream of CR3 is a

conserved negative charge regulatory region (N-region), it is here that activation of the Raf proteins is induced^{274,275}.

RTK-mediated phosphorylation of S299, Y301 and Y302 in the N-region of A-Raf begins the activation cascade in CR3 priming A-Raf for Ras interaction^{274,275}. Activated A-Raf then goes on to activate the MAPK/Erk pathway leading to progression of the cell cycle and proliferation^{272,273}. Furthermore, A-Raf induced Erk activation leads to the initiation of ARF6-mediated endocytic recycling²⁷⁶. Unique to the Raf family, A-Raf can perform roles independent of its kinase activity^{272,273}. For example, A-Raf inhibits the proapoptotic tumour suppressor protein MST2 and enhances the activity of PKM2, a protein involved in the Warburg effect in cancer^{277,278,279}. Finally, using the cBioportal for Cancer Genomics database we found that pA-Raf and pShc (section 1.8.4) are present in excess in breast cancer samples with high PI4KIII β ^{280,281,282}.

1.8.4 Shc

The adapter Shc protein has 3 different splice-variant (46, 52 and 66kDa) isoforms, with varying cellular functions. All 3 isoforms have conserved phosphotyrosine binding regions (PTB and SH2) and a central region containing tyrosine phosphorylation sites²⁸³. Shc is activated by receptor-ligand induced tyrosine phosphorylation, promoting the interaction of Shc with Grb2²⁸⁴. With regards to the 46 and 52kDa isoforms, Shc-Grb2 then binds with the Ras-GEF, Sos, to generate active Ras-GTP at the plasma membrane and activate the MAPK/Erk pathway²⁸⁴. Conversely, the 66kDa isoform inhibits the complete activation of the MAPK/Erk pathway by inhibiting the activation of the downstream fos

promoter^{285,286}. Dysregulation in the production and activation of Shc proteins is associated with cancers^{287,288}.

1.9 Endocytic regulation of plasma membrane receptor signaling

As discussed previously (section 1.7), the endocytic system is important for the uptake and vesicular movement of cargo throughout the cell, however, it is also tightly linked to control of plasma membrane signaling activation^{289,290}. The most prevalent method of endocytic regulation of plasma membrane receptor signaling is the control of receptor availability on the plasma membrane surface^{289,290}. Activated receptors are internalized and can then either be recycled back to the surface or sorted for degradation, propagating or terminating signaling, respectively^{289,290}. For instance, phosphorylation of GPCRs initiates signaling at the plasma membrane surface, while also recruiting β -arrestins to bind and stop the signaling^{291,292}. Desensitized GPCRs are internalized then, in the early endosome, dephosphorylated and recycled back to the plasma membrane surface, where they are able to reinitiate signaling, allowing for sustained pathway activation^{291,292}. On the other hand, some receptors require ubiquitination in order to be internalized, while others are ubiquitinated post-internalization, in either case, at the ERC, ESCRT complexes sort these receptors into MVBs for degradation^{20,293,294}. Furthermore, signaling events can even be started and/or ended on the surface of endosomes, due to the unique and constantly changing environments of endosomes (section 1.10).

1.10 Signaling endosomes

Besides the endocytic regulation of signaling through recycling and degradation, so-called 'signaling endosomes' provide sites for unique signal activation and propagation²⁸⁹.

The diverse and evolving environments of endosomes provide opportunities for signal propagation that would be unlikely or impossible to occur at the plasma membrane²⁸⁹.

Quite simply, endosomes have smaller surface areas and volumes than the plasma membrane of a cell, as such, there are increased chances for a receptor to interact with ligand or adaptor proteins to initiate or propagate signaling^{290,295}. Beyond size and coincidence interactions, endosomes provide unique signaling modulator or adaptor proteins, acidic environments, and plasticity which can all act to augment signaling events²⁸⁹.

Some signaling adaptor proteins, necessary for activation, can only be found on the surface of endosomes^{289,290}. For example, the late-endosome specific lipid raft adaptor protein p18 anchors the p14-MP1 complex to late endosomes, acting as a scaffold for MAPK/Erk pathway activation²⁹⁶. MVBs, late endosomes, and lysosomes are destinations for degradation and as such have acidic pHs^{170,297,298}. NOTCH signaling at both the plasma membrane and endosome surfaces requires cleavage by γ -secretase, which is optimally active at low pHs^{299,300}. As such it is speculated that physiological NOTCH signaling requires the sequestration of NOTCH and γ -secretase in acidic endosomes^{299,301}. The maturation of endosomes can regulate signaling by the recruitment and dismissal of proteins integral for specific phases of endosome progression, for example, when ligand-bound EGFR is internalized into the cell via an early endosome, Rab5 on the early endosome recruits APPL

proteins to EGFR, thereby promoting the activation of Akt^{168,302}. As the endosome matures APPL is replaced by EEA1 and the Akt signaling is terminated on that endosome³⁰³.

1.11 Research hypothesis and objectives

Regulation of plasma membrane signaling through the endocytic system is responsible for proper intensity and duration of many signaling events. Dysregulation of this system is responsible for aberrant signaling that has been demonstrated to contribute to the development and progression of cancers. PI4KIII β is implicated in the pathogenesis of breast cancer, but the mechanism through which it acts to promote oncogenesis is not clear. Mounting evidence suggests a role for PI4KIII β -mediated oncogenesis being linked with the endocytic system. This thesis will examine the roles of PI4KIII β in the regulation of endocytic rates and oncogenic signaling activation, in order to provide a better understanding of how PI4KIII β contributes to breast cancer oncogenesis.

1.11.1 Hypothesis

We hypothesize that PI4KIII β has a novel role as a regulator of the endocytic system. We believe that PI4KIII β cooperates with the endocytic regulatory protein Rab11a to control the rate of endocytic kinetics. In addition, we hypothesize that this PI4KIII β -mediated control of the endocytic system provides increased opportunities for plasma membrane receptor activation both at the plasma membrane and on the surface of endosomes. Finally, we hypothesize that deletion of PI4KIII β expression will decrease tumour growth in mice.

1.11.2 Statement of objectives

1. To determine the role of PI4KIII β in endocytic dynamics
2. To examine how PI4KIII β affects oncogenic signaling pathway activation
3. To investigate how PI4KIII β expression affects tumour growth in mice

Chapter 2: Materials and Methods

2.1 Cell culture

The BT549 human breast ductal carcinoma, NIH3T3 mouse embryo fibroblast and Phoenix human embryo kidney cell lines were purchased from American Tissue Culture Collection (ATCC, Manassas, VA, USA). The 4T1 mouse mammary carcinoma cell line, constitutively expressing the firefly luciferase gene, was a gift from Dr. John Bell (University of Ottawa, Canada). BT549 cells were grown in RPMI-1640 from Life Technologies (Thermo Scientific, Burlington, Canada) supplemented with 10% FBS (Thermo Scientific), 1mmol/L sodium pyruvate (Thermo Scientific), 10nmol/L HEPES buffer (VWR International, Mississauga, Canada), 0.023 IU/mL insulin from bovine pancreas (catalog no. I-5500, Sigma-Aldrich, Oakville, Canada), and penicillin-streptomycin (Thermo Scientific). NIH3T3 and Phoenix cells were cultured in Dulbecco's modified Eagle's medium (Thermo Scientific) supplemented with 10% FBS, 1mmol/L sodium pyruvate, and penicillin-streptomycin. BT549 cell lines stably expressing ectopic wildtype, kinase-inactive (D656A), or Rab11a-binding deficient (N162A) PI4KIII β were created using pLXSN (wildtype and kinase-inactive) or pLPC (Rab11a-binding deficient) helper-dependent retroviral systems. The plasmids (20 μ g) were transfected into Phoenix cells via CaCl₂-mediated transfection. The Phoenix cells were given at least 8 hours to generate virus which was then collected from the supernatant and used to infect the BT549 and NIH3T3 cell lines. Each cell line was given 2 titres of the virus. Infected cells were selected in 0.4mg/mL G418 (Bioshop, Burlington, Canada) for 2 weeks. BT549, NIH3T3 and 4T1 cell lines with PI4KIII β deletion by

CRISPR/Cas9 were generated by transfecting wildtype cells with CRISPR/Cas9 plasmid (Santa Cruz Biotechnology, Mississauga, Canada) followed by single cell fluorescent-activated cell sorting (FACS) of green fluorescent cells into 96-well plates with a MoFlo Astrios sorter. Single cells were grown to colonies and the deletion was verified by western blot and immunofluorescence. All cell lines were grown in a 37°C humidified incubator with 5% CO₂.

2.2 Plasmids

The wildtype and kinase-inactive (D656A) PI4KIII β plasmids were generated as described previously¹³⁵. Rab11a-binding deficient (N162A) PI4KIII β plasmid (a gift from Dr. Roger Williams, University of California, San Francisco, CA, USA) was full-length human PI4KIII β with a single amino acid mutation (N162A) cloned into the pLPC retroviral vector in the PacI and ClaI sites.

The PI4KIII β targeted gRNA sequences for CRISPR/Cas9 deletion were 5'-CCCTGATGGCGATCGGCAAG-3', 5'-TCCTGCCAGCCGGCGCCTTT-3', 5'-TATGAGCCAGCTGTTCCGAA-3' (catalog no. sc-4185251) and 5'-CAGACCGTGACTCCGAATT-3', 5'-GGCTCCCTACCTGATCTACG-3', 5'-ATAAGCTCCCTGCCCAGAGTC-3' (catalog no. sc-430739) for human and mouse, respectively. Through double-stranded DNA breaks and repair by non-homologous end joining at three locations throughout the gene, we were able to delete the *PI4KB* or *Pi4kb* genes from these cell lines.

2.3 Western blot

Cells were lysed in radioimmunoprecipitation assay buffer (Tris-HCl, pH 7.4, 50mM; NaCl, 150mM; NP-40 1%; sodium deoxycholate, 0.5%; sodium dodecyl sulfate, 0.1%; ethylenediaminetetraacetic acid, 2mM; sodium fluoride, 50mM) supplemented with protease and phosphatase inhibitor cocktails (Roche, Mississauga, Canada). Protein concentrations were determined by Bradford protein assay (Bio-Rad, Mississauga, Canada). Loading buffer (5x) was added to 30µg of protein lysate and resolved by SDS-PAGE. The protein was then transferred onto polyvinylidene difluoride membrane (Millipore, Toronto, Canada) and probed for targets. The bands were detected with a MicroChemiluminescent system (DNR Bio-Imaging Systems, Toronto, Canada) and intensities were quantified by densitometry using GelQuant (DNR Bio-Imaging Systems). The antibodies used for western blotting were PI4KIIIβ (catalog no. 611817) from BD Biosciences (Mississauga, Canada), vinculin (catalog no. sc-25336) from Santa Cruz Biotechnologies and tubulin (catalog no. 3873), IGF-IRβ (catalog no. 3027), phosphoIGF-IRβ (Y1135/1136; catalog no. 3024), A-Raf (catalog no. 4432), phosphoA-Raf (S299; catalog no. 4431), Shc (catalog no. 2432), phosphoShc (Y317; catalog no. 2431) from Cell Signaling Technology (Whitby, Canada). The secondary antibodies used were all purchased from Cell Signaling Technology and include anti-mouse HRP-linked (catalog no. 7076), anti-rabbit HRP-linked (catalog no. 7074).

2.4 Growth factor stimulation assays

Stable BT549 and NIH3T3 cell lines were plated at 80,000cells/well to reach ~60-70% confluency the following day and then washed twice with PBS and starved overnight in media containing 0.1% BSA. The following day the cells were stimulated with IGF-I (100ng/mL) (catalog no. I3769, Sigma-Aldrich) or EGF (50ng/mL) (catalog no. E9644, Sigma-Aldrich) for the time periods indicated. To inhibit endocytosis, after overnight starvation the cells were incubated with 10µg/mL chlorpromazine (catalog no. C8138, Sigma-Aldrich) for 1 hour prior to and during IGF-I or EGF stimulation. The lysate was then collected and subjected to SDS-PAGE as described earlier.

2.5 Transferrin pulse-chase and uptake assays

To reach ~70% confluency by the following day, BT549 cells were plated at 80,000cells/dish in 35mm µ-Dishes from ibidi and incubated overnight. The following day, or once cells were the desired confluency, they were washed two times with PBS to remove all growth media and incubated in serum-free uptake medium (SFUM; RPMI-1640 with 10nmol/L HEPES buffer, penicillin-streptomycin, and 0.1% BSA) for 1 hour at 37°C to deplete transferrin. The cells were then incubated in SFUM containing 25µg/mL transferrin AlexaFluor®488 (for overexpression and CRISPR deletion experiments) or 546 (for rescue cell line experiments) (Thermo Scientific) for 30 minutes at 37°C to internalize (pulse-chase assay) or 1 hour at 4°C to bind (uptake assay). The cells were then washed twice with SFUM and incubated at 37°C with SFUM containing 1mg/mL unlabelled holo-transferrin (Sigma-Aldrich) (pulse-chase) or transferred to 37°C (uptake) for the specified times. At the

indicated times the cells were placed on ice and washed 3 times with PBS followed by a 6-minute wash with an acid wash buffer (20mM acetic acid, 500mM NaCl, pH 3.0). The cells were then fixed with 4% paraformaldehyde for 10 minutes at room temperature and permeabilized with 0.25% saponin, 1% BSA-PBS for 10 minutes on ice. Finally, the cells were incubated with 1 μ g/mL DAPI for 5 minutes and mounted with ibidi mounting medium. Images were acquired with a Zeiss LSM 510 META/AxioVert 200 confocal microscope with a 63x Plan-Apochromat 1.4 NA oil objective and Zen 2009 software. ImageJ was used to quantify the corrected total cellular fluorescence (CTCF). For each time point and cell line at least 25 cells were outlined and the total integrated density was determined. CTCF was determined as previously described by multiplying the area of selected cell by the mean fluorescence of 4 background readings and subtracting that from the integrated density (CTCF = integrated density – (area of selected cell x mean fluorescence of background readings)³⁰⁴. For the pulse-chase assays the CTCF values were normalized to the average CTCF of the 0-time point and inverted in order to determine the relative transferrin recycled. For the uptake assays, the CTCF values then had the average CTCF for the negative control subtracted and were normalized to the average CTCF of the binding control in order to determine the relative transferrin internalized.

2.6 Immunofluorescence

BT549 and NIH3T3 cells were plated at 80,000cells/well or dish on 22 x 22mm #1.5 coverslips or ibidi (Madison, WI, USA) 35mm μ -Dishes for confocal experiments or World Precision Instruments (Sarasota, FL, USA) 35mm Fluorodishes for TIRF experiments and fixed with 3.7% paraformaldehyde in PHEM buffer for 10 minutes at 37°C. The cells were

permeabilized with 0.5% Triton X-100 PBS for 10 minutes. The cells were blocked in Abdil (0.1% Triton X-100, 2% BSA-PBS) for 10 minutes. The cells were then incubated with primary antibodies in Abdil for 1 hour. Cells were washed 5 times with 0.1% Triton X-100 PBS and incubated with appropriate secondary antibodies in Abdil for 45 minutes. When dual staining, the previous steps were repeated from the blocking step with the necessary antibodies. The cells were stained with 1 μ g/mL DAPI for 5 minutes. The coverslips were then mounted on slides with Dako mounting medium and the dishes were mounted with ibidi mounting medium. The antibodies used for immunofluorescence staining were PI4KIII β (catalog no. AP8030a) from Abgent, (Mississauga, Canada), PI4KIII β (catalog no. 611817), EEA1 (catalog no. 610457), GM130 (catalog no. 610822) from BD Biosciences, and Rab11a (catalog no. ab170134, ab3612), Rab5 (catalog no. ab18211), Rab7 (catalog no. ab50533), and TGN46 (catalog no. ab50595) from Abcam (Cambridge, MA, USA). The secondary antibodies used were all purchased from Invitrogen (Burlington, Canada) and include anti-mouse AlexaFluor[®]488 (catalog no. A-11029), anti-mouse AlexaFluor[®]546 (catalog no. A-11003), anti-rabbit AlexaFluor[®]488 (catalog no. A-11008), anti-rabbit AlexaFluor[®]546 (catalog no. A-11010). All antibodies were used as per the manufacturer's recommendations. Confocal images were acquired with a Zeiss LSM 510 META/AxioVert 200 confocal microscope with a 63x Plan-Apochromat 1.4 NA oil objective and Zen 2009 software. TIRF images were acquired with a TIRF-Spinning Disk Spectral Discovery System with a 63x Plan-Apochromat 1.4 NA oil objective and MetaMorph software. All analysis of images was performed using ImageJ. Colocalization analysis was performed by measuring the plot profile of at least 50 vesicles in the periphery of the cells. Analysis of the TIRF

images was performed by circling the outline of the cell and measuring the number, size, area and circularity of the particles after background subtraction and thresholding.

2.7 Proximity ligation assay

BT549 cells were plated at 15,000cells/well in ibidi 8-well μ -slides then fixed with 4% paraformaldehyde for 10 minutes at room temperature. The cells were subjected to proximity ligation assays (PLAs) using the Duolink[®] system (Sigma-Aldrich). The cells were blocked and permeabilized with blocking buffer (5% BSA, 0.1% Tween-20 in PBS) for 45 minutes. The blocking buffer was removed and primary antibody for both proteins was added (1:20 dilution per antibody) in blocking buffer and incubated at 4°C overnight. The negative control was achieved by incubating overnight in blocking buffer alone without primary antibodies. The following day, Duolink[®] PLA Probe anti-mouse MINUS (catalog no. DUO92004), Duolink[®] PLA Probe anti-rabbit PLUS (catalog no. DUO92002) were mixed (1:20 dilution per probe) in ddH₂O. The PLA probes were mixed gently by vortex and incubated in an Eppendorf tube for 30 minutes before use. Primary antibody was removed and the cells washed with PBS. The PLA probe mixture was added to the cells and incubated for 1 hour at 37°C in a moisture chamber. After 1 hour, the PLA probes were removed from the cells, which were then washed twice with wash buffer A (0.01M Tris, 0.15M NaCl, 0.05% Tween-20 in ddH₂O) for 5 minutes per wash. A ligation solution was prepared from 5x ligation stock (1:10 dilution) in ddH₂O and immediately before adding to cells, ligase (1:150 dilution) was added. The cells were incubated in the ligation solution for 30 minutes at 37°C in a humidity chamber. After 30 minutes, the ligation solution was removed and the cells were washed once with wash buffer A. An amplification solution was

prepared from 5x amplification stock (1:10 dilution) in ddH₂O and immediately before adding to cells, polymerase (1:150) was added. The cells were incubated in the amplification solution for 2 hours at 37°C in a humidity chamber. The amplification solution was removed and the cells were wash twice with wash buffer B (0.2M Tris, 0.1M NaCl in ddH₂O) for 10 minutes each, then again for 3 minutes in 1:10 diluted wash buffer B. The nuclei were then stained by incubation with 1µg/mL DAPI for 5 minutes. Finally, the samples were mounted with ibidi mounting medium. Epifluorescent images were acquired with a Zeiss AxioObserver.D1 microscope with a 63x Plan-Apochromat 1.4 NA oil objective and AxioVision 4.8 software. Confocal images were acquired with a Zeiss LSM 510 META/AxioVert 200 confocal microscope with a 63x Plan-Apochromat 1.4 NA oil objective and Zen 2009 software. The antibodies used for proximity ligation assays were PI4KIIIβ (catalog no. AP8030a) from Abgent and Rab11a (catalog no. ab170134) from Abcam. Additionally, the and Duolink® Detection Reagents Red (catalog no. DUO92008) were purchased from Sigma-Aldrich.

2.8 *In vivo* tumour studies

Animals were housed and handled according to Canadian Council on Animal Care standards and policies. 100,000 4T1 cells/mouse in PBS were injected into the mammary fat pads of 7-9 week old Balb/c mice. For imaging, mice were injected *i.p.* with 200µL of 15mg/mL D-Luciferin (PerkinElmer, Waltham, MA, USA) in PBS and luminescence quantitated 5 minutes later using a PerkinElmer IVIS SpectrumCT.

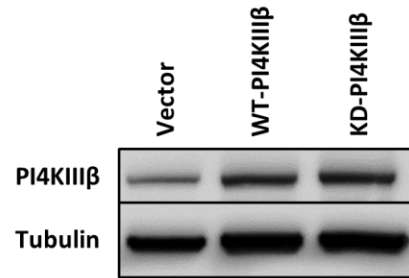
Chapter 3: Results

3.1 PI4KIII β enhances the intensity and/or duration of IGF-IR β , A-Raf, and Shc activation

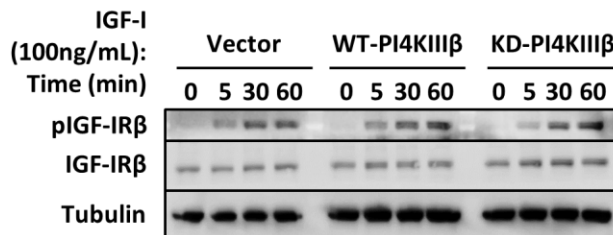
Our lab has previously shown that PI4KIII β protein is highly expressed in ~20% of human breast cancers and that ectopic expression of PI4KIII β activates Akt in a kinase-independent manner in breast cell lines¹³⁵. We next wanted to determine whether or not PI4KIII β affects additional signaling pathways. We decided to investigate how ectopic PI4KIII β expression affected the activation of IGF-IR β , due to its well-characterized role in the development and progression of breast cancer, and A-Raf and Shc, due to their coincident increased activation observed in breast cancer samples with high PI4KIII β ^{270,280,281,282}. In order to determine whether or not PI4KIII β expression affects these signaling pathways, we expressed ectopic wildtype or kinase-inactive (D656A) PI4KIII β in BT549 human breast ductal carcinoma cell lines (Figure 3.1a).

We observed a significant 2-3-fold increase in IGF-IR β activation when either wildtype or kinase-inactive PI4KIII β was highly expressed activation compared to controls (Figure 3.1b and c). PI4KIII β overexpression caused a 50% increase in A-Raf activation, but only if PI4KIII β was catalytically active (Figure 3.1d and e). Furthermore, cell lines with high wildtype or kinase-inactive PI4KIII β expression had increased intensity and duration of activation of all 3 Shc isoforms (Figure 3.2). Overall, this demonstrates that PI4KIII β activates insulin and EGF signaling. Moreover, it augments IGF-IR β and Shc activation independent of PI4P generation.

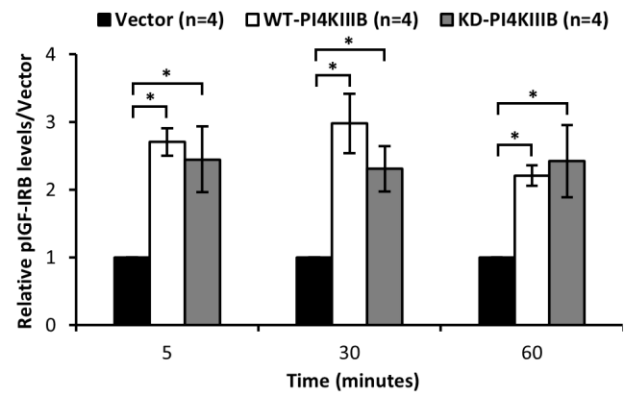
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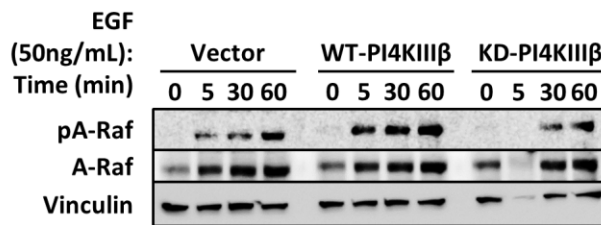
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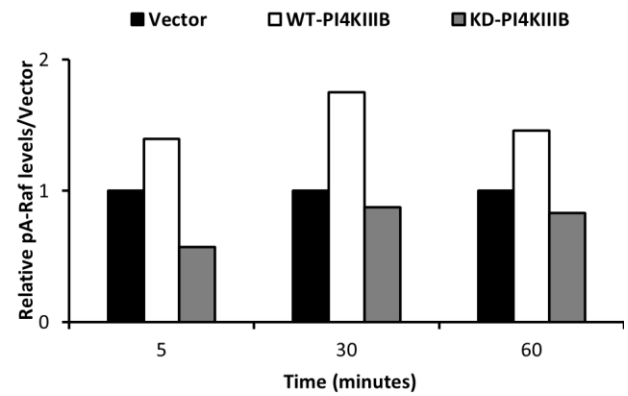


Figure 3.1 PI4KIII β increases the intensity of IGF-IR β and A-Raf activation.

(a) Western blot analysis showing levels of PI4KIII β (110kDa) with Tubulin (55kDa) as a loading control in BT549 vector control (Vector), PI4KIII β -overexpressing (WT-PI4KIII β), and kinase-inactive PI4KIII β -expressing (KD-PI4KIII β) human breast ductal carcinoma cell lines. Each lane contains 30 μ g of total protein. (b-e) Vector, WT-PI4KIII β , and KD-PI4KIII β cell lines were serum starved overnight followed by stimulation with (b and c) IGF-I (100ng/mL) or (d and e) EGF (50ng/mL) for the indicated time periods. The lysate was collected and subjected to western blot analysis to determine levels of (b) pIGF-IR β (95kDa) and IGF-IR β (95kDa), or (d) pA-Raf (68kDa) and A-Raf (68kDa) with (b) Tubulin (55kDa) or (d) Vinculin (120kDa) as loading controls. Each lane contains 30 μ g of total protein. (c and e) Protein levels were quantified by densitometry and the data shown represents the mean $\pm$ SE of the mean from (c) 4 independent trials comparing the pIGF-IR β levels relative to the IGF-IR β levels or (e) 1 trial comparing the pA-Raf levels relative to the A-Raf levels in each cell line, followed by normalization to the Vector. Statistical significance (*, $P \leq 0.05$, one-way ANOVA with multiple comparison tests) is indicated.

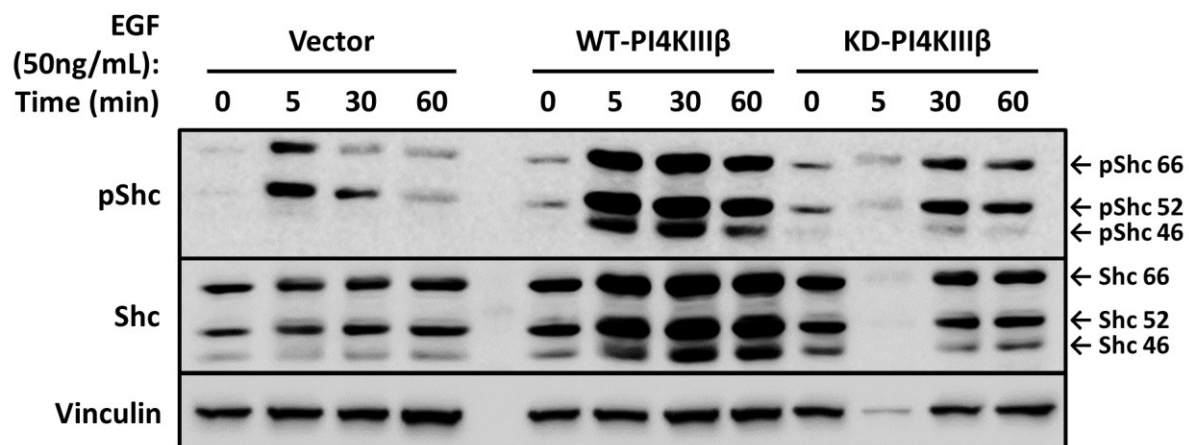
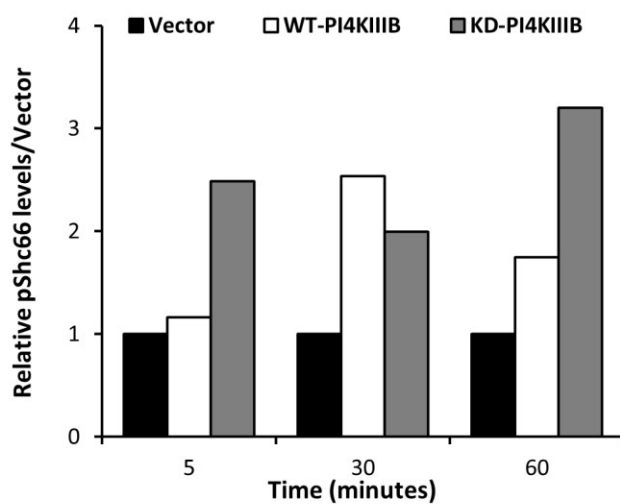
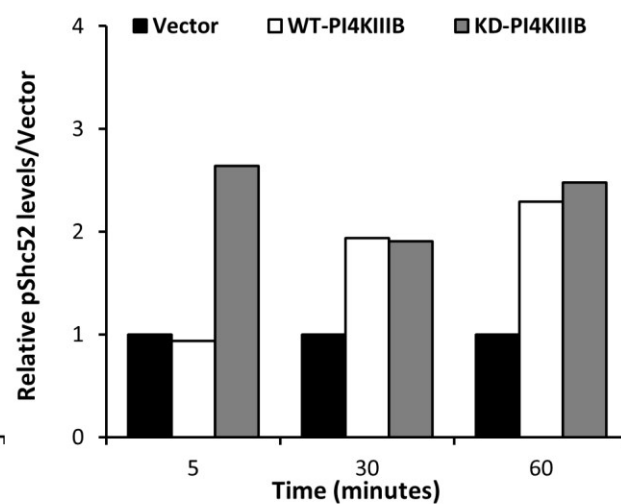
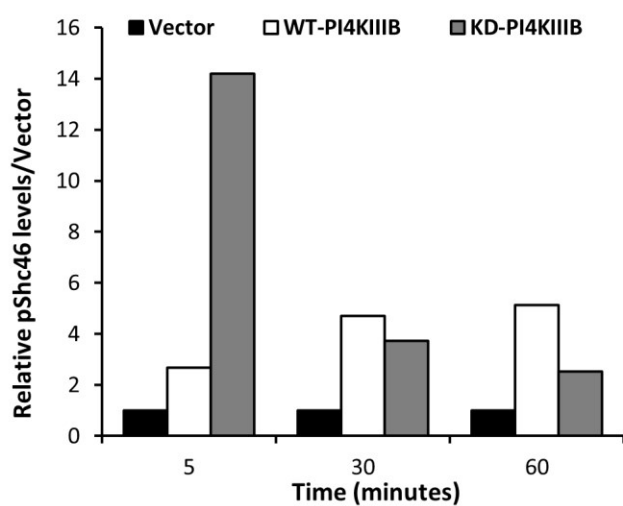
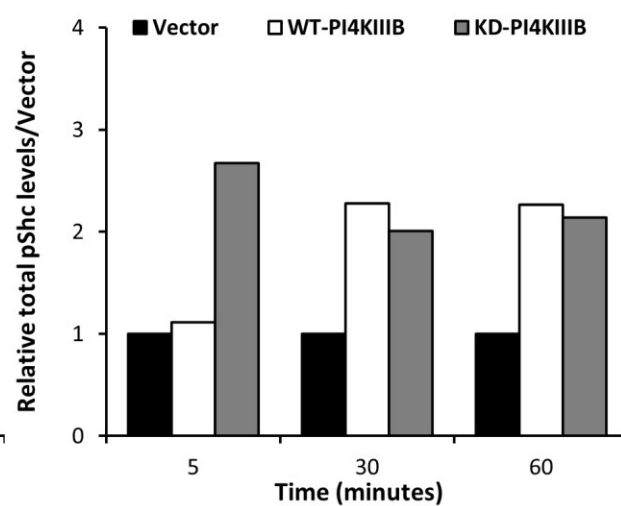
a**b****c****d****e**

Figure 3.2 PI4KIII β increases the intensity and duration of Shc activation.

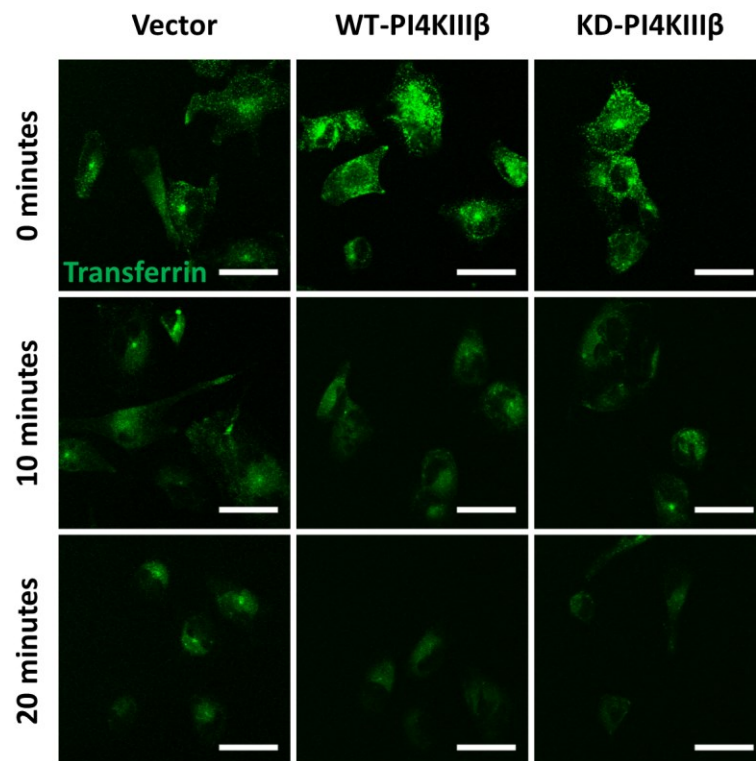
(a-e) BT549 vector control (Vector), PI4KIII β -overexpressing (WT-PI4KIII β), and kinase-inactive PI4KIII β -expressing (KD-PI4KIII β) human breast ductal carcinoma cell lines were serum starved overnight followed by stimulation with EGF (50 ng/mL) for the indicated time periods. (a) The lysate was collected and subjected to western blot analysis to determine levels of pShc (46, 52, 66kDa) and Shc (46, 52, 66kDa) with Vinculin (120kDa) as a loading control. Each lane contains 30 μ g of total protein. (b-e) Protein levels were quantified by densitometry and the data shown represents the mean from 1 trial comparing the pShc levels relative to the Shc levels for the (b) 66kDa isoform, (c) 52kDa isoform, (d) 46kDa isoform, or (e) all isoforms in each cell line, followed by normalization to the Vector.

3.2 PI4KIII β expression alters endocytic kinetics

The activation of Akt by PI4KIII β previously established by our lab was shown to be dependent upon the presence of Rab11a¹³⁵. Because Rab11a regulates endosome recycling, we hypothesized that the effects of PI4KIII β on breast cancer oncogenesis may be due to a regulatory role in endosome function^{200,202}. To determine whether PI4KIII β might affect endosome kinetics we used transferrin pulse-chase assays with microscopy to measure the rate of endosomal recycling. We observed that cell lines overexpressing wildtype or kinase-inactive PI4KIII β were able to recycle out relatively twice as much internalized transferrin as the vector control (Figure 3.3). Next, we used transferrin uptake assays with microscopy to observe the rate at which cells were able to internalize fluorescent transferrin. BT549 cells with ectopic wildtype or kinase-inactive PI4KIII β were able to internalize significantly more transferrin (4-5x) than the vector control (Figure 3.4).

To further explore the importance of PI4KIII β on endosome function, we created *PI4KB*-null cell lines using CRISPR/Cas9 in BT549 wildtype cells (Figure 3.5a). *PI4KB*-null cells are viable and appear to proliferate normally. Surprisingly, we did not observe any disruption of the gross morphology of the Golgi in PI4KIII β -deleted BT549 or NIH3T3 cells (Figure 3.5b and c and Figure 3.6). This suggests that PI4KIII β deletion does not cause dysregulation of Golgi structure or function and any change in endocytic kinetics or signaling is not due to Golgi dysfunction. In order to investigate how the deletion of PI4KIII β might affect endocytic kinetics, we repeated the transferrin recycling and uptake assays in *PI4KB*-null cell lines. We found that BT549 cells with PI4KIII β deletion were able to recycle only half as much transferrin as the wildtype cells (Figure 3.7). Furthermore, cells with

a



b

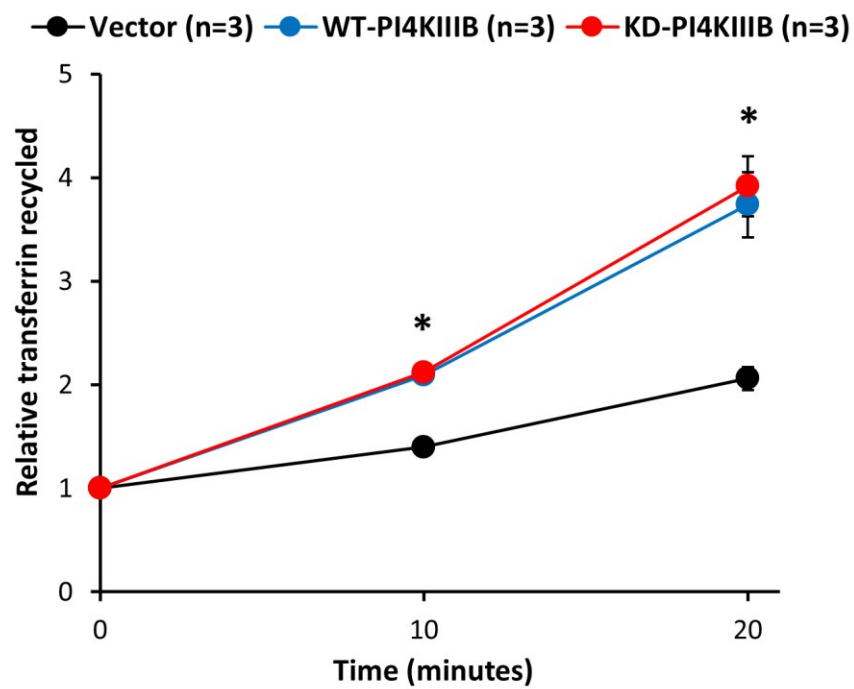


Figure 3.3 High expression of PI4KIII β increases endocytic recycling.

(a) Representative confocal images of paraformaldehyde fixed BT549 vector control (Vector), PI4KIII β -overexpressing (WT-PI4KIII β), and kinase-inactive PI4KIII β -expressing (KD-PI4KIII β) human breast ductal carcinoma cell lines after undergoing transferrin pulse-chase assay for specified times. 63x magnification. Scale bars, 50 μ m. (b) The relative transferrin recycled in BT549 Vector, WT-PI4KIII β , and KD-PI4KIII β cell lines was calculated from images obtained through confocal microscopy by determining the average corrected total cellular fluorescence for 25 cells in each condition per trial. The relative transferrin recycled is presented as the mean $\pm$ SE of the mean, from 3 independent trials, following background subtraction and normalization to the initial time point. Statistical significance (*, $P \leq 0.05$, one-way ANOVA with multiple comparison tests) is indicated.

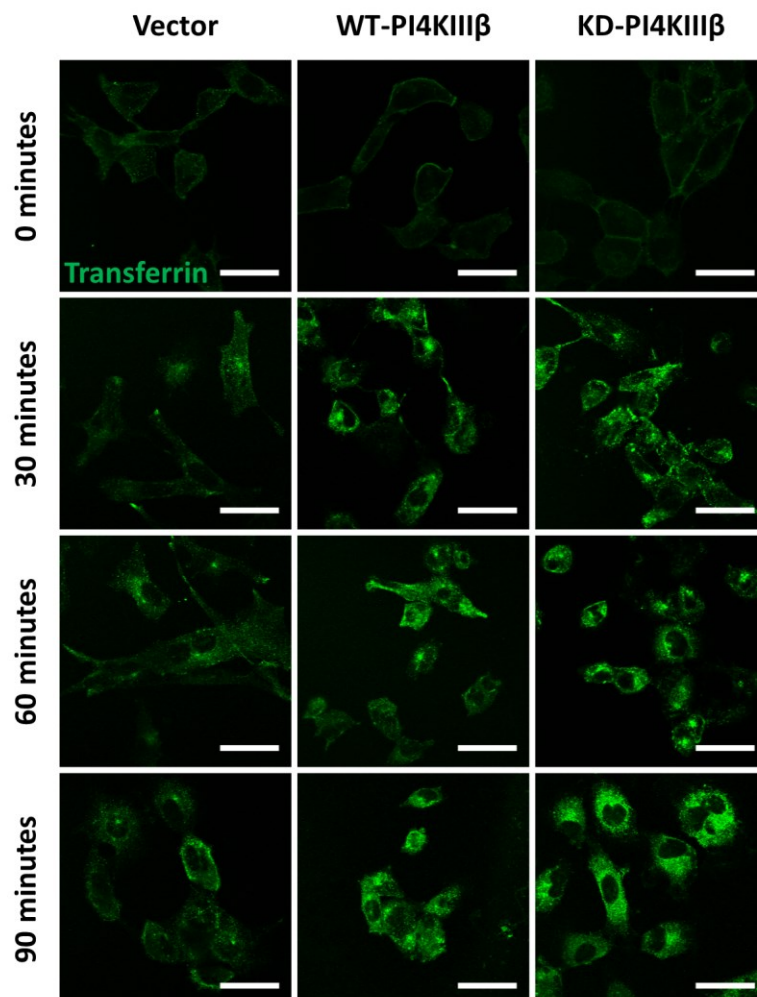
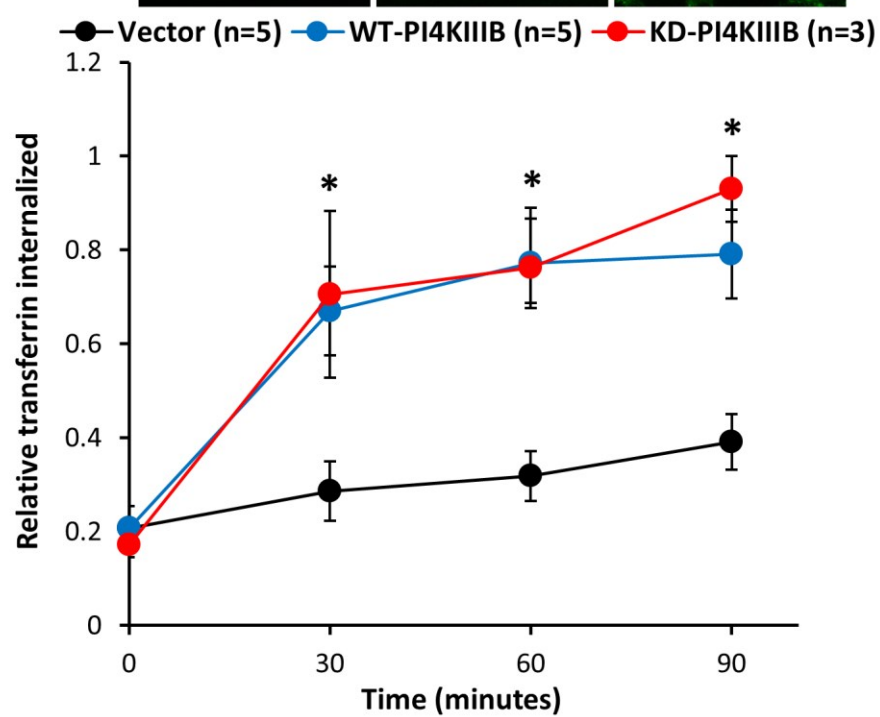
a**b**

Figure 3.4 High PI4KIII β expression increases the rate of endocytic internalization.

(a) Representative confocal images of paraformaldehyde fixed BT549 vector control (Vector), PI4KIII β -overexpressing (WT-PI4KIII β), and kinase-inactive PI4KIII β -expressing (KD-PI4KIII β) human breast ductal carcinoma cell lines after undergoing transferrin uptake assay for specified times. 63x magnification. Scale bars, 50 μ m. (b) The relative transferrin internalized in BT549 Vector, WT-PI4KIII β , and KD-PI4KIII β cell lines was calculated from images obtained through confocal microscopy by determining the average corrected total cellular fluorescence for 25 cells in each condition per trial. The relative transferrin internalized is presented as the mean $\pm$ SE of the mean, from 5 (Vector and KD-PI4KIII β) or 3 (WT-PI4KIII β) independent trials, following background subtraction and normalization to the binding control. Statistical significance (*, $P \leq 0.05$, one-way ANOVA with multiple comparison tests) is indicated.

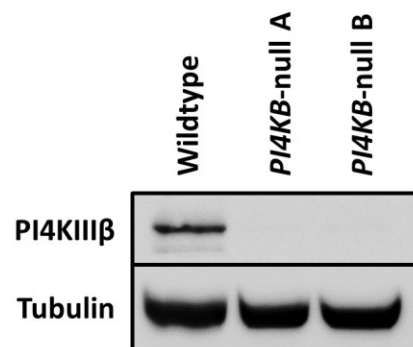
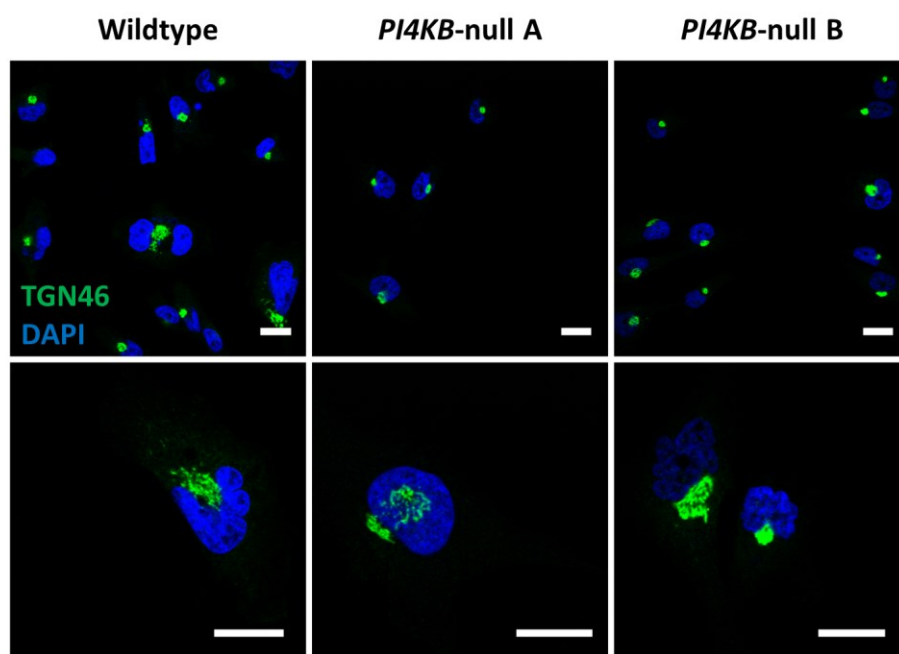
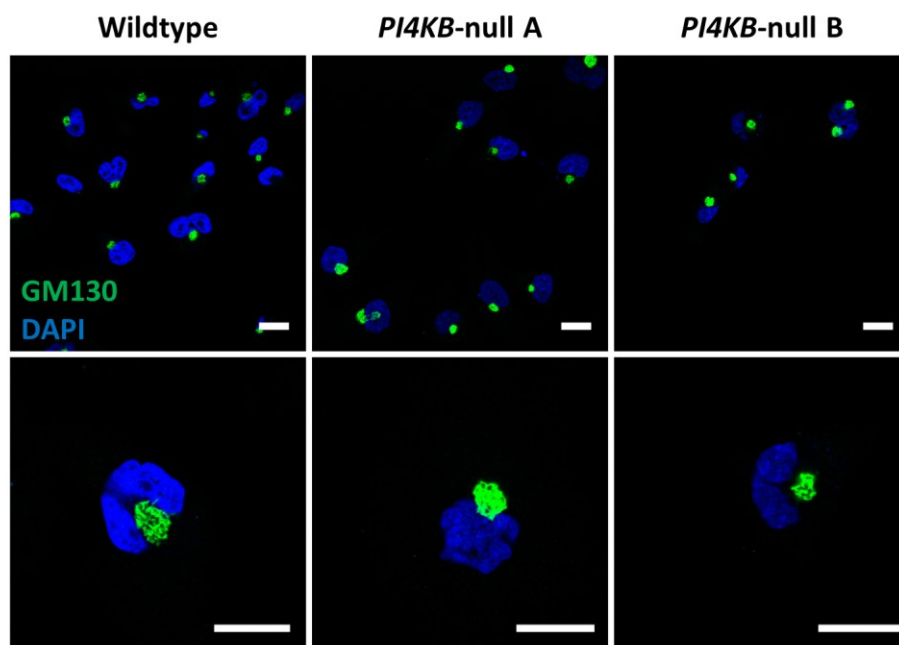
a**b****c**

Figure 3.5 Deletion of PI4KIII β does not affect Golgi structure in BT549 cells.

(a) Western blot analysis showing levels of PI4KIII β (110kDa) with Tubulin (55kDa) as a loading control in BT549 wildtype (Wildtype) and two independent CRISPR/Cas9 knockouts of PI4KIII β (*PI4KB*-null A and *PI4KB*-null B) human breast ductal carcinoma cell lines. Each lane contains 30 μ g of total protein. (b and c) Representative confocal images of paraformaldehyde fixed BT549 Wildtype, *PI4KB*-null A, and *PI4KB*-null B cell lines following immunofluorescence of the (b) *trans*-Golgi marker, TGN46, or (c) *cis*-Golgi marker, GM130. 63x magnification. Scale bars, 20 μ m.

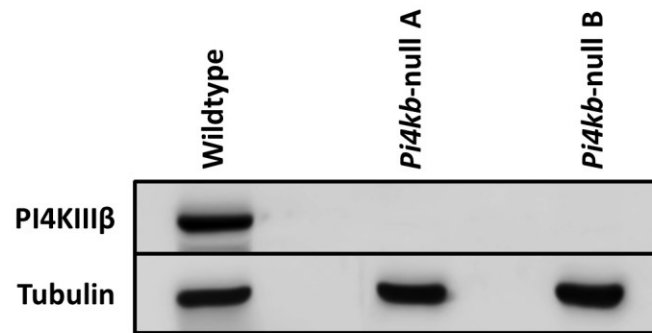
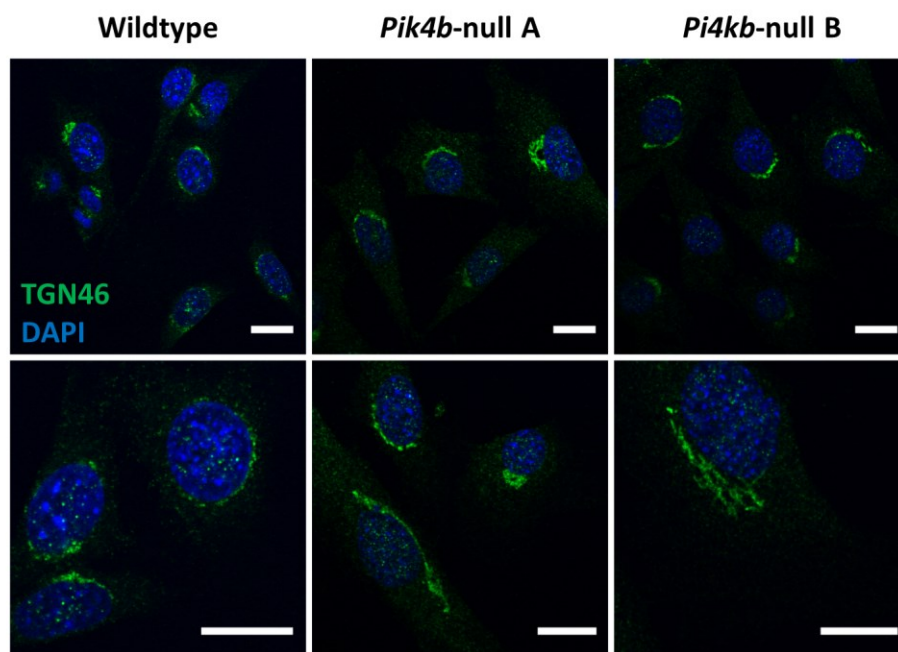
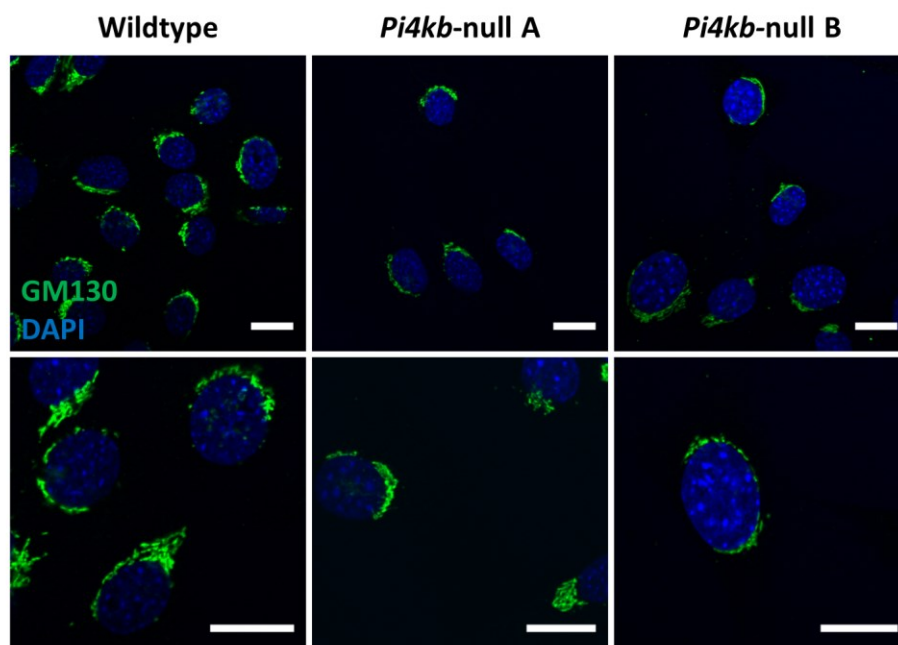
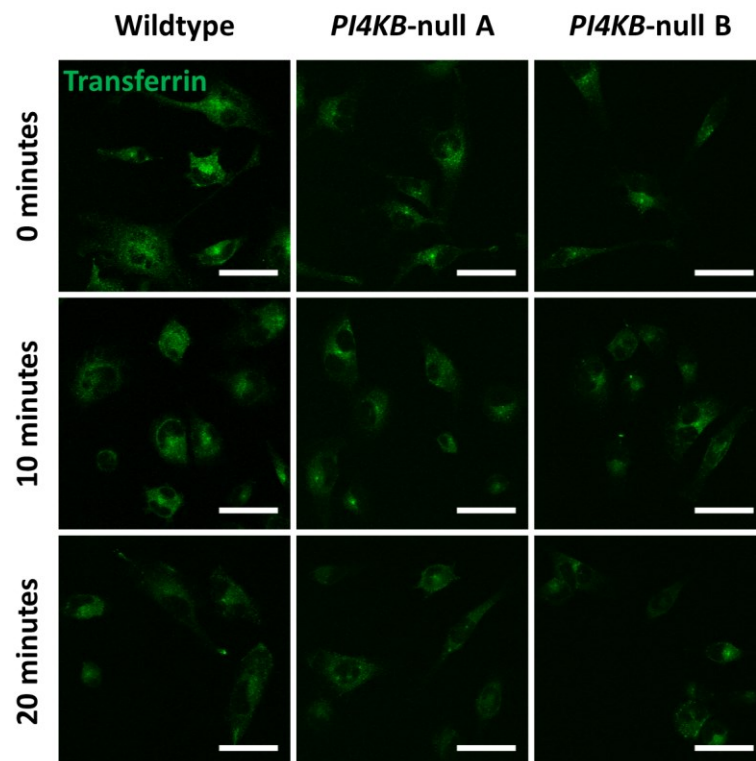
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Figure 3.6 Deletion of PI4KIII β does not affect Golgi structure in NIH3T3 cells.

(a) Western blot analysis showing levels of PI4KIII β (110kDa) with Tubulin (55kDa) as a loading control in NIH3T3 wildtype (Wildtype) and two independent CRISPR/Cas9 knockouts of PI4KIII β (*Pi4kb*-null A and *Pi4kb*-null B) mouse embryo fibroblast cell lines. Each lane contains 30 μ g of total protein. (b and c) Representative confocal images of paraformaldehyde fixed NIH3T3 Wildtype, *Pi4kb*-null A, and *Pi4kb*-null B cell lines following immunofluorescence of the (b) *trans*-Golgi marker, TGN46, or (c) *cis*-Golgi marker, GM130. 63x magnification. Scale bars, 20 μ m.

a



b

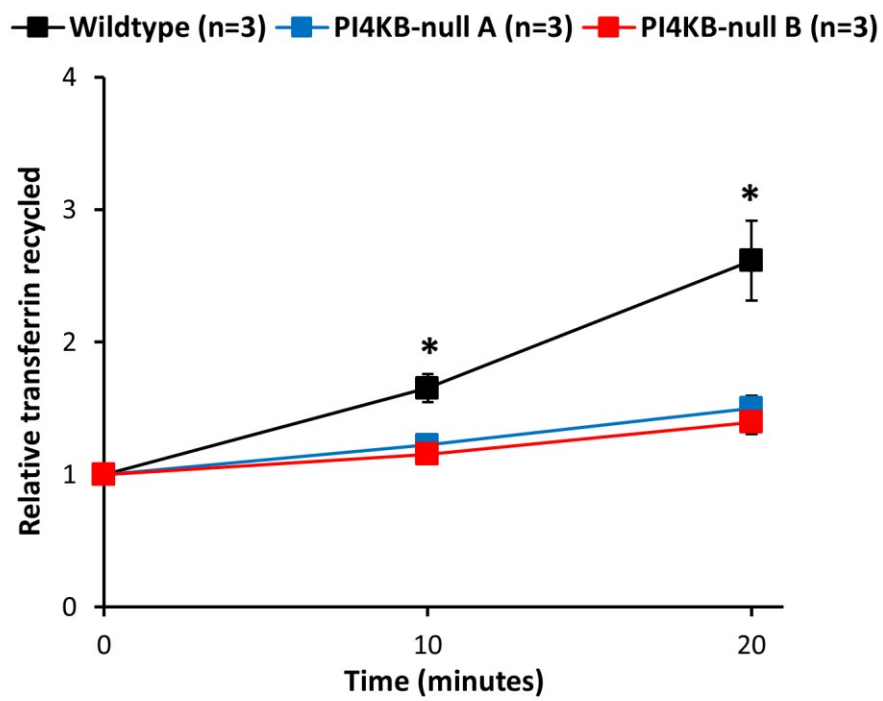


Figure 3.7 PI4KIII β deletion decreases endocytic recycling.

(a) Representative confocal images of paraformaldehyde fixed BT549 wildtype (Wildtype) and two independent CRISPR/Cas9 knockouts of PI4KIII β (*PI4KB*-null A and *PI4KB*-null B) human breast ductal carcinoma cell lines after undergoing transferrin pulse-chase assay for specified times. 63x magnification. Scale bars, 50 μ m. (b) The relative transferrin recycled in BT549 Wildtype, *PI4KB*-null A, and *PI4KB*-null B cell lines was calculated from images obtained through confocal microscopy by determining the average corrected total cellular fluorescence for 25 cells in each condition per trial. The relative transferrin recycled is presented as the mean $\pm$ SE of the mean, from 3 independent trials, following background subtraction and normalization to the initial time point. Statistical significance (*, $P \leq 0.05$, one-way ANOVA with multiple comparison tests) is indicated.

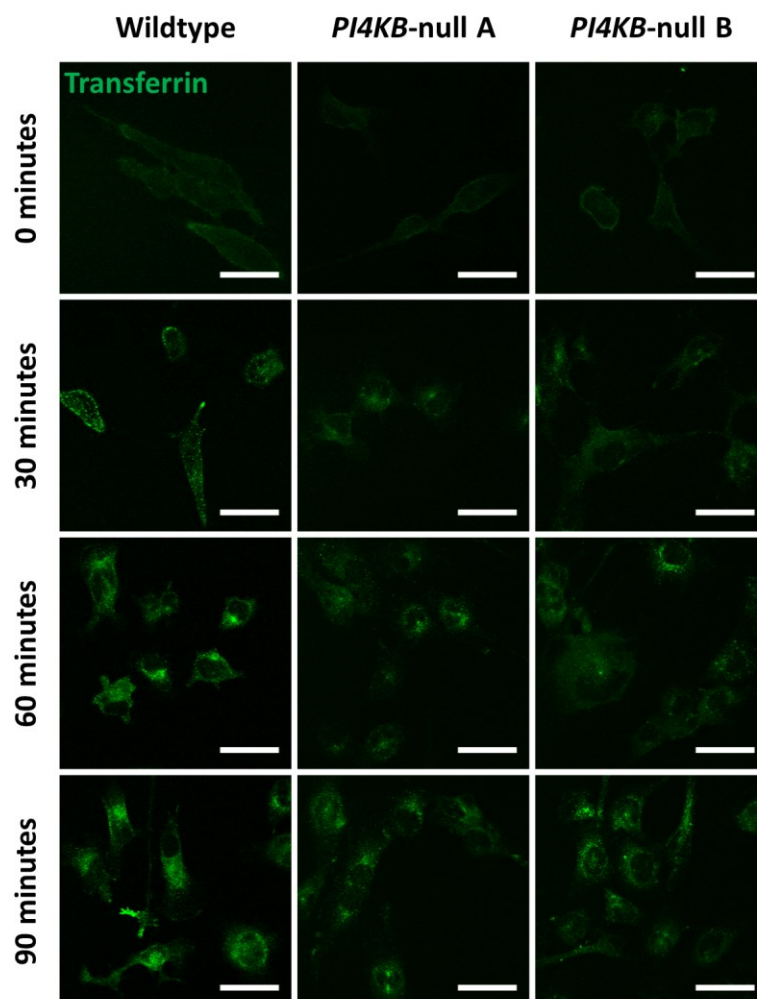
PI4KIII β deletion internalized significantly less transferrin (4-5x) than the wildtype (Figure 3.8). Overall, these results are consistent with a novel role for PI4KIII β in controlling endocytic internalization, that is independent of its function in regulating PI4P.

3.3 Lipid kinase-independent control of endocytic function

PI4KIII β is a multifunctional protein and we next wanted to determine which aspect of PI4KIII β regulated endocytic function. To this end, we expressed wildtype, kinase-inactive (D656A) and Rab11a-binding deficient (N162A) PI4KIII β in the BT549 *PI4KB*-null CRISPR/Cas9 cell lines (Figure 3.9). We then used the transferrin pulse-chase and uptake assays to measure endocytic function. We found that the reintroduction of wildtype and kinase-inactive PI4KIII β was able to rescue the rate of transferrin recycling (Figure 3.10). However, the Rab11a-binding deficient PI4KIII β was unable to do so (Figure 3.10). This suggests that endocytic recycling is dependent upon the functional interaction between PI4KIII β and Rab11a but is independent of PI4P generation.

On the other hand, we found that the rate of transferrin uptake was only fully rescued if the reintroduced PI4KIII β was catalytically active and also able to interact with Rab11a (Figure 3.11). In the absence of endogenous PI4KIII β , kinase-inactive or Rab11a-binding deficient PI4KIII β resulted in only a partial (~30-50%) rescue of the transferrin uptake rate (Figure 3.11). This result is in contrast to those obtained earlier, where we observed a similar increase in uptake between ectopically expressed wildtype and kinase-inactive PI4KIII β (Figure 3.4). We suggest that this may be due to the lower levels of expression of kinase-inactive PI4KIII β in our *PI4KB*-null rescue cell lines, compared to the

a



b

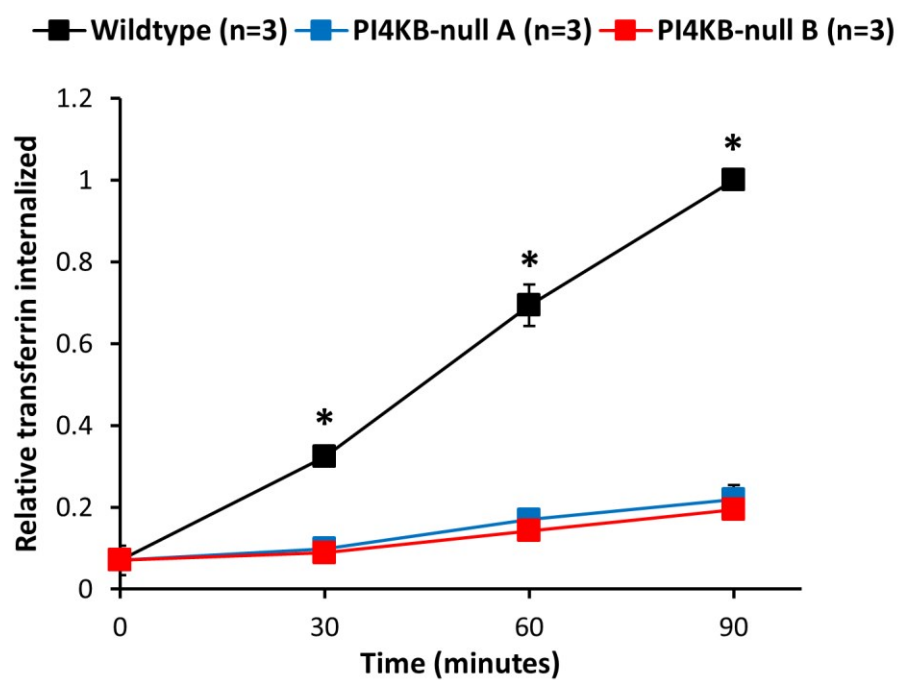


Figure 3.8 Deletion of PI4KIII β decreases endocytic internalization.

(a) Representative confocal images of paraformaldehyde fixed BT549 wildtype (Wildtype) and two independent CRISPR/Cas9 knockouts of PI4KIII β (*PI4KB*-null A and *PI4KB*-null B) human breast ductal carcinoma cell lines after undergoing transferrin uptake assay for specified times. 63x magnification. Scale bars, 50 μ m. (b) The relative transferrin internalized in BT549 Wildtype, *PI4KB*-null A, and *PI4KB*-null B cell lines was calculated from images obtained through confocal microscopy by determining the average corrected total cellular fluorescence for 25 cells in each condition per trial. The relative transferrin internalized is presented as the mean $\pm$ SE of the mean, from 3 independent trials, following background subtraction and normalization to the binding control. Statistical significance (*, $P \leq 0.05$, one-way ANOVA with multiple comparison tests) is indicated.

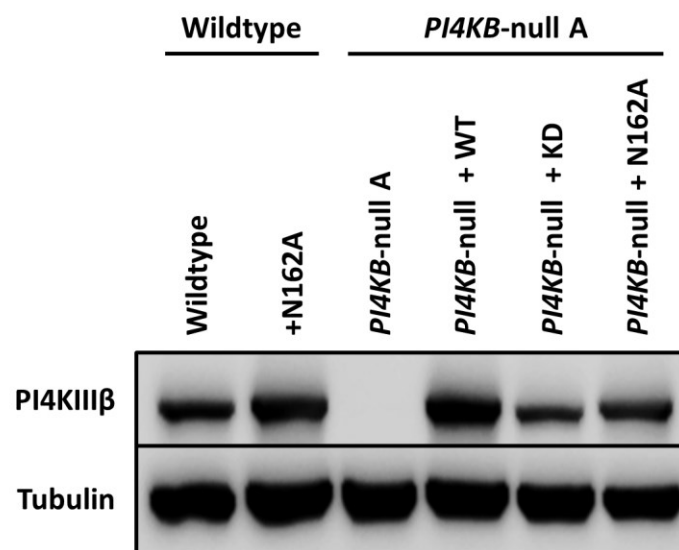


Figure 3.9 PI4KIII β rescue cell lines.

Western blot analysis showing levels of PI4KIII β (110kDa) with Tubulin (55kDa) as a loading control in BT549 wildtype (Wildtype) cell line transfected with PI4KIII β unable to bind Rab11a (+N162A) and BT549 CRISPR/Cas9 knockout of PI4KIII β (*PI4KB*-null A) cell line transfected with wildtype PI4KIII β (*PI4KB*-null + WT), kinase dead PI4KIII β (*PI4KB*-null + KD), or PI4KIII β unable to bind Rab11a (*PI4KB*-null + N162A) plasmids followed by antibiotic selection in order to generate stable cell lines. Each lane contains 30 μ g of total protein.

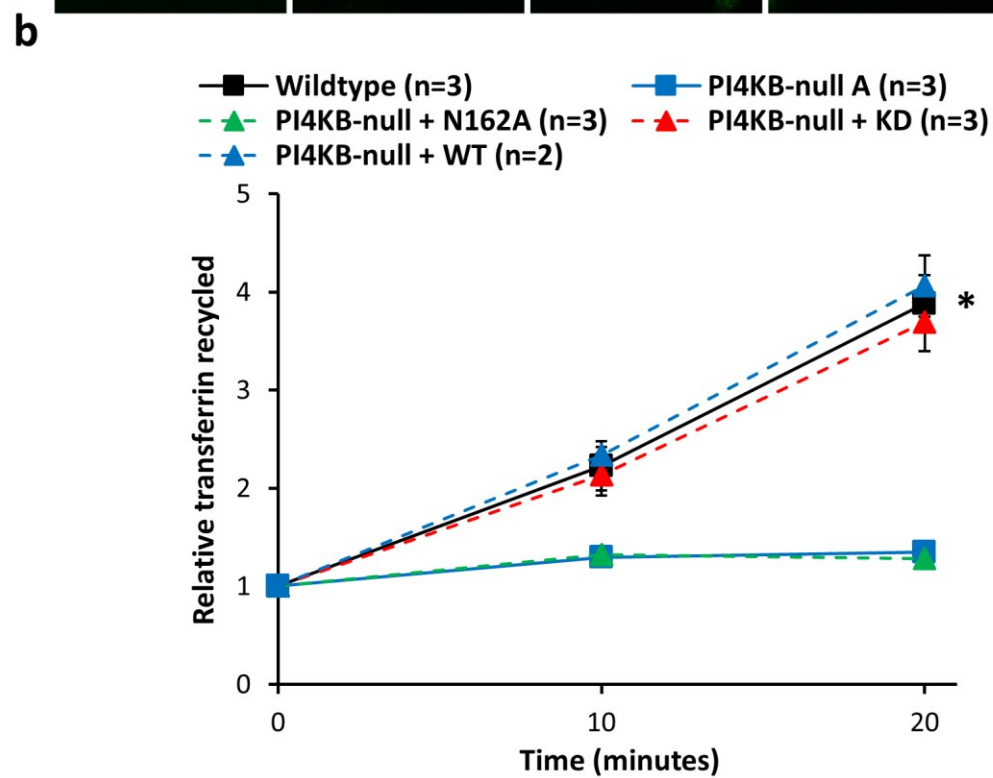
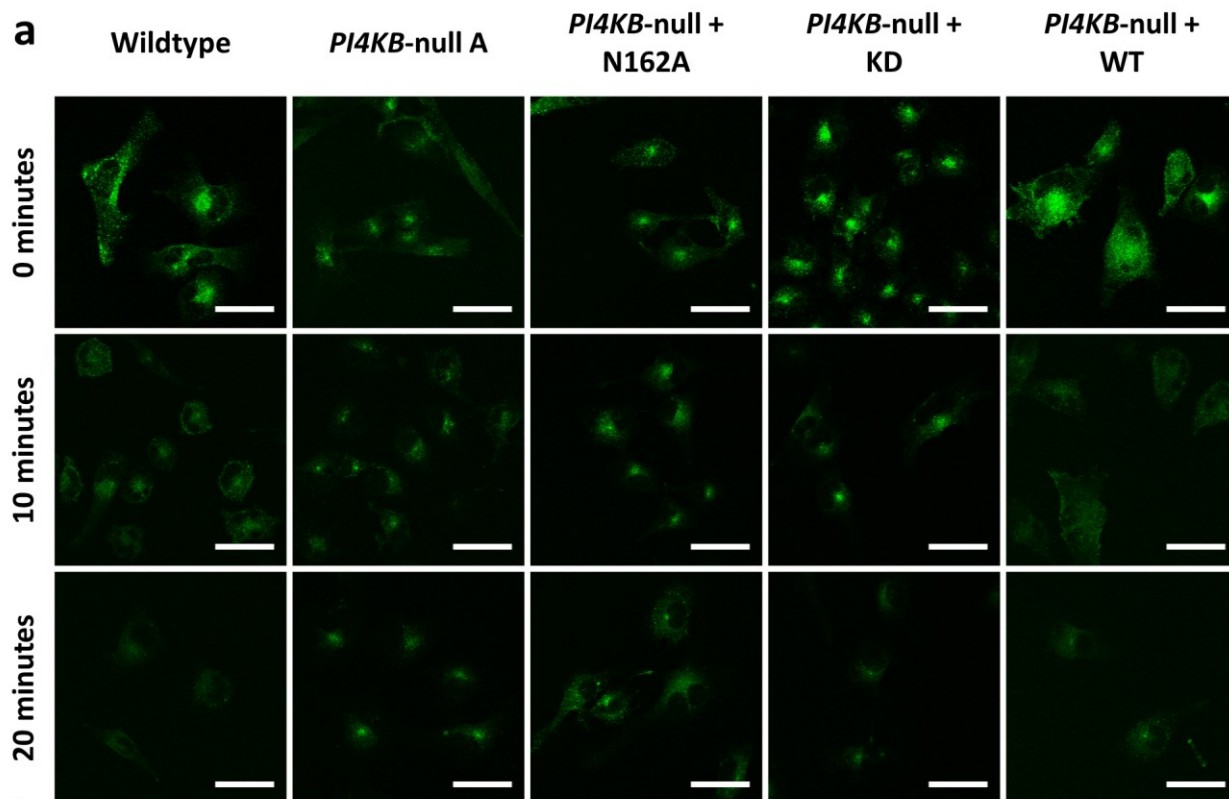


Figure 3.10 PI4KIII β -Rab11a interaction rescues endocytic recycling.

(a) Representative confocal images of paraformaldehyde fixed BT549 wildtype (Wildtype), CRISPR/Cas9 knockout of PI4KIII β (*PI4KB*-null A), PI4KIII β unable to bind Rab11a rescue (*PI4KB*-null + N162A), kinase dead PI4KIII β rescue (*PI4KB*-null + KD), and wildtype PI4KIII β rescue (*PI4KB*-null + WT) human breast ductal carcinoma cell lines after undergoing transferrin pulse-chase uptake assay for specified time. 63x magnification. Scale bars, 50 μ m. (b) The relative transferrin recycled in BT549 Wildtype, *PI4KB*-null A, *PI4KB*-null + N162A, *PI4KB*-null + KD, and *PI4KB*-null + WT cell lines was calculated from images obtained through confocal microscopy by determining the average corrected total cellular fluorescence for 25 cells in each condition per trial. The relative transferrin recycled is presented as the mean $\pm$ SE of the mean, from 3 (Wildtype, *PI4KB*-null A, *PI4KB*-null + N162A, and *PI4KB*-null + KD) or 2 (*PI4KB*-null + WT) independent trials, following background subtraction and normalization to the initial time point. Statistical significance (*, $P \leq 0.05$, one-way ANOVA with multiple comparison tests) is indicated.

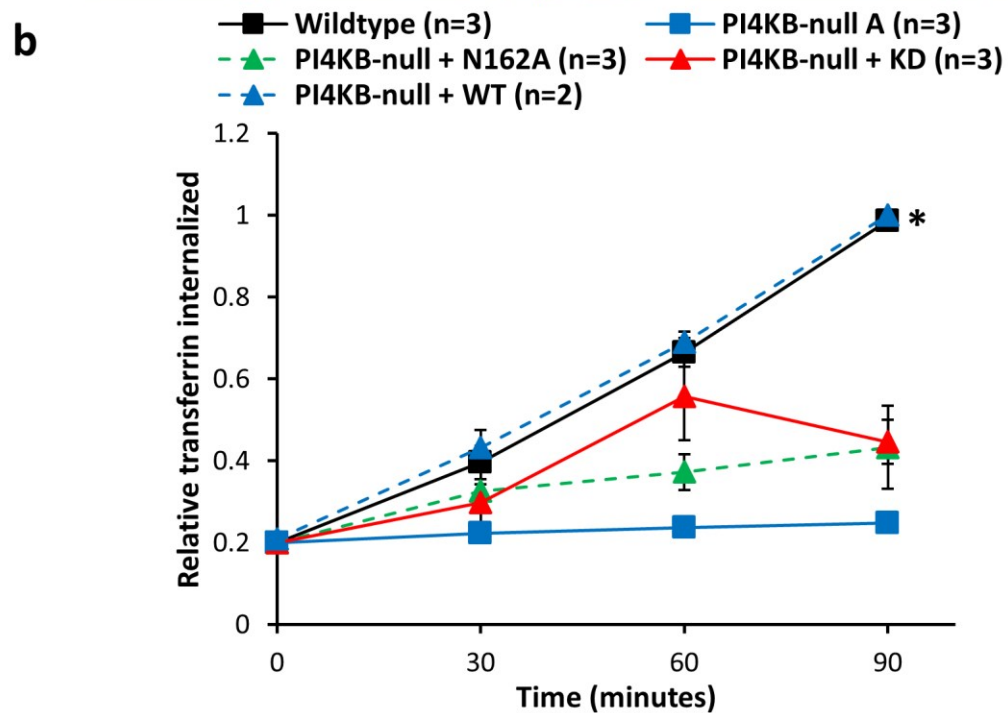
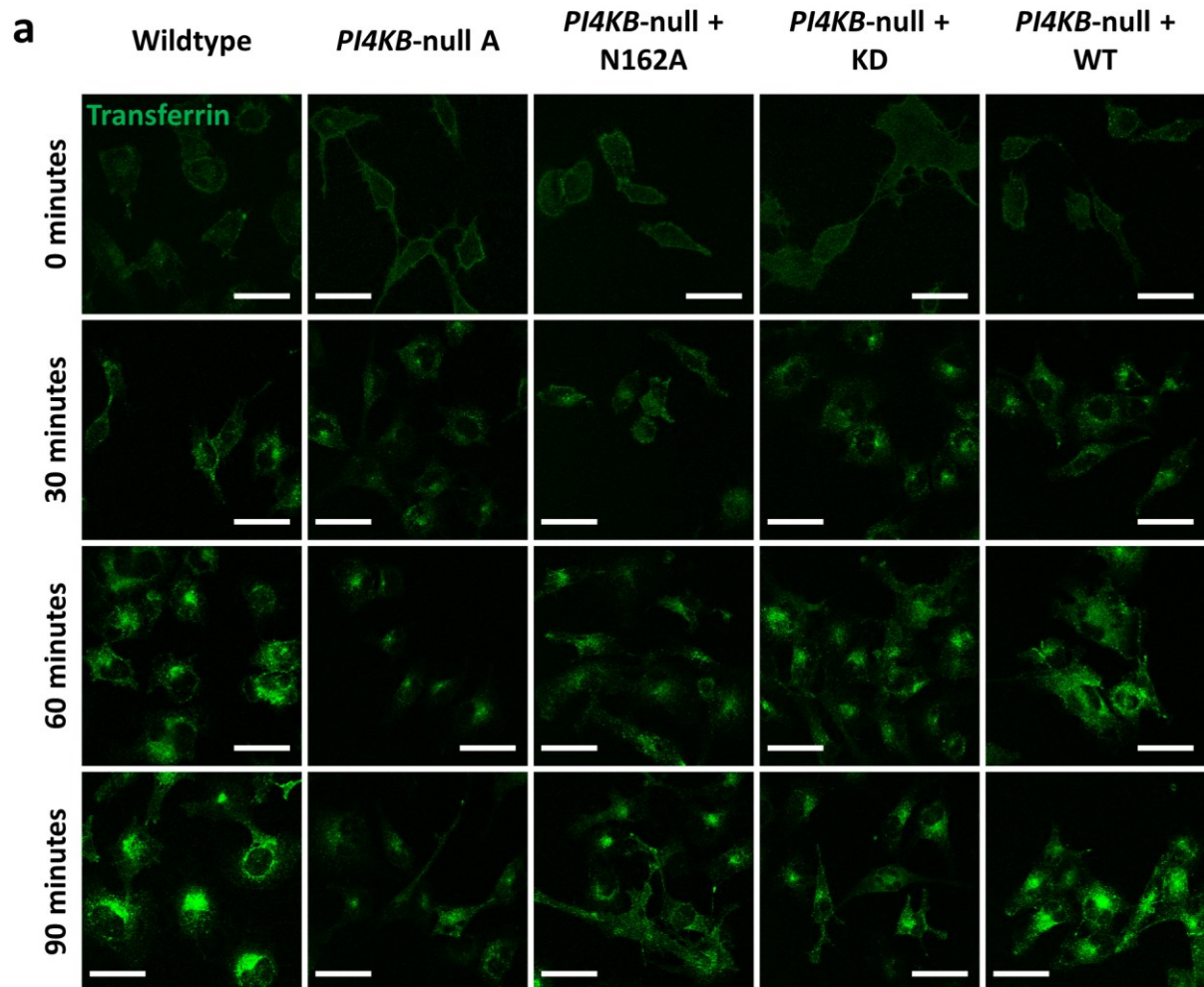


Figure 3.11 Catalytically active PI4KIII β rescues endocytic internalization.

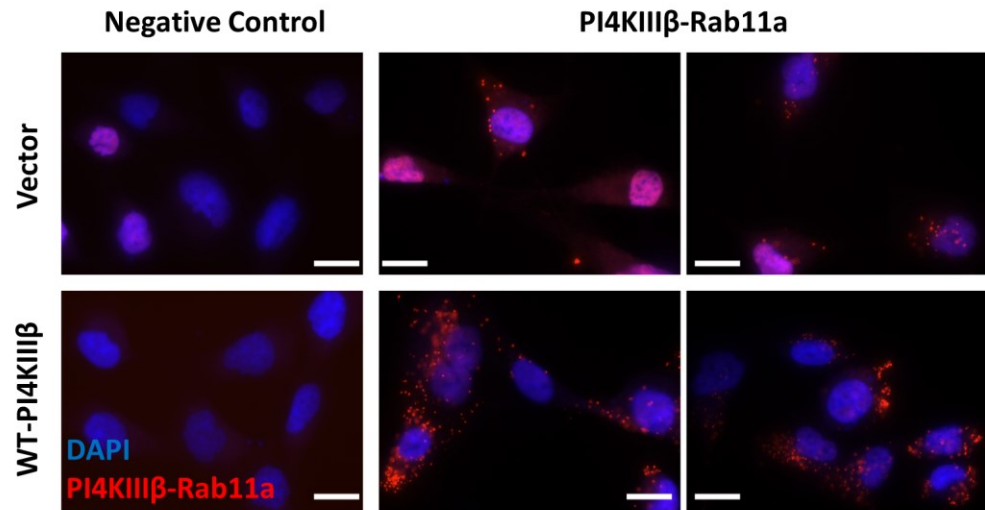
(a) Representative confocal images of paraformaldehyde fixed BT549 wildtype (Wildtype), CRISPR/Cas9 knockout of PI4KIII β (*PI4KB*-null A), PI4KIII β unable to bind Rab11a rescue (*PI4KB*-null + N162A), kinase dead PI4KIII β rescue (*PI4KB*-null + KD), and wildtype PI4KIII β rescue (*PI4KB*-null + WT) human breast ductal carcinoma cell lines after undergoing transferrin uptake assay for specified time. 63x magnification. Scale bars, 50 μ m. (b) The relative transferrin internalized in BT549 Wildtype, *PI4KB*-null A, *PI4KB*-null + N162A, *PI4KB*-null + KD, and *PI4KB*-null + WT cell lines was calculated from images obtained through confocal microscopy by determining the average corrected total cellular fluorescence for 25 cells in each condition per trial. The relative transferrin internalized is presented as the mean $\pm$ SE of the mean, from 3 (Wildtype, *PI4KB*-null A, *PI4KB*-null + N162A, and *PI4KB*-null + KD) or 2 (*PI4KB*-null + WT) independent trials, following background subtraction and normalization to the binding control. Statistical significance (*, $P \leq 0.05$, one-way ANOVA with multiple comparison tests) is indicated.

amount of wildtype PI4KIII β (Figure 3.9). Alternatively, the kinase-dependent and -independent functions of PI4KIII β may cooperate in regulating endosome internalization. Interestingly, this level of kinase-inactive PI4KIII β expression fully rescued recycling, indicating that the kinase function of PI4KIII β is dispensable for endosome recycling. Overall, this suggests that the Rab11a interaction with PI4KIII β promotes endocytic uptake and that this function is additionally enhanced by the catalytic activity of PI4KIII β .

3.4 PI4KIII β interacts with Rab5, Rab7, and EEA1

As we determined that PI4KIII β has an impact on both endosomal recycling and internalization, we speculated that the increased activation of signaling pathways may be due to alterations in the endocytic pathways. We previously reported that ectopically expressed PI4KIII β , both wildtype and kinase-inactive, colocalizes with Rab11a¹³⁵. This is further corroborated by our use of proximity ligation assays, where we observed increased interactions between Rab11a and PI4KIII β in BT549 cells overexpressing wildtype PI4KIII β compared to the vector control (Figure 3.12). This observation, in conjunction with our previous results, suggests that ectopic PI4KIII β engages in a functional interaction with Rab11a¹³⁵. However, PI4KIII β has not been reported to interact with other endosomal proteins, so we used immunofluorescence to further explore this idea. We found that PI4KIII β colocalizes with EEA1 and Rab5, markers of early endosomes, and Rab7, a marker of late endosomes (Figure 3.13). This demonstrates that PI4KIII β binds with other endocytic regulating proteins in addition to Rab11a. We have shown representative images of the colocalization observed in BT549 cells overexpressing wildtype PI4KIII β (Figure 3.13a-c),

a



b

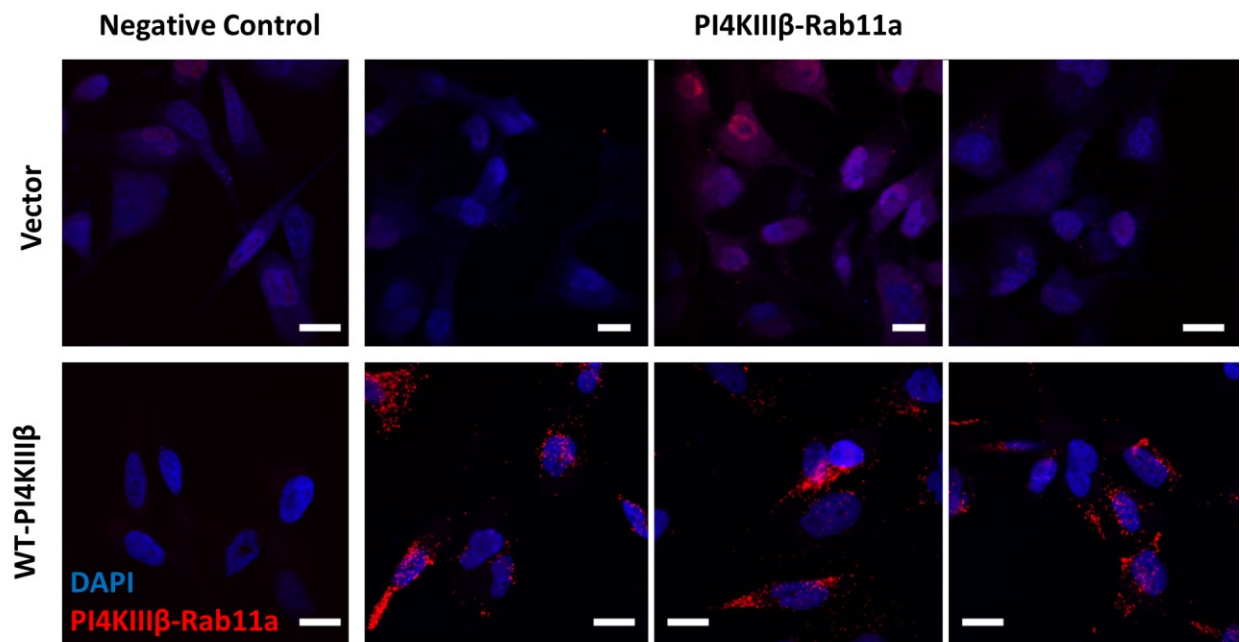


Figure 3.12 Rab11a interacts with ectopic PI4KIII β .

(a) Epifluorescent and (b) confocal images of paraformaldehyde fixed BT549 vector control (Vector) and PI4KIII β -overexpressing (WT-PI4KIII β) human breast ductal carcinoma cell lines following proximity ligation assay. Negative control represents an assay where the primary antibodies for PI4KIII β and Rab11a were not used. A red signal indicates protein-protein interactions within 40nm. 63x magnification. Scale bars, 20 μ m.

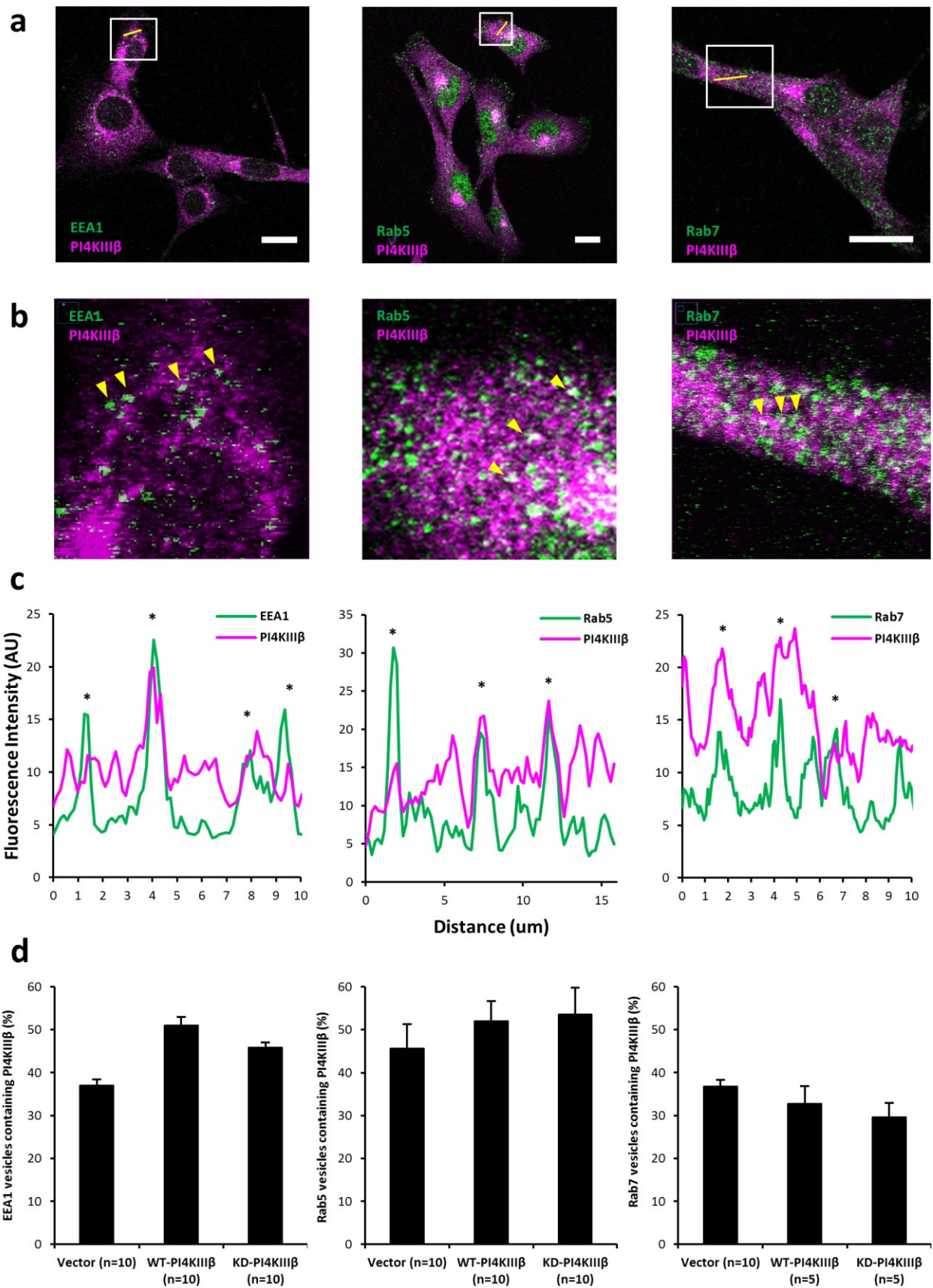


Figure 3.13 PI4KIII β colocalizes with compartments of the endocytic system.

(a) Representative confocal images of EEA1, Rab5, Rab7 (left to right) (green), and PI4KIII β (magenta) in paraformaldehyde fixed BT549 vector control (Vector), PI4KIII β -overexpressing (WT-PI4KIII β) and kinase-inactive PI4KIII β -expressing (KD-PI4KIII β) human breast ductal carcinoma cell lines (images shown are from WT-PI4KIII β cells). 63x magnification. Scale bars, 20 μ m. (b) Zoomed in image of white insets in Figure 3.13a with colocalization marked (yellow arrows). (c) Intensity profile for EEA1, Rab5, Rab7 (left to right) (green), and PI4KIII β (magenta) across the yellow line drawn in Figure 3.13a. Colocalization (*) is indicated. (d) The percentage of EEA1, Rab5, or Rab7 (left to right) vesicles that co-localize with PI4KIII β in BT549 Vector, WT-PI4KIII β , and KD-PI4KIII β cell lines. At least 50 EEA1, Rab5, or Rab7 vesicles were counted per cell and the number of cells counted for each type is shown.

however, this colocalization appears similar between BT549 cells overexpressing wildtype or kinase-inactive PI4KIII β and the vector control (Figure 3.13d).

The colocalization of PI4KIII β with many endocytic proteins strongly supports our model that PI4KIII β plays a role in regulating endosomes. Given the previous evidence for the importance of the PI4KIII β -Rab11a interaction, we focused on the role of PI4KIII β in regulating Rab11a-positive endosomes^{125,132,135,195}. To understand how PI4KIII β expression affects Rab11a recycling endosomes we used immunofluorescence to observe changes in the Rab11a-positive endosomes (Figure 3.14a). We found that there was no difference in the amount, size or shape of the Rab11a vesicles in cells with no, endogenous or high PI4KIII β expression (Figure 3.14b-e). This finding supports our hypothesis that PI4KIII β augments the rate of endocytic recycling by demonstrating that the interaction of PI4KIII β with Rab11a is not related to changes in the gross morphology or number of endosomes.

3.5 PI4KIII β mediated activation of IGF-IR β is dependent on endosome function

Because we saw an increase in the activation of IGF-IR β in cells with high PI4KIII β expression regardless of their kinase activity (Figure 3.1b and c), we next wanted to determine whether or not PI4KIII β activates signaling pathways through endosome function. To this end, we used chlorpromazine, an inhibitor of the formation of clathrin-coated pits, to halt clathrin-dependent endocytosis in BT549 cells³⁰⁵. We observed that chlorpromazine decreases the activation of IGF-IR β in all cell lines (Figure 3.15). This suggests that endocytosis regulates IGF-IR β activation and that the increased activation

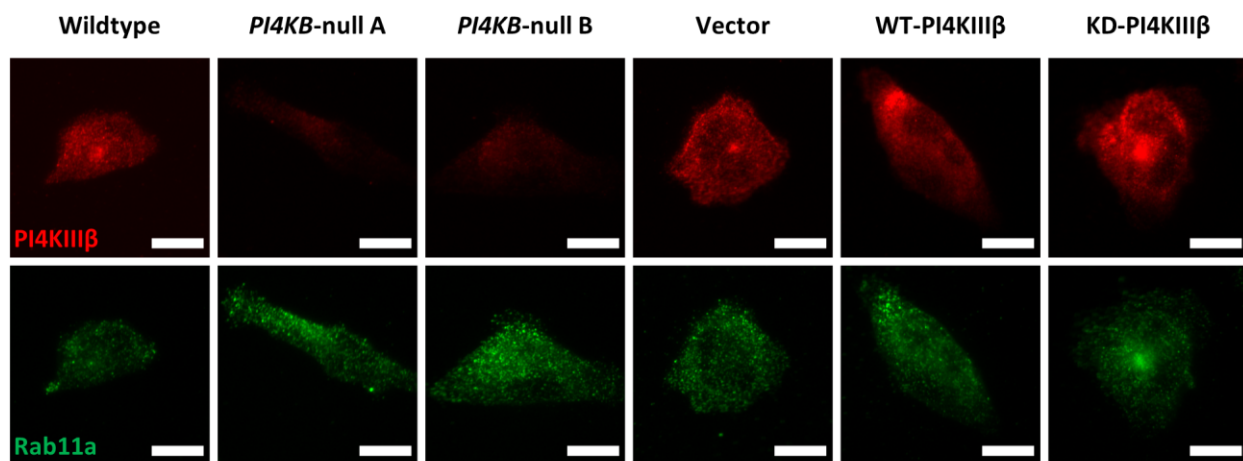
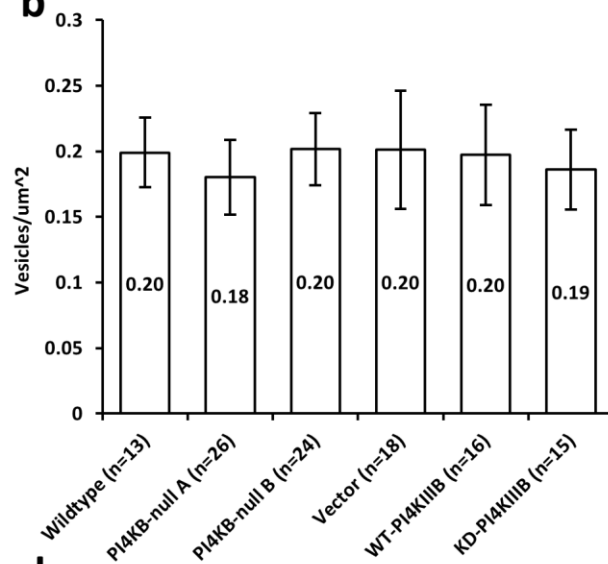
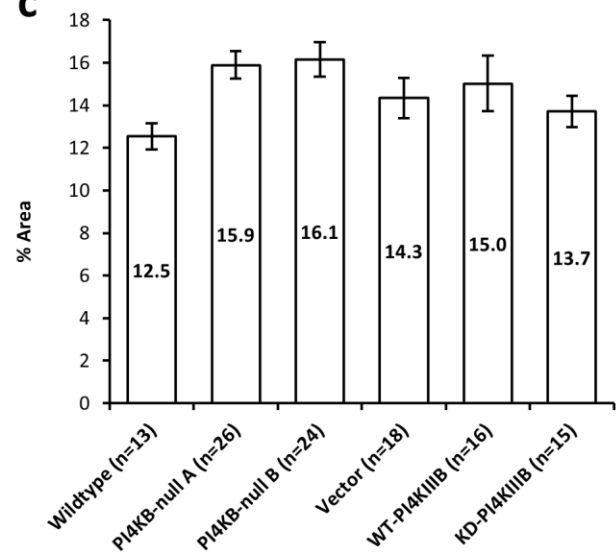
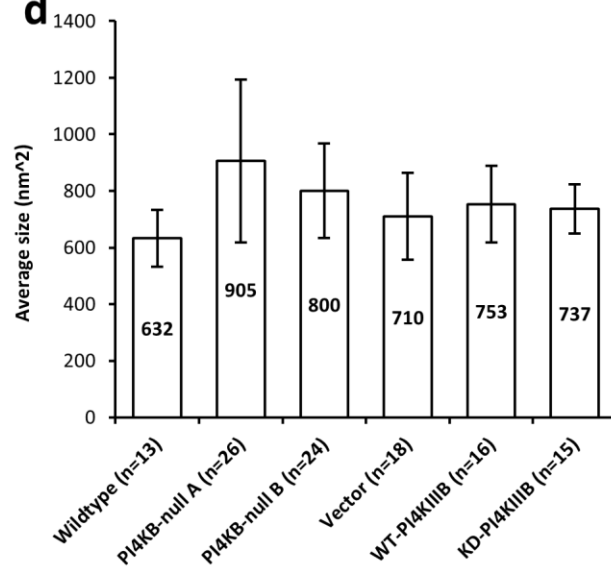
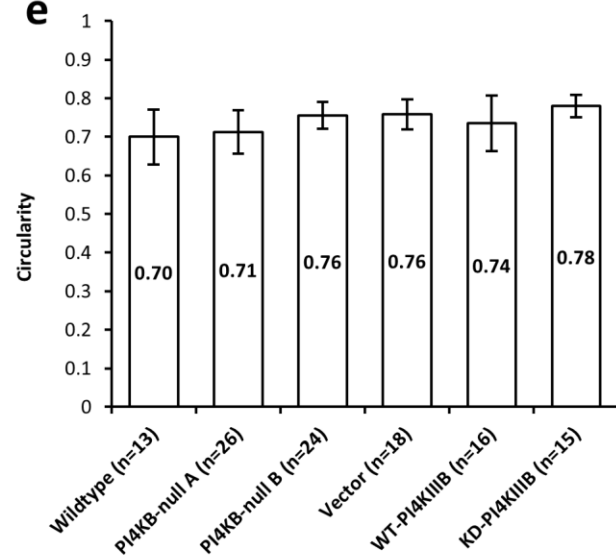
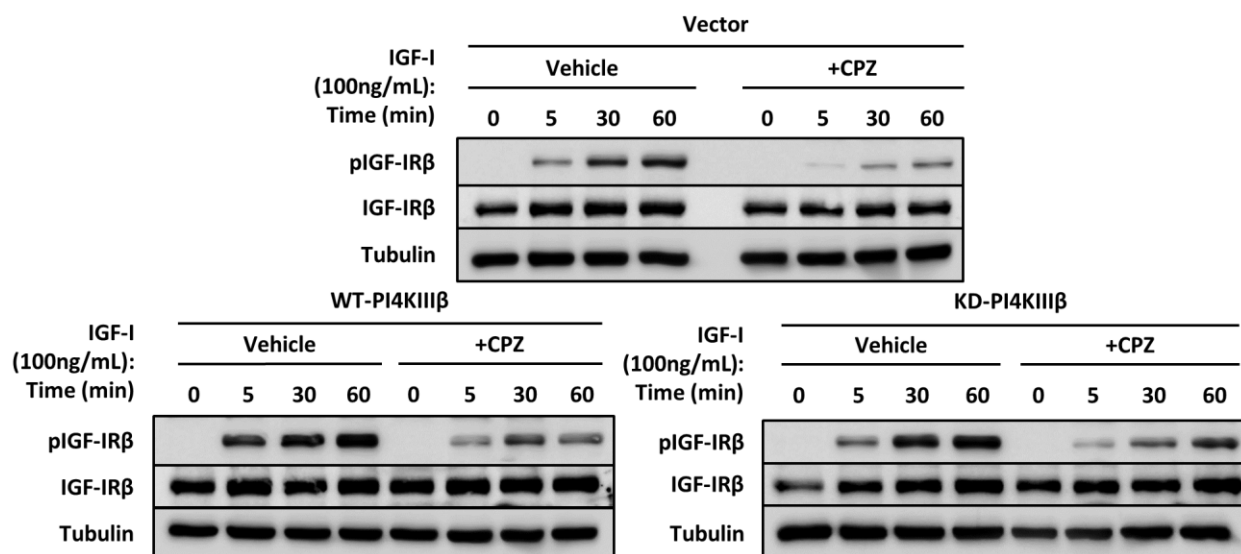
a**b****c****d****e**

Figure 3.14 PI4KIII β does not affect Rab11a recycling endosome number, shape or size.

(a) Representative TIRF images of PI4KIII β (red) and Rab11a (green) in paraformaldehyde fixed BT549 wildtype (Wildtype), two independent CRISPR/Cas9 knockouts of PI4KIII β (*PI4KB*-null A and *PI4KB*-null B), vector control (Vector), PI4KIII β -overexpressing (WT-PI4KIII β), and kinase-inactive PI4KIII β -expressing (KD-PI4KIII β) human breast ductal carcinoma cell lines. 63x magnification. Scale bars, 20 μ m. (b-e) ImageJ was used to quantify the (b) total number of Rab11a vesicles per μ m² of cell, (c) percentage of area of a cell that is occupied by Rab11a vesicles, (d) the average size of Rab11a vesicles and (e) the circularity, with 1 being a perfect circle and 0 being a straight line, of the specified number of cells for each cell line.

a



b

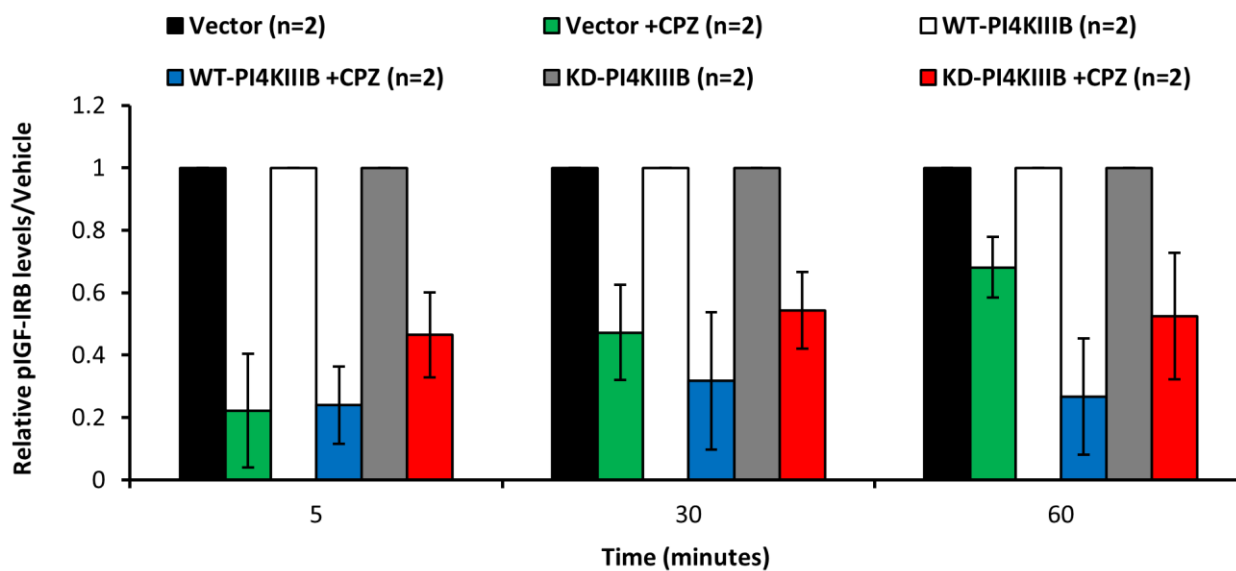


Figure 3.15 Ectopic PI4KIII β regulates IGF-IR β signaling dependent on endosome function.

(a and b) BT549 vector control (Vector), PI4KIII β -overexpressing (WT-PI4KIII β), and kinase-inactive PI4KIII β -expressing (KD-PI4KIII β) human breast ductal carcinoma cell lines were serum starved overnight followed by stimulation with IGF-I (100 ng/mL) in the presence or absence of endocytic inhibitor, chlorpromazine (CPZ) (10 μ g/mL) for the indicated time periods. (a) The lysate was collected and subjected to western blot analysis to determine levels of pIGF-IR β (95kDa) and IGF-IR β (95kDa) with Tubulin (55kDa) as a loading control. Each lane contains 30 μ g of total protein. (b) Protein levels were quantified by densitometry and the data shown represents the mean $\pm$ SE of the mean from 2 independent trials comparing the pIGF-IR β levels relative to the IGF-IR β levels in each cell line in the presence of CPZ, followed by normalization to the vehicle control for the same cell line. Statistical significance (*, $P \leq 0.05$, one-way ANOVA with multiple comparison tests) is indicated.

observed in the PI4KIII β overexpressing cell lines (Figure 3.1b and c) is dependent on endosome function.

3.6 PI4KIII β deletion decreases IGF-IR β activation

Next, we wanted to see how PI4KIII β deletion affected plasma membrane and endosome signaling. To this end, we used the BT549 CRISPR/Cas9 deletion cells we generated (Figure 3.5a). Initially, we observed no significant difference in the activation of IGF-IR β when comparing cell lines with endogenous and deleted PI4KIII β (Figure 3.16a and b). However, when looking at the initial total IGF-IR β expression in these cell lines we found that the CRISPR PI4KIII β deleted cell lines seem to have 1.5-2 times more expression than the wildtype (Figure 3.17). Taking this into consideration suggests that if the total IGF-IR β was equivalent between the cell lines we would see 1.5-2 times less IGF-IR β activation in the PI4KIII β deleted cell lines. Ultimately, our data suggests that PI4KIII β expression regulates IGF-IR β activation.

Next, we wanted to see if IGF-IR β activation was still dependent on endosome function in the absence of PI4KIII β . Again, we used chlorpromazine to inhibit clathrin-dependent endocytosis. Once more, we found that there was a relatively equivalent decrease in IGF-IR β in the wildtype and PI4KIII β -deleted cell lines (Figure 3.16a and c). Overall, this suggests that IGF-IR β activation is dependent on both PI4KIII β expression and endosome function.

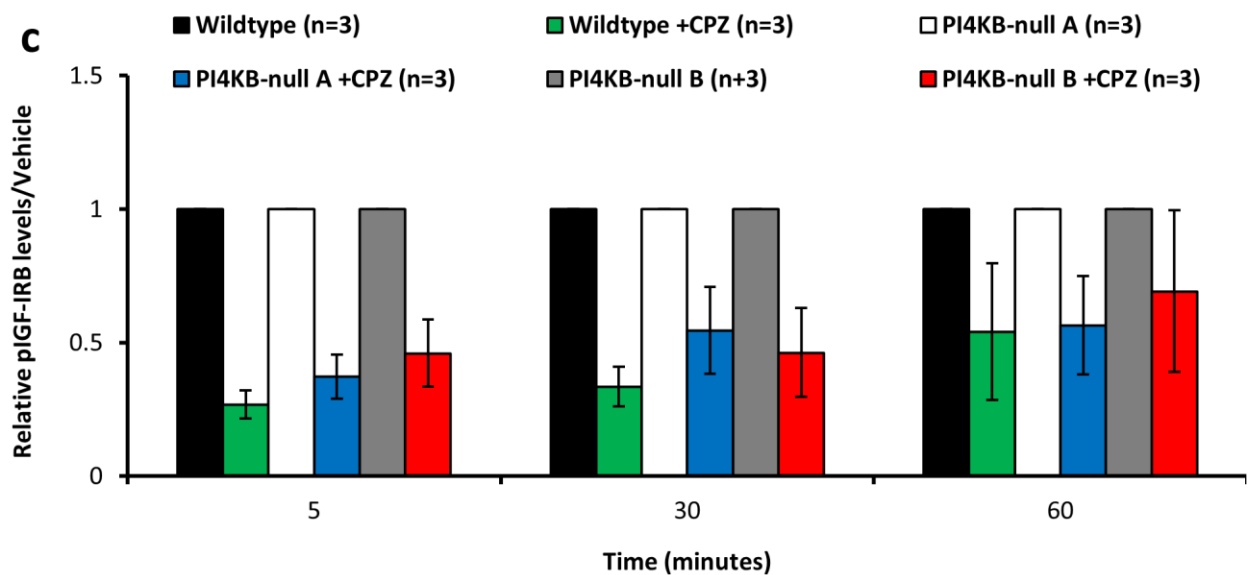
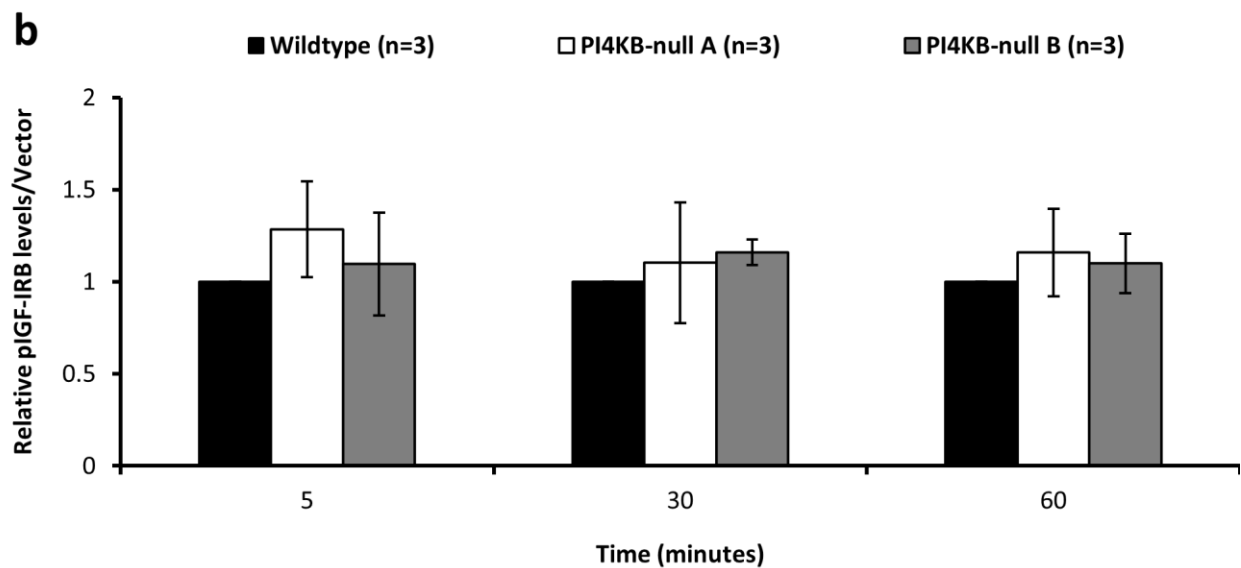
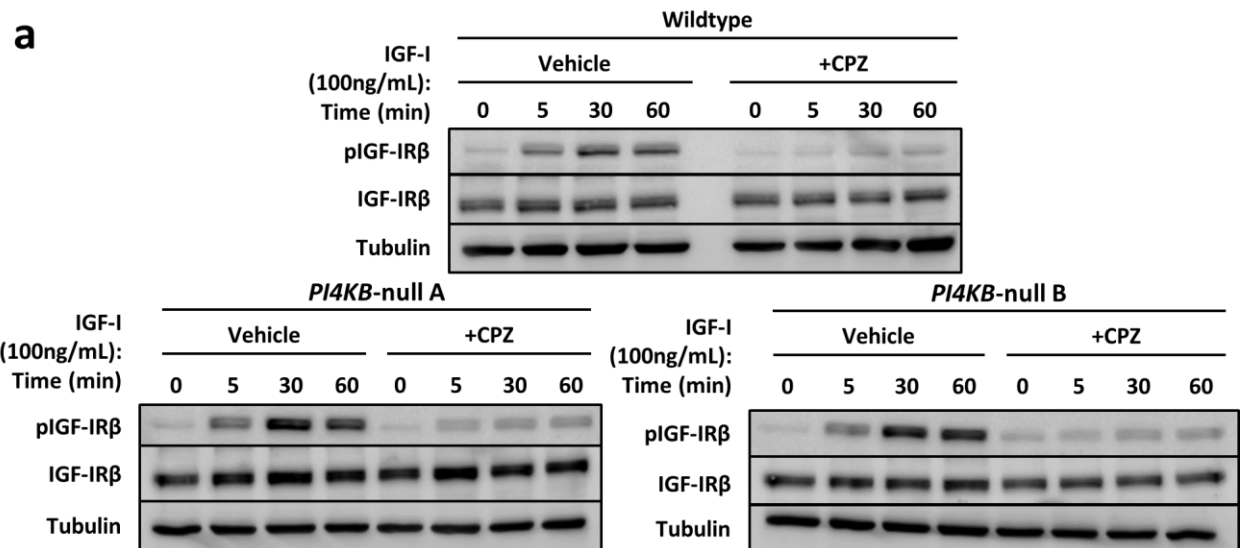


Figure 3.16 IGF-IR β signaling does not appear to be affected by PI4KIII β knockout.

(a-c) BT549 wildtype (Wildtype) and two independent CRISPR/Cas9 knockouts of PI4KIII β (PI4KB-null A and PI4KB-null B) human breast ductal carcinoma cell lines were serum starved overnight followed by stimulation with IGF-I (100 ng/mL) in the presence or absence of endocytic inhibitor, chlorpromazine (CPZ) (10 μ g/mL) for the indicated time periods. (a) The lysate was collected and subjected to western blot analysis to determine levels of pIGF-IR β (95kDa) and IGF-IR β (95kDa) with Tubulin (55kDa) as a loading control. Each lane contains 30 μ g of total protein. (b) Protein levels were quantified by densitometry and the data shown represents the mean $\pm$ SE of the mean from 3 independent trials comparing the pIGF-IR β levels relative to the IGF-IR β levels in each cell line in the absence of CPZ, followed by normalization to the Wildtype. Statistical significance (*, $P \leq 0.05$, one-way ANOVA with multiple comparison tests) is indicated. (c) Protein levels were quantified by densitometry and the data shown represents the mean $\pm$ SE of the mean from 3 independent trials comparing the pIGF-IR β levels relative to the IGF-IR β levels in each cell line in the presence of CPZ, followed by normalization to the vehicle control for the same cell line. Statistical significance (*, $P \leq 0.05$, one-way ANOVA with multiple comparison tests) is indicated.

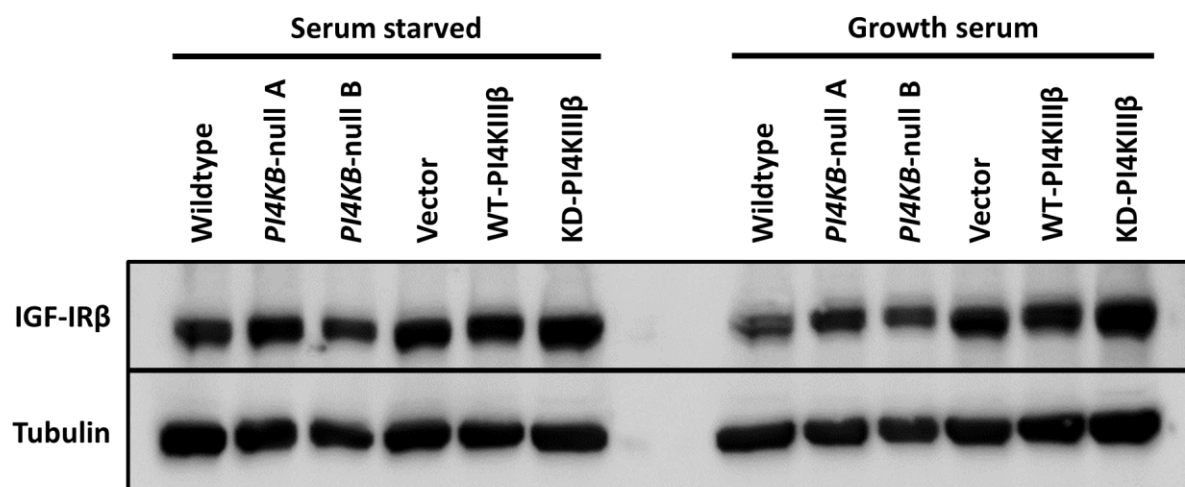
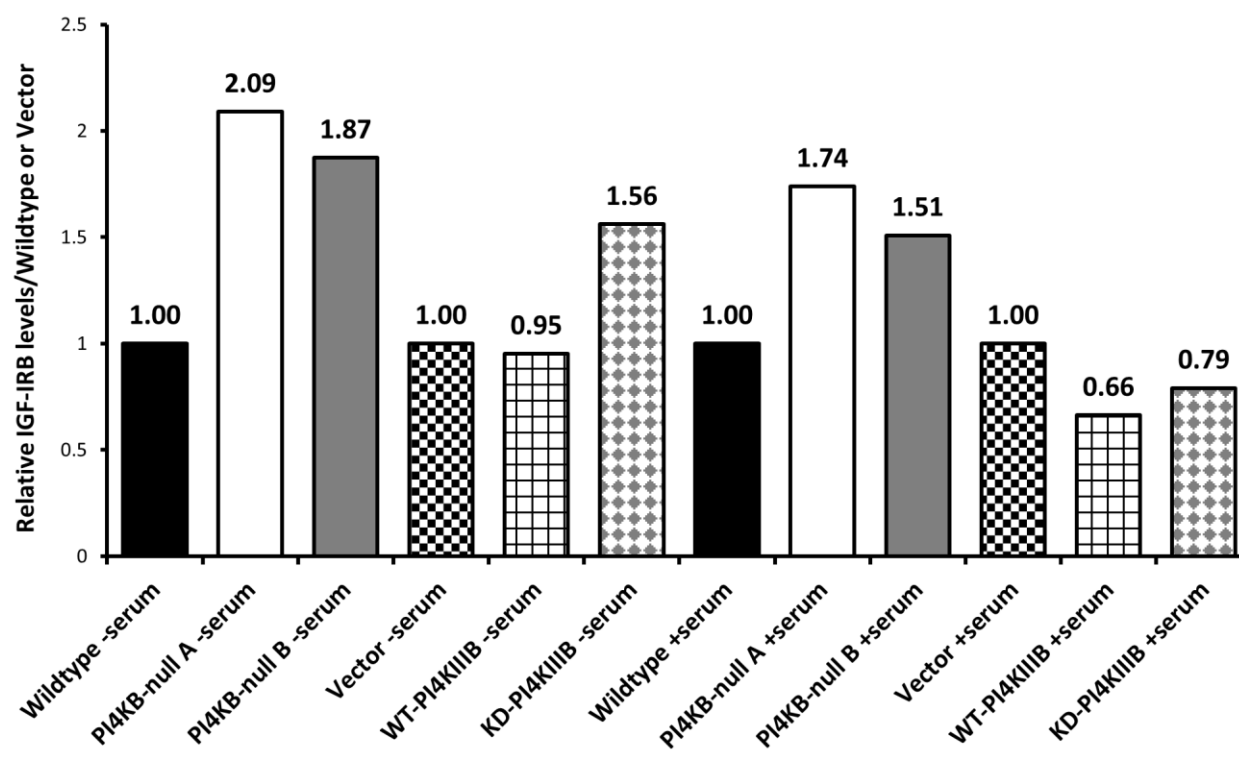
a**b**

Figure 3.17 PI4KIII β deletion causes increased total IGF-IR β .

(a and b) BT549 vector control (Vector), PI4KIII β -overexpressing (WT-PI4KIII β), kinase-inactive PI4KIII β -expressing (KD-PI4KIII β), wildtype (Wildtype), and two independent CRISPR/Cas9 knockouts of PI4KIII β (*PI4KB*-null A and *PI4KB*-null B) human breast ductal carcinoma cell lines were either serum starved or given full growth serum overnight. (a) The lysate was collected and subjected to western blot analysis to determine levels of IGF-IR β (95kDa) with Tubulin (55kDa) as a loading control. Each lane contains 30 μ g of total protein. (b) Protein levels were quantified by densitometry and the data shown represents the mean from 1 trial comparing IGF-IR β levels relative to the Wildtype (*PI4KB*-null A and *PI4KB*-null B) or Vector (WT-PI4KIII β and KD-PI4KIII β) for the same serum condition.

3.7 PI4KIII β regulates mammary tumourigenesis

Finally, we wanted to determine whether or not PI4KIII β might have a role in primary mammary tumour development. To this end, we used CRISPR/Cas9 to delete *Pi4kb*, the mouse *PI4KB* homolog, from the mouse mammary 4T1 tumour cell line (Figure 3.18b). 4T1 is a metastatic derivative of a spontaneous mammary tumour from Balb/c mice³⁰⁶. When injected into the mammary fat pad of syngeneic mice they grow as solid tumours and this model is commonly used to study *in vivo* tumour growth³⁰⁷. Tumour growth was monitored over 15 days post injection. Two independent lines of 4T1 that lack *Pi4kb* either grew substantially slower than control cells or had a delayed onset of growth (Figure 3.18a, c, and d). This retarded tumorigenesis is consistent with the idea that PI4KIII β function is important in mammary tumour development as predicted by our previous studies^{135,136}.

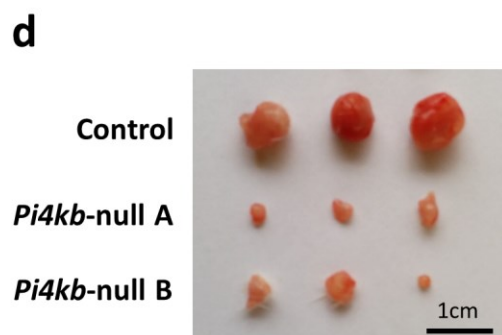
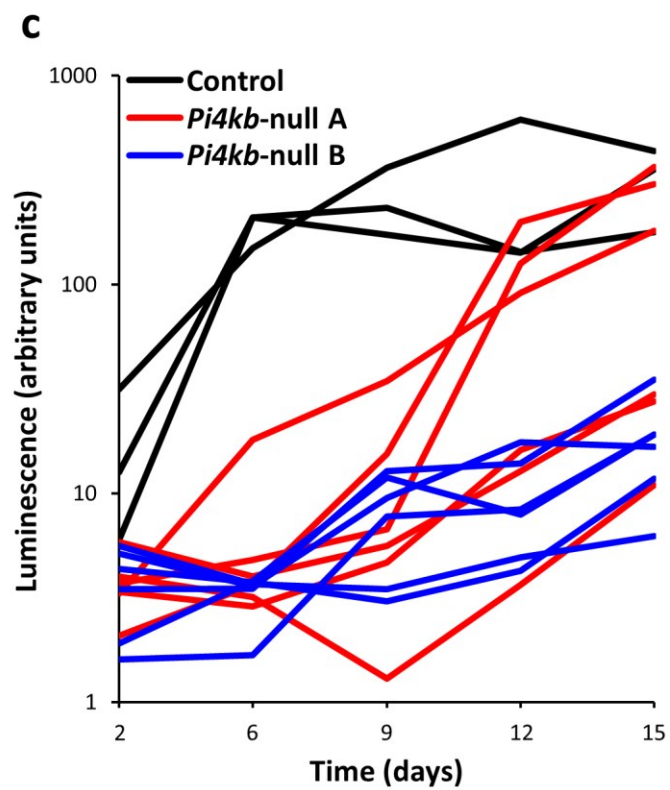
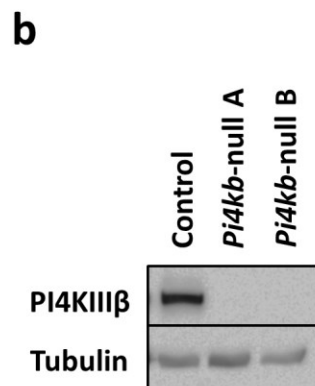
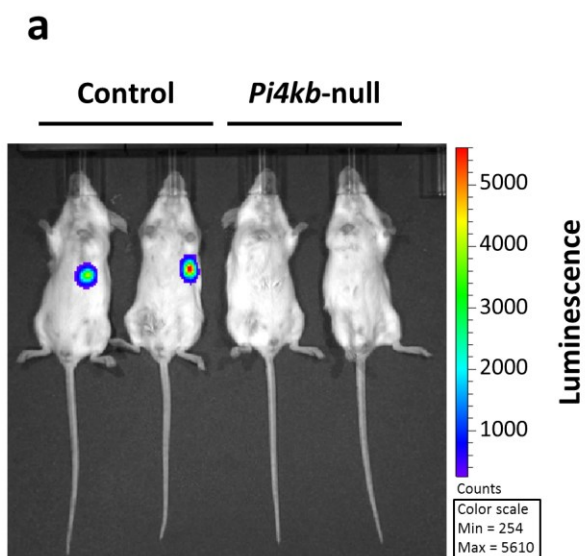


Figure 3.18 *Pi4kb* deletion inhibits mammary tumorigenesis in mice.

(a) Representative image of tumour formation in Balb/c mice injected with control 4T1 cells (Control) and mice injected with *Pi4kb* deleted 4T1 cells (*Pi4kb*-null). The tumours shown are from day 7 post injection. Tumour volume is indicated colorimetrically. (b) Western blot analysis showing levels of PI4KIII β (110kDa) with Tubulin (55kDa) as a loading control in 4T1 Control and two independent CRISPR/Cas9 knockouts of *Pi4kb* gene (*Pi4kb*-null A and *Pi4kb*-null B) cell lines. (c) Tumour development as a function of time in 4T1 Control (black lines), *Pi4kb*-null A (red lines), and *Pi4kb*-null B (blue lines) cell lines. Mice were euthanized at day 15 post injection. The data shown represents the mean from 4 independent experiments. (d) Representative tumours from Control, *Pi4kb*-null A, and *Pi4kb*-null B injected 4T1 cell lines. Tumours are from day 15 post injection.

Chapter 4: Discussion

In this study, we establish an important role for PI4KIII β in the regulation of the endocytic system. We show that ectopic wildtype or kinase-inactive PI4KIII β expression increases the rates of both internalization and recycling of transferrin in BT549 cells. PI4KIII β deletion has the opposite effect, decreasing both internalization and recycling rates. This suggests that PI4KIII β expression regulates the rates of both endocytic internalization and recycling, independent of PI4P generation. Our results are consistent with previous reports from our lab, which found increased colocalization between PI4KIII β and transferrin receptor in cells with high PI4KIII β expression compared to wildtype¹³⁵. Since the transferrin receptor travels through the entire internalization and recycling process, like our chosen marker, transferrin, the colocalization of PI4KIII β and transferrin receptor suggests that PI4KIII β is present throughout this entire process as well.

Interestingly, we find that PI4KIII β -deletion does not appear to affect the gross morphology of the Golgi. PI4KIII β -mediated production of PI4P at the TGN interacts with GOLPH3 and MYO18A to anchor the TGN to the actin cytoskeleton and maintain the TGN structure³⁰. However, a recent study has shown that the reduction or deletion of MYO18A does not disturb Golgi structure, suggesting that the PI4P-GOLPH3-MYO18A complex interaction with F-actin may not be as important for Golgi morphology as previously believed³⁰⁸. In both human breast cancer and mice fibroblast cell lines we saw similar *cis*- and *trans*-Golgi structures between wildtype and PI4KIII β -deleted cell lines. There does, however, appear to be some collapse of the Golgi in the BT549 cell lines relative to the

more well-defined tubular structure observed in the NIH3T3 lines. The collapsed Golgi in the breast cancer cells is consistent with Golgi enlargement and fragmentation, often seen in the Golgi of cancer cells³⁰⁹. We hypothesize that in cells lacking PI4KIII β , PI4KII α compensates for reduced TGN PI4P-pools by producing its own. PI4KII α localizes to both the TGN and endocytic vesicles but has been mostly studied in regards to its role in endosome trafficking from the TGN to the plasma membrane or late endosomes^{117,118,119}. As a PI4K, PI4KII α generates PI4P from PtdIns, therefore, we suspect that when TGN PI4P-pools are depleted due to loss of PI4KIII β , PI4KII α produces PI4P to maintain Golgi structure, in addition to its roles in endocytic trafficking^{117,118,119}. However, the recent evidence demonstrating the dispensability of MYO18A in Golgi morphology suggests that PI4P may also not be necessary for Golgi structure³⁰⁸. In which case, whether or not PI4KII α compensates for decreased PI4KIII β function would be inconsequential, with regards to Golgi morphology. Importantly, the decreased internalization and recycling in PI4KIII β -deleted cell lines is not due to dysregulated Golgi morphology.

Since we see that PI4KIII β expression is proportional to endocytic internalization and recycling rates, we next wanted to see which of the documented roles of PI4KIII β are involved in regulating endocytic function. Using BT549 cells with deleted PI4KIII β , we expressed, wildtype, kinase-inactive, or Rab11a-binding deficient PI4KIII β mutants. Our results suggest that the ability of PI4KIII β to regulate recycling is Rab11a-dependent because the Rab11a-binding deficient PI4KIII β is completely unable to rescue transferrin recycling, but that it is kinase-independent, due to the capacity for the kinase-inactive PI4KIII β to rescue recycling. On the other hand, internalization is partially dependent on

both Rab11a and PI4P generation because we only see a partial rescue of transferrin internalization in Rab11a-binding deficient and kinase-inactive PI4KIII β . However, these results are not entirely consistent with what we had previously seen in our ectopic PI4KIII β expressing cell lines, where both recycling and internalization rates were affected by PI4KIII β expression, independent of PI4P generation. Thus the role of PI4P in PI4KIII β -dependent internalization is likely to be complex.

A possible explanation for this inconsistency is that we were only able to rescue about half as much PI4KIII β in the kinase-inactive rescue cell lines as the wildtype (Figure 3.9), possibly causing the discrepancies observed in internalization. However, recycling does not seem to be affected by this difference in PI4KIII β expression, and therefore, this explanation is unlikely. A more probable explanation for the discrepancy could be because our ectopic overexpressing PI4KIII β cell lines have endogenous wildtype PI4KIII β , as well as ectopic expression of wildtype or kinase-inactive PI4KIII β . Therefore, our kinase-inactive overexpressing cell line does still contain PI4KIII β with kinase activity, while our kinase-inactive rescue cell line has only kinase activity-impaired PI4KIII β . We see that internalization is about 50% dependent on kinase activity, so in our ectopic kinase-inactive cell lines it is possible that the endogenous wildtype PI4KIII β present fulfills the kinase-dependent requirement for internalization and the ectopic kinase-inactive PI4KIII β increases the internalization rate. This would suggest cooperation between the kinase-dependent and -independent roles of PI4KIII β in the regulation of endocytic internalization.

We were surprised to see that PI4KIII β expression had an effect on endocytic internalization. One possible explanation for the PI4KIII β -mediated internalization observed

could be that if the transferrin bound to ferric ions is recycled before the ferric ions dissociate from transferrin or the ions themselves are recycled, the cell has not received any iron and therefore will need to internalize more transferrin to meet its iron needs. In this example, the transferrin is either still bound or in close proximity to the ferric ions, thus interaction and internalization would be facilitated rapidly. Alternatively, internalization is partially dependent on the lipid kinase activity of PI4KIII β . PI4P metabolites, PI(3,4)P₂ and PI(4,5)P₂, are both involved in the formation and internalization of clathrin-coated pits^{59,69,84}. Therefore, it is possible, that either of these explanations separately or in combination, could contribute to the PI4KIII β -mediated increased internalization observed.

Interestingly, we find that both internalization and recycling are dependent, to some extent, on the functional interaction of PI4KIII β with Rab11a. With regards to recycling, this is consistent with the PI4KIII β -Rab11a interaction being required for Rab11a localization and function^{125,132,199}. However, Rab11a has no known role in endocytic internalization, suggesting that the disruption in Rab11a-binding we made in our PI4KIII β (N162A) mutant may be disrupting an interaction between PI4KIII β and another Rab. This led us to explore, how PI4KIII β interacts with the endocytic system. Using proximity ligation assays we corroborated previous reports from our lab and show that not only do PI4KIII β and Rab11a colocalize, they are within 40nm of each other and directly interacting¹³⁵. We also observe that this interaction, again, like our previous reports, tends to be in the periphery of the cell when PI4KIII β is overexpressed¹³⁵. This suggests, that PI4KIII β overexpression causes not only increased interactions with Rab11a but that these interactions occur outside of the typical perinuclear localization²⁰¹. However, we find that

there is no detectable difference in shape, size or number of Rab11a-positive vesicles, regardless of PI4KIII β expression, or activity. Overall, our data suggest, that PI4KIII β expression alters the activity and distribution of Rab11a-positive vesicles, but not the appearance of the vesicles themselves.

Through further investigation of PI4KIII β interactions with the endocytic system, we find that PI4KIII β colocalizes with EEA1 and Rab5, associated with early endosomes, and Rab7, associated with late endosomes^{162,173}. Although we did not find any difference in the amount of colocalization with these endocytic regulating proteins between endogenous and ectopic PI4KIII β expressing cell lines, the novel interactions suggest further potential roles for PI4KIII β in the endocytic system. Therefore, we hypothesize that our Rab11a-binding deficient PI4KIII β cell lines may have had partial inhibition of binding with other Rab proteins, such as Rab5, in addition to the intended Rab11a. Thus, the possible unintended inhibition of PI4KIII β -Rab5 interaction provides another possible explanation for endocytic internalization being, seemingly, partially dependent on the PI4KIII β -Rab11a interaction that we observed. However, further work must be done using mutant PI4KIII β proteins that more specifically inhibit the interaction between Rab11a and the other Rab proteins.

Our data strongly suggests a novel kinase-independent role for PI4KIII β in the regulation of endocytic recycling in cooperation with Rab11a. We hypothesize that PI4KIII β -Rab11a interactions localized at the periphery of cells with high PI4KIII β expression, as in a subset of breast cancer patients, interact with internalized cargo, such as transferrin, sooner than typical, and causes rapid recycling back to the plasma membrane. We propose a novel role for PI4KIII β as a regulator of endocytic kinetics (Figure 4.1). In cells with

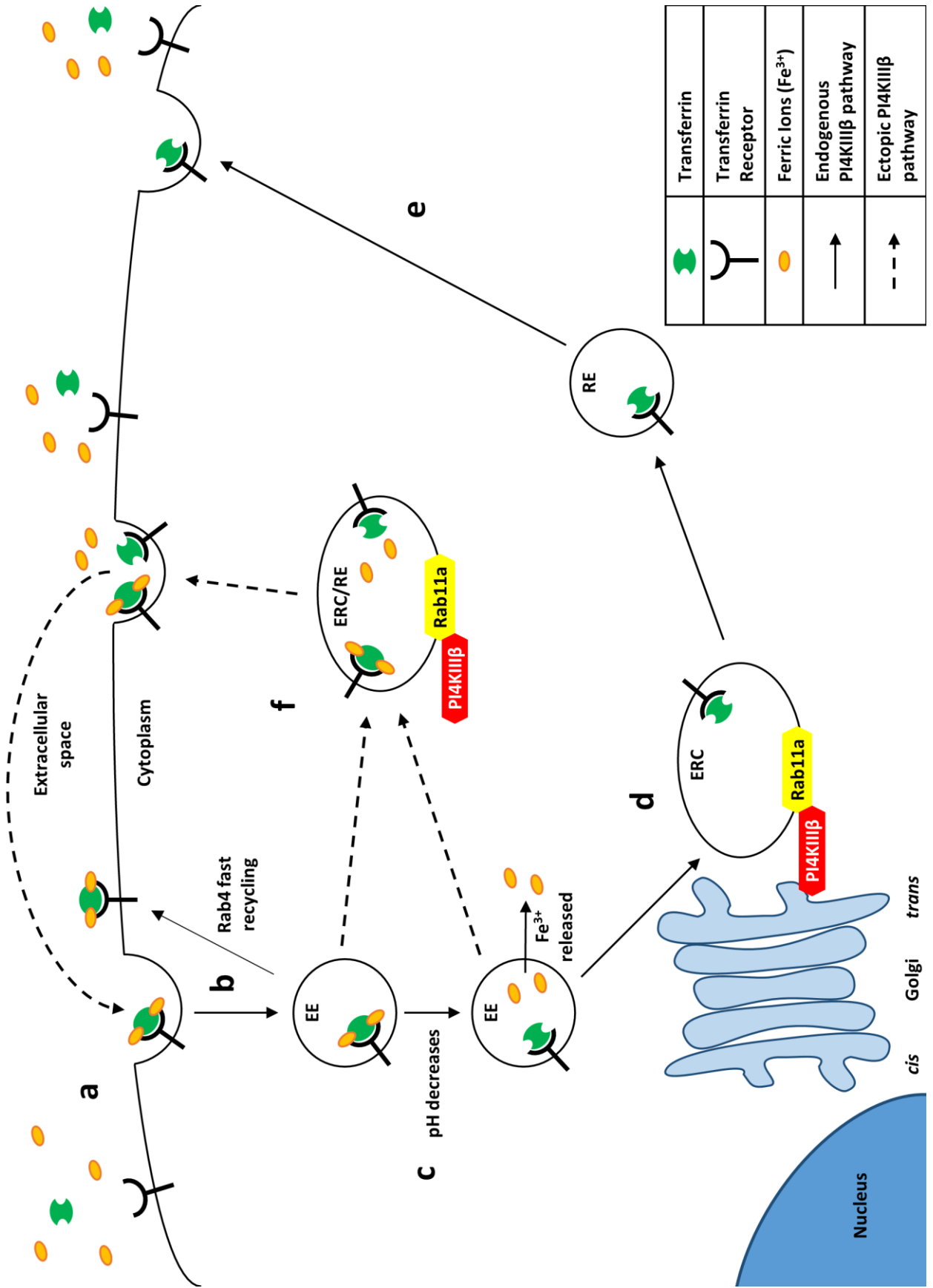


Figure 4.1 PI4KIII β -mediated regulation of endocytic recycling.

(a) Transferrin binds two ferric ions and travels to the plasma membrane where it binds transferrin receptor. (b) The transferrin complex is internalized via clathrin-mediated endocytosis. Endosomes can undergo rapid Rab4-mediated recycling, or continue through the internalization pathway. (c) Early endosomes (EE) mature and their internal pH decreases, causing the release of ferric ions into the cytoplasm. (d) Maturing endosomes travel toward the perinuclear endocytic recycling compartment (ERC), where the transferrin complex will be sorted into recycling endosomes (RE). (e) Recycling endosomes return the transferrin complex to the plasma membrane, where it can gather more ferric ions. (f) Rapidly after internalization, early endosomes interact with PI4KIII β -Rab11a-positive endocytic recycling compartments/recycling endosome-like structures located near the periphery of the cell, where the transferrin complex is recycled back to the plasma membrane to bring in more ferric ions and be internalized again.

endogenous PI4KIII β , transferrin follows a typical route where it is internalized upon binding two ferric ions bring them to the transferrin receptor at the plasma membrane (Figure 4.1a). The transferrin-ferric ions-transferrin receptor complex is internalized via CME. In the early endosomes, some transferrin complex will be rapidly recycled via the Rab4 recycling pathway, but the majority will continue on in the early endosome as it matures (Figure 4.1b). As the early endosome matures, it undergoes a decrease in pH which causes the dissociation of the ferric ions from transferrin, which are transported into the cytoplasm (Figure 4.1c). Transferrin and transferrin receptor remain bound. The transferrin complex is then trafficked toward the perinuclear region, where it enters the ERC. In the ERC cargo is sorted for recycling or degradation. In the case of transferrin complex, the majority is sorted for recycling (Figure 4.1d). Transferrin complexes are then returned to the plasma membrane to continue the cycle (Figure 4.1e). On the other hand, in cells with high PI4KIII β expression, we hypothesize that the internalized transferrin complex will interact with the abundant PI4KIII β -Rab11a interaction-positive ERC/RE-like structures near the periphery of the cell. This will facilitate the rapid recycling of the transferrin complex out of the cell to repeat the cycle (Figure 4.1f).

To further verify this model the next step would be to track transferrin movement through the endocytic system with live cell microscopy. Initially, we would start by creating cells with fluorescently labelled PI4KIII β and/or Rab11a, then we would incubate the cells with labelled transferrin. This would provide us with data such as, on average how long transferrin takes to be taken up and recycled, and where the transferrin goes in the cell and what happens there. For example, we could determine if transferrin recycling occurs more

at the TGN or at the peripherally located PI4KIII β -Rab11a interaction sites, if at all. A more elegant, but potentially difficult solution would be to use a protein-fragment complementation assay in combination with the live cell tracking of transferrin movement³¹⁰. By attaching part of a marker, for example, GFP, to PI4KIII β and another part to Rab11a, we would only see a signal when the proteins are directly interacting³¹⁰. This way we would be certain that if transferrin passes through areas of signal, it is from the PI4KIII β -Rab11a interaction and not just their colocalization. Finally, although Rab11a appears to have a large role in the PI4KIII β -mediated recycling and potentially internalization as well, our data suggests that PI4KIII β may be more extensively involved in the endocytic system. Therefore, we could perform similar experiments to these described, but with different Rab proteins, such as Rab4, Rab5, and Rab7, to explore the rapid recycling, uptake, and degradation pathways, respectively.

Signaling endosomes and PI4KIII β

Two primary locations of biochemical signaling have been identified; the plasma membrane and the surface of endosomes²⁴⁷. At the plasma membrane, in general, ligand binds to the extracellular domain of a transmembrane protein, leading to a conformational change in the receptor protein promoting signal transduction^{249,250,255}. Signaling on the surface of endosomes can involve the same receptors, however, due to endocytosis, it occurs in endosomes rather than at the plasma membrane^{168,289}. The unique and evolving environments of endosomes provide opportunities for signal transduction that cannot occur at the plasma membrane^{168,289}. Signaling is largely regulated by the availability of receptors at the plasma membrane to bind ligand and activate signaling cascades

throughout the cell^{289,290}. The control of receptor availability is through the endocytic system^{289,290}. When a cell requires more activation of a pathway, internalized receptors will be recycled back to the plasma membrane^{289,290}. However, when the cell no longer requires signal activation the receptors will be sent to the lysosome and degraded^{289,290}. Additionally, the control of signaling on signaling endosomes occurs by the constant change of the endosome composition and environment as they mature and travel through the endocytic cycle²⁸⁹. Our lab has previously shown that PI4KIII β is involved in Akt activation, and here, in this thesis, we demonstrate its role in endocytic dynamics¹³⁵. We next sought to determine whether PI4KIII β has a role in the regulation of signaling pathways, through its control of the endocytic system.

Initially, we wanted to see if PI4KIII β was able to activate any additional signaling pathways, as our lab had previously demonstrated with Akt¹³⁵. We find that PI4KIII β expression regulates IGF-IR β activation, independent of the generation of PI4P. We see a significant increase in the intensity of IGF-IR β activation in ectopic wildtype or kinase-inactive PI4KIII β expressing cell lines, suggesting the kinase-independent role of PI4KIII β . Concomitantly, we see that there does not appear to be a difference in IGF-IR β signaling between PI4KIII β -deletion and wildtype cell lines. However, when we look at total IGF-IR β , we find that cell lines lacking PI4KIII β have 1.5-2 times more than the wildtype cell line. This suggests that in PI4KIII β -null cell lines, more IGF-IR β is produced in order to compensate, as less is being activated. Thus, if the western blots were re-done so that total IGF-IR β was equivalent, rather than tubulin, we would find decreased activation of IGF-IR β . Overall, this suggests that PI4KIII β promotes the activation of IGF-IR β .

Additionally, we observe that ectopic wildtype PI4KIII β expressing cell lines increases the intensity of A-Raf activation compared to ectopic kinase-inactive PI4KIII β expressing and vector control cell lines, suggesting a kinase-dependent role for PI4KIII β -mediated activation. Shc, on the other hand, is more complicated due to its multiple isoforms, but overall, has both increased intensity and duration of activation of ectopic wildtype or kinase-inactive expressing PI4KIII β cell lines compared to the vector. Shc has three isoforms with both overlapping and unique roles²⁸³. We see that ectopic PI4KIII β expression causes the largest increase in activation in Shc46, at 3-4 times that of the vector cell line. Shc46 and Shc 52 are responsible for the activation of the MAPK/Erk, which promotes cell division and proliferation²⁸⁴. Conversely, Shc66 inhibits MAPK/Erk activation^{285,286}. Since we only have data from one trial both A-Raf and Shc activation and the 5-minute time point for the kinase-inactive PI4KIII β expressing cell lines is inadequately loaded, we cannot make any conclusive remarks about these results. That being said, we speculate that further examination of A-Raf, Shc, and their downstream effectors in the MAPK/Erk pathway would provide valuable insight into how deep PI4KIII β expression penetrates signal transduction pathways.

In addition to activating downstream signaling pathways, IGF-IR β , upon activation is internalized via CME²¹⁴. Once internalized, receptors have been shown to continue signal transduction on the surface of signaling endosomes²⁸⁹. Since we observe that PI4KIII β is able to activate multiple signaling pathways both originating extra- and intracellularly we wanted to see whether endocytosis was involved in these pathways. Our data shows that inhibition of CME by chlorpromazine had a relatively equivalent diminishing effect on IGF-

IR β activation between the PI4KIII β overexpressing and vector cell lines and the PI4KIII β -deletion and wildtype cell lines. This suggests that regardless of PI4KIII β expression a relatively equal proportion of IGF-IR β activation is occurring after internalization, on the surface of signaling endosomes. Because this signaling endosome activity is relatively proportional and we see increasing IGF-IR β activation with increasing PI4KIII β expression, this suggests that as PI4KIII β expression increases so does the amount and/or activity of signaling endosomes. We have only used chlorpromazine to inhibit CME, as this is the primary route of internalization for RTKs, such as IGF-IR β ^{214,305}. However, RTKs are able to enter via other mechanisms, such as CDE, to a lesser extent ²¹⁴. It is possible that when we inhibited CME, the cells compensated and internalized IGF-IR β through one of the other pathways of internalization. Therefore, it would be prudent, in the future, to investigate if this is the case by using inhibitors of other internalization pathways alone or in concert and observe how IGF-IR β activation is affected. Additionally, since A-Raf, Shc, and Akt are intracellular second messengers it would be interesting to investigate how endocytic inhibition affects them. Like it has for IGF-IR β , endocytic inhibition, would allow us to determine if the PI4KIII β -mediated increased A-Raf, Shc, and Akt is at the plasma membrane or on signaling endosomes ¹³⁵.

Building on our proposed model of PI4KIII β -mediated control of endocytosis (Figure 4.1), we propose that this control of endocytosis, in turn, regulates cell signaling (Figure 4.2). In cells with endogenous PI4KIII β transmembrane receptors, such as RTKs or GPCRs, bind extracellular ligand, dimerize and transautophosphorylate their intracellular catalytic domains (Figure 4.2a). The phosphorylation of the catalytic domains leads to the activation

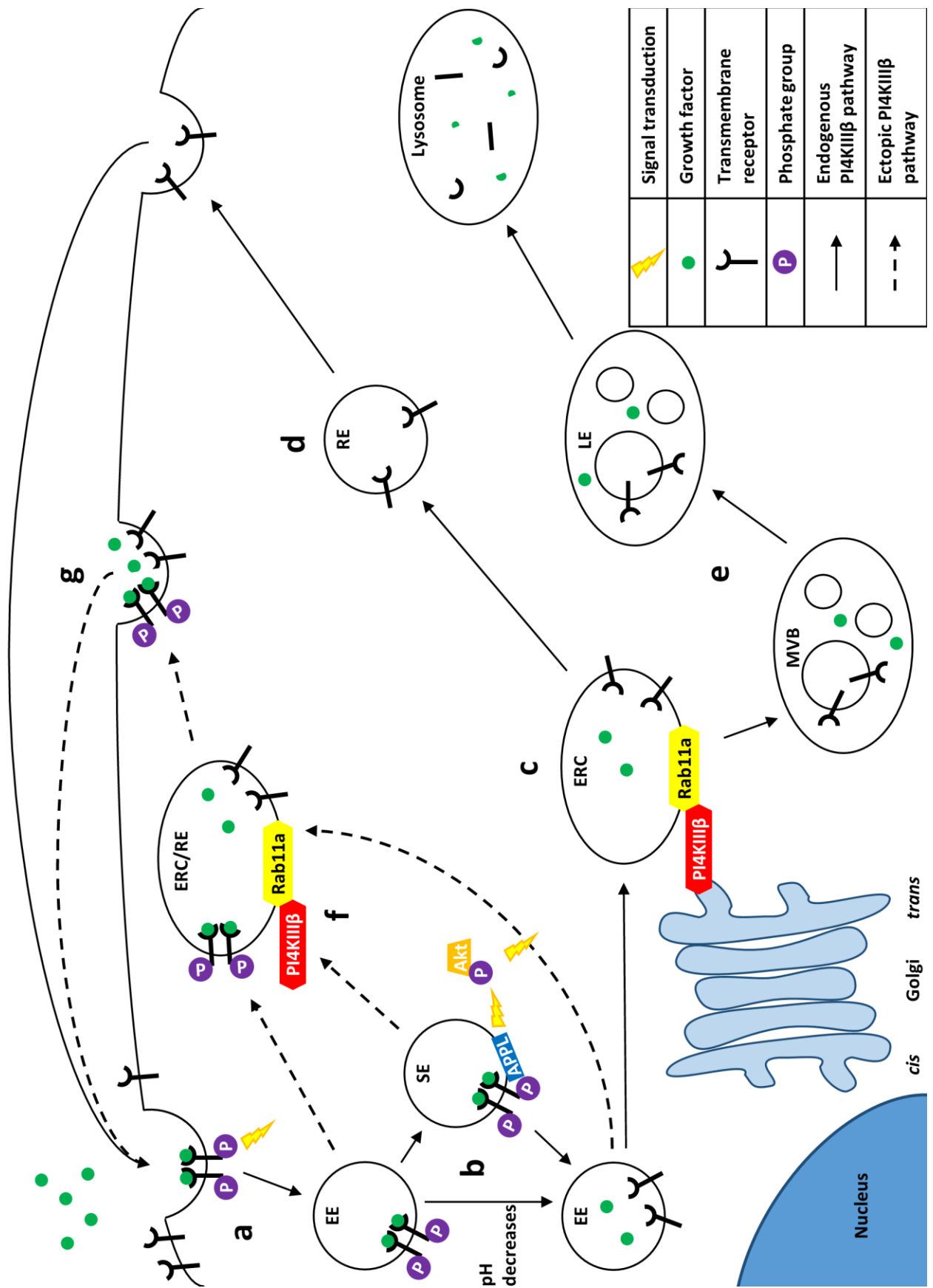


Figure 4.2 PI4KIII β -mediated receptor signaling via regulation of endocytosis.

(a) Transmembrane receptors bind ligand and dimerize, causing transautophosphorylation of intracellular domains and propagation of signal transduction. The receptor complex is internalized via clathrin-mediated endocytosis. (b) Early endosomes (EE) mature and their internal pH decreases, causing the dissociation of ligand and dimerized receptors. The diverse environment of endosomes can provide opportunities for unique signal transduction events to occur on the surface of endosomes, called signaling endosomes. Numerous signaling pathways occur via signaling endosomes; APPL-mediated Akt activation is shown in this figure. (c) Maturing endosomes travel toward the perinuclear endocytic recycling compartment (ERC), where the receptor complex and ligand will be sorted into recycling endosomes (RE) or multivesicular bodies (MVB). (d) Recycling endosomes return the transmembrane receptors to the plasma membrane, where they can bind more ligand and continue signal transduction. (e) Multivesicular bodies mature into late endosomes and fuse with a lysosome, where the receptor complex and ligand are degraded to terminate signal transduction. (f) Rapidly after internalization, early and signaling endosomes interact with PI4KIII β -Rab11a-positive endocytic recycling compartments/recycling endosome-like structures located near the periphery of the cell, where the receptor complex and ligand are recycled back to the plasma membrane. (g) Still bound or close-proximity ligand-receptor complexes are reactivated, initiating further signal transduction and internalization.

of downstream effector proteins that can have numerous effects on the cell, including promoting survival, proliferation, and invasion. Eventually, the dimerized receptor-ligand complex is internalized via CME. In early endosomes, the receptor complexes have a few different possible fates (Figure 4.2b). They can be quickly recycled back out of the cell via Rab4-mediated rapid recycling (not shown in Figure 4.2) or as the endosome matures, the decreasing pH causes dissociation of the receptor complex and terminates signaling. Alternatively, before dissociation it is possible for unique signaling events to take place on the surface of the endosome. APPL-mediated activation of Akt is shown in Figure 4.2, but there are numerous different pathways that can be activated depending on the plasma membrane receptor activated and the composition of the endosome compartment it enters (see section 1.10). Ultimately, however, the dissociated ligand and complex will traffic toward the perinuclear ERC (Figure 4.2c). Here, the cargo will enter either the recycling pathway, where the components will be brought back to the plasma membrane to allow for further signaling (Figure 4.2d) or enter the degradative pathway to be catabolized and terminate signaling (Figure 4.2e). However, like with the movement of transferrin, in cells with high expression of PI4KIII β , we hypothesize that shortly after internalization the endosomes containing receptor complexes will interact with the PI4KIII β -Rab11a-positive ERC/RE-like structures, that are located near the periphery of the cell in abundance in PI4KIII β -high cells. Here, the ligand-receptor complexes or close-proximity ligand and receptor will be recycled back to the plasma membrane, where they will be promptly reactivated and internalized.

The rapid reactivation of receptor complexes will have two major effects on signaling; increased intensity and duration. First, it will cause the activation of the signaling pathways at the plasma membrane surface, as well as the generation of numerous signaling endosomes propagating their own signaling events, ultimately, these events in cooperation will lead to more intense signaling events. Recently, Rohatgi *et al* have shown, similarly, that regulation of endocytic kinetics can increase the duration and intensity of signaling events³¹¹. Similarly, the upregulation of PI4KIII β we present here, Rohatgi *et al* demonstrated how knockdown of beclin 1 prevents the maturation of early endosomes³¹¹. Beclin 1 stimulates the production of PI3P, which causes early endosomes to mature and shed APPL when stimulated with growth factors³¹¹. In breast tumours, beclin 1 is often downregulated, and as such, early endosomes are unable to shed APPL³¹¹. As discussed previously, one mechanism of signaling endosomes is the recruitment and activation of Akt by APPL, and the termination of this signaling when APPL is shed^{168,302,303}. Beclin 1 downregulation is associated with increased in Akt and Erk activation³¹¹. In this study, increased PI4KIII β could be acting similarly, however, instead of prolonging signaling intensity and duration on endosomes by halting maturation, PI4KIII β does it by promoting rapid recycling and internalization of receptors, causing constant generation of numerous signaling endosomes.

Second, if the receptors are constantly being internalized and recycled, there is likely decreased degradation, leading to prolonged signaling duration. Receptors are often ubiquitinated either as a means of internalization or later for degradative targeting^{293,294}. Cbl is an E3 ubiquitin ligase that binds to phosphotyrosines, such as those on activated

RTKs, and promotes their ubiquitination³¹². ESCRT complexes sort ubiquitinated proteins toward the lysosomes for degradation^{20,293,294,313}. Cancer cells have adapted methods to evade receptor ubiquitination³¹⁴. For example, mutations in RTKs, such as a mutation in EGFR on the Cbl binding site that impedes internalization and promotes recycling of receptor that is internalized, rather than degradation^{315,316}. Additionally, cells can alter Cbl activity either by altering Cbl binding domains, or sequestering Cbl away from RTKs, both preventing Cbl from interaction with RTKs^{317,318}. In addition to impaired ubiquitination, abnormal ESCRT complex activity is frequently found in human cancers and is capable of transforming normal cells into invasive cancer cells^{319,320}. It is possible that PI4KIII β regulation of endocytic dynamics, negatively affects some aspect of endocytic degradation, in addition to the increase in internalization and recycling rates we observe.

However, further work must be done to investigate a role for PI4KIII β in endocytic degradation. As mentioned earlier, we found that PI4KIII β regulates the rate of both endocytic internalization and recycling. We also saw that PI4KIII β is likely more involved in the endocytic system than just that, due to its interactions with endocytic regulating proteins such as Rab7, associated with the degradative pathway. As a major mechanism of signaling regulation is through the recycling or degradation of receptors and ligands, it will be important in the future, to look at whether PI4KIII β has a role in the degradative pathway of endocytosis. PI4KII α is involved in the trafficking of EGFR for degradation via lysosomes, therefore it is possible a similar role may exist for PI4KIII β ¹¹⁹. EGFR, unlike transferrin, is often degraded, rather than recycled, therefore, fluorescently labelled EGFR could be used to follow the degradative pathway in order to explore how PI4KIII β affects

this aspect of endocytosis¹⁸². Additionally, we see that PI4KIII β regulation of both endocytic internalization and recycling requires a functional interaction with Rab11a, which is consistent with PI4KIII β -mediated activation of Akt requiring the presence of Rab11a¹³⁵. Since our data suggests that PI4KIII β regulates the IGF-IR β pathway through endocytosis, further work must be done exploring whether the PI4KIII β -Rab11a interaction is involved in this signaling pathway as well. We can use our Rab11a-binding deficient PI4KIII β cell lines, in order to examine how the loss of interaction between PI4KIII β and Rab11a affects signaling pathways.

PI4KIII β and cancer

Both the PI4KIII β protein and *PI4KB* gene are frequently highly expressed or amplified in a subset of primary breast tumours^{134,135}. Although PI4KIII β is the least well-studied PI4K in regards to its role in cancer, evidence, much of it produced in Dr. Lee's lab, suggests PI4KIII β has an important role in breast and ovarian cancer¹³³. PI4KIII β acts downstream of the oncogene, eEF1A2, which is, itself, frequently upregulated in both breast and ovarian cancers^{321,322,323}. eEF1A2-mediated activation of PI4KIII β is responsible for the movement of cancer cells through the formation of filopodia¹³⁷. Additionally, PI4KIII β expression inhibits apoptosis in MDA-MB-231 breast cancer cells and disrupts acinar morphogenesis in normal breast cells^{136,138}. Finally, as mentioned, our lab has recently shown that PI4KIII β is responsible for direct activation of Akt¹³⁵. Tissue invasion, apoptotic evasion, disruption of morphogenesis, and sustaining proliferative signaling are all hallmarks of cancer³²⁴. PI4KIII β has roles in all of these hallmarks and as such is likely an oncogene. However, despite the mounting evidence implicating PI4KIII β as an oncogene,

there is no previous data showing how PI4KIII β affects tumour growth *in vivo*. Here, although the exact mechanism has yet to be uncovered, our data reveals a novel role for PI4KIII β in the regulation of endocytosis. Endocytosis controls signaling, primarily by controlling the intensity and duration^{289,290}. As such, dysregulated endocytosis has been implicated in the transformation of cancer cells through proliferative signaling, loss of polarity and invasive capabilities^{314,325}. This lead us to investigate how PI4KIII β affects the formation of tumours in mice.

Here we find that *Pi4kb* deletion in mouse mammary cancer cells inhibits their ability to initiate tumour growth and development in syngeneic mice. The decreased capacity of *Pi4kb*-null cells to invade and initiate tumour growth, suggests that when these cells were injected into the mammary fat pads they were less capable of invading the normal mammary tissue than the *Pi4kb*-expressing cells. We can conclude that neither the *Pi4kb*-null or *Pi4kb*-expressing cells traveled to distal locations and colonize there, as we do not see any tumours in other locations in these mice. This suggests that the *Pi4kb*-null cells took longer to invade and initiate tumour growth and that those cells unable to may have undergone apoptosis. These results are consistent with previous literature suggesting eEF1A2 promotes invasion and formation of filopodia through PI4KIII β and that PI4KIII β has anti-apoptotic effects^{135,137,138,326}. Furthermore, dysregulation of endocytosis can lead to the formation of filopodia and invasive capabilities in cancer cells³²⁷. Consistent with our previous results, cells lacking PI4KIII β have decreased endocytic activity and therefore, may be less motile and invasive. This may explain why the *Pi4kb*-null cells were less capable of invasion than the *Pi4kb*-expressing cells. Although we see that PI4KIII β -deletion reduces the

rate of tumour development in a syngeneic mouse model, an important next step would be to develop transgenic mice colonies with variable, low, endogenous, and high, PI4KIII β expression in their mammary tissue in order to observe how PI4KIII β expression affects spontaneous tumour formation and mammary epithelial morphogenesis.

Conclusion

We propose a novel role for PI4KIII β as a regulator of endocytic kinetics, further implicating it as a breast cancer oncogene. In this thesis, we have demonstrated that PI4KIII β regulates both endocytic internalization and recycling, with this regulation being partially or completely independent of the lipid kinase role of PI4KIII β , respectively. Additionally, we discovered that through this control of endocytosis, PI4KIII β also regulates receptor signaling, both at the plasma membrane and the surface of endosomes. Finally, we provide the first *in vivo* evidence that expression of PI4KIII β in cancer cells promotes the initiation and growth of tumours in mice.

Future directions

More work must be done to have a better understanding of the role of PI4KIII β in both endocytosis and cancer. We found that in order to regulate both endocytic internalization and recycling, PI4KIII β required a functional interaction with Rab11a. This is consistent with previously documented roles for Rab11a in recycling. However, Rab11a has no role in internalization²²⁸. As such, further work must be done to explore the role of PI4KIII β and its interactions with other Rab proteins, including Rab5 and Rab7, in order to determine if our Rab11a-binding deficient PI4KIII β was not preventing the binding of

Rab11a exclusively, or if we have uncovered a novel role for Rab11a in the internalization of endosomes. Additionally, we hypothesize that the PI4KIII β -Rab11a interactions at the periphery of the cell are shunting endosomes and cargo away from the degradative pathway and keeping them in the recycling pathway, however, transferrin is rarely degraded²¹⁵. Therefore, further experiments must be done investigating the role of PI4KIII β in the degradative pathway. PI4KII α is required for the trafficking of EGFR to the late endosomes and lysosomes for degradation, however, if PI4KIII β has a similar role at endogenous or ectopic expression levels remains to be determined¹¹⁹. Thus, similar uptake and pulse-chase experiments could be performed, however, substituting transferrin with EGFR. This would provide the opposite point of view, as EGFR is primarily degraded and occasionally recycled¹⁸². Additionally, the tracking of transferrin movement with live cell microscopy would provide us with the opportunity to understand the differences in endocytosis between cells with variable PI4KIII β expression levels through the divergence in the paths transferrin takes.

Furthermore, we determined that PI4KIII β expression promotes the activation of IGF-IR β , A-Raf, and Shc signaling, in addition to the previously documented increase in Akt signaling¹³⁵. Additionally, we found that the activation of the plasma membrane receptor, IGF-IR β , was subdued when CME was inhibited. From this, we concluded that PI4KIII β promotes receptor signaling both at the plasma membrane and the surface of endosomes. Through the use of chlorpromazine, we inhibited CME, which is the primary method of internalization for RTKs and GPCRs^{214,305}. It is possible that when one pathway is inhibited, RTKs are internalized more rapidly through other pathways in order to compensate^{214,305}.

Thus, it would be interesting to see how the inhibition of other internalization pathways, alone, or in combination, would affect signaling activation. That being said, we only investigated how CME inhibition affected the IGF-IR β pathway. IGF-IR β is the only transmembrane protein that binds an extracellular ligand that we investigated in this thesis²⁶⁹. A-Raf, Shc, and the previously explored Akt are all intracellular and are activated by plasma membrane receptors, regardless of their internalization^{135,265,275,284}. Therefore, an investigation into the effects of endocytic inhibition on these other pathways is necessary. By inhibiting endocytosis and observing the effect PI4KIII β expression has on these intracellular protein pathways, we would be able to determine if the increased activation we are observing is at the plasma membrane alone or also on intracellular signaling endosomes. Finally, as we see that PI4KIII β regulation of endocytosis is dependent on its interaction with Rab11a, further work exploring how the PI4KIII β -Rab11a interaction affects signaling activation should be done. Using the Rab11a-binding deficient PI4KIII β rescue cell lines we could observe whether the inability to bind to Rab11a has any effect on signaling activation.

Finally, we determined that deletion of PI4KIII β in cancer cells inhibits their ability to form and develop tumours in syngeneic mice. The next step is to create transgenic mice with variable expression of PI4KIII β in mammary tissue. This would allow us to monitor *in vivo* differences in mammary epithelial morphogenesis in cells with low, endogenous or high PI4KIII β expression, including developmental defects, the rate of spontaneous tumour formation, and the size and type of tumours generated.

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Contributions of collaborators

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