Isoform Specific Functions of Protein Kinase D3 (PKD3) in Breast Cancer Cell Proliferation and Motility

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There is no such thing as a failed experiment, only experiments with unexpected outcomes.

Richard Buckminster Fuller (1895- 1983)

Eidesstattliche Erklärung

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Schwaikheim, den 14.10.13	Bettina Noll (geb. Huck)
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Abbreviations

°C	degree celsius
4E-BP1	eIF4E-binding protein 1
a	anti
Ab	antibody
Akt/PKB	protein kinase B
AMPK	adenine monophosphate-activated protein kinase
AP	alkaline phosphatase
APS	ammonium persulfate
Arf	ADP ribosylation factor
ATG	autophagy-related protein
BCIP	5-Bromo-4-chloro-3-indolyl phosphate
ca	catalytically active
CAMK	calcium/calmodulin-dependent protein kinase
Cdc24	cell division control protein 42 homolog
CERT	ER-to-Golgi ceramide transport protein
conc.	concentration
DAG	diacylglycerol
DMEM	Dulbecco's Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGF	epidermal growth factor
EGFP	enhanced green fluorescent portein
EGFR	epidermal growth factor receptor
ER	endoplasmic reticulum
ERK	extracellular regulated kinase
EtBr	ethidium bromide
FA	focal adhesion
FACS	fluorescence activated cell sorting
FAK	focal adhesion kinase
FCS	fetal calf serum
Fig.	figure
GAP	GTPase activating protein
GDP	guanosine diphosphate
GEF	guanine nucleotide exchange factor
GIT1	G-protein coupled receptor kinase-interacting protein 1
GOLPH3	golgi phosphoprotein 3

GST	glutathione-S-transferase
GTP	guanosine Triphosphate
h	hour
HER2	human epidermal growth factor receptor 2 (ErbB2)
HM	hydrophobic motif
HRP	horseradish peroxidase
IF	immunfluoreszenz
IP	immunoprecipitation
IP3	inositol trisphosphate
KD	kinase dead, inactive
kDa	kilo Dalton
LacZ	beta-galactosidase gene in *E. coli*
LC3	microtubule-associated protein light chain 3
LKB1	leading to liver kinase B1
m	molar; mol/l
mA	milliampere
mAb	monoclonal antibody
MAPK	mitogen-activated protein kinase
max	maximal
MEK	mitogen-activated protein kinase kinase (MAPKK)
mg	milligram
min	minute
ml	milliliter
mM	millimolar
MMP	matrix metalloprotease
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
mTORC1	mTOR complex 1
mTORC2	mTOR complex 2
NaDoc	sodium deoxycholate
Na_3VO_4	sodium orthovanadate
NaF	sodium fluoride
NLS	nuclear localization sequence
NBT	nitro blue tetrazolium chloride
PA	phosphatidic acid
PAK	p21-activating kinase
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDBu	phorbol 12,13-dibutyrate
PDGF	platelet-derived growth factor
PDK1	3-phosphinositide-dependent protein kinase 1
PDZ	density-95/discs large/zonula occludens-
PFA	paraformaldehyde
p-GIT1	phosphorylated GIT1, detected with pCERT antibody
pH	potential of hydrogen

PH	pleckstrin homology
PI3K	phosphoinositide 3-kinase
PI4P	phosphatidylinositol-4-phosphate
PI4KIIIβ	phosphatidylinositol 4-kinase IIIβ
PIP2	phosphatidylinositol 4, 5-bisphosphate
PIX	p21-activated kinase interacting exchange factor
PKC	protein kinase C
PKD	protein Kinase D
PLCγ	phospholipase Cγ
PLD	phospholipase D
PMSF	phenylmethylsulfonylfluorid
PVDF	polyvinylidene difluoride
Rac	Ras-related C3 botulinum toxin substrate
Ras	abbreviation that originated from rat sarcoma; a GTPase protein
Rheb	Ras homologue enriched in brain
Rho proteins	Rho GTPase proteins (e.g. RhoA, Rac1 and Cdc42)
RIN1	Ras and Rab interactor 1
RIPA	radio Immuno Precipitation Assay
RNA	ribonucleic acid
RNAi	RNA interference
RNase	ribonuclease
Rpm	rotations per minute
RPMI	medium named after Roswell Park Memorial Institute
RT	room temperature
S6K1	S6 kinase 1
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS- polyacrylamide gel electrophoresis
sec	second
SEM	standard error of the mean
Ser/S	serine
SFM	serum free medium
SGK1	glucocorticoid-induced protein kinase 1
SHD	Spa2-homology domain
siRNA	small interfering RNA
Src	proto-oncogene tyrosine-protein kinase Src
SSH1L	slingshot homolog 1
TAE	tris-actetate-EDTA
TEMED	N,N,N',N'-Tetramethylethylendiamin
TGN	trans-Golgi network
TNBC	triple-negative breast cancer
Tris	tris-(hydroxylmethyl)-amino methane
TSC1	tuberous sclerosis 1
TSC2	tuberous sclerosis 1
ULK1	UNC-51 like kinases 1
ULK2	UNC-51 like kinases 2

Vps34	class III phosphoinositide 3-kinase
v/v	volume per volume
w/v	weight per volume
WB	Western Blot
Wt	wild type
µg	microgram
µl	microliter
µm	micrometer
µM	micromolar

Abstract

The protein kinase D (PKD) family of serine/threonine kinases belongs to the family of calcium/calmodulin-dependent protein (CAM) kinases and comprises three family members, PKD1/PKCμ, PKD2, and PKD3/PKCν. By the phosphorylation of numerous protein substrates, PKDs regulate a variety of different cellular functions such as proliferation and differentiation, membrane and vesicle transport, apoptosis, gene expression, adhesion, motility and invasion. Most studies on PKDs have focused on the cellular regulation and functions of PKD1 and PKD2 and in general, the three PKD isoforms are thought to possess overlapping substrate specificities and function, at least in part, redundantly. However, emerging evidence suggests that isoform specific differences exist. Due to its structural characteristics PKD3 appears to play a unique role among the three isoforms, nevertheless it still is the least studied family member. The aim of this study was therefore to reveal isoform specific functions of PKD3 in the context of breast cancer biology.

In the first part of this thesis, the regulation of cell adhesion and motility by PKD3-mediated phosphorylation of the G-protein coupled receptor kinase-interacting protein 1 (GIT1) was investigated. The continuous assembly and disassembly of focal adhesions (FA) is required for efficient cell spreading and migration. GIT1 is a multidomain protein whose dynamic localization to sites of cytoskeletal remodeling is critically involved in the regulation of these processes. Mass spectrometry analysis identified a novel phosphorylation site within GIT1 at serine 46, the phosphorylation of which was abrogated by PKD3, but not PKD1 and/or PKD2 depletion, thereby identifying GIT1 as the first PKD3-specific substrate. Functional experiments further provide support that PKD3-mediated GIT1 phosphorylation on serine 46 represents a molecular switch by which GIT1 localization and thus paxillin trafficking, cellular protrusive activity and motility of breast cancer cells are regulated.

In the second part of this thesis the consequences of elevated expression of PKD3 in triple-negative breast cancer (TNBC) cells were investigated. Using a phospho-kinase signaling array, PKD3 was identified to trigger the activation of S6 kinase 1 (S6K1), a main downstream target of the mammalian target of rapamycin (mTOR) signaling pathway. Accordingly, PKD3 knockdown in TNBC cells led to reduced S6K1 phosphorylation, which was associated with impaired activation of mTOR at lysosomal membranes and the accumulation of the autophagy marker LC3. PKD3 depletion strongly inhibited proliferation of TNBC cells, most likely in part through the suppression of mTOR activation. These findings uncover a novel role for PKD3 in the homeostasis of the

endolysosomal system and implicate this kinase as a potential molecular therapeutic target in TNBC.

In summary, the experimental studies performed in this thesis provide novel insights into isoform specific functions of PKD3 in the context of breast cancer cell motility and the progression of TNBC.

Zusammenfassung

Die Proteinkinase D (PKD) Familie der Serin/Threoninkinasen gehört zur Familie der Calcium/Calmodulin-abhängigen (CAM) Kinasen und umfasst die drei Mitglieder PKD1/PKCμ, PKD2, und PKD3/PKCν. Durch die Phosphorylierung zahlreicher Proteinsubstrate regulieren PKDs eine Vielzahl an verschiedenen zellulären Funktionen wie beispielsweise Proliferation und Differenzierung, Membranen- und Vesikeltransport, Apoptose und Genexpression sowie Adhäsion, Motilität und Invasion. Viele Studien über PKD haben sich mit der zellulären Funktion und Regulation der ersten beiden Isoformen, PKD1 und PKD2 beschäftigt und im Allgemeinen geht man davon aus, dass die PKD Isoformen überlappende Substratspezifitäten und zumindest teilweise redundante Funktionen aufweisen. Jedoch deuten neueste Erkenntnisse darauf hin, dass auch Isoform-spezifische Unterschiede existieren. Aufgrund ihrer strukturellen Besonderheiten wird vermutet, dass PKD3 eine gesonderte Funktion innerhalb der Kinasefamilie spielt, dennoch ist PKD3 die bisher am wenigsten untersuchte Isoform. Das Ziel dieser Promotionsarbeit liegt daher auf der Enthüllung PKD3-spezifischer Funktionen im Rahmen der Biologie von Brustkrebs.

Im ersten Teil dieser Doktorarbeit wurde die Regulation von Zelladhäsion und Zellmotilität durch PKD3-vermittelte Phosphorylierung des G-protein gekoppelten Rezeptor Kinase Interaktionsproteins 1 (GIT1) untersucht. Der kontinuierliche Aufbau und Abbau von Fokalen Adhäsionen (FA) ist Voraussetzung für eine effiziente Zellausbreitung und Migration. GIT1 ist ein multidomänes Protein, dessen subzelluläre Lokalisation zu Positionen innerhalb der Zelle, an denen das Zytoskelett aktiv umgebaut wird, kritisch mit der Regulation dieser Prozesse verbunden ist. Massenspektrometrische Analysen identifizierten in GIT1 eine neue Phosphorylierungsstelle an Serin 46, deren Phosphorylierung nur durch PKD3, nicht aber PKD1 und /oder PKD2 Verlust aufgehoben werden konnte, wodurch GIT1 als das erste PKD3-spezifische Substrat ermittelt wurde. Weitere funktionelle Experimente zeigen auf, dass die Phosphorylierung von GIT1 an Serin 46 durch PKD3 einen molekularen Schalter darstellt, durch den die Lokalisation von GIT1 und dadurch der Transport von Paxillin sowie die zelluläre Ausbreitung und Motilität von Brustkrebszellen reguliert wird.

Im zweiten Teil der Doktorarbeit wurden die Konsequenzen erhöhter PKD3 Expression in dreifach-negativen Brustkrebszellen (TNBC) untersucht. Durch einen phospho-spezifischen Kinasearray wurde herausgefunden, dass PKD3 die Aktivierung der S6 Kinase 1 (S6K1) induzierte, welche ein Hauptsubstrat des mammalian target of rapamycin (mTOR) Signalwegs darstellt. Folglich führte der Knockdown von PKD3 in dreifach-negativen Brustkrebszellen zu einer verminderten Phosphorylierung der S6K1, welche mit

einer verschlechterten Aktivierung von mTOR an lysosomalen Membranen und der Akkumulation des Autophagiemarkers LC3 assoziiert war. Der Verlust von PKD3 hemmte deutlich die Proliferation von TNBC Zellen, vermutlich teilweise durch die Unterdrückung der Aktivierung von mTOR. Diese Ergebnisse decken eine neue Rolle von PKD3 in der Homöostase des endolysosomalen Systems auf und weisen darauf hin, dass PKD3 ein molekulares, therapeutisches Ziel in dreifach-negativem Brustkrebs darstellen kann.

Zusammengefasst bieten die experimentellen Studien dieser Doktorarbeit neue Einblicke in Isoform-spezifische Funktionen von PKD3 bezüglich der Motilität von Brustkrebszellen und der Tumorprogression von dreifach-negativem Brustkrebs.

1 Introduction

1.1 Breast cancer

Cancer is a broad group of diseases and belongs to the major chronic health problems worldwide. In 2008, almost 8 million people died from cancer all over the world and this number is estimated to increase up to 13 million deaths in 2030 (Ferlay, Shin et al. 2010). In cancer, cell transformation leads to uncontrollable cell growth resulting in the formation of tumors and metastases and the destruction of surrounding healthy tissue. Cell transformation is a highly complex multi-step process in which genetic and environmental factors together alter critical cell regulatory pathways and lead to new cellular characteristics. These new characteristics allow the cells to overcome programmed cell death, grow independently of growth factors, facilitate angiogenesis, and invade into surrounding tissues, all processes which are normally tightly controlled in untransformed cells.

Among women, breast cancer accounts for 23% of all cancers and is the most common cancer diagnosed in Europe and the USA. Breast cancer is a heterogeneous disease including clinical, morphological and molecular very distinct entities. It can be classified into several distinct subtypes according to different parameters such as histological grade, tumor size, lymph node involvement, receptor status or affected signaling pathways (Eroles, Bosch et al. 2012). In general, classification of breast cancer comprises luminal A, luminal B, HER2-enriched, normal breast, claudin-low and basal-like subtypes. Basal-like breast cancers frequently lack expression of the estrogen, progesterone and HER2/ErbB receptors and these cancers are referred to as triple negative (TNBC). This subtype accounts for 10-20% of all breast carcinomas and is correlated with poor prognosis, survival rate and a high metastatic potential (Irvin and Carey 2008). Due to the negative hormone receptor and HER2/ErbB2 status of TNBC, treatment options are limited and thus efforts are being made to identify TNBC-associated deregulated signaling pathways for the development of improved targeted and combinatorial therapies. In addition to chemotherapy which the tumors are sensitive for, several targeted agents have been tested so far which may lead to improved clinical outcomes, among them are inhibitors of epidermal growth factor receptor, poly(ADP-ribose) polymerase, or mammalian target of rapamycin (Tan and Swain 2008). Nevertheless, the cellular and molecular heterogeneity of breast cancer and the large number of deregulated genes and signaling pathways emphasize the importance of studying the underlying molecular mechanisms of cell transformation to reveal novel leads for the treatment of breast cancer patients.

1.2 The protein kinase D (PKD) family

Protein Kinase D (PKD) is a family of serine/threonine kinases comprising three family members, PKD1/PKCμ, PKD2, and PKD3/PKCν. Kinases are specialized enzymes catalyzing phosphorylation events, in which proteins are modified by chemically adding of phosphate groups to serine, threonine, and tyrosine residues within the molecule. Reversible phosphorylation of proteins is one of the most important post-translational modifications within the cell and can lead to conformational changes of the protein structure and thereby regulate protein activity in a positive or negative manner. Moreover it can also alter the subcellular localization of proteins or have an impact on protein-protein interaction. The human genome contains 518 protein kinase genes and together with phosphatases, kinases are known to regulate the majority of cellular pathways, especially those involved in signal transduction processes (Cohen 2002; Manning, Whyte et al. 2002).

The PKD family is a protein kinase family within the calcium/calmodulin-dependent protein kinase (CAMK) group and was initially described to belong to the protein kinase C (PKC) family (Rozengurt, Rey et al. 2005). However, structural analysis revealed that the catalytic domain of PKD shows very low homology to the conserved kinase domain of the PKCs and displays a distinct inhibitor and substrate specificity which led, based on highest sequence similarities of the kinase domain to CAMKs, to the classification of a novel subgroup of the CAMK family (Manning, Whyte et al. 2002).

PKD1/ PKCμ, is the best studied member and was identified in 1994 (Johannes, Prestle et al. 1994; Valverde, Sinnett-Smith et al. 1994), PKD3/PKCν was found in 1999 (Hayashi, Seki et al. 1999) and the characterization of PKD2 followed in the year 2001 (Sturany, Van Lint et al. 2001). PKDs play an important role in cellular processes such as the organization of the Golgi complex, vesicle transport, remodeling of the cytoskeleton and consequently in migration and invasion (Van Lint, Rykx et al. 2002; Rykx, De Kimpe et al. 2003; Olayioye, Barisic et al. 2013). However, only a few physiological substrates have been identified so far. The substrate requirement for PKD shows a high selectivity for leucine, isoleucine or valine in the -5 and arginine in the -3 position relative to the serine or threonine to be phosphorylated (consensus sequence: L/I/V x R x x S/T) (Doppler, Storz et al. 2005). Most of the studies in recent years focused on PKD1 and/or PKD2 isoforms, whereas relatively little is known about PKD3.

1.2.1 Structural characteristics of PKD

The PKD isoforms share a similar modular structure consisting of a regulatory domain at the N-terminus and a catalytic domain at the C-terminus (Fig. 1). The regulatory domain of all isoforms comprises a tandem repeat of zinc finger-like cysteine-rich motifs (CYS1 and

CYS2) at the N-terminus (C1 domain), which is responsible for diacylgylcerol (DAG) or phorbol ester binding and recruitment of PKD to the Golgi complex and plasma membrane (Maeda, Beznoussenko et al. 2001; Rey, Young et al. 2001). The regulatory domain contains further a region with acidic amino acids (AC) and a pleckstrin homology (PH) domain, which is important for the interaction with other proteins and membrane lipids. The PH and C1 domains also exhibit an inhibitory effect on the catalytic kinase activity, as deletion or mutations of the PH domain or deletion of the C1 domain lead to full activation of the kinase (Iglesias and Rozengurt 1998; Jamora, Yamanouye et al. 1999; Vertommen, Rider et al. 2000). The catalytic domain at the C-terminus shares homology with the calmodulin-dependent kinases and contains the kinase activity of the protein (Fig. 1). High homology between the three isoforms exists, particularly in the catalytic and C1 domains, but they also show some structural differences in the N-terminal region and in the regions flanked by the C1 and PH domains, which may confer isoform-specific functions (Rykx, De Kimpe et al. 2003; Rozengurt, Rey et al. 2005). The N-terminus of PKD1 and PKD2 starts with a apolar region, rich in alanine and/or prolin, which is responsible for recruitment to the Golgi complex and is missing in PKD3 (Hausser, Link et al. 2002). PKD2 possess an additional serine-rich stretch in the linker region between the two zinc finger-like cysteine-rich motifs in the C1 domain. In contrast to PKD1 and PKD2, PKD3 does not seem to have an autophosphorylation site at its C-terminus. Furthermore, PKD3 also lacks a postsynaptic density-95/discs large/zonula occludens- (PDZ)-binding motif which is present at the C-terminus in PKD1 and PKD2. The PDZ motif has been shown to be necessary for protein interaction by binding to the PDZ domain of substrate or scaffold proteins. For instance, it is required for an appropriate surface localization of Kidins220, the first identified PKD substrate in brain and neurons (Sanchez-Ruiloba, Cabrera-Poch et al. 2006).

Due to their structural similarities, the current notion is that the PKD isoforms possess overlapping substrate specificity and function redundantly. However emerging evidence suggests that the isoforms also regulate a distinct set of substrates. Thus, isoform specific differences presumably determine substrate and signaling specificities within different pathways and tissues, thereby allowing the PKD isoforms to interact with and modulate a plethora of proteins in a variety of signaling pathways.

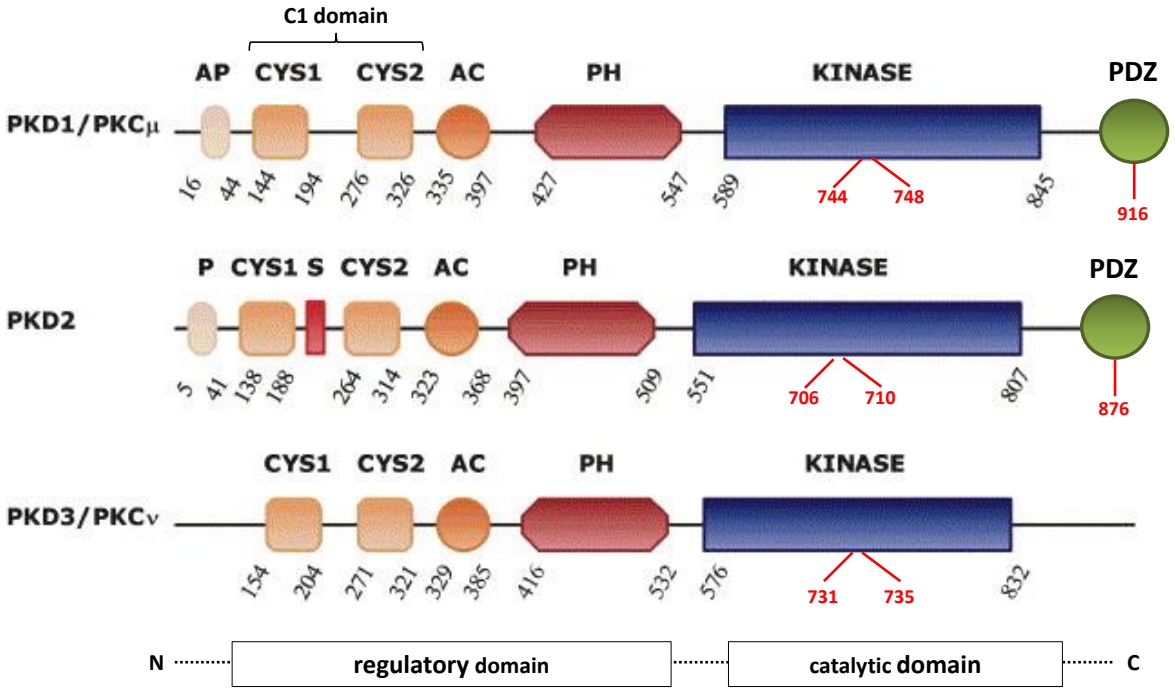


Figure 1: Modular structure of PKD family members

Numbers correspond to amino acid position of PKD1/PKCμ (mouse), PKD2 (human), PKD3/PKC (human). Serines within the activation loop that become phosphorylated via nPKCs are highlighted in red. AP, alanine- and proline-rich domain; P, proline-rich domain; S, serine-rich domain; CYS, cysteine-rich Zn finger domain; AC, acidic domain; PH, pleckstrin homology domain; KINASE, kinase catalytic domain. Modified from (Rykx, De Kimpe et al. 2003).

1.2.2 PKD activation

The most important pathway for PKD activation is the protein kinase C (PKC)-PKD signaling pathway. Here, PKD activity is regulated by the second messenger signaling lipid DAG, which recruits the kinase to cellular membranes and stimulates its activation by PKC-dependent phosphorylation of the activation loop (Rykx, De Kimpe et al. 2003; Wang 2006). DAG is a glyceride consisting of two fatty acid chains covalently bound to a glycerol molecule through ester linkages and is involved in a variety of signaling pathways. DAG and inositol trisphosphate (IP3) are the hydrolysis products of the phospholipid phosphatidylinositol 4, 5-bisphosphate (PIP2), this reaction is catalyzed by the membrane-bound enzyme phospholipase C (PLC). This hydrolysis is initiated by ligand binding to growth factors, G-protein coupled receptors (GPCR) or B cell and T cell receptors (BCR, TCR), consequently activating PLC (Waldron, Iglesias et al. 1999; Matthews, Rozengurt et al. 2000; Yuan, Slice et al. 2000; Yuan, Rey et al. 2005). Generated DAG then recruits PKD (via binding to the C1 domain) and PKC to membranes and activates PKC, which in turn binds PKD at the PH domain and transphosphorylates PKD at two serine residues within the activation loop ((Waldron, Rey et al. 2001) and Fig. 1). Concurrently IP3 leads to the release of Ca^{2+} from intracellular storages and

contributes to Ca^{2+}-dependent activation of PKCs. Activation loop phosphorylation of PKD triggers the release of autoinhibition of the PH domain leading to full activation of kinase activity (Waldron and Rozengurt 2003). Novel PKC (nPKC) isoforms such as PKCδ, ε, θ and η are strong PKD activators; however, classical PKCs like PKCα can also activate PKD (Li, O'Connor et al. 2004). The specific PKC isoforms that phosphorylate PKD vary with the cell context and stimuli, thereby offering flexibility for the activation of PKD at various intracellular sites in response to different extracellular stimuli (Wang 2006). The basis for the isoform-selective interactions between PKC and PKD might reside in the PH domain of PKD isoforms (Waldron, Iglesias et al. 1999).

Activated PKD1 and PKD2 further autophosphorylate S916 (mouse PKD1) and S876 (human PKD2) at the C-terminus (Fig. 1). This autophosphorylation is not required for activation but rather regulates the conformation of PKD and the duration of kinase activity, therefore serves as a measure for the activation status of PKD (Matthews, Rozengurt et al. 1999; Rybin, Guo et al. 2009). As mentioned before, the corresponding autophosphorylation site in PKD3 is missing. In PKD1, another autophosphorylation site within the regulatory domain was identified, which is required for binding of 14-3-3 proteins (Hausser, Storz et al. 1999) and attenuates PKD1 activity by altering its subcellular localization (Van Lint, Rykx et al. 2002). Recently, an additional phosphorylation site (S244) has been mapped in human PKD2, which is targeted by casein kinase I and facilitates nuclear accumulation of the protein (von Blume, Knippschild et al. 2007).

Apart from DAG-PKC dependent activation of PKD, there are some other pathways which can activate PKD independently of PKC signaling. Tyrosine phosphorylation at Y463 and Y95 within the regulatory domain of PKD1 (analogous tyrosines have been mapped in PKD2) by the Src-Abl pathway activate PKD in response to oxidative stress (Storz, Doppler et al. 2003; Doppler and Storz 2007). Alternatively, caspase-mediated proteolytic cleavage of PKD1 leads to activation of kinase activity (Vantus, Vertommen et al. 2004) and during osteoblast differentiation, morphogenetic protein 2 (BMP-2) can induce activation of PKD by the activation of the stress mitogen-activated protein kinases JNK and p38 (Lemonnier, Ghayor et al. 2004).

1.2.3 Localization of PKD

In response to cell stimulation, PKD family members undergo rapid redistribution and are recruited to a variety of subcellular compartments. The intracellular redistribution is regulated by the interaction of the regulatory subunits with other cellular proteins or lipids and consists of several translocation steps. Thus, the localization of PKD to specific compartments and the correlated tight regulation of its activation are of prime importance for the ability of the protein to execute multiple functions at distinct subcellular locations

leading to different cellular responses. At basal levels, the PKD isoforms are distributed in the cytosol and to some extent, in the nucleus, at the Golgi complex and mitochondria. Activated PKD isoforms are known to mainly localize to the Golgi complex, the plasma membrane and they have also been reported to shuttle to the nucleus (Rozengurt, Rey et al. 2005). In response to receptor activation induced by growth factors, GPCR agonists, B cell receptor ligands and phorbol esters (Matthews, Iglesias et al. 1999; Rey, Young et al. 2001), PKDs can be recruited via DAG binding to the CYS2 to the plasma membrane where they get activated (Rozengurt, Rey et al. 2005). PKC-mediated activation within the activation loop is required for translocation of the protein back into the cytosol and for redistribution to other organelles (Rey, Young et al. 2001), where it regulates major signaling pathways and remains active for hours (Matthews, Iglesias et al. 2000). Via a nuclear localization sequence (NLS) and a nuclear export signal (NES) within the regulatory domain, activated PKDs possess the ability to shuttle between the cytoplasm and the nucleus where they regulate gene transcription (Rey, Young et al. 2001; Rey, Yuan et al. 2003; Auer, von Blume et al. 2005). Binding of DAG to CYS1 of PKD directly recruits the protein to the trans-Golgi network (TGN) of the Golgi apparatus, where it gets activated by Golgi-associated PKCη and regulates the fission of transport carriers specifically destined to the cell surface (Liljedahl, Maeda et al. 2001). Of note, this recruitment is independent of the kinase activity as kinase dead PKD mutants also get recruited to the Golgi complex (Liljedahl, Maeda et al. 2001; Rey, Young et al. 2001). Golgi recruitment can only be abolished by mutating the CYS1 domain or by reducing the level of DAG at the Golgi membranes (Baron and Malhotra 2002). Recently, it has been further demonstrated that activation loop phosphorylated PKDs are associated with centrosomes, spindles and midbody suggesting a regulatory role of these activated kinases in mitosis and cell division (Papazyan, Doche et al. 2008). At the mitochondria PKD was identified as a sensor for oxidative stress signaling. In response to increased levels of reactive oxygen species (ROS) PKD is involved in the communication of signals to the nucleus leading either to NF-κB dependent gene transcription or to the induction of mitochondria-detoxifying enzyme MnSOD expression (Storz, Doppler et al. 2005; Storz 2007).

Most localization studies have focused on PKD1 and/or PKD2, whereas relatively little is known about PKD3. Due to their similarity, the PKD isoforms have long been thought to have similar localizations and therefore to function redundantly. However, emerging evidence suggests that the isoforms vary regarding their expression and subcellular localization and that the activation of the different PKD isoforms might have distinct cellular outcomes (Vantus, Vertommen et al. 2004; Fu and Rubin 2011; Olayioye, Barisic et al. 2013). So, unlike the predominate cytosolic distribution of PKD1 and PKD2, PKD3 is also present in the nucleus of unstimulated cells. This distribution of PKD3 results from its continuous shuttling between both compartments by a mechanism that requires a nuclear

import receptor and a competent CRM1-nuclear export pathway (Rey, Papazyan et al. 2006). It was further shown by Rey and colleagues that the nuclear import of PKD3 requires its catalytic activity (Rey, Papazyan et al. 2006). Stimuli including GPCR agonists (e.g. neurotensin) and B cell antigen receptor engagement induce a rapid and reversible plasma membrane translocation of PKD3 and GPCR activation enhances the rate of PKD3 entry into the nucleus (Matthews, Dayalu et al. 2003; Rey, Yuan et al. 2003). Thus, the differences in the intracellular distribution of the different PKD isoforms may confer the ability to execute multiple functions at distinct subcellular locations.

1.2.4 PKD signaling and biological function

The PKD family of proteins has been implicated in the regulation of a remarkable array of fundamental normal and abnormal biological processes including cell proliferation, polarity, migration and differentiation, membrane trafficking and secretion, pain transmission, inflammation, angiogenesis, cardiac contractility, hypertrophy and cancer (Rozengurt 2011). This is achieved by the regulation of a plethora of targets in a variety of subcellular localizations resulting in different cellular outcomes. Among these manifold biological processes, the role of PKD at the Golgi complex and its major role in the control of actin dynamics are the best studied ones.

At the Golgi complex, PKD regulates vesicular traffic to the plasma membrane, whereas at the plasma membrane PKD is involved in the regulation of cell shape, movement and invasion (Fu and Rubin 2011; Olayioye, Barisic et al. 2013). The budding and fission of secretory vesicles from the TGN and the transport to the plasma membrane are regulated by PKD-mediated phosphorylation of Golgi-localized substrates such as phosphatidylinositol 4-kinase IIIβ (PI4KIIIβ), a key player required for fission of TGN-to-plasma membrane carriers (Liljedahl, Maeda et al. 2001; Yeaman, Ayala et al. 2004; Hausser, Storz et al. 2005). PI4KIIIβ generates phosphatidylinositol-4-phosphate (PI4P), which is responsible for the recruitment of the machinery that is required for carrier fission (Ghanekar and Lowe 2005). In addition to secretion, the regulation of Golgi cargo to the plasma membrane has also been implicated in Rac1-dependent leading edge activity and integrin recruitment to newly formed focal adhesions (FA, see chapter 1.3), both processes which are important for cell motility (Prigozhina and Waterman-Storer 2004; Woods, White et al. 2004). However, the contribution of PKD to cell motility and actin dynamics is complex and depends on the cell context and exposure to specific stimuli. PKDs phosphorylate several proteins directly or indirectly involved in actin rearrangements such as Ras and Rab interactor 1 (RIN1), rhotekin, cortactin, G-protein coupled receptor kinase-interacting protein 1 (GIT1), and members of the cofilin pathway including p21 activated kinase 4 (PAK4) and slingshot homolog 1 (SSH1) (Olayioye, Barisic et al. 2013). PKD1 was reported to complex with the actin-binding protein cortactin

and paxillin in invadopodia of breast cancer cells (Bowden, Barth et al. 1999) regulating tumor cell invasion. Cortactin was then identified as a PKD1 substrate and PKD1 itself was shown to directly interact with F-actin *in vitro* (Eiseler, Schmid et al. 2007). In pancreatic cells, overexpression of wild type PKD1 inhibited migration whereas expression of a dominant negative PKD1 variant increased migration (Eiseler, Schmid et al. 2007). In fibroblasts, PKD1 has furthermore been implicated in the PDGF-induced short loop recycling of αβ3 integrins, whereby the migratory behavior of cells was modulated (Woods, White et al. 2004). It thus appears that PKDs are critically involved in the regulation of both cytoskeletal remodeling and membrane trafficking through the phosphorylation of specific substrates of which some still remain to be identified.

As mentioned before, most studies concerning signaling pathways do not distinguish between the three isoforms and have focused on PKD1 and/or PKD2, only few studies deal with PKD3. Furthermore, recent research revealed that the PKD isoforms vary regarding their expression, subcellular localization and regulation and therefore could also function non-redundantly. For example, PKD1/2-mediated phosphorylation of substrates such as slingshot SSH1L and RIN1 leads to the inhibition of cell migration (Eiseler, Doppler et al. 2009; Peterburs, Heering et al. 2009; Ziegler, Eiseler et al. 2011) and reduced PKD1 expression levels were observed by immunohistological analyses of invasive breast cancer samples (Hayashi, Seki et al. 1999; Eiseler, Doppler et al. 2009), suggesting that the loss of PKD1 may be associated with breast tumor progression. By contrast, Chen and colleagues showed that PKD3 is upregulated in primary prostate cancers and prostate cancer cell lines and stimulates prosurvival pathways, indicating a positive correlation between PKD3 expression and tumorigenesis (Chen, Deng et al. 2008). In mouse exocrine pancreas, PKD3 was further shown to be the predominant PKD isoform expressed promoting hormone-induced amylase secretion whereas PKD1 and PKD2 were more abundantly expressed in the pancreatic islets (Chen, Li et al. 2009). This implies that PKD3 regulates a distinct set of substrates to fulfill such functions and makes it obvious that more attention needs to be focused on the question of how the substrate specificity of the PKD isoforms is achieved and on the analysis of the corresponding substrates and signaling pathways.

Of note, most studies concerning the functions of PKDs have been performed *in vitro* by using tissue cultures and biochemical approaches. For *in viv*o studies, PKD mouse models are a useful tool. Here, conditional knockout or tissue specific transgene expression of different PKD isoforms and variants allow insights into the physiological role of the PKD isoforms in the context of a living organism. During mouse embryogenesis it was shown by *in situ* hybridization techniques that the expression pattern of the three PKD isoforms was partially overlapping (Oster, Abraham et al. 2006). A further study demonstrated that PKD1 and PKD3 play a redundant role in the regulation of class II histone deacetylases in B lymphocytes (Matthews, Liu et al. 2006). These data suggest

that the isoforms are to some extent functionally redundant *in vivo*. By contrast, Matthews and colleagues postulated unique functions for PKD1 and PKD2 in mammalian cells (Matthews, Navarro et al. 2010). It was shown that proper embryonic development of mice in early stages required PKD1 but not PKD2 activity as depletion of PKD1 or transgenic expression of a kinase dead PKD1 variant was lethal whereas the loss of PKD2 activity did not affect embryonic development (Fielitz, Kim et al. 2008; Matthews, Navarro et al. 2010). PKD2 was instead found to be important for the adaptive immunity (Navarro, Sinclair et al. 2012), raising the possibility that the isoforms additionally possess non-redundant functions *in vivo*. One study addressed the expression pattern of PKD3 during mouse embryogenesis by immunohistochemistry analysis and revealed that PKD3 was differentially expressed during organogenesis (Ellwanger, Pfizenmaier et al. 2008). In early embryogenesis, PKD3 was most prominently expressed in the heart suggesting an important role of the kinase in fetal cardiac myocytes. They additionally demonstrated apparent PKD3 expression in the developing skeleton and a progressive expression of PKD3 in neuronal cells as well as in the connective tissue and skeletal muscle from stage E12.5 on, pointing towards a specific function of the kinase during development of these tissues. However, in late embryonic stages, PKD3 was more or less ubiquitously expressed suggesting a basic housekeeping function which is in line with the mRNA expression pattern of PKD3 observed in adult organs and tissues (Hayashi, Seki et al. 1999; Oster, Abraham et al. 2006). These data imply that PKD3 might play a specific role *in vivo* during organogenesis probably caused by its specific structural characteristics. However, there are no data available up to date in the literature about a PKD3-specific knockout mouse.

1.3 Cell migration and adhesion

Many biological processes like embryogenesis, inflammation, wound healing and tumor metastasis are based on cell migration and adhesion (Vicente-Manzanares and Horwitz 2011). Both processes require the coordinated activation and targeting of both structural and signaling molecules as well as the coordinated remodeling of membranes and the actin cytoskeleton (Fig. 2). Cell adhesion is essential for maintaining multicellular structures, cell homeostasis and for the formation of new adhesions during migration events. Adhesion is achieved by cell-cell and cell-extracellular matrix (ECM) contacts which are composed of cell adhesion molecules like selectins, integrins, or cadherins and a variety of additional molecules with which adhesion molecules interact, thereby forming large, multi-protein adhesion complexes (Parsons, Horwitz et al. 2010). Focal contacts are initial small and transient adhesion complexes; focal adhesions (FA) are mature focal contacts and contain integrins, signaling molecules such as the focal adhesion kinase

(FAK), adaptor proteins like vinculin and paxillin and proteins that are directly coupled to the actin filament. All components of adhesion complexes undergo rapid and regulated turn-over to maintain a pool of molecules needed for the building of new complexes at the front of a migrating cell (Petit and Thiery 2000). Cell migration is a highly integrated, multistep cycle process and usually starts with the disruption of cell-cell contacts in response to an external signal. This is followed by a front to rear polarization of the cell and the extension of a protrusion at the leading edge in the direction of movement which is driven by actin polymerization. The formation of integrin mediated adhesions attaches the protrusion to the substratum on which the cell is migrating and serves as a traction point for migration. Actomyosin-driven contraction then moves the cell body forward and disassembly of adhesion complexes at the cell rear results in a successive forward gliding of the cell body and completes the cycle (Ridley, Schwartz et al. 2003).

Numerous proteins and signal molecules are known to be involved in cell migration and adhesion, but in many cases the complex interplay and the regulation of the protagonists are only poorly understood.

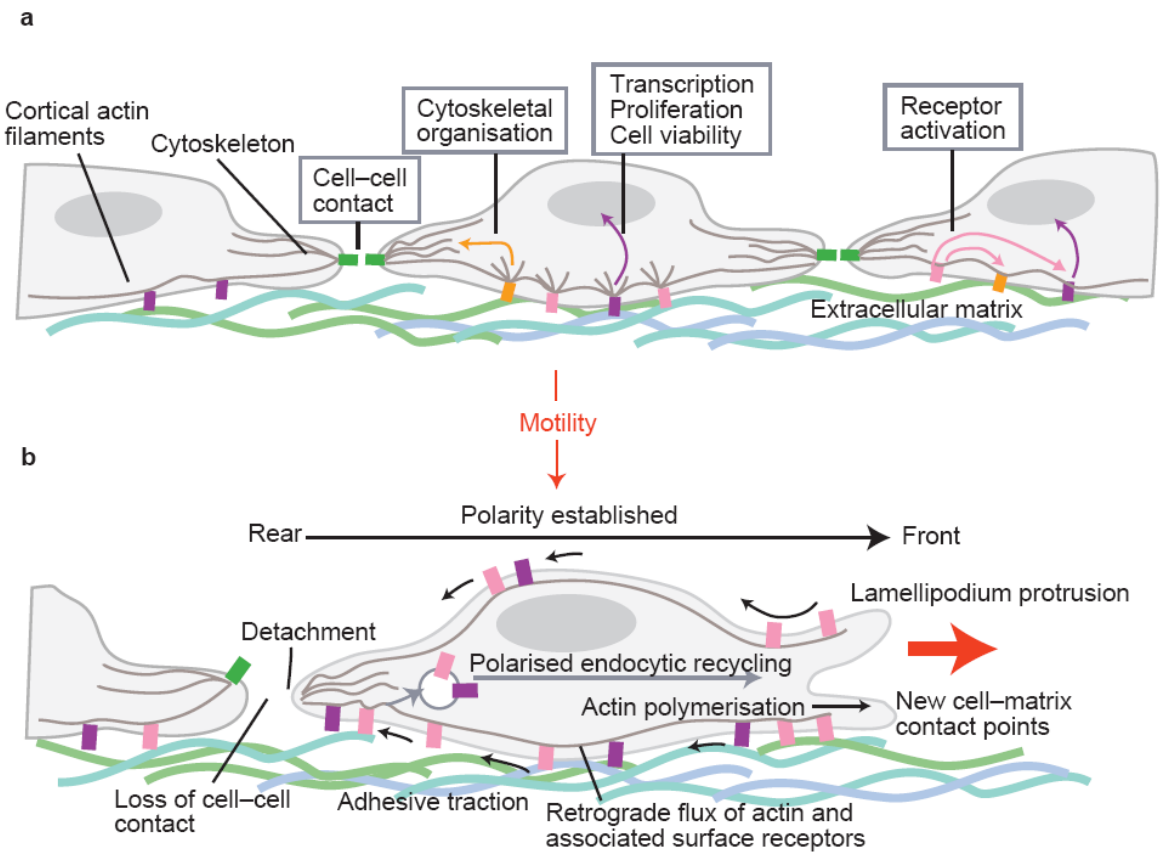


Figure 2: Cell adhesion and cell migration

(A) The shape and behavior of cells is determined by a complex series of dynamic interactions that are mediated by a heterogeneous population of transmembrane adhesion molecules. In addition to mediating cell–cell and cell–extracellular-matrix attachment, cell-adhesion molecules facilitate indirectly cytoskeletal–membrane interaction and signal-transduction processes, which control cell

viability, cytoskeletal organization, cell motility and receptor activation. (B) In many cases, cell motility is physically and biochemically restricted by cadherin-mediated cell–cell interactions. Disruption of these interactions enhances the motility of cells *in vitro* and contributes to the invasive nature of tumor cells *in vivo*. The sequential phases of cell movement *in vitro* include the induction of a well-defined front–rear polarity, which determines the direction of motility. Membrane extension at the front of the cell is mediated by the combined effects of actin polymerization and actin movement mediated by myosin. Receptor proteins (such as integrins) that are inserted into the front of the cell bind to components of the extracellular matrix (ECM), thus preventing membrane retraction and providing adhesive traction for cell movement. The physical process of cell movement results from the retrograde flux of polymerised actin (and associated membrane receptors) and the cyclical flux of membrane from the rear to the front of the cell. The final stage of cell movement involves the disruption or severing of matrix attachments at the rear of the cell and retraction of the trailing edge of the cell. Taken from (Sanderson and Smith 1999).

1.4 G-protein coupled receptor kinase-interacting protein 1 (GIT1)

One of the proteins that has been extensively investigated over the last few years in the context of cell migration is the G-protein coupled receptor kinase-interacting protein 1 (GIT1). It belongs to the GIT family which also comprises GIT2, both proteins are widely expressed, show a complex domain structure and form homo- and heterodimers. GIT1 exists almost entirely in its full length form whereas GIT2 is extensively alternatively spliced in a tissue specific manner (Premont, Claing et al. 2000). GIT1 is a ubiquitous multidomain protein that plays an important role in different cellular processes such as vesicle transport, cell adhesion, migration, organization of the cytoskeleton and receptor internalization (Premont, Claing et al. 1998; Hoefen and Berk 2006). Thus, most of its functions are connected to the control of cytoskeletal dynamics and membrane trafficking between the plasma membrane and recycling endosomes (Randazzo, Inoue et al. 2007; Frank and Hansen 2008). Due to its multidomain structure GIT1 is able to complex with a plethora of signaling proteins such as PLCγ, p21-activated kinase interacting exchange factor (PIX), PAK, FAK, paxillin and extracellular regulated kinase (ERK), conferring functions mainly associated with the regulation of cell morphology. Because of its ability to integrate signaling pathways by controlling the spatial activation of several signaling molecules, GIT1 is considered to act as a scaffold protein (Manabe, Kovalenko et al. 2002). GIT1 furthermore owns a GTPase activating domain with specificity for ADP-ribosylation factor 6 (Arf6), a small GTPase mainly involved in the regulation of membrane trafficking and actin remodeling. The complex multidomain structure of GIT1 comprises the N-terminal Arf GTPase activating protein domain (Arf-GAP), three ankyrin repeats, a Spa2 homology domain (SHD) that mediates binding to the Rac/Cdc42-specific exchange factor PIX and further signaling molecules including mitogen-activated protein kinase kinase (MEK), FAK and PLCγ, a coiled coil domain including a leucine zipper that

mediates homo- and heterodimerization, and a C-terminal paxillin-binding site ((Hoefen and Berk 2006) and Fig. 3).

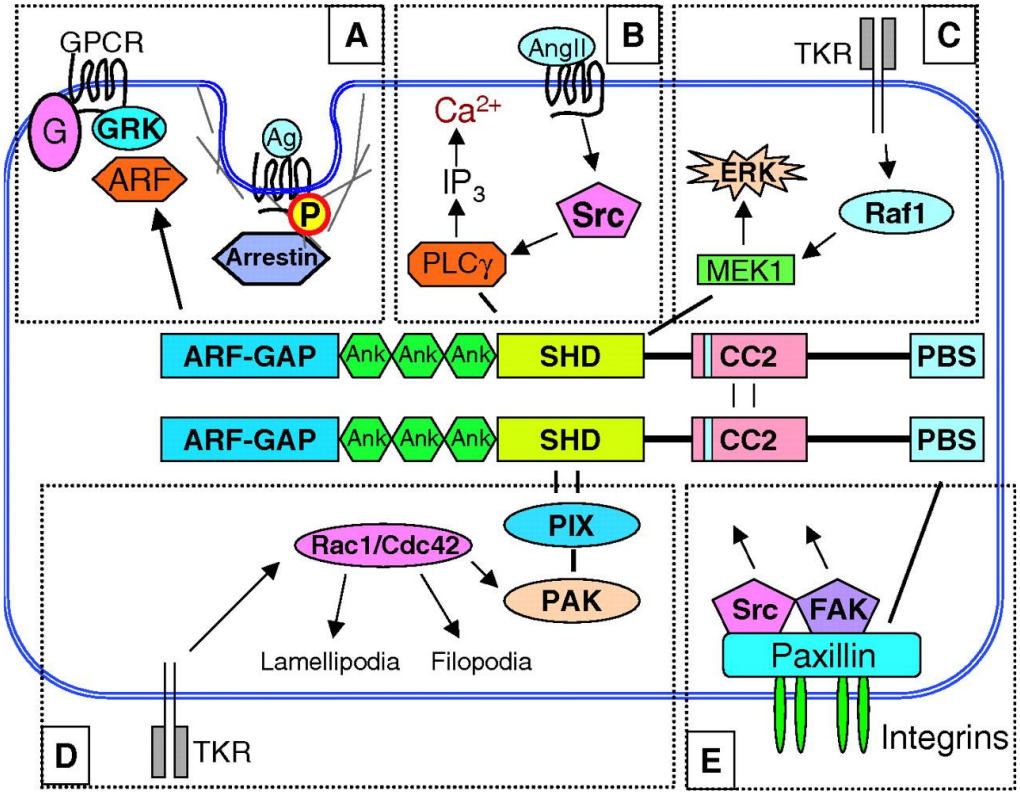


Figure 3: Summary of GIT1 protein interactions and functions

Major signaling pathways include (A) agonist (Ag)-receptor (GPCR) endocytosis; (B) angiotensin-II (AngII)-stimulated PLCγ activation and Ca2+ mobilization; (C) scaffolding MEK1 activation in response to TRK activation; (D) cytoskeletal regulation through PAK and Rho-family GTPases; and (E) focal complex assembly and disassembly. The following abbreviations are used: G, heterotrimeric G proteins; GRK, GPCR kinase; GPCR, G-protein-coupled receptor; IP3, inositol (1,4,5)-trisphosphate; P, phosphorylation; TKR, tyrosine kinase receptor. Taken from (Hoefen and Berk 2006).

1.4.1 GIT1 protein domain structure and interaction partners

GIT1 exhibits Arf-GAP activity towards all Arf members in vitro, but reports with intact cells suggest specificity mainly for Arf6 (Albertinazzi, Za et al. 2003; Hoefen and Berk 2006; Meyer, Deliot et al. 2006). Arf proteins are small GTP-binding proteins (GTPases) that regulate membrane traffic by interacting with vesicular coat proteins and phospholipid-metabolizing enzymes and modulate the structure of actin at membrane surfaces (D'Souza-Schorey and Chavrier 2006). GTP-binding proteins cycle between an inactive (GDP-bound) and an active (GTP-bound) state and their nucleotide state is regulated by additional proteins. Guanine exchange factors (GEFs) facilitate the exchange of GDP for GTP and therefore activate GTPases, whereas GTPase activating proteins (GAPs)

promote GTP hydrolysis and inactivation. Thus, GIT1 negatively regulates Arf6 GTPase signaling by stimulating the intrinsic Arf6 GTP hydrolysis activity converting Arf6 to the inactive, GDP-bound state. The regulation of Arf proteins is important because their functions in intracellular trafficking as well as recycling and secretory processes are tightly linked to secretion, endocytosis and cell adhesion, migration and invasion (Randazzo and Hirsch 2004; Randazzo, Inoue et al. 2007).

Additionally to Arf-GAP dependent functions, which are mediated by the GAP domain of GIT1, several protein binding partners have been identified to interact with different domains of GIT1 mediating functions mainly connected to the regulation of cell morphology. These functions require complex formation of GIT1 with additional interaction partners and the regulated targeting of GIT1 to FAs. One of those interactions is between the SHD domain of GIT1 and PIX (Zhao, Manser et al. 2000). PIX proteins are homodimeric GEFs for the small GTPases Rac and Cdc42 and to a lesser extent for Rho, all members of the Rho-GTP binding protein family and known to be involved in the regulation of actin dynamics and cell motility (Heasman and Ridley 2008). Because of their ability to form homodimers, GIT1 and PIX tend to form large oligomers or aggregates in cells (Premont, Perry et al. 2004). The PIX-GIT complex functions to integrate and coordinate signaling among Arf, Cdc42, and Rac proteins in response to signals emanating from integrins, heterotrimeric G proteins, receptor tyrosine kinases, and cell-cell interactions. A concept that emerges from the literature is that the PIX-GIT complex serves as a cassette to elicit changes in cell shape essential for polarized cell responses in a wide range of biological contexts (Frank and Hansen 2008).

The SHD domain of GIT1 also interacts with the mitogen-activated protein kinase 1 (MAPK1) (Yin, Haendeler et al. 2004) and PLCγ (Haendeler, Yin et al. 2003). Subsequently to GIT-PIX binding, the serine/threonine kinase PAK, an effector of the Rho family of GTPases, interacts with PIX and is recruited to and activated by GIT-PIX oligomers, a process that is potentiated by activated Rac and Cdc42. Rac is activated by cell attachment and active Rac induces membrane ruffling, formation of lamellipodia and the polarization of cells during directed migration (Sahai and Marshall 2002; Burridge and Wennerberg 2004), whereas Cdc42 regulates filopodial extension (Ridley and Hall 1992; Nobes and Hall 1999). Rac1 and Cdc42 have also been implicated in the formation of invadopodia (Nakahara, Otani et al. 2003). Thus, the GIT-PIX complex plays an essential role in Rac/Cdc42 mediated activation of PAK kinase activity (Brown, West et al. 2002). It has been additionally shown that the Arf-GAP domain of GIT1 is also crucial for GIT1-stimulated activation of PAK, but interestingly the GAP activity is not (Loo, Ng et al. 2004).

The focal adhesion protein paxillin binds the PBS (paxillin binding site) domain of GIT1 via its LD4 motif (Turner, Brown et al. 1999) and this interaction is mainly responsible for the localization of GIT1 to FAs. Paxillin is a multidomain scaffolding protein that functions in

the recruitment of both signaling and structural molecules to focal adhesions (Turner 2000; Deakin and Turner 2008). Paxillin contains five leucine- and aspartate-rich LD motifs, of which LD4 directly binds GIT1-PIX signaling complexes. The PBS domain of GIT1 is thought to be unavailable for paxillin binding due to an intramolecular interaction between the N and C terminus of GIT1 masking the PBS domain (Totaro, Paris et al. 2007). The release of the intramolecular interaction is triggered by the binding of PIX to GIT1 (Brown, West et al. 2002). Furthermore, phosphorylation of paxillin within the LD4 motif by PAK appears to promote binding of GIT1 to paxillin (Nayal, Webb et al. 2006). Figure 3 shows a schematic overview of GIT1 protein interactions and functions.

1.4.2 GIT1 localization, role in regulation of cell shape, actin remodeling and migration

GIT1 has been observed at different subcellular locations, including FAs, membrane protrusions, adherens junctions, and the centrosome. Translocation of GIT1 to the cell periphery, induced by complex formation with paxillin, PIX, PAK and Rac, inhibits Rac1 activity, thus acting as a negative feedback that prevents prolonged Rac activity through an undefined mechanism that requires Arf-GAP activity (Randazzo, Inoue et al. 2007; Frank and Hansen 2008). This model is supported by the observation that fibroblasts expressing a paxillin mutant deficient in GIT1 binding, exhibit abnormal membrane protrusion dynamics that are caused by sustained global Rac activity (West, Zhang et al. 2001). These cells are also defective in polarized cell migration (West, Zhang et al. 2001; Brown and Turner 2004) and focal adhesion disassembly (Webb, Donais et al. 2004). GIT1 has been suggested to restrict local Rac activation by inhibiting Arf6 which in turn downregulates Rac activity resulting in a Rac-GTP gradient from the front to the back of the cell (Nishiya, Kiosses et al. 2005). On the other hand, GIT1 expression has also been found to promote membrane protrusions and motility by PIX-mediated recruitment and activation of PAK and Rac independently of GIT's GAP activity (Zhao, Manser et al. 2000; Manabe, Kovalenko et al. 2002; Loo, Ng et al. 2004), suggesting that the precise effects of GIT1 may depend on the cell type and experimental setup.

PAK is a serine/threonine kinase that is activated by the small GTPases Rac and Cdc42 and promotes the recruitment of GIT-PIX oligomers to focal complexes (Hoefen and Berk 2006; Frank and Hansen 2008). Since PAK lacks a focal adhesion targeting motif, its recruitment to FAs requires binding to PIX, which, besides its nucleotide exchange activity for Rac and Cdc42, functions as an adaptor protein. A PAK mutant that is unable to bind PIX blocks localization of GIT-PIX to focal complexes (Brown, West et al. 2002). The down regulation of Rac by FA-localized GIT1 could then act as a negative feedback preventing prolonged Rac activation. It thus appears that the dynamically controlled localization of GIT1 is crucial for the regulation of cellular polarity and motility.

GIT1 was found to cycle between the leading edge and FAs in motile punctate cytoplasmic structures (Manabe, Kovalenko et al. 2002). These structures were described as supramolecular protein complexes containing paxillin, PIX and PAK that were not associated with any membrane markers (Manabe, Kovalenko et al. 2002). However, in other studies GIT1 was observed in vesicular structures that stained positively for the endosomal markers Rab11 and transferrin. Furthermore, GAP-inactive variants of GIT1 accumulate in membrane-bound structures also positive for Rab11 and transferrin, characteristic of recycling endosomes, suggesting that Arf-GAP activity is necessary to maintain proper membrane trafficking and peripheral localization of GIT1 (Di Cesare, Paris et al. 2000; Matafora, Paris et al. 2001; Albertinazzi, Za et al. 2003). The subcellular distribution of GIT1 thus appears to differ in distinct cell types, perhaps due to differential expression of binding partners and/or posttranslational modification of the protein. Regardless of the precise nature of these structures, a major function of GIT1 is the delivery of signaling complexes to cell adhesions and membrane protrusions to regulate cell shape and motility.

There are several reports on the regulation of cell motility by GIT1, both positive and negative, which are likely mediated by GAP-dependent and GAP-independent functions. Owing to its GAP activity towards Arf6 on the one hand and the interaction with paxillin, PIX and PAK and the regulation of Rac via GAP-independent mechanisms on the other hand, GIT1 may affect cell migration in multiple ways. Apart from controlling cytoskeletal remodeling, GIT1 could influence cell migration by modulating receptor internalization and recycling.

1.4.3 Regulation of GIT1 by phosphorylation

All these important GIT1-mediated events discussed above make it obvious that GIT1 activity must be regulated in a secured manner. Regulation of protein activity and alteration of subcellular localization can commonly be achieved by molecular mechanisms such as protein-protein interaction or posttranslational modifications, e.g. phosphorylation. Mass spectrometry analysis identified multiple serine, threonine, and tyrosine residues to be phosphorylated in GIT1 (Webb, Mayhew et al. 2006). It has been shown that GIT1 becomes phosphorylated in fibroblasts upon adhesion of cells to fibronectin (Bagrodia, Bailey et al. 1999). Several growth factors including EGF and PDGF induce tyrosine phosphorylation of GIT1 by Src (Haendeler, Yin et al. 2003). Furthermore, it was recently reported that GIT1 is phosphorylated on tyrosine 321 downstream of FAK and Src kinases in platelet-derived growth factor-stimulated osteoblasts, leading to increased association with and activation of FAK and cell motility (Ren, Yu et al. 2012). Another report describes that phosphorylation of GIT1 on serine 709, which is located within the PBS, increases paxillin binding and cellular protrusive activity (Webb, Kovalenko et al. 2006) but in most

cases the functional significance of GIT1 phosphorylation and the upstream kinases are unknown.

1.5 Mammalian target of rapamycin (mTOR)

The mammalian target of rapamycin (mTOR) is an important serine/threonine protein kinase of the phosphoinositide 3-kinase (PI3K)-related kinase family, which functions as an environmental sensor and regulates organismal growth, cell physiology and homeostasis by integrating a variety of cellular stimuli. mTOR is the catalytic subunit of two distinct complexes, mTOR complex 1 and mTOR complex 2 (mTORC1 and mTORC2), which consist of several additional regulatory proteins. The subunit composition of each mTORC dictates its substrate specificity. mTORC1 responds to amino acids, stress, oxygen, energy, and growth factors and is acutely sensitive to rapamycin. It promotes cell growth by inducing and inhibiting anabolic and catabolic processes, respectively, and also drives cell-cycle progression. mTORC2 responds to growth factors and regulates cell survival and metabolism, as well as the cytoskeleton and is insensitive to acute rapamycin treatment, however, chronic exposure to the drug was shown to disrupt its structure. Due to its important role in coupling energy and nutrient abundance to the execution of cell growth, division and homeostasis, deregulation of the mTOR signaling pathway is implicated in an increasing number of pathological conditions including obesity, type 2 diabetes, aging, neurodegeneration and cancer (Dann, Selvaraj et al. 2007; Laplante and Sabatini 2012).

1.5.1 mTOR substrates and downstream signaling

Main substrates of mTORC1 are S6 Kinase 1 (S6K1) and eIF4E-binding protein 1 (4E-BP1), both implicated in the regulation of mRNA and protein synthesis (Fig. 4). S6K1 belong to the AGC kinase family, exists in four isoforms (the main isoforms being p70 and p85 kDa, but p60 and p31 kDa isoforms have also been described) and is regulated by complex multi-site phosphorylation. Maximal S6K1 activity requires T-loop phosphorylation by 3-phosphinositide-dependent protein kinase 1 (PDK1) at threonine 229 (Thr229) and, more importantly, hydrophobic motif (HM) site phosphorylation by mTORC1 at Thr389 (Magnuson, Ekim et al. 2012). Emerging evidence suggests that aberrant mTORC1-S6K1 signaling contributes to cancer (Dann, Selvaraj et al. 2007). Besides the mTORC1-S6K1 axis, mTORC1 also controls the synthesis of lipids, regulates cellular metabolism and ATP production, inhibits autophagy and negatively regulates the biogenesis of lysosomes ((Laplante and Sabatini 2012), Fig. 4). mTORC2 controls several members of the AGC subfamily of kinases including Akt, serum- and glucocorticoid-induced protein kinase 1 (SGK1) and protein kinase C-α (PKCα) and is thereby implicated

in the regulation of cell survival, cell cycle progression and anabolism (Laplante and Sabatini 2012), Fig. 4). Among AGC kinases, Akt is especially important because of its role in the pathogenesis of cancer. Through phosphorylation of several effectors, Akt regulates cellular processes such as metabolism, survival, apoptosis, growth and proliferation. It is directly phosphorylated by mTORC2 at serine 473 (Ser473) within the hydrophobic motif, which causes, together with phosphorylation of Thr308 within the activation loop by PDK1, maximal activation of Akt (Sarbassov, Guertin et al. 2005). Recent research support the importance of the mTOR pathway in cancer progression, because oncogenic activation of mTOR signaling has been shown to induce several processes required for cancer cell growth, survival and proliferation (Fig. 4).

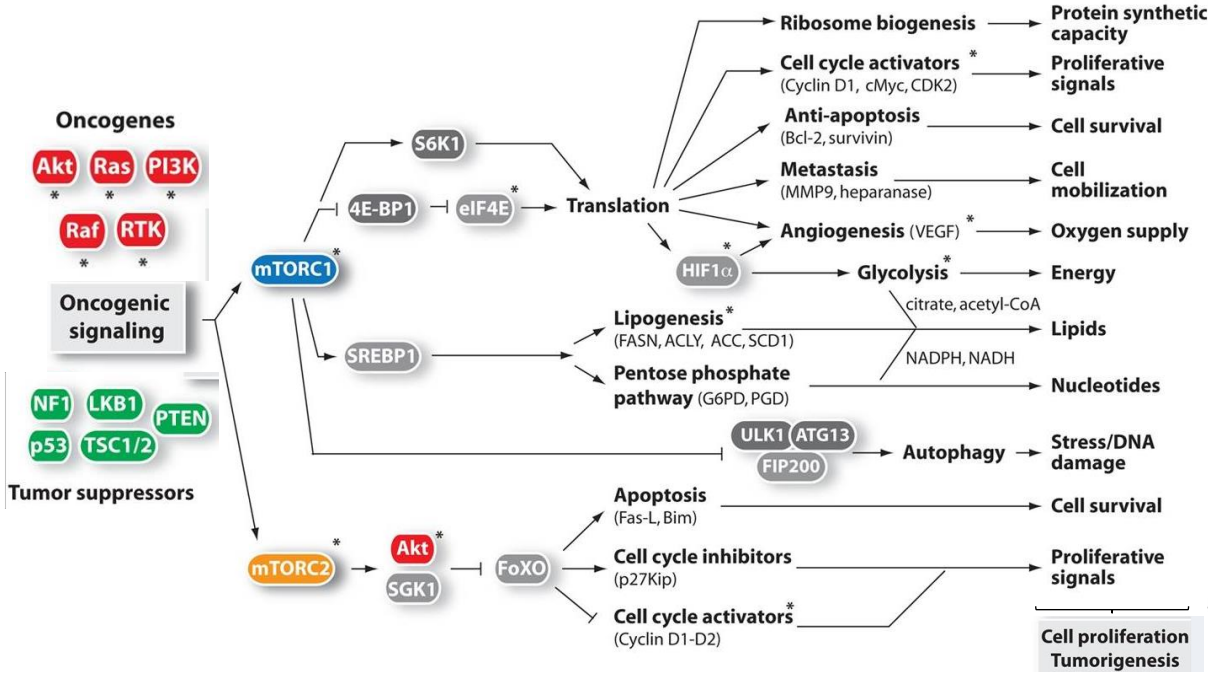


Figure 4: mTOR substrates and the connection to cancer

Shown are the substrates of mTORC1 (blue) and mTORC2 (orange) and how mTOR signaling promotes tumorigenesis. mTORC1 regulates a plethora of biological processes through the phosphorylation of several proteins, S6K1 and 4E-BP1 are by far the best-characterized substrates of mTORC1. mTORC2 regulates survival/metabolism and the cytoskeleton through the phosphorylation of many AGC kinases including Akt, SGK1, and PKC-α. Oncogenes (red) or tumor suppressors (green) implicated in the control of mTOR signaling are also indicated. An asterisk (*) denotes proteins currently targeted for cancer therapy. The following abbreviations are used: ACC, acetyl-coA carboxylase; ACLY, acyl-coA lyase; Bcl-2, B cell CLL/lymphoma 2; Bim, BCL2-like 11; CDK2, cyclin-dependent kinase 2; cMyc, v-myc myelocytomatosis viral oncogene homolog; Fas-L, Fas ligand; FASN, fatty acid synthase; G6PD, glucose-6-phosphate dehydrogenase; MMP9, matrix metallopeptidase 9; NADH, nicotinamide adenine dinucleotide; NADPH, nicotinamide adenine dinucleotide phosphate; p27Kip, cyclin-dependent kinase inhibitor 1B; PGD, phosphogluconate dehydrogenase; SCD1, stearoyl-Coenzyme A desaturase 1; VEGF, vascular endothelial growth factor. Taken from (Laplante and Sabatini 2012).

1.5.2 Upstream signaling of mTOR

The upstream signaling of mTOR is quite complex and many regulators of different signaling pathways are involved including insulin/PI3K, Ras/MAPK and PLD signaling. mTORC1 is the better studied member and integrates signals from nutrients (mainly amino acids), growth factors (insulin and insulin-like growth factors), energy and stress (hypoxia and DNA damage), whereas mTORC2 seems to be only regulated by growth factors (Zoncu, Efeyan et al. 2011). Figure 5 gives an overview of the upstream signaling events.

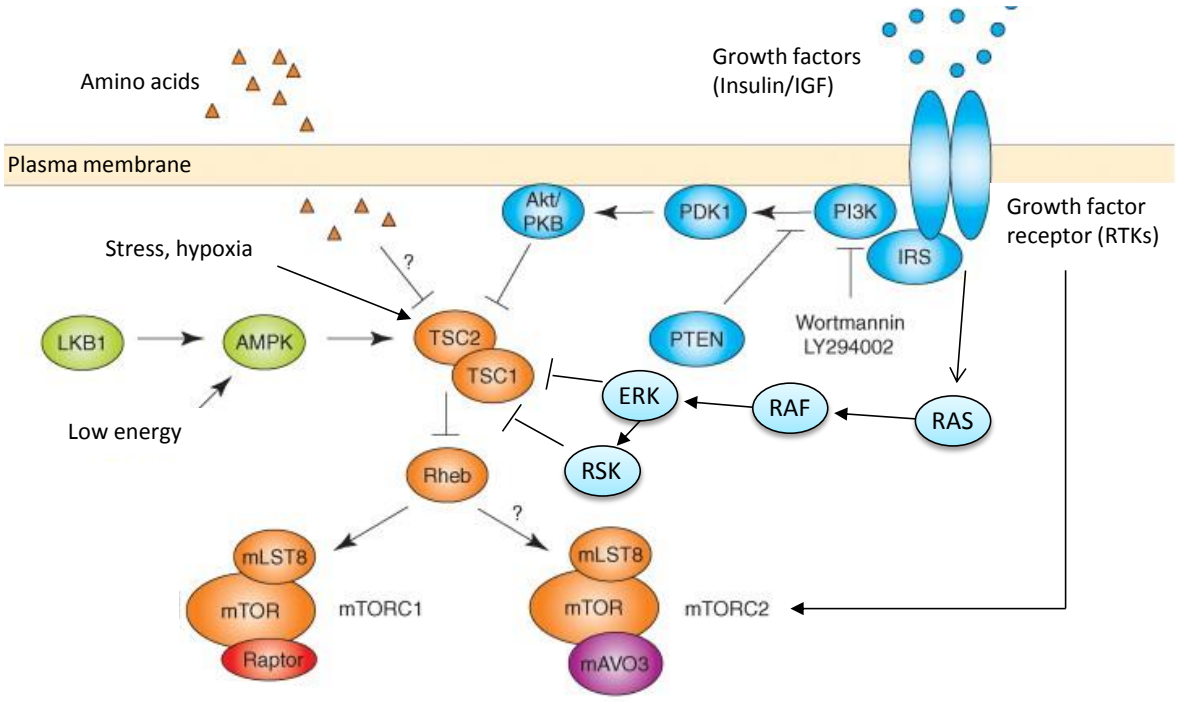


Figure 5: Schematic signaling pathway upstream of mTOR

Signal integration of mTORC1 occurs at the level of the TSC1-TSC2 complex. Akt and Erk phosphorylate TSC2, thus inhibiting the GAP activity of the TSC1-TSC2 complex towards Rheb, which stimulates the kinase activity of mTORC1 when loaded with GTP. By contrast, phosphorylation of TSC2 by AMPK results in the activation of TSC1-TSC2 complex in response to energy stress. Hypoxic stress inhibits mTORC1 by inducing the assembly and activation of TSC1-TSC2. mTORC2 is less defined and supposed to be directly or indirectly regulated by growth factor signaling. Modified from Current Opinion in Cell Biology.

Growth factors bind to and stimulate receptor tyrosine kinases (RTKs), which can then activate mTORC1 through both, the PI3K-Akt and Ras-Erk signaling pathway. Both pathways inhibit the tuberous sclerosis 1 (TSC1)-TSC2 complex by phosphorylation, thus inhibiting the GAP activity of TSC1-TSC2 complex towards Ras homologue enriched in brain (Rheb), allowing Rheb-GTP to accumulate and activate mTORC1. Cellular energy depletion is sensed by mTORC1 through a rise in intracellular AMP, leading to liver kinase B1 (LKB1) dependent activation of adenine monophosphate-activated protein kinase

(AMPK). AMPK also signals through the TSC1-TSC2 complex, but on the contrary enhances the activity of the complex, thereby inhibiting mTORC1 in response to energy stress. Hypoxic stress promotes the assembly and activation of TSC1-TSC2 complex, also leading to inhibited mTORC1 activity. A second level of integration occurs at the lysosomes (not shown in Fig. 5) where mTOR gets recruited to and activated in response to amino acid withdrawal by Rag GTPases (Sancak, Bar-Peled et al. 2010).

1.6 Autophagy

Autophagy is an evolutionarily conserved, self-degrading process of cellular components that facilitates the recycling of organelles and proteins into their nutrient components by the autophagosomal-lysosomal pathway. Under poor nutrient availability, this process serves to degrade and recycle macromolecules (e.g. proteins, glycogen, lipids and nucleotides) and organelles in order to replenish intracellular nutrients for cell survival and homeostasis during growth unfavorable conditions (Mizushima, Levine et al. 2008; Parkhitko, Favorova et al. 2013). Autophagy also serves as a cellular quality control process for the degradation of unneeded or mal-functional cytoplasmic organelles (Wong and Cuervo 2010), plays a role in immunity and inflammation (Levine, Mizushima et al. 2011), in cellular development and differentiation (Levine and Klionsky 2004) and is linked to a variety of diseases including cancer, obesity and neurodegenerative disorders. The regulation of autophagy is quite complex and requires the cooperation of numerous proteins and signaling pathways. Autophagy consists of several sequential steps, including induction, autophagosome formation (initiation, elongation and closure), autophagosome-lysosome fusion (maturation) and degradation (Fig. 6). mTOR as a central regulator of cell growth and homeostasis, plays a key role in regulating the balance between cell growth and autophagy in response to nutritional status, growth factors and stress signals. Under nutrient-rich conditions, mTORC1 suppresses autophagy by inhibiting a conserved protein complex containing the UNC-51 like kinases 1 and 2 (ULK1 and ULK2). This complex is required for the induction of autophagy in mammalian cells and shows high homology to the autophagy-related protein (ATG) kinases containing complex in yeast (Jung, Ro et al. 2010; Neufeld 2010). A second level of autophagy induction includes a complex containing Vps34, a class III PI3K and ATG6/Beclin (Simonsen and Tooze 2009). Later steps of autophagy are regulated by two ubiquitin-like proteins, microtubule-associated protein light chain 3 (LC3/ATG8) and ATG12 , both of which are involved in the formation of autophagosomes ((Geng and Klionsky 2008), Fig. 6).

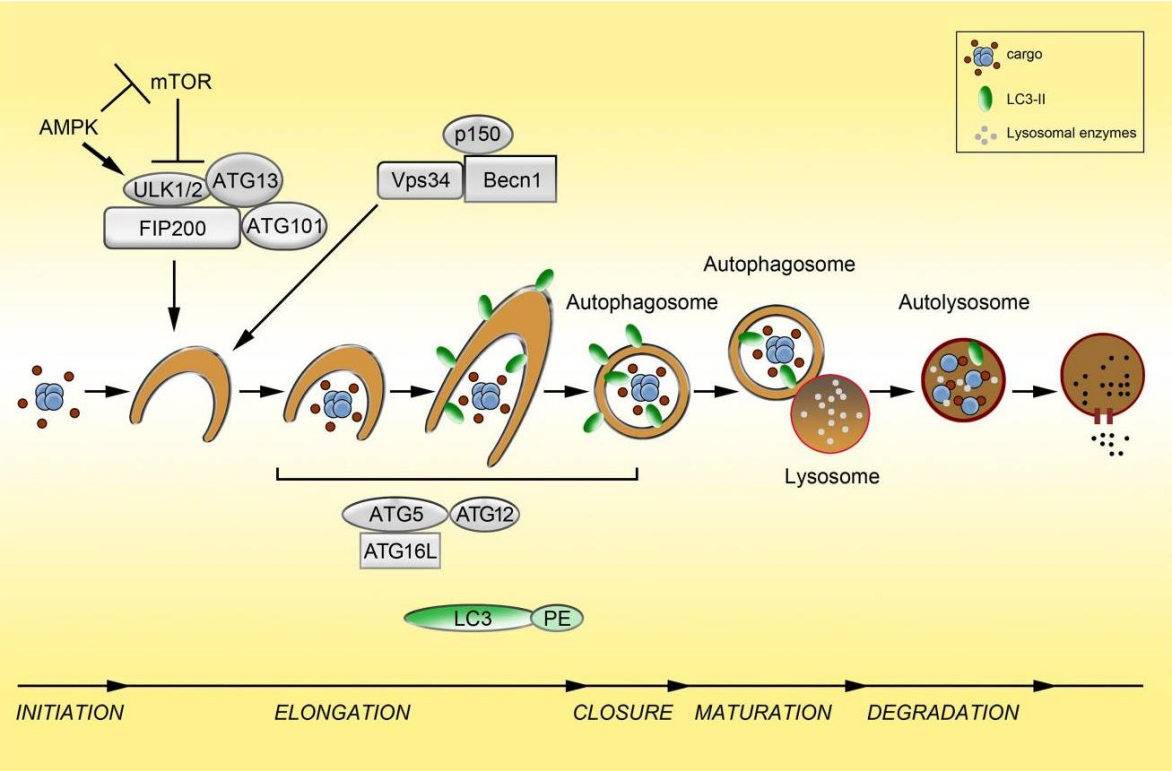


Figure 6: The process and regulation of autophagy

The stages of autophagy (initiation, elongation, closure, maturation, and degradation) are depicted. Cargo is sequestered in a double-membrane vesicle that forms the autophagosome. This fuses to the lysosome (autolysosome), where the cargo is degraded by lysosomal enzymes and degradation products are recycled back into the cytosol by lysosomal permeases. mTOR is a key regulator of autophagy in response to changes in nutrient availability. During nutrient-replete conditions, mTOR is activated and autophagy is inhibited through repression of ULK1/2 (the mammalian homologs of ATG1). Upon nutrient depletion, ULK1/2 is activated and can promote autophagy initiation. ULK is also activated in states of low energy (increased AMP/ATP ratio) by phosphorylation by AMPK as well as repression of mTORC1. Also critical to autophagy initiation is the production of phosphatidylinositol-3-phosphate (PI3P) by the class III PI3K Vps34, which is in a complex with ATG6/Beclin1 and p150 (Vps15). This complex is an additional level of regulation and, depending on the particular proteins bound, can activate or repress Vps34 activity. The ATG5–ATG12 complex as well as LC3 conjugated to PE, known as LC3-II, act downstream from Vps34 and ULK1/2 and, along with other proteins, have roles in autophagosome membrane elongation. LC3 is present on the outer and inner surfaces of the autophagosome (depicted as a green oval) and is a commonly used marker to monitor autophagosomes. Taken from (Kimmelman 2011).

The role of autophagy in tumorigenesis is complex and has been discussed to either promote or inhibit tumorigenesis depending on tumor type, stage and genetic context (Kimmelman 2011). In cells of established tumors which experience metabolic and nutritional stress, autophagy is thought to have prosurvival effects by enabling the cells to evade apoptosis (Mathew, Karantza-Wadsworth et al. 2007). On the other hand, autophagy is considered as a process that suppresses malignant transformation as

defective initiation and inhibition of autophagy in early stages of tumorigenesis could promote tumor progression and elevated autophagy may contribute to enhanced cell death (Debnath, Baehrecke et al. 2005).

1.7 Goals

PKDs are important regulators of a variety of cellular functions and deregulation of PKD activity is linked to an increasing number of pathological diseases including cancer. However, the precise role of PKDs in the context of breast cancer cell biology is not fully understood and especially in the case of PKD3 no specific substrates as well as deregulated signaling pathways have been identified so far.

The objective of this thesis was therefore to gain insights into isoform selective functions and substrate specificities of PKD3 in the context of breast cancer biology. In the first part of this thesis I analyzed the regulation of the Arf-GAP protein GIT1 by PKD3-mediated phosphorylation and investigated in breast cancer cells the physiological consequences of GIT1 phosphorylation using a combination of biochemical and cellular assays. In the second part of the thesis I focused my attention on a potential oncogenic role of PKD3 in the progression of TNBC and the identification of associated downstream signaling pathways.

2 Materials and Methods

2.1 Materials

2.1.1 Equipment

Table 1: List of equipment used in this thesis

Equipment	Company
Amaxa biosystems Nucelofector II	Amaxa
Axiovision software	Zeiss, Jena
Beckman J2-MC Centrifuge (Rotor J20) (for amounts up to 250 ml)	Beckman Coulter, Krefeld
Beckman Coulter Avanti J-30I Centrifuge (Rotor J20) (for amounts up to 250 ml)	Beckman Coulter, Krefeld
Casy (cell counter)	Schärfe System
CK2 (standard light microscope)	Olympus, Hamburg
Curix 60 processor	Agfa, Düsseldorf
Cytomics FC 500 (FACS)	Beckman Coulter, Krefeld
Eppendorf Centrifuge 5415R (Rotor F45-24-11) (for amounts up to 1.5 ml)	Eppendorf, Hamburg
Eppendorf Centrifuge 5415D (Rotor F45-24-11) (for amounts up to 1.5 ml)	Eppendorf, Hamburg
Jouan GR4i Centrifuge (Rotor Jouan 2) (for amounts up to 500 ml)	Jouan Quality system
Jouan CR422 Centrifuge (low speed for cell culture)	Jouan Quality system
LSM 710 (confocal laser scanning microscope	Zeiss, Jena
MKR 13 (orbital microplate shaker)	HLC BioTech, Bovenden
NanoDrop® ND-1000 (Spectrophotometer)	peQLab, Erlangen
Nuaire IR Autoflow, CO2 Water-Jacketed Incubator	Zapf Instruments
Pipettes (1 - 20 µl / 20 - 200 µl / 200 - 1000 µl)	Gilson / Eppendorf
Robo Cycler Gradient 96 (PCR cycler)	Stratagene
semi-dry blotting chamber	Phase, Luebeck
Sonopuls HD 200 (sonyfier)	Bandelin, Berlin
Spectramax 340PC (96-well plate spectrophotometer)	Molecular Devices, Sunnyvale, USA

Equipment	Company
TCS SL (Confocal laser scanning microscope)	Leica, Wetzlar
Tecan Infinite 200 Reader	Tecan, Crailsheim
vertical gel electrophoresis chamber	Phase, Luebeck
Vortex Genie 2 (vortex mixer)	Scientific Industries, Bohemia, USA
xCELLigence device	Roche Diagnostics, Mannheim
Zeiss Axiovision system (Plan-Apochromat 63x/1.4 oil DIC M27 objective)	Zeiss, Jena
Zeiss Observer system (Plan-Apochromat 40x/0.8 NA M27 objective)	Zeiss, Jena

2.1.2 Consumables

Table 2: List of consumables used in this thesis

Consumable	Company
0.2 µm filter for sterile filtration	Sarstedt, Nümbrecht
6-well glass bottom cell culture dishes	MatTek Corporation
96-well E-plates	Roche Diagnostics, Mannheim
blotting paper, 3 mm Whatman	Schleicher Schuell, Dassel
cell culture dishes	Greiner, Frickenhausen
cell culture flasks	Greiner, Frickenhausen
cell culture plates	Greiner, Frickenhausen
Cryo vials 1ml	Greiner, Frickenhausen
falcon tubes (15 ml and 50 ml)	Greiner, Frickenhausen
Glass beads, acid-washed	Sigma-Aldrich, München
glass bottom cell culture dishes (35,0/10 mm)	Greiner BIO-ONE
glass coverslips 18 mm x 18 mm	Carl Roth GmbH & Co, Karlsruhe
pipette tips (1 - 20 µl; 20 - 200 µl; 200 - 1000 µl)	Greiner, Frickenhausen
pipettes (5 ml, 10 ml and 25 ml)	Corning Incorporated, Costar®
PVDF blotting membrane	Carl Roth GmbH & Co, Karlsruhe
reaction tubes 1.5 ml (standard and safe-lock)	Eppendorf, Hamburg
replica dishes	Sterilin Limited, Newport, UK
Transwells (24-well plate plus inserts) for cell migration assays, (0.8 µm pore size	Costar/Vitaris AG, Baar, Germany
X-ray films	CEA, Strangnas, Sweden

2.1.3 Kits

Table 3: List of kits used in this thesis

Kit	Company
DC protein assay Kit	Bio Rad, Munich, Germany
First Strand cDNA Synthesis Kit	MBI Fermentas, La Jolla, CA, USA
Human Phospho-Kinase Antibody Array	R&D System
HRP SuperSignal® West substrate pico	Pierce/Thermo, Rockford, USA
HRP SuperSignal® West substrate dura	Pierce/Thermo, Rockford, USA
PureLink™ HiPure plasmid DNA purification Kit	Invitrogen, Karlsruhe, Germany
PureLink™ PCR Purification Kit	Invitrogen, Karlsruhe, Germany
PureLink™ Quick Gel Extraction Kit	Invitrogen, Karlsruhe, Germany
QuikChange site-directed PCR mutagenesis Kit	Stratagene, La Jolla, CA, USA
REDTaq® ReadyMix™ PCR Reaction Mix	Sigma-Aldrich, Taufkirchen, Germany

2.1.4 Buffers and solutions

Table 4: List of buffers and solutions used in this thesis

Buffer/ Solution	composition
0,8% agarose gel	0,8 % agarose, 1:25000 x dilution ethidium bromide (10 mg/ml, Sigma) in 1x TAE buffer
10% APS solution	10% (w/v) APS in H 2 O
AP buffer	100 mM Tris pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$
Blocking solution (microscopy)	5% (v/v) goat serum, 0.1% (v/v) Tween20 in PBS
Blocking solution (Western blot)	0.5% (v/v) blocking solution, 0.05% (v/v) Tween20, 0.01% (v/v) thimerosal in PBS
Blotting buffer	200 mM glycine, 25 mM Tris base, 20% (v/v),methanol
Coomassie blue solution	0.1% Comassie blue R-250 in 40% Methanol, 10% acetic acid
Kinase assay buffer	50 mM Tris, pH 7.5, 10 mM MgCl2, 2 mM DTT
Laemmli SDS sample buffer (5x)	400 mM Tris, pH 6.8, 500 mM dithiothreitol, 50% (v/v) glycerol, 10% (w/v) SDS, 0.2% (w/v), bromophenol blue
LB Medium liquid	0.5% Bacto yeast extract, 1% Trypton, 1% Natrium Chlorid, pH 7.0
Lysis buffer (TEB)	50 mM Tris pH 7.5, 150 mM NaCl, 10 mM NaF, 20 mM ß-glycerolphosphate, 1 mM EDTA, 1% (v/v) Triton-X-100, 1mM Na_3VO_4, 0.5 mM PMSF, Complete protease inhibitor cocktail (EDTA-free, 1:25)

Buffer/ Solution	composition
Lysis buffer (RIPA)	50 mM Tris pH 7.5, 150 mM NaCl, 10 mM NaF, 20 mM ß-glycerolphosphate, 1 mM EDTA, 1% (v/v) Triton X-100. 0,1% (v/v) SDS, 0,5% (v/v) NaDOC,1mM Na_3VO_4, 0.5 mM PMSF, Complete protease inhibitor cocktail (EDTA-free, 1:25)
Phosphate Buffered Saline (PBS)	140 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4
PBS-Tween	0.05 % (v/v) Tween20 in PBS
PFA	4 % (w/v) in PBS
S1 resuspension buffer	50 mM Tris pH 8.0, 10 mM EDTA, 0.1 mg/ml RnaseA
S2 lysis buffer	200 mM NaCl, 1% SDS in ddH 2 O
S3 neutralization buffer	2.8 M potassium acetate in ddH 2 O, pH 5.1
Separating gel solution	8%,10%, 12% or 15% (v/v) acrylamide, 375 mM Tris, pH 8.8, 0.1% (v/v) SDS, 0.1% APS, 0.1% TEMED in H_2O
SDS-PAGE running buffer	25 mM Tris pH 8.8, 192 mM glycine, 0.1% SDS,
Stacking gel solution	5 % (v/v) acrylamide, 130 mM Tris, pH 6.8, 0.1% (v/v) SDS, 0.1% APS, 0.1% TEMED in H_2O
Tris-Acetat-EDTA (TAE), pH 8,0	40 mM Tris-acetate, 1 mM EDTA, pH 8.3
TE buffer (10x)	M Tris-HCl, 10 mM EDTA, pH7.5

2.1.5 Oligonucleotides (Primers and siRNAs)

Table 5: List of primers used in this thesis for mutagenesis or sequencing

Primer for cloning/ mutagenesis	Sequence 5'-3'
GIT1-S46A-for (Forward)	5'-CTGGGACGCCACATCGCCATTGTCAAGCACC-3'
GIT1-S46A-rev (Reverse)	5'-GGTGCTTGACAATGGCGATGTGGCGTCCCAG-3'
GIT1-S46D-for (Forward)	5'-GCCTGGGACGCCACATCGACATTGTCAAGCACC-3'
GIT1-S46D-rev (Reverse)	5'-GGTGCTTGACAATGTCGATGTGGCGTCCCAGGC-3'
PKD3 cDNA for (Forward)	5'-GGCCTCGAGATGTCTGCAAATAATTCC-3'
PKD3 cDNA rev (Reverse)	5'-GGCTCTAGATTAAGGATCTTCTTCCAT-3'
pEGFPN1-PKD3 for (Forward)	5'-GATCTCGAGCTCATGTCTGCAAATAATTCC-3'
pEGFPN1-PKD3 rev (Reverse)	5'-TGGATCCCGGGAAGGATCTTCTTCCATATC-3'
PKD3-S731E-for (Forward)	5'-ATCATTGGTGAAAAGGAATTCAGGAGATCTGTG-3'
PKD3-S735E-for (Forward)	5'-AAGGAATTCAGGAGAGAAGTGGTAGGAACTCCA-3'

Primer for sequencing	Sequence 5'-3'
CMV-For (Forward)	5'-CGCAAATGGGCGGTAGGCGTG-3'
pEGFP-C2 RP (Reverse)	5'-TTTAAAGCAAGTAAAACCTC-3'
GIT1-F750 (Forward)	5'-ATGGCTGACAGATCTCGGC-3'
pEGFP-C2-FP (Forward)	5'-GATCACATGGTCCTGCTG-3'

Table 6: List of siRNAs used in this thesis

siRNA/ smartpool siRNA	Sequence 5'-3'
siLacZ	5'-GCGGCUGCCGGAAUUUACC-3'
siPKD3-1	5'-GCAGAGUGAAAGUUCCACACACAUU-3'
siPKD3-2	5'-GCUGCUUCUCCGUGUUCAAGUCCUA-3'
siPKD1	5'-GUCGAGAGAAGAGGUCAAATT-3'
siPKD2	5'-GCAAAGACUGCAAGUUUAATT-3'
ON-TARGETplus SMARTpool control (siNon)	unknown

2.1.6 Plasmids, vectors and constructs

Table 7: List of vectors, plasmids and constructs used in this thesis

Plasmid, vector, construct	Source
pEGFP-C1 (empty vector)	Clontech, Mountain View, CA, USA
pEGFP-N1 (empty vector)	Clontech, Mountain View, CA, USA
pEFr Puro Flag A (empty vector)	Kindly provided by Jane Visvader, Walter and Eliza Hall Institute of Medical Research, Australia
pCR3.V62-Met-Flag (empty vector)	Kindly provided by Angelika Hauser, IZI, University of Stuttgart, Germany
GIT1-Flag (pEGFP-N1)	Addgene, Rick Horwitz (Manabe, Kovalenko et al. 2002)
GFP-GIT1 (pEGFP-C1)	Addgene, Rick Horwitz (Manabe, Kovalenko et al. 2002)
GIT1-Flag S46A (pEGFP-N1)	Quikchange of GIT1-Flag (pEGFP-N1)
GFP-GIT1 S46A (pEGFP-C1)	Quikchange of GFP-GIT1 (pEGFP-C1)
GIT1-Flag S46D (pEGFP-N1)	Quikchange of GIT1-Flag (pEGFP-N1)
GFP-GIT1 S46D (pEGFP-C1)	Quikchange of GFP-GIT1 (pEGFP-C1)
pCR3.V62-Met-Flag-PKD3	Kindly provided by Angelika Hauser, IZI, University of Stuttgart, Germany

Plasmid, vector, construct	Source
pEGFPN1-PKD3	Kindly provided by Angelika Hauser, IZI, University of Stuttgart, Germany
pCR3.V62-Met-Flag-PKD3 ca (S731/735E)	Quickchange of pEGFPN1-PKD3
pEGFPN1-PKD3 ca (S731/735E)	Quickchange of pEGFPN1-PKD3
pCMV 6M myc-PAK1 wt	Kindly provided by Ora Bernard (St. Vincent Institute of Medical Research, 9 Princes Street Fitzroy, Victoria 3065, Australia)
Flag-betaPIXa	Addgene (#15234) (Mayhew, Webb et al. 2006)
Flag-CERT	Kindly provided by Juan Saus, Spain
Flag-CERT S132A	Quickchange of Flag-CERT

2.1.7 Antibodies (primary and secondary)

Table 8: List of primary antibodies used in this thesis

Antibody	Species	Dilution	Company
anti-EEA1	rabbit pAb	1:100 (IF)	Cell Signaling, Frankfurt (Main)
anti-GST	goat pAb	1:2000 (WB)	GE Healthcare
anti-Flag	mouse mAb	1:1000 (WB)	Sigma-Aldrich, Taufkirchen, Germany
anti-GFP	mouse mAb	1:2000 (WB)	Roche Biosciences
anti-LAMP1	mouse mAb	1:400 (IF)	Developmental Studies Hybridoma Bank
anti-LC3B	rabbit mAb	1:200 (IF)	Cell Signaling, Frankfurt (Main)
anti-GIT1	mouse mAb	1:250 (WB)	BD, Heidelberg
anti-mTOR	rabbit mAb	1:200 (IF)	Cell Signaling, Frankfurt (Main)
anti-c-Myc (9E10)	mouse mAb	1:2000 (WB)	Kindly provided by Heiner Böttinger, IZI, University of Stuttgart, Germany
anti-p70 S6 Kinase	rabbit pAb	1:2000 (WB)	Cell Signaling, Frankfurt (Main)
anti-paxillin	rabbit pAb	1:500 (IF)	Santa Cruz Biotechnology, Santa Cruz, USA
anti-phospho-Akt (Ser473)	rabbit mAb	1:1000 (WB)	Cell Signaling, Frankfurt (Main)
anti-phospho CERT (pCERT)	rabbit pAb	1:2000 (WB)	phosphospecific antiserum that was raised against the PKD consensus site Ser132 in the lipid transfer protein CERT (Animal 3, day 90)

Antibody	Species	Dilution	Company
anti-phospho-mTOR (Ser2448)	rabbit mAb	1:50 (IF)	Cell Signaling, Frankfurt (Main)
anti-phospho-S6 ribosomal protein (Ser240/244)	rabbit pAb	1:1000 (WB)	Cell Signaling, Frankfurt (Main)
anti-phospho-(Ser/Thr) PKD substrates antibody	rabbit pAb	1:1000 (WB)	Cell Signaling, Frankfurt (Main)
anti-phospho-p44/42 MAPK (Erk1/2, Thr202/204)	rabbit pAb	1:1000 (WB)	Cell Signaling, Frankfurt (Main)
anti-phospho-p70 S6 Kinase (Thr389)	mouse mAb	1:1000 (WB)	Cell Signaling, Frankfurt (Main)
anti-p230 trans Golgi	Mouse mAb	1:250 (IF)	BD, Heidelberg
anti-PKD1 (C20)	rabbit pAb	1:2000 (WB)	Santa Cruz Biotechnology, Santa Cruz, USA
anti-PKD2	rabbit pAb	1:2000 (WB)	Calbiochem, S. Francisco, USA
anti-PKD3	rabbit pAb	1:1000 (WB)	Cell Signaling, Frankfurt (Main)
anti-αtubulin	mouse mAb	1:2000 (WB)	Sigma-Aldrich, Taufkirchen, Germany
anti-transferrin receptor	mouse mAb	1:1000 (WB)	Invitrogen, Karlsruhe, Germany
anti-vinculin	mouse mAb	1:400 (IF)	Sigma-Aldrich, Taufkirchen, Germany

Table 9: List of secondary antibodies used in this thesis

Antibody	Recognized IgG	Species	Dilution	Company
Alexa Fluor® 488	anti-mouse	goat	1:500 (IF)	Invitrogen, Karlsruhe, Germany
Alexa Fluor® 488	anti-rabbit	goat	1:500 (IF)	Invitrogen, Karlsruhe, Germany
Alexa Fluor® 546	anti-mouse	goat	1:500 (IF)	Invitrogen, Karlsruhe, Germany
Alexa Fluor® 546	anti-rabbit	goat	1:500 (IF)	Invitrogen, Karlsruhe, Germany
Alexa Fluor® 633	anti-mouse	goat	1:500 (IF)	Invitrogen, Karlsruhe, Germany
Alexa Fluor® 633	anti-rabbit	goat	1:500 (IF)	Invitrogen, Karlsruhe, Germany
Horseradish peroxidase (HRP)	anti-mouse	sheep	1:10000 (WB)	GE Healthcare, Munich

Antibody	Recognized IgG	Species	Dilution	Company
Horseradish peroxidase (HRP)	anti-rabbit	donkey	1:10000 (WB)	GE Healthcare, Munich
Horseradish peroxidase (HRP)	anti-goat	donkey	1:100000 (WB)	Santa Cruz Biotechnology, Santa Cruz, USA

2.1.8 Enzymes

Table 10: List of enzymes used in this thesis

Enzyme	Company
Calf Intestine Alkaline Phosphatase (CIAP)	MBI Fermentas, St. Leon-Rot, Germany
Dpn1	MBI Fermentas, St. Leon-Rot, Germany
GST-tagged recombinant human PKD3	Invitrogen, Karlsruhe, Germany
PfuUltra™ High-Fidelity DNA Polymerase AD	Stratagene, La Jolla, CA, USA
Restriction enzymes	MBI Fermentas, St. Leon-Rot, Germany
RNase A stock solution (20 mg/ml)	Invitrogen, Karlsruhe, Germany
T4 DNA Ligase	MBI Fermentas, St. Leon-Rot, Germany

2.1.9 Chemicals, solvents and reagents

Table 11: List of chemicals, solvents and reagents used in this thesis

Chemical/ solvent	Company
Acrylamide, Rotiphorese Gel 30	Carl Roth GmbH & Co, Karlsruhe
Adenosine-5‘-triphosphate (ATP)	Sigma-Aldrich, München
Agarose	Carl Roth GmbH & Co, Karlsruhe
Alexa Fluor 546® -labeled phalloidin	Molecular Probes /Invitrogen, Carlsbad, USA
Alexa Fluor 633® -labeled phalloidin	Molecular Probes /Invitrogen, Carlsbad, USA
Ammonium persulfate (APS)	Carl Roth GmbH & Co, Karlsruhe
Ampicillin	Sigma-Aldrich, München
Anti-Flag M2 Affinity Gel	Sigma-Aldrich, München
Blocking reagent	Roche Diagnostics, Mannheim
Bromphenol blue	Serva, Heidelberg
Calcium chloride ($CaCl_2$)	Merck, Darmstadt
Complete Protease Inhibitor Cocktail (EDTA free)	Roche Diagnostics, Mannheim

Chemical/ solvent	Company
Crystal violet	Merck, Darmstadt
Dimethylsulfoxide (DMSO)	Carl Roth GmbH & Co, Karlsruhe
Disodium hydrogen phosphate (Na_2HPO_4)	Carl Roth GmbH & Co, Karlsruhe
Dithiothreitol (DTT)	Carl Roth GmbH & Co, Karlsruhe
DNAorange	Sigma-Aldrich, München
DRAQ5™	Biostatus Limited, Leicestershire, United Kingdom
Ethanol (EtOH)	Carl Roth GmbH & Co, Karlsruhe
Ethidium bromide (EtBr)	Roche Diagnostics, Mannheim
Ethylene diamine tetraacetic acid (EDTA)	Carl Roth GmbH & Co, Karlsruhe
Fluoromount-G	Southern Biotech, Birmingham, USA
GeneRuler 1 kb DNA ladder)	MBI Fermentas, St. Leon-Rot
glutathione beads	Pierce / Thermo, Rockford, USA
Glycerol	Carl Roth GmbH & Co, Karlsruhe
Glycine	Carl Roth GmbH & Co, Karlsruhe
Goat Serum	Invitrogen, Karlsruhe
Hydrochloric acid (HCl)	Carl Roth GmbH & Co, Karlsruhe
Isopropanol	Carl Roth GmbH & Co, Karlsruhe
Kanamycin	Carl Roth GmbH & Co, Karlsruhe
Methanol (MeOH)	Carl Roth GmbH & Co, Karlsruhe
N,N,N,N-Tetramethylethyldiamine (TEMED)	Carl Roth GmbH & Co, Karlsruhe
NP-40 (Nonidet P40)	Sigma-Aldrich, München
PageRuler prestained protein ladder	MBI Fermentas, St. Leon-Rot
Paraformaldehyde (PFA)	Carl Roth GmbH & Co, Karlsruhe
Phenylmethylsulphonyl fluoride (PMSF)	Sigma-Aldrich, München
Potassium acetate	Sigma-Aldrich, München
Potassium chloride (KCl)	Carl Roth GmbH & Co, Karlsruhe
Propidium iodide (PI)	Invitrogen, Karlsruhe
Protein G Sepharose beads	KPL, Gaithersburg, USA
Sodium azide (NaN_3)	Sigma-Aldrich, München
Sodium chloride (NaCl)	Carl Roth GmbH & Co, Karlsruhe
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co, Karlsruhe
Sodium fluoride (NaF)	Carl Roth GmbH & Co, Karlsruhe

Chemical/ solvent	Company
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, München
Thimerosal	Carl Roth GmbH & Co, Karlsruhe
Tris-hydroxymethyl-aminomethane (Tris)	Carl Roth GmbH & Co, Karlsruhe
Triton X-100	Carl Roth GmbH & Co, Karlsruhe
Tween 20	Carl Roth GmbH & Co, Karlsruhe
β-Glycerophosphate	Sigma-Aldrich, München
β-Mercaptoethanol	Carl Roth GmbH & Co, Karlsruhe

2.1.10 Transfection reagents

Table 12: List of transfection reagents used in this thesis

Transfection reagent	Company
Lipofectamine™ 2000	Invitrogen, Karlsruhe, Germany
Lipofectamine® RNAiMAX	Invitrogen, Karlsruhe, Germany
Oligofectamine™	Invitrogen, Karlsruhe, Germany
TransIT® 293	Mirus, Madison, USA

2.1.11 Cell culture reagents and media

Table 13: List of cell culture reagents and media used in this thesis

Cell culture reagent/ media	Company
Alexa Fluor 546 phalloidin	Invitrogen, Karlsruhe
Collagen R solution	Serva, Heidelberg
DMEM	Invitrogen, Karlsruhe
Fetal calf serum (FCS)	PAA Laboratories, Pasching, Austria
G418	Invitrogen, Karlsruhe
Gö6976	Calbiochem, S. Francisco, USA
Gö6983	Calbiochem, S. Francisco, USA
Lysotracker Red DND-99	Invitrogen, Karlsruhe
NB-172-40 (PKD-Inhibitor)	Tocris Bioscience, Bristol, UK
OptiMEM®	Invitrogen, Karlsruhe
phorbol-12, 13-dibutyrate (PDBu)	Enzo Life Sciences
RPMI 1640 + L-glutamine (RPMI 1640)	Invitrogen, Karlsruhe

Cell culture reagent/ media	Company
RPMI 1640 + L-glutamine, phenol red-free	Invitrogen, Karlsruhe
staurosporine	Alexis Biochemicals
Trypsin-EDTA (10 x stock)	Invitrogen, Karlsruhe

2.1.12 Bacterial strain

The competent bacteria strain DH5α (*E. coli*) was from Invitrogen, Karlsruhe in Germany.

2.1.13 Human cell lines

Fresh cultures of the human cell lines were established every three months from frozen stocks stored in liquid nitrogen.

Table 14: List of humans cell lines used in this thesis

Cell line	Originally obtained from
BT474	Nancy Hynes ,Friedrich Miescher Institute, Basel, Switzerland
HEK293T	Angelika Hauser, IZI, University of Stuttgart, Germany
HS-578T	Bernhard Lüscher, RWTH Aachen University, Germany
MCF7	Cornelius Knabbe, Institute of Clinical Pharmacology, Stuttgart, Germany
MDA-MB-134	Cornelius Knabbe, Institute of Clinical Pharmacology, Stuttgart, Germany
MDA-MB 157	Cornelius Knabbe, Institute of Clinical Pharmacology, Stuttgart, Germany
MDA-MB 231	CLS, Heidelberg, Germany
MDA-MB 453	Jane Visvader, The Walter and Eliza Hall Institute, Melbourne, Australia
MDA-MB 468	Bernhard Lüscher, RWTH Aachen University, Germany
SKBR3	Nancy Hynes, Friedrich Miescher Institute, Basel, Switzerland
T47D	Cornelius Knabbe, Institute of Clinical Pharmacology, Stuttgart, Germany
ZR751	Bernhard Lüscher, RWTH Aachen University, Germany

2.2 Methods

2.2.1 DNA cloning and constructs

Full-length GIT1 wild type plasmids (based on pEGFP-N1 and pEGFP-C1 vectors from Clontech) were obtained from Addgene and either contained a Flag (pEGFP-N1) or GFP (pEGFP-C1) tag fused to the cDNA and kanamycin as a selection marker. The plasmids were kindly provided by the principal investigators Manabe and colleagues (Manabe,

Kovalenko et al. 2002). Both plasmids were modified by QuickChange Site-directed polymerase chain reaction (PCR) Mutagenesis (Stratagene) to introduce point mutations in order to create GIT1 S46A and S46D mutants. The full-length PKD3 cDNA was PCR-amplified using cDNA from HEK293T cells as a template. The PCR product was cloned into the pCR3.V62-Met-Flag vector by XhoI/XbaI restriction. pEGFPN1-PKD3 was generated by PCR amplification of the PKD3 cDNA using pCR3.V62-Met-Flag-PKD3 as a template, and cloned into the pEGFPN1 vector by SacI/XmaI restriction. To obtain the PKD3-ca (S731/735E) cDNA, sequential site-directed PCR mutagenesis was carried out. Plasmid pCMV 6M encoding myc-tagged full-length PAK1 (pCMV6M-myc-PAK1) was kindly provided from Ora Bernard (St. Vincent Institute of Medical Research, 9 Princes Street Fitzroy, Victoria 3065, Australia) and the Flag-betaPIXa expression plasmid was obtained from Addgene (#15234) (Mayhew, Webb et al. 2006).

The amplified cDNAs were verified by sequencing and all oligonucleotides were purchased from MWG Biotech. All primers used for PCR, mutagenesis and sequencing are listed in Table 5 and all plasmids and vectors used in the study are listed in Table 7.

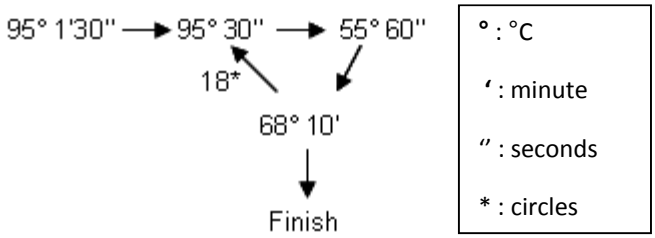
2.2.1.1 QuickChange Site directed PCR Mutagenesis

Point mutations were generated by site directed PCR mutagenesis according to the manufacturer's instructions using specific designed mutagenic primers (Table 5). The mutagenic primer design was accomplished according to the following requirements: Primers should be between 25 and 45 bases in length with a melting temperature (Tm) of the primer-template complex of at least 78°C. The desired mutation should be in the middle of the primer with approximately 10-15 bases of correct sequence on both sides. The optimal GC content should be around 40% and the primers should terminate with one or more C and G bases. The reagents for the PCR samples were combined as described in Table 15.

Table 15: Reagents for PCR

	Volume [µl]	Indication
Buffer	5	10 x Pfu Ultra Reaction Buffer AD, Stratagene
Primer 1 (Forward)	1.5	See table 5 (1:10 dilution, 10 pmol/µl)
Primer 2 (Reverse)	1.5	Complementary to forward primer (1:10 dilution, 10 pmol/µl)
Template	1	plasmid DNA (~20 ng)
dNTP	1.5	10 mM dNTP-Mix, Stratagene
enzyme	1	Pfu-Ultra DNA Polymerase, 2,5 U/µl, 500 U, Stratagene
dH2O	38.5	Autoclaved distilled water (ddH_2O)

The following PCR-program was used to introduce the mutations:



The amplification of the PCR product was controlled on a 0.8 % agarose gel (DNA-Marker: GeneRuler 1kb DNA Ladder, 0.5 µg/µl, Fermentas).

The methylated, nonmutated parental DNA template in the PCR product was digested with Dpn I (10 U/µl, 500 U, Fermentas). The Dpn I endonuclease is specific for the target sequence 5´-Gm6ATC-3` in methylated and hemi-methylated DNA (DNA from *E. coli* DH5α strain is dam methylated) and allowed for the selection of PCR-amplified DNA containing the desired mutation. 1.5 µl of Dpn I was added to each PCR product, the samples were incubated for 2-4 h at 37°C.

2.2.1.2 Transformation of competent bacteria

The digested PCR product was used to transform competent bacteria DH5α (*E. coli*). 100 µl of competent cells (stored at -80°C) were thawed on ice, 3 µl of digested PCR product were added, mixed by tapping and left for 10 min on ice. The transformation mix was incubated for 1 min at 42°C, for 1-2 min on ice, 1 ml of LB medium was added, the mix was incubated at 37°C with shaking for 1 hour and the cells were spun down (5000 rpm, 1 min, 25 °C) after recovery. The supernatant was discarded and 100 µl were left to resuspend the cells. The cell suspension was plated onto LB plates with the appropriate antibiotic (1x kanamycin or ampicillin) and incubated overnight at 37 °C.

2.2.1.3 Purification of plasmid DND (Mini-Prep)

Two colonies of the transformed *E.coli* were incubated overnight in 2 ml of LB medium in the presence of the appropriate antibiotic (1x kanamycin or ampicillin). Afterwards the plasmid DNA of the overnight *E. coli* cultures was isolated and purified. 1.5 ml of the overnight cultures were transferred to a 1.5 ml microfuge tube and spun down (13000 rpm, 2 min, and 25°C). The pellet was resuspended in 150 µl buffer S1, 150 µl of buffer S2 were added and the solution was carefully mixed by inverting. After incubation on ice for maximal 5 min, 150 µl of buffer S3 were added and the solution was mixed by inverting, incubated on ice for 5 min and centrifuged for 10 min at 13000 rpm. The supernatant was transferred to a new tube and centrifuged again for 2 min at 13000 rpm. The clear supernatant was then mixed with 1 ml of 100% EtOH, incubated on ice for 10 min and the precipitated DNA was pelleted by centrifugation (2min, 13000 rpm, 4°C). The pellet was washed with 70% EtOH, dried and resolved in 30 µl of sterile water.

2.2.1.4 Sequencing of mutated constructs

The mutated plasmids were sequenced to make sure that the introduced mutation was at the right position and to exclude other unwanted mutations. Therefore the samples (template: 30-100 ng) and the sequencing primers (Table 5) were sent to GATC Biotech and the sequencing results were analyzed with HUSAR Genius Bioinformatic Labs using the wild type sequence as a reference.

2.2.1.5 Purification of plasmid DNA (Midi-Prep)

To obtain high amounts of pure plasmid DNA for transformations, a midi preparation (Midi-Prep) was performed using the PureLink HiPure plasmid DNA purification Kit from Invitrogen according to the manufacturer's instructions with one modification. Instead of cell lysate clearance by centrifugation, a filtration through filter paper (Macherey-Nagel, Ø 125 mm) was used to separate the aqueous phase from the mixture. Afterwards, the plasmid DNA concentration was determined and adjusted to 0.5 µg/µl. All plasmids and constructs are listed in Table 7.

2.2.2 Cell culture

HEK293T cells were kindly provided by Dr. Angelika Hausser (IZI, University of Stuttgart, Germany). MDA-MB 231 cells were obtained from CLS, Heidelberg, Germany. MCF7, T47D, MDA-MB 157, and MDA-MB-134 cells were obtained from Cornelius Knabbe (Institute of Clinical Pharmacology), BT474 and SKBR3 cells from Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland), MDA-MB 453 cells from Jane Visvader (The Walter and Eliza Hall Institute, Melbourne, Australia), and HS-578T, ZR751, and MDA-MB 468 cells from Bernhard Lüscher (RWTH Aachen University). MDA-MB-231 cells were cultivated under sterile conditions in DMEM (Invitrogen), all other cell lines were cultured in RPMI 1640 (Invitrogen), supplemented with 10% fetal calf serum (FCS; PAA) and incubated in a humidified atmosphere of 5% CO2 at 37°C. Before reaching confluence, cells were passaged using trypsin-EDTA for cell detachment and maintained in culture for not longer than three month. New cultures of each cell line were established from frozen stocks containing the respective culture medium supplemented with 10% DMSO which were stored in liquid nitrogen. The cell number per ml was determined by using the CASY® cell counter.

2.2.3 Cell transfection

HEK293T cells were transiently transfected with TransIT® 293 (Mirus Bio) according to the manufacturer's instructions. Briefly HEK293T cells were seeded in order to reach 80% confluence the day of transfection and allowed to adhere overnight. For transfection in 6 well format, 2 µl TransIT® reagent (per 1 µg DNA) and 100 µl OptiMEM® medium were

mixed and incubated for 15 min at RT. 1 µg of DNA was added to the mixture and after 15 min incubation, the mix was carefully added drop-wise to the cells. Cells were used for further studies 24-48 h after transfection.

MCF-7 and MDA-MB-231 cells were transiently transfected with Lipofectamine™ 2000 (Invitrogen) according to the manufacturer's instructions using a ratio of DNA to Lipofectamine™ 2000 of 1:2.5. Briefly, cells were seeded in order to reach 90% confluence the day of transfection and allowed to attach overnight. Before transfection, medium was changed and for transfection in 12-well format, 0.25 µg plasmid were mixed with 50 µl OptiMEM® and incubated for 5 min at RT. Additionally, Lipofectamine™ 2000 (2,5 µl per 1 µg DNA) was mixed with 50 µl Optimen and added to the DNA/Optimen mixture. After 20 min incubation, the mixture was carefully added to the cells and changed 6 h later to remove the transfection reagents. After one day, cells were used for further studies.

For RNA interference experiments, cells were transiently transfected with short interfering RNAs (siRNAs) using Oligofectamine™ or Lipofectamine® RNAiMAX (Invitrogen) according to the manufacturer's instructions. All the required materials and reagents (tips, medium, water, pipettes, siRNA) needed to be RNAse-free to avoid contaminations with RNA-degrading enzymes. For oligofection in 6 well format, cells were seeded in order to reach 30%-40% confluence the day of transfection and allowed to attach overnight. Before oligofection, growth medium was replaced with 800 µl of serum free media, and 2 µl Oligofectamine™ was diluted in 13 µl OptiMEM®, incubated at RT for 5 min and mixed with 185 µl of a siRNA/OptiMEM® solution (180 µl OptiMEM® and 5 µl siRNA of 20 µM stock, siRNA end concentration was 100 nM) and incubated for 15 min at RT to form complexes. The transfection solution was added drop wise to the cells and after 4 hours, 500 µl of RPMI containing 30% FCS were added to the cells, yielding a final concentration of 10 % FCS. Cells were used for further studies 48-72 h after transfection. For reverse Lipofectamine® RNAiMAX transfection in 6 well format, a cell suspension of 250000 cells in 2.5 ml medium per well was prepared. Next, 1.5 µl siRNA of a 20 µM stock solution (siRNA end concentration was 10 nM) were diluted in 500 µl OptiMEM®, mixed and 5 µl Lipofectamine® RNAiMAX were added and the mixture was incubated for 15 min at RT to form siRNA-lipid complexes. The transfection solution and cell suspension were added simultaneously to the well and cells were used for further studies 48-72 h after transfection.

Transfection experiments performed in culture dishes with different formats compared to the ones mentioned above were scaled up or down according to the surface area. All plasmids and siRNAs used in the study are listed in the corresponding materials part.

2.2.4 Generation of stable cell lines

Stable MCF7 sublines cells were generated by nucleofection according to the manufacturer's instructions (program P020, Kit V; Amaxa) with pEGFPC1 GIT1 expression vectors followed by selection with 1 mg/ml G418 (Calbiochem) and FACS sorting of GFP-positive cells (medium expression intensity). Briefly, cells were harvested by centrifugation and counted. For each nucleofection sample, 1.75 million cells were mixed with 100 µl nucleofection solution and added to 3 µg DNA. After nucleofection, 500 µl of medium was added, the cells were transferred to 10 cm tissue culture plates containing 10 ml medium and after 48 h of recovery selection was started with 1 mg/ml G418 and continued for at least 2 weeks with regular medium change. Stable expression of the GFP-tagged GIT1 proteins in the MCF7 sublines was verified by frequent FACS analysis and immunoblotting.

2.2.5 Cell lysis

The medium was carefully removed, cells were washed with cold PBS and lysed in 350 µl lysis buffer (RIPA or 1% TEB) per 6 well or 175 µl per 12 well, transferred to a microfuge tube and briefly vortexed. The lysate was incubated for 10 min on ice and clarified by centrifugation at 13 000 rpm for 10 min (4°C). The supernatant was then transferred to a new microfuge tube.

2.2.6 Quantification of protein amounts

Total protein amount of cell lysates was determined by using the DC Protein Assay (Bio-Rad). The standard protocol was as follows: 5 µl of cell lysates were transferred to 96-well plates and incubated with 25 µl provided Reagent A and Reagent S mixture (1:50 ratio) and 200 µl of Reagent B. After 15 min, the color development due to the amino acids tyrosine and tryptophan was read at 750 nm. By comparing the absorbance with a BSA standard curve, the protein concentration of the lysates was determined.

2.2.7 Immunoprecipitation

For immunoprecipitation, equal amounts of protein were incubated with specific antibodies for 2-4 h at 4°C with gentle agitation. Immune complexes were collected with protein G-Sepharose (KPL) and washed 3x with TEB buffer. Precipitated proteins were released by boiling in 1x Laemmli SDS sample buffer for 5 min at 95°C.

2.2.8 SDS polyacrylamide gel electrophoresis (SDS-PAGE)

Equal protein amounts (usually 50 or 70 µg) of cell lysates were denaturated in 1x Laemmli SDS sample buffer for 5 min at 95°C and loaded onto Tris/glycine SDS

polyacrylamide gels. The gels consisted of an 8%, 10%, 12% or 15% polyacrylamide running gel for high and low molecular weight proteins, respectively, and a stacking gel with 5% polyacrylamide. Electrophoresis was carried out by using a vertical gel electrophoresis chamber (Phase, Luebeck) and the gels were run in 1x SDS running buffer for approximately 1h 30 min at 50 mA. After electrophoretic protein separation Western blotting was performed.

2.2.9 Western blotting

For Western blot analysis, the proteins separated by SDS PAGE were transferred to polyvinylidine difluoride membrane (PVDF, Roth). The PVDF membrane was hydrated with 100% methanol and then equilibrated with blotting buffer. The proteins were blotted on membrane by semi-dry transfer using a semi-dry blotting chamber (Phase, Luebeck) and a constant current of approximately 150 mA (1.5- 1.6 mA/cm^2) for 2 hours at room temperature. The membrane was blocked with 0.5 % blocking reagent (Roche) in PBS containing 0.1% Tween at RT for 1 hour and incubated overnight at 4°C with the primary antibody (diluted according to the manufacturer's instructions in blocking solution). The membrane was washed three times with 1x PBS-T and incubated with the appropriate secondary antibody (Horseradish Peroxidase (HRP) conjugate, 1:10000 dilution in 0,5% blocking solution) for 1 hour at RT. After washing (3x with 1x PBS-T) the membrane was treated with a peroxidase substrate (enhanced chemiluminescence (ECL) detection system from Pierce) according to the manufacturer's instructions to visualize the signals and exposed to an X-ray film for different time points.

2.2.10 Mass spectrometry analysis

HEK293T cells transiently expressing Flag-tagged GIT1 (5x 10cm dishes) were stimulated with PDBu, lysed in 1% TEB and GIT1 was immunoprecipitated with anti-Flag M2 agarose (Sigma Aldrich). The beads were washed with TEB and PBS, followed by elution with 0.1 M glycine, pH 2.5 and neutralization with 1/10 volume of 1 M Tris, pH 8. Eluted protein was subjected to SDS-PAGE and the gel was stained with Colloidal Coomassie Blue (Roth). GIT1 expression and phosphorylation was verified by parallel Western blotting. The Coomassie stained Flag-tagged GIT1 band was in gel digested with trypsin as described previously (Borchert, Dieterich et al. 2010). Acetonitrile was added to the peptide mixture to a final concentration of 30% and the pH was adjusted to 2-3. Enrichment of phosphopeptides by titanium dioxide chromatography was done as described previously (Olsen and Macek 2009) with the following modifications: phosphopeptide elution from the beads was performed three times with 100 µl 40% ammonia hydroxide solution in 60% acetonitrile at a pH of >10.5. Analysis of the phosphopeptides was done on a Proxeon Easy-LC system (Proxeon Biosystems,

Denmark) coupled to a LTQ-Orbitrap-XL (Thermo Fisher Scientific, Germany) equipped with a nanoelectrospray ion source (Proxeon Biosystems, Denmark) as described previously (Koch, Krug et al. 2011). The five most intense precursor ions were fragmented by activation of neutral loss ions at –98, –49, and –32.6 relative to the precursor ion (multistage activation). Additionally an inclusion list containing the doubly charged ions of tryptic GIT1 peptides that include serine 46 in the phosphorylated form was applied. Mass spectra were analyzed using the software suite MaxQuant, version 1.0.14.3 (Cox, Matic et al. 2009). The data were searched against a target-decoy human database (ipi.HUMAN.v3.77) containing 182695 forward protein sequences and 262 frequently observed contaminants. Trypsin was set as protease in which two missed cleavage sites were allowed. Beside acetylation at the N-terminus and oxidation of methionine, phosphorylation of serine, threonine, and tyrosine were set as variable modifications. Carbamidomethylation of cysteine was set as fixed modification. Initial precursor mass tolerance was set to 7 parts per million (ppm) at the precursor ion and 0.5 Da at the fragment ion level. Phosphorylation events with a localization probability of at least 0.75 were considered to be assigned to a specific residue. Spectra of modified peptides were manually validated.

2.2.11 Phosphorylation assay

HEK293T cells were transiently transfected with plasmids encoding Flag-GIT1 wt and S46A. Proteins were immunoprecipitated from whole cell lysates with Flag-specific mouse mAb and Protein G agarose and washed with kinase buffer. Immunoprecipitated proteins were incubated in the presence (+) or absence (-) of purified GST-tagged PKD3 with 20 µM ATP in kinase buffer for 30 min at 37°C. The reaction was stopped by addition of sample buffer and proteins were separated by SDS-PAGE and analyzed by immunoblotting.

2.2.12 Immunofluorescence microscopy

Cells (either parental or transiently transfected with expression vectors or siRNAs) grown on glass coverslips coated with 25 µg/ml collagen R solution (Serva, diluted in PBS for 2 h at 37°C) were washed once with PBS containing $CaCl_2$/$MgCl_2$, fixed with 4% PFA for 10 min, incubated with 150 mM glycine, permeabilized with PBS containing 0.2% Triton X-100 for 5 min and blocked with 5% goat serum (Invitrogen) in PBS containing 0.1% Tween-20 (PBS-T) for 30 min. Cells were then incubated for 2 h with primary antibodies in blocking buffer, followed by washing steps with PBS-T and incubation with secondary antibody in blocking buffer for 1 h. Coverslips were mounted in Fluoromount G (Southern Biotechnology) and analyzed on a confocal laser-scanning microscope (TCS SL, Leica) using 488 nm, 543 nm and 633 nm excitation and a 63x HCX Plan Apo CS oil objective

lens. Images were processed with Adobe Photoshop. Alternatively, a Zeiss LSM 700 confocal laser-scanning microscope using 488, 561 and 633 nm excitation with the oil objective lense Plan-Apochromat 63x/1.40 DIC M27 was used and images were processed with the ZEN software.

2.2.13 Live cell imaging

MCF7 cells stably expressing GFP-GIT1 S46D were plated at low density on collagen-coated glass bottom cell culture dishes (35,0/10 mm, Greiner BIO-ONE). After overnight cell adhesion, recording was started by acquiring fluorescence (488 nm) images every 40 s for a duration of 20 min with a Zeiss Axiovision system equipped with a heated incubation chamber (5% CO2, 37°C) using a Plan-Apochromat 63x/1.4 oil DIC M27 objective lense and the Axiovision software.

2.2.14 Spreading assay

MCF7 cells stably expressing GIT1 wt, S46A and S46D were seeded at low density (10.000 cells) into collagen-coated (10 µg/ml) 96-well E-plates (Roche). For quantitative initial spreading analysis, the impedance of cells was measured using an xCELLigence device (Roche) and the mean cell index was plotted.

2.2.15 Protrusion assay

MCF7 cells stably expressing GIT1 wt, S46A and S46D were plated at low density on collagen-coated 6-well glass bottom cell culture dishes (MatTek Corporation). After overnight cell adhesion, recording of single cells was started by acquiring fluorescence (488 nm) and bright field (phase contrast) images every 30 min for several hours with a Zeiss Observer system equipped with a heated incubation chamber (5% CO2, 37°C) using a Plan-Apochromat 40x/0.8 NA M27 objective and Zeiss Axiovision software. Then, GFP-positive cells were outlined using a paintboard (Waco) at the beginning and the end of a 30 min time interval as described previously (Manabe, Kovalenko et al. 2002). The outlined images were overlaid and the change in total cell area normalized to the initial cell size was calculated using Image J software.

2.2.16 Migration assay

MCF7 cells stably expressing GIT1 wt, S46A and S46D were harvested, washed twice with 5 ml medium supplemented with 0.5% FCS, counted and 50.000 cells in 100 µl medium containing 0.5% FCS were seeded into Transwells (0.8 µm pore size; Costar) coated with 10 µg/ml collagen on the underside and allowed to migrate overnight. The bottom chamber contained medium supplemented with 10% FCS. Cells on the top surface

of the membrane were removed using a cotton swab and cells on the underside of the membranes were fixed with 4% PFA, stained with 0.2% crystal violet and counted in 5 independent microscopic fields at a 20-fold magnification. Experiments were performed in triplicates.

2.2.17 Microarray data analysis

Normalized gene expression microarray data from datasets GSE3744/GDS2250 (Richardson, Wang et al. 2006) and GSE1561/GDS1329 (Farmer, Bonnefoi et al. 2005) was retrieved from NCBI gene expression omnibus (GEO). Expression values for PKD3, PKD1 and PKD2 (also called PRKD3, PRKD1 and PRKD2) were extracted and boxplots were generated in concordance with published subgroup classifications of each dataset. Student T-test analysis was performed in order to assess significance of PKD expression between subgroups.

2.2.18 Human Phospho-Kinase Antibody Array

The Human Phospho-Kinase Antibody Array was purchased from R&D Systems and was performed according to the manufacturer's instructions. Briefly, MCF7 cells were transiently transfected by nucleofection with an expression vector encoding for GFP-PKD3 ca (catalytically active, S731/735E) or a control vector. After 48 h, cells were lysed in array lysis buffer and the protein concentration was determined by Bio-Rad DC protein assay. Equal amounts of cell lysates were diluted in array buffer and incubated overnight with the Human Phospho-Kinase Array. After washing, the array was incubated with a cocktail of biotinylated detection antibodies, washed again, followed by application of streptavidin-horseradish peroxidase and chemiluminescent detection reagents. Quantification of spot intensity on the developed films was performed using Image J.

2.2.19 Crystal violet proliferation assay

Cells were transiently transfected with PKD3-specific siRNAs. Cells transfected with control siRNA as well as mock-transfected cells were used as controls. 24h after transfection, 2500 cells were seeded in sextublicate into 96-well plates and then fixed with Roti®-Histofix (4%) and stained with crystal violet (0.2% in ddH2O) after 6 h, or 3 and 5 days later. After rinsing and drying, the dye was dissolved in 200 µl methanol and absorption was measured at 550 nm using a microplate reader (Tecan Infinite 200 Reader). The absorption on days 3 and 5 was normalized to the absorption measured 6 h after plating.

3 Results

In the first part of the study we addressed the question whether the GIT1 protein activity is regulated by PKD-mediated phosphorylation and identified PKD3 to be exclusively responsible for GIT1 phosphorylation at serine 46. We further investigated the physiological consequences of GIT1 phosphorylation at this site in breast cancer cells using a combination of biochemical and cellular assays.

3.1 GIT1 phosphorylation by PKD3

3.1.1 GIT1 is phosphorylated downstream of PKD3

PKD substrates have successfully been identified in the past using a phosphospecific antibody that recognizes the PKD consensus motif (Doppler, Storz et al. 2005). To investigate whether GIT1 is a substrate for the PKD family members, we used phorbol-12,13-dibutyrate (PDBu), an analog of diacylglycerol, to stimulate cellular PKD activity. PDBu activates novel PKCs, which in turn phosphorylate PKD family members on two specific serines within the activation loop. In addition, PDBu activates PKD directly by high-affinity binding to cysteine-rich motifs within the protein. HEK293T cells transiently expressing Flag-tagged GIT1 were stimulated with PDBu, followed by immunoblotting of GIT1 immunoprecipitates with a commercially available phospho-(Ser/Thr) PKD substrate antibody. A basal signal corresponding to GIT1 was obtained that increased upon PDBu stimulation (Fig. 7A, top panel). Preincubation of cells with specific pharmacological inhibitors of the PKC/PKD pathway abrogated GIT1 detection with the phosphospecific antibody (Fig. 7A, top panel), indicating that GIT1 was recognized in a phosphorylation-dependent manner. Gö6976 inhibits PKD family members directly, whereas Gö6983 can inhibit PKD indirectly by inhibiting novel PKCs. Staurosporine, which blocks a wide range of kinases including PKCs and PKD, was used as a control. Since preincubation of cells with Gö6983 did not affect GIT1 recognition by the phosphospecific antibody, this indicates that GIT1 phosphorylation downstream of PKD occurs independently of novel PKCs. Identical results were obtained when GIT1 immunoprecipitates were immunoblotted with a phosphospecific antiserum that was originally raised against the PKD consensus site (Ser132) in the lipid transfer protein CERT (Fugmann, Hausser et al. 2007), (Fig. 7A, middle panels). This antibody recognizes CERT only when phosphorylated on serine 132 (Fig. 7B). Due to its crossreactivity, this antiserum was used for the detection of phosphorylated GIT1 (pGIT1) in all further experiments.

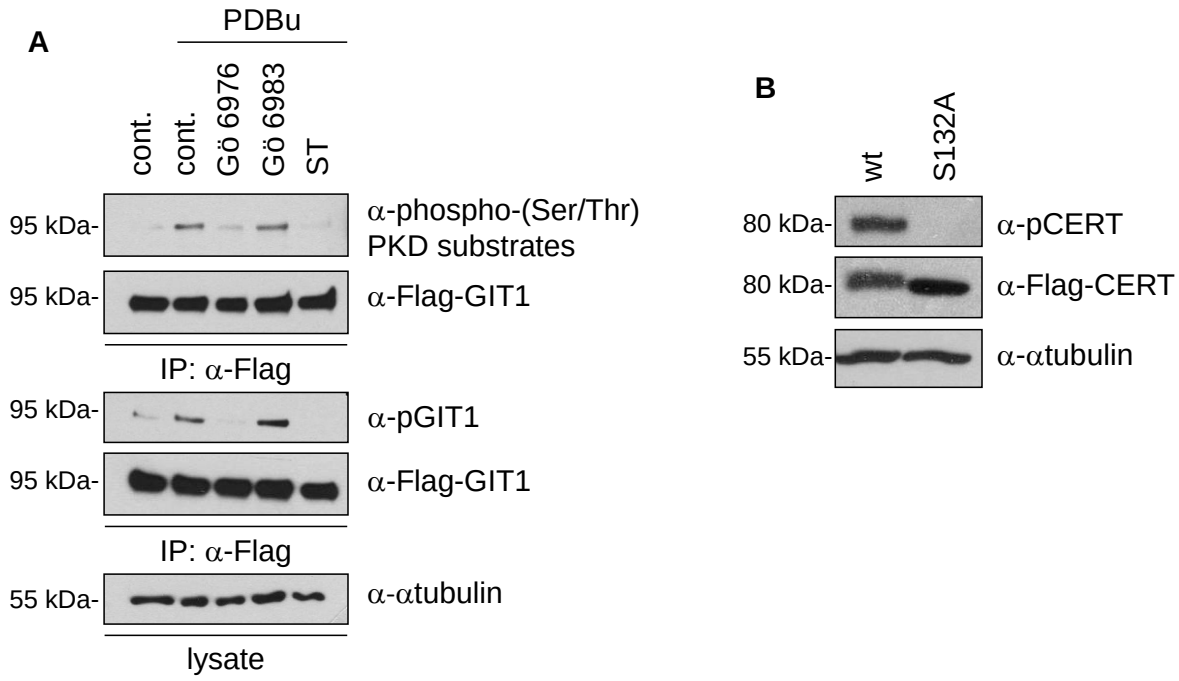


Figure 7: Pharmacological PKD-Inhibition prevents GIT1 phosphorylation

(A) HEK293T cells were transiently transfected with an expression vector encoding Flag-GIT1. The next day, cells were treated with 5 µM Gö6976 and Gö6983, and 1 µM staurosporine (ST) for 3 h. DMSO was used as a control. Cells were then stimulated with 1 µM PDBu for 15 min. Cells were lysed and GIT1 was immunoprecipitated with Flag-specific antibody and analyzed by immunoblotting with a phospho(Ser/Thr) PKD substrates antibody (Cell Signaling; top panel) and a phosphospecific antiserum originally raised against the PKD consensus sequence in CERT (pGIT1; middle panel), followed by detection with Flag-specific antibody to ensure equal GIT1 expression and precipitation. Equal loading was confirmed by immunoblotting of whole cell lysates with anti-α-tubulin antibody (bottom panel). (B) HEK293T cells were transiently transfected with Flag-tagged CERT wt and S132A and cells were lysed after 24h. Lysates were immunoblotted with the phosphospecific antiserum (pCERT; top panel), and Flag- and α-tubulin-specific antibodies (middle and bottom panels) as an expression and loading control, respectively.

To investigate whether GIT1 phosphorylation could also be detected at the endogenous level, MCF7 cells were stimulated with PDBu, followed by incubation of cell lysates with the phosphospecific antiserum. Immunoblotting of precipitated proteins with a GIT1-specific antibody revealed increased GIT1 protein levels upon PDBu stimulation (Fig. 8), providing evidence that endogenous GIT1 is phosphorylated in a PDBu-dependent manner.

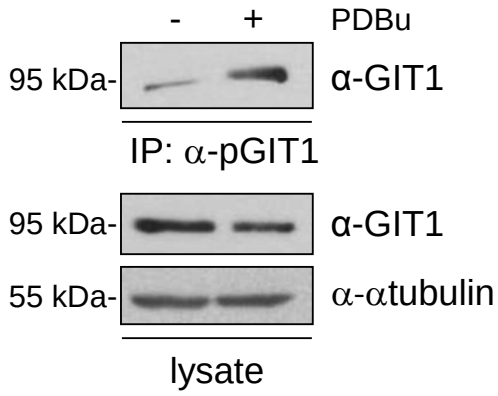


Figure 8: GIT1 is phosphorylated on endogenous level

MCF7 cells were stimulated with 1 µM PDBu for 15 min and endogenous GIT1 was immunoprecipitated with the pGIT1 antibody and analyzed by immunoblotting with a GIT1-specific antibody (top panel). Equal GIT1 expression and loading were confirmed by immunoblotting of whole cell lysates with anti-GIT1 and α-tubulin antibodies, respectively (middle and bottom panels).

To identify the PKD isoform mediating GIT1 phosphorylation, we used specific siRNAs to knockdown the different PKD isoforms. Cells transfected with a beta-galactosidase-specific siRNA (siLacZ) were used as a negative control. Knockdown of PKD1 and PKD2, either singly or in combination, did not affect the detection of basal and PDBu-stimulated GIT1 with the phosphospecific antibody (Fig. 9A). Interestingly, knockdown of PKD3 with two independent siRNAs reduced the detection of GIT1 with the phosphospecific antibody in response to PDBu stimulation (Fig. 9B, top). Efficient silencing of PKD3 was confirmed by immunoblotting of immunoprecipitated PKD3 with a PKD3-specific antibody (Fig. 9B, middle).

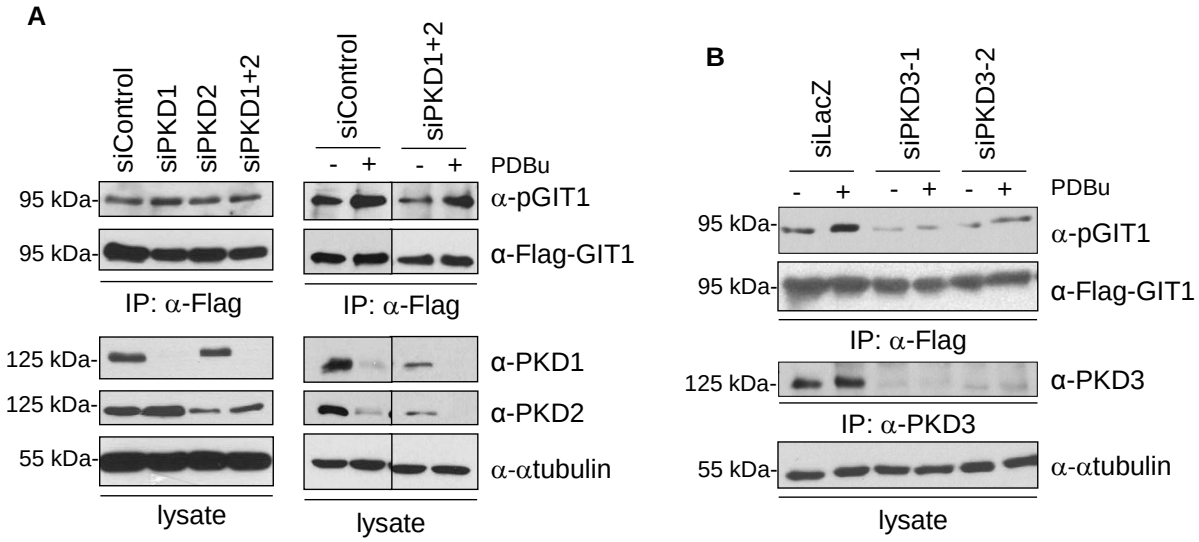


Figure 9: GIT1 is downstream of PKD3

(A) HEK293T cells were transiently transfected with siRNAs or On-Target Plus Smartpools specific for PKD1 and PKD2, either alone or in combination, or with control siRNA. One day later, cells were transiently transfected with a Flag-GIT1 expression vector. After 24 h, cells were left untreated or stimulated with 1 µM PDBu for 15 min as indicated (right panels) and lysed. GIT1 was immunoprecipitated with a Flag-specific antibody and analyzed by immunoblotting with the pGIT1 antibody, followed by detection with Flag-specific antibody to ensure equal GIT1 expression and precipitation. The right panels shown are from the same gel. To confirm the knockdown of PKD1/2, equal amounts of total cell lysate were analyzed by immunoblotting using PKD1- and PKD2-specific antibodies. Of note, the PKD1- and PKD2-specific antibodies only poorly recognize the active, phosphorylated kinases. Equal loading was verified by reprobing the membranes with α-tubulin-specific antibody. In (B), HEK293T cells were transiently transfected with two independent PKD3-specific and LacZ-specific siRNA. One day later, cells were transiently transfected with a Flag-GIT1 expression vector. After 24 h, cells were stimulated with PDBu, lysed and GIT1 was immunoprecipitated with Flag-specific antibody and analyzed by immunoblotting with the pGIT1 antibody, followed by detection with Flag-specific antibody. To confirm the knockdown of PKD3, PKD3 was immunoprecipitated with a PKD3-specific antibody, followed by immunoblotting using a PKD3-specific antibody. Equal loading was verified by immunoblotting of whole cell lysates with α-tubulin-specific antibody.

Next, we coexpressed wild type (wt) and a catalytically active (ca) PKD3 variant (S731/735E) together with GIT1 in HEK293T cells (Fig. 10). Coexpression of both PKD3-wt and PKD3-ca strongly enhanced the recognition of immunoprecipitated GIT1 by the phosphospecific antibody, indicating that ectopic PKD3 expression is sufficient to trigger GIT1 phosphorylation. Taken together, these data provide strong evidence that GIT1 is specifically phosphorylated downstream of PKD3.

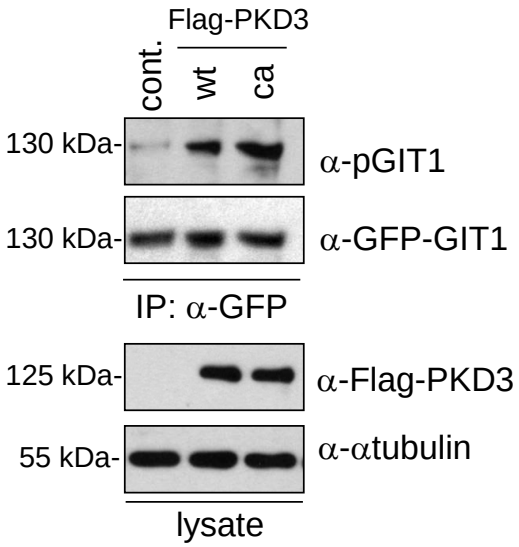
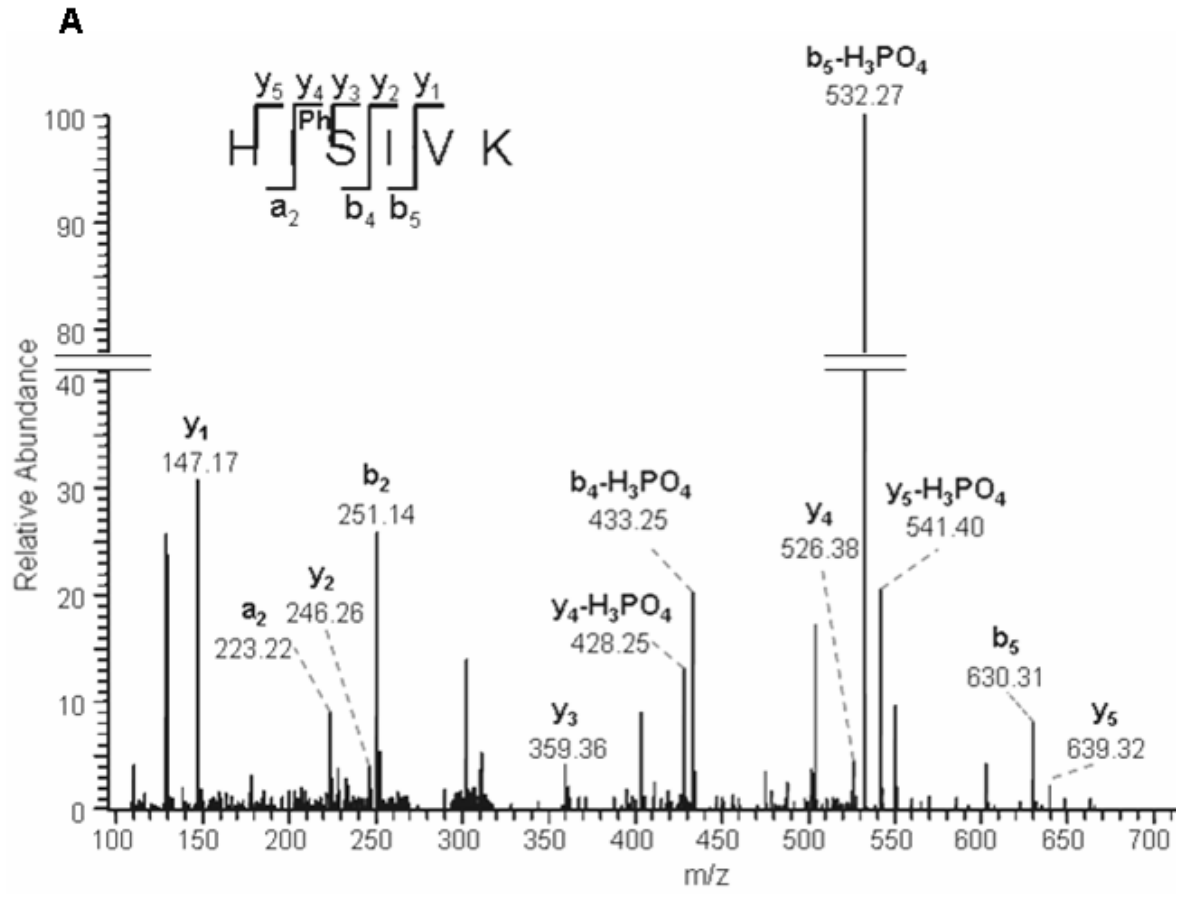


Figure 10: Overexpression of PKD3 induces GIT1 phosphorylation

GFP-GIT1 was coexpressed with empty vector as a control, Flag-tagged PKD3 wild type (wt) and catalytically active (ca, S731/735E) in HEK293T cells. GIT1 was immunoprecipitated with GFP-specific antibody and analyzed by immunoblotting with the pGIT1 antibody, followed by detection with GFP-specific antibody. Expression of the PKD3 variants was confirmed by immunoblotting of whole cell lysates with Flag-specific antibody. Equal loading was verified by immunoblotting of whole cell lysates with α-tubulin-specific antibody.

3.1.2 PKD3 directly phosphorylates GIT1 on serine 46

To identify the PKD3-specific phosphorylation site within GIT1, we subjected GIT1 to mass spectrometry analysis with a specific focus on identifying phosphopeptides that match the PKD consensus motif. To this end, Flag-tagged GIT1 was transiently expressed in HEK293T cells and cells were stimulated with PDBu prior to lysis and isolation of Flag-GIT1 with anti-Flag immunoaffinity resin (M2 agarose). Phosphorylated peptides were enriched by titanium dioxide chromatography and then subjected to mass spectrometry. A singly phosphorylated peptide with a parent mass of 388.71 ([M+H]+2), corresponding to amino acids 44-49 (HIpSIVK) in GIT1, was detected and fragmented. The resulting fragmentation spectrum showed good sequence coverage by y- and b-ions and the a2-ion, unambiguously identifying serine 46 to be phosphorylated (Fig. 11A). This serine residue is contained within a perfect PKD consensus motif, namely L/I/VxRxxS/T, with x denoting any amino acid (Fig. 11B; note that the PKD motif is fully conserved in mouse, rat, bovine and avian GIT1 (data not shown)). Additional phosphosites identified are listed in Table 16.



B

PKD motif	**L**/I/V X R X X **S**/T
GIT1 (41-46)	**L** G **R** H I **S**

Figure 11: Identification of phosphorylation site serine 46 in GIT1 by mass spectrometry

(A) Flag-tagged GIT1 was purified from HEK293T cell lysates transiently expressing the protein with M2 agarose and subjected to SDS-PAGE. The Colloidal Coomassie-stained band corresponding to GIT1 was in-gel digested with trypsin. Phosphopeptides were enriched by titanium dioxide chromatography and then subjected to mass spectrometry. The spectrum shows the fragmentation pattern of the phosphopeptide HIpSIVK corresponding to amino acids 44-49. (B) Consensus motif of the PKD family of proteins and GIT1 alignment (amino acids 41-46).

Table 16: Phosphosites identified in GIT1 by mass spectrometry

Phosphosite	Identified phosphopeptide
S261	CMpSQSLDLSELAK
S281*	SLSSPTDNLELpSLR
S368*	pSLSpSPTDNLELSLR
S371	SLSpSPTDNLELSLR
T373*	SLSSPpTDNLELSLR
S379*	SLSSPTDNLELpSLR
S397	SQSDLDDQHDYDSVApSDEDTDQEPLR
S457	VNpSSLSDELR
T489	QPPGPVPpTPPLPSER
T500*	AEHpTPMAPGGSTHR
S507	AEHTPMAPGGpSTHRR
S605	HGSGADpSDYENTQSGDPLLGLEGK
Y607	HGSGADSDpYENTQSGDPLLGLEGK

The identified phosphosites and corresponding peptides are listed. Only phosphosites with a location probability above 0.75 are shown. Spectra of modified peptides were manually validated. Novel sites that were not previously detected by (Webb, Mayhew et al. 2006) are marked with an asterisk (*).

To prove that PKD3 directly phosphorylates GIT1 on serine 46, an *in vitro* kinase assay was performed. Flag-tagged wild type GIT1 and a serine-to-alanine point mutant (S46A) were expressed in HEK293T cells, immunoprecipitated from cell lysates and incubated with recombinant purified PKD3 in the presence of ATP (Fig. 12A). Compared with the wild type protein, the detection of GIT1 S46A with the phosphospecific antibody was strongly reduced (Fig. 12A, top panel), indicating that the antibody binds GIT1 when phosphorylated on serine 46. To finally test if PKD3 also phosphorylates GIT1 on serine 46 in intact cells, GIT1 wild type and S46A were coexpressed with or without PKD3-ca in HEK293T cells, followed by analysis of GIT1 phosphorylation with the phosphospecific antibody (Fig. 12B). As shown in figure 12B, substitution of serine 46 with alanine abrogated the PKD3-induced phospho-signal (top panel). Together these data suggest that PKD3 directly phosphorylates GIT1 on serine 46 both *in vitro* and in intact cells.

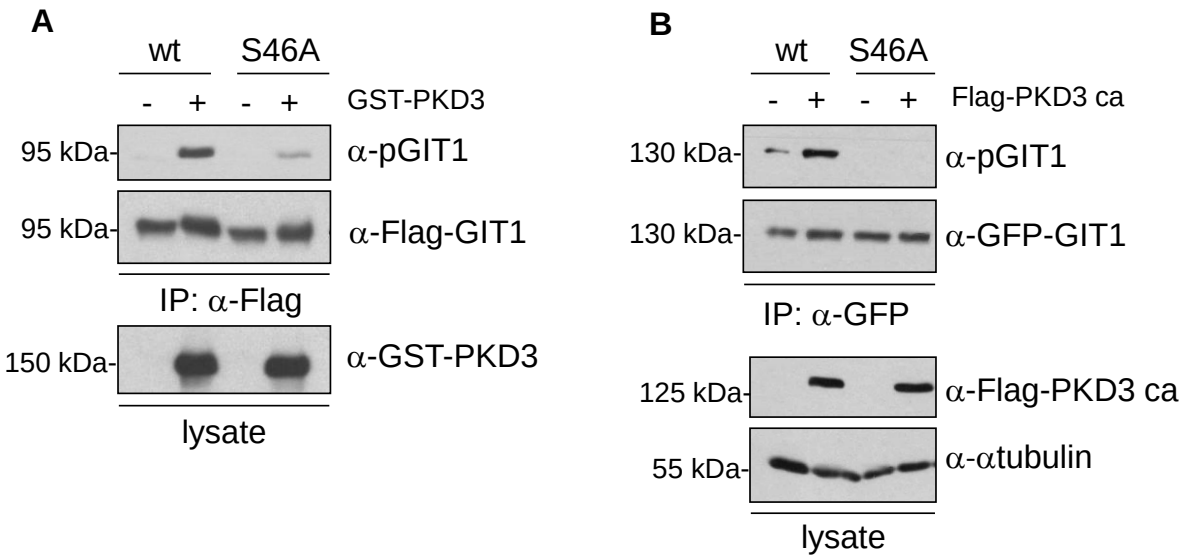


Figure 12: GIT1 is directly phosphorylated by PKD3 on serine 46

(A) GIT1 was immunoprecipitated with anti-Flag antibody from HEK293T cell lysates transiently expressing Flag-tagged GIT1 wt or S46A and incubated with ATP in the absence (-) or presence (+) of purified GST-tagged PKD3. Samples were subjected to SDS-PAGE, proteins were transferred to membrane and analyzed by Western blotting with pGIT1-, Flag- and GST-specific antibodies. In (B), GFP-GIT1 wt and the mutant S46A were coexpressed with Flag-tagged PKD3 ca in HEK293T cells. GIT1 was immunoprecipitated with GFP-specific antibody and analyzed by immunoblotting with the pGIT1 antibody (top panel), followed by detection with GFP-specific antibody to ensure equal GIT1 expression and precipitation. Expression of PKD3 variants was confirmed by immunoblotting of whole cell lysates with Flag-specific antibody. Equal loading was confirmed by immunoblotting of whole cell lysates with anti-α-tubulin antibody.

3.1.3 GIT1 phosphorylated on serine 46 locates to motile cytoplasmic paxillin-positive complexes

Depending on the cell type and condition, different subcellular localizations have been reported for GIT1. We therefore analyzed whether GIT1 phosphorylation at serine 46 affects its localization within the cell. GFP-tagged variants of GIT1 (wt, S46A, S46D) were expressed in MCF7 breast cancer cells and fixed cells were analyzed by confocal laser scanning microscopy (Fig. 13). The wild type GIT1 protein was found to localize to paxillin-positive structures at the cell periphery, which showed a typical focal adhesion appearance at the bottom of the cell (Fig. 13, top). GIT1 also localized to a few distinct paxillin-positive cytoplasmic structures (Fig. 13, top), which have been described previously (Matafora, Paris et al. 2001; Manabe, Kovalenko et al. 2002). Interestingly, cells expressing GIT1 S46D, which contains a negatively charged aspartic acid introduced to mimic the phosphorylated state, were filled with these cytoplasmic structures that further appeared to recruit cytosolic paxillin (Fig. 13, middle). By contrast, the focal adhesion adaptor protein vinculin did not co-localize with GIT1 S46D in cytoplasmic

complexes, indicating a specific effect on the distribution of paxillin and ruling out the disruption of focal adhesions in general (Fig. 14, upper panel). The GFP-GIT1 S46A mutant localized only to focal adhesions and no cytoplasmic complexes were observed (Fig. 13, bottom), indicating that dephosphorylation of serine 46 favors association with cytoskeletal structures. PKD3 was observed to localize to VAMP2-positive vesicles in HEK 293 and HCC1806 cells (Lu, Chen et al. 2007), but PKD3 did not colocalize with GIT1 S46D in MCF7 cells (data not shown).

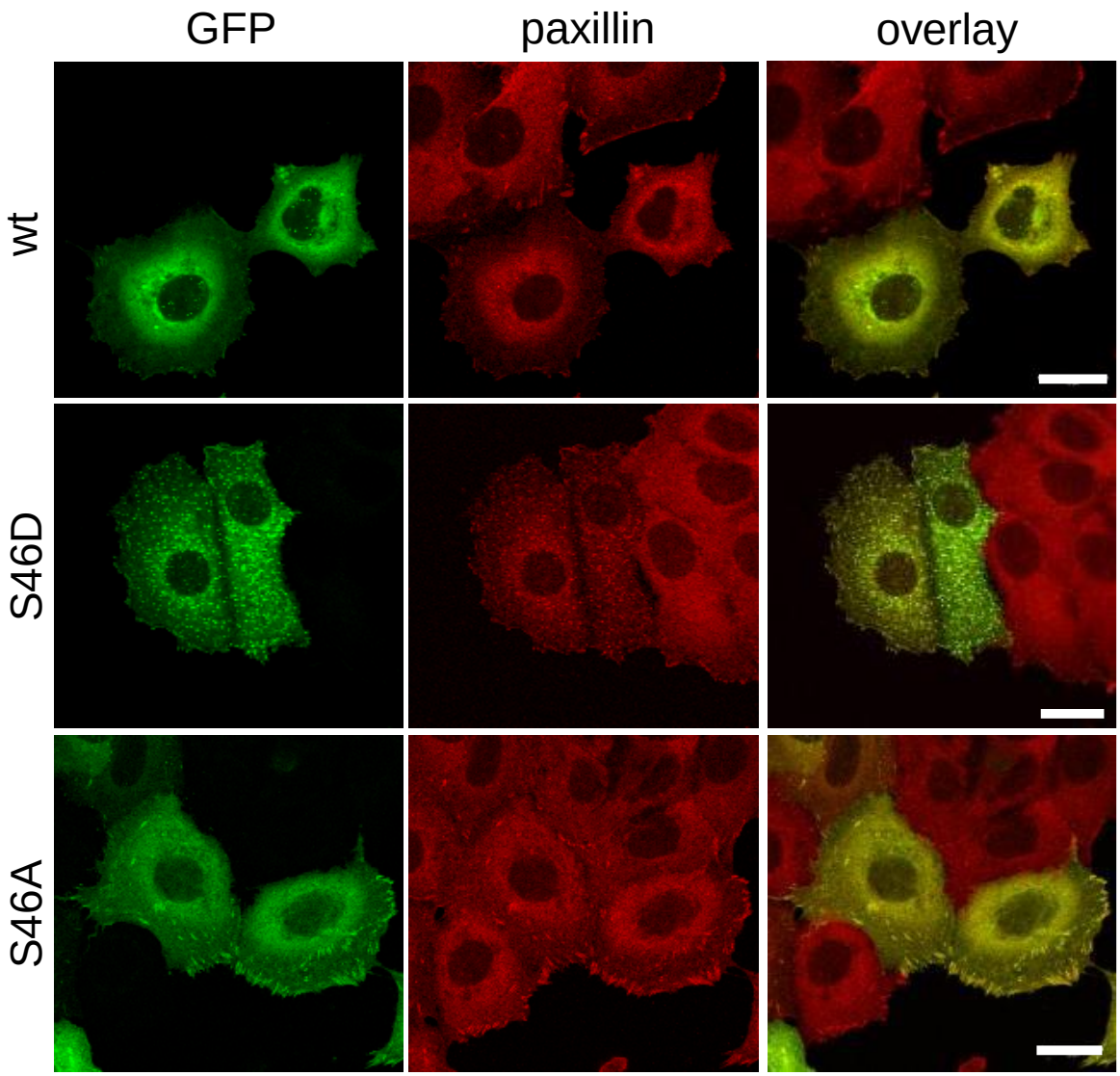


Figure 13: GIT1 S46D localizes to paxillin-positive cytoplasmic complexes

MCF7 cells were plated onto collagen-coated coverslips and transiently transfected with expression vectors encoding GFP-GIT1 wt, S46A and S46D as indicated. The next day, cells were fixed and stained with paxillin-specific primary antibody, followed by Alexa Fluor 546-conjugated secondary antibody (red). The images shown are projections of several confocal sections. Scale bar, 20 µm.

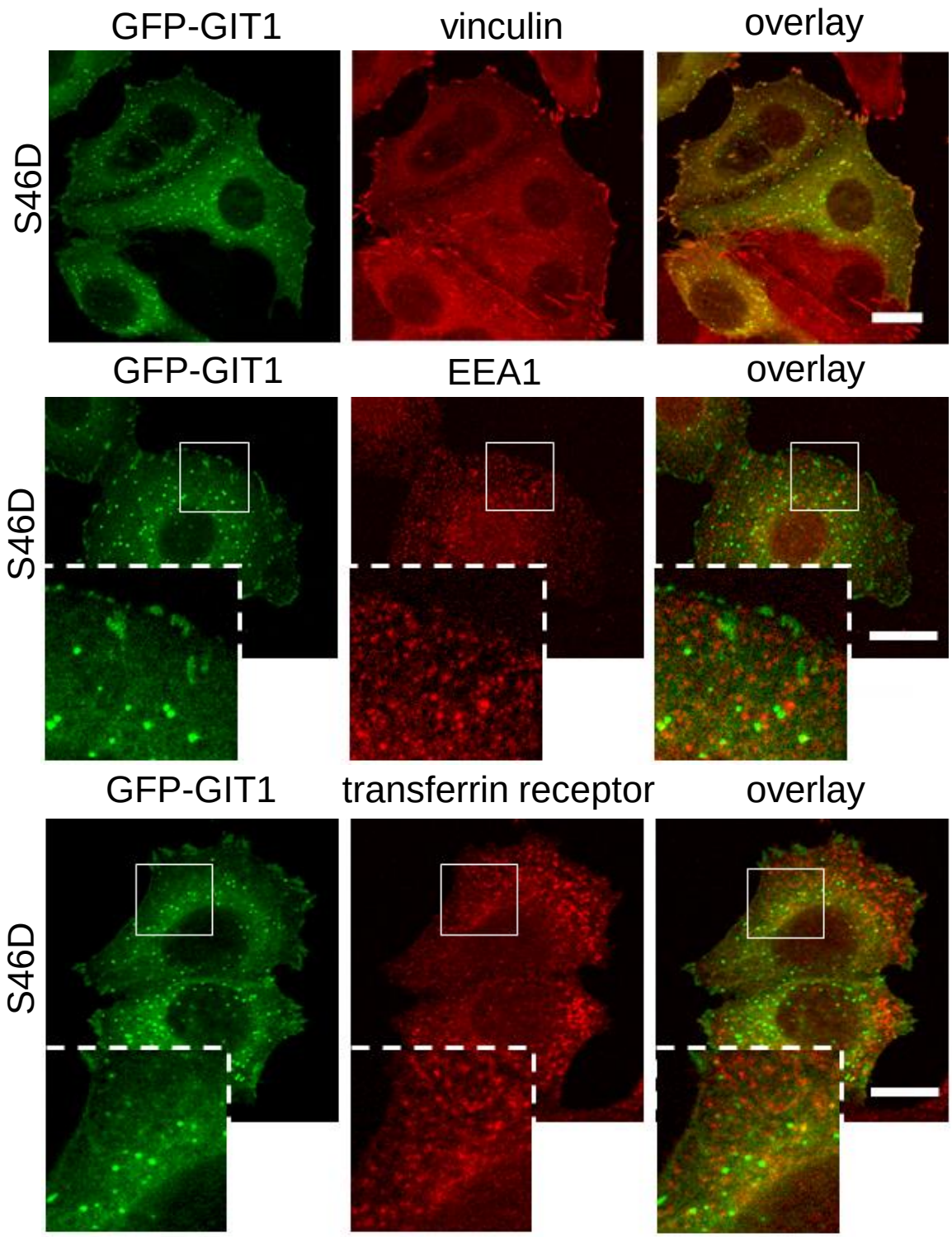


Figure 14: GIT1 S46D cytoplasmic complexes do not colocalize with vinculin, EEA1 or transferrin receptor

MCF7 cells were plated onto collagen-coated coverslips and transiently transfected with expression vectors encoding GFP-GIT1 wt, S46A and S46D as indicated. The next day, cells were fixed and stained with vinculin-, EEA1- and transferrin receptor-specific primary antibodies, followed by Alexa Fluor 546-conjugated secondary antibody (red). The images shown are projections of several confocal sections. Scale bar 10 µm.

PDBu stimulation of cells expressing GFP-GIT1 wild type induced the formation of cytoplasmic complexes, as determined by scoring cells with a low (<10) and a higher number (>10) of complexes (Fig. 15), supporting the notion that GIT1 subcellular localization is regulated by serine 46 phosphorylation.

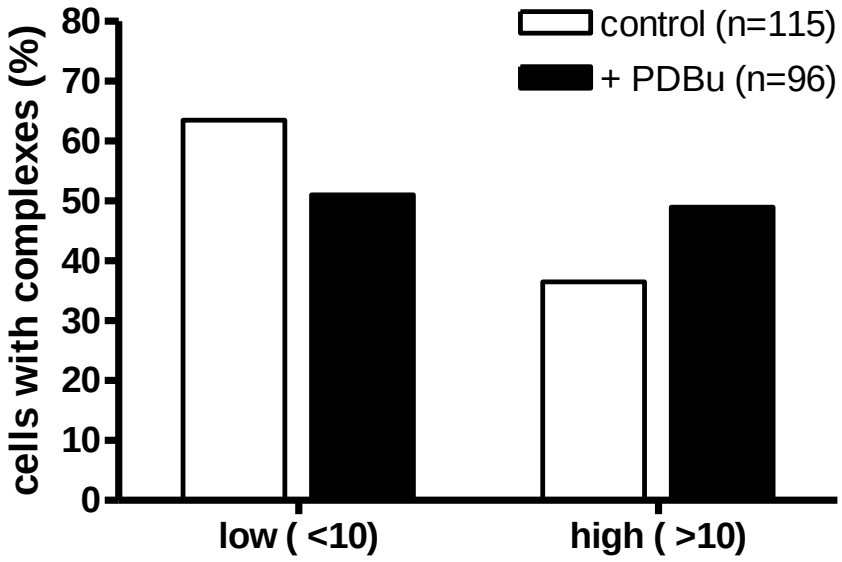


Figure 15: Pharmacological PKD activation induces formation of cytoplasmic complexes

MCF7 cells were plated onto collagen-coated coverslips and transiently transfected with an expression vector encoding GFP-GIT1 wt. Prior fixation, cells were stimulated with 1 µM PDBu for 15 min. Cells were analyzed by confocal microscopy and scored according to their number of GIT1 cytoplasmic complexes.

The precise nature and function of the cytoplasmic GIT1 structures, as to whether they are recycling endosomes or motile supramolecular protein complexes, has been discussed controversially. Because we could not detect any colocalization with endosomal markers such as EEA1 and the transferrin receptor (Fig. 14, middle and bottom), the GIT S46D containing structures observed in MCF7 cells most likely resemble the supramolecular complexes described by Manabe et al. (Manabe, Kovalenko et al. 2002). However, costainings revealed that PAK1 was absent from GIT1 S46D cytoplasmic complexes and colocalized only with FA-localized GIT1 (Fig. 16, top), whereas PIX colocalized with both GIT1 S46A and S46D (Fig. 16, bottom).

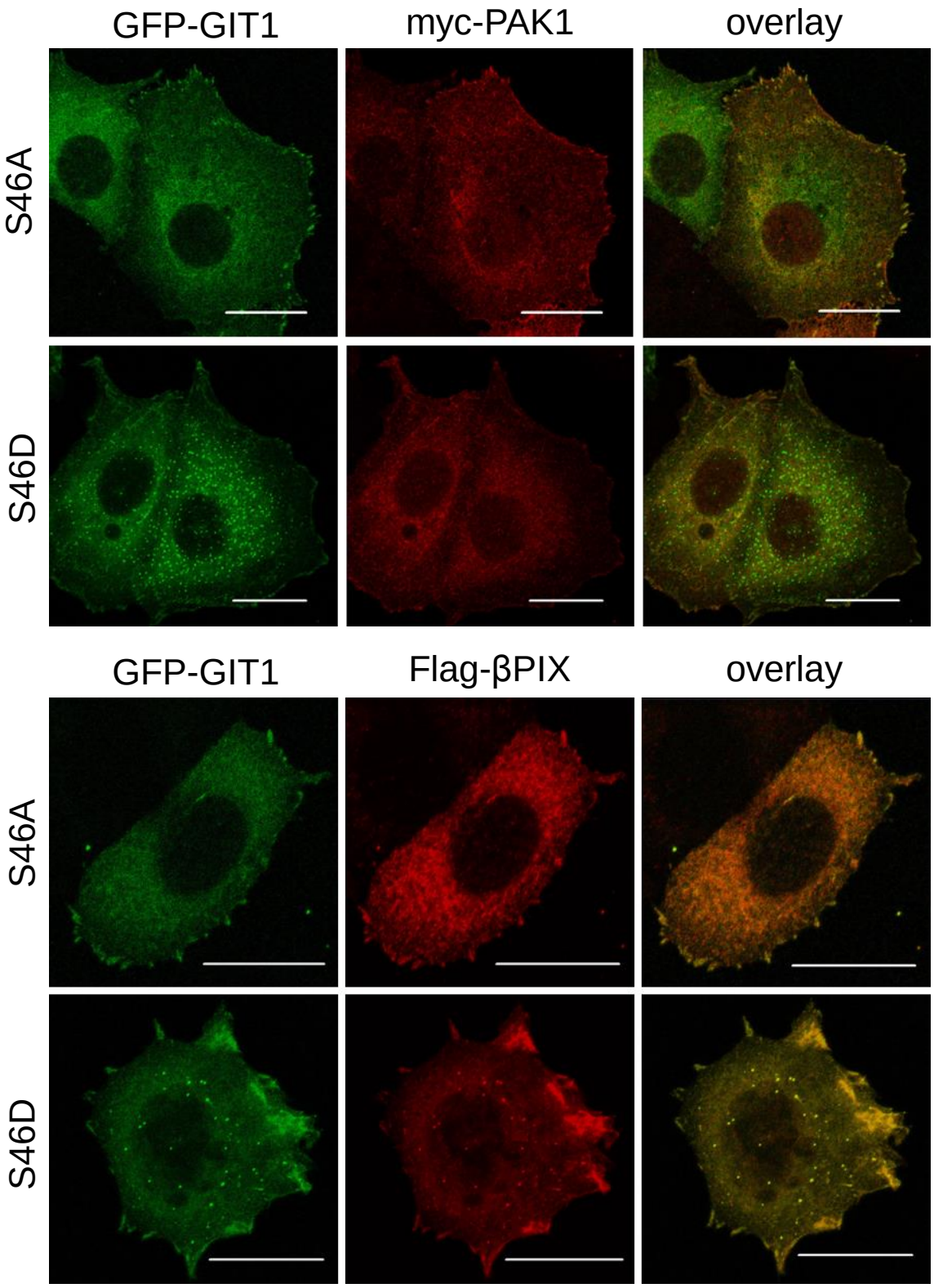


Figure 16: GIT1 S46D cytoplasmic complexes contain betaPIX but not PAK1

MCF7 cells were plated onto collagen-coated coverslips and transiently co-transfected with expression vectors encoding GFP-GIT1 S46A or S46D and either myc-tagged PAK1 or Flag-tagged betaPIX. The next day, cells were fixed and stained with myc- or Flag-specific primary and Alexa Fluor 546-conjugated secondary antibodies (red). The images shown are projections of several confocal sections. Scale bar, 20 µm.

GIT1 cytoplasmic complexes have been reported to be involved in paxillin trafficking (Manabe, Kovalenko et al. 2002). We therefore analyzed MCF7 cells expressing GFP-GIT1 S46D in more detail by time lapse video microscopy (Fig. 17). Pictures of cells were captured every 40 seconds for the duration of 20 min and the movement of individual GFP-GIT1 S46D complexes was analyzed. Figure 17 shows for a chosen GIT1 S46D positive complex (arrow) the movement towards to the cell periphery. It thus appears that phosphorylation at serine 46 shifts the localization of GIT1 from focal adhesions to highly motile, paxillin-transporting cytoplasmic complexes.

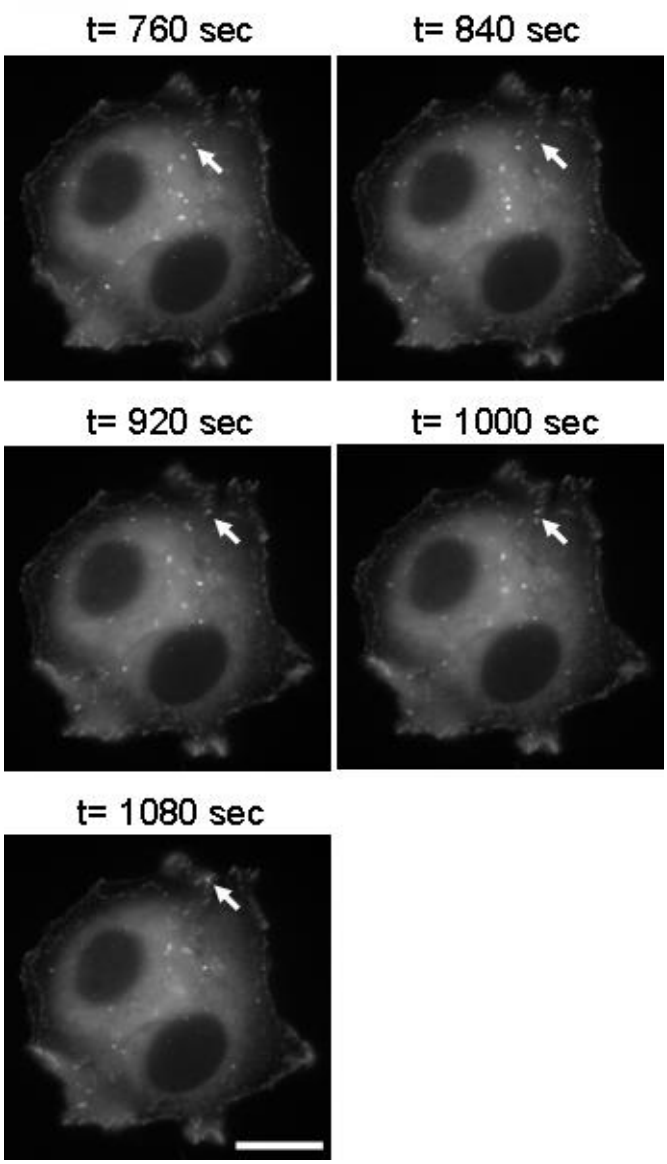


Figure 17: GIT1 S46D cytoplasmic complexes are motile

MCF7 cells stably expressing GFP-GIT1 S46D were plated at low density onto collagen-coated glass bottom cell culture dishes and fluorescence images of cells were captured using a video microscope every 40 sec for a duration of 20 min. Representative photos (time points as indicated) of the movement of a chosen complex (arrow) to the cell periphery are shown. Scale bar, 20 µm.

3.1.4 GIT1 phosphorylation on serine 46 increases spreading, protrusive activity and migration of MCF7 cells

GIT1 is known to be important for cell spreading and migration processes which rely on paxillin trafficking and focal adhesion turnover. We therefore investigated whether GIT1 phosphorylation on serine 46 modulates these biological activities using MCF7 cells stably expressing GIT1 wt, S46D and S46A. Importantly, phosphorylation of GIT1 on serine 46 also depends on PKD3 in MCF7 cells (Fig. 18).

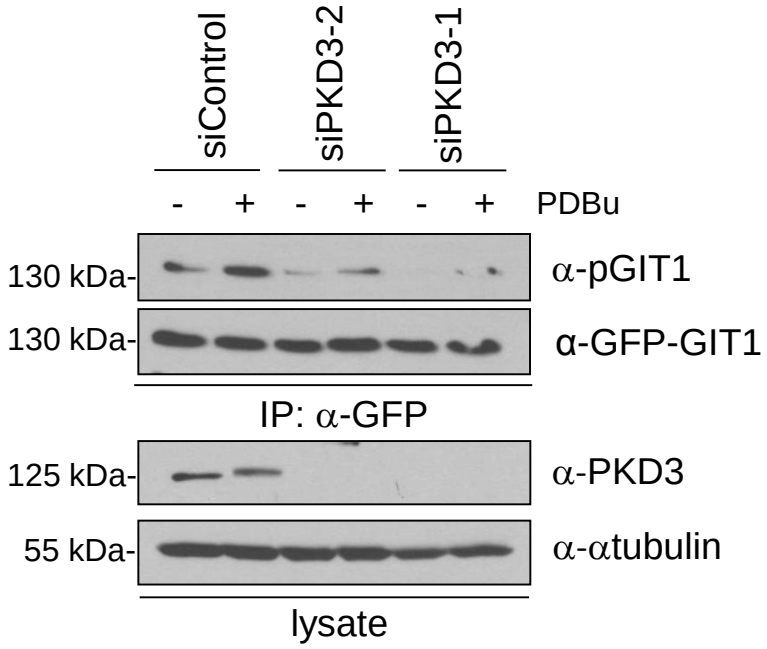


Figure 18: PKD3 phosphorylates GIT1 on serine 46 in MCF7 cells

MCF7 cells stably expressing GFP-GIT1 wt were transiently transfected with siRNAs specific for PKD3 or a control siRNA. Two days later, cells were stimulated with 1 µM PDBu for 15 min and lysed. GIT1 was immunoprecipitated with a GFP-specific antibody and analyzed by immunoblotting with the pGIT1 antibody, followed by detection with GFP-specific antibody to ensure equal GIT1 expression and precipitation. To confirm the knockdown of PKD3, equal amounts of total cell lysate were analyzed by immunoblotting using a PKD3 specific antibody. Equal loading was verified by reprobing the membrane with α-tubulin-specific antibody.

To analyze initial cell adhesion and spreading, cells were plated at low density onto collagen-coated microtiter plates arrayed with electrodes (E-plates) and impedance (cell index) was measured using an xCELLigence device. The cell index is proportional to the surface area covered by the cells and therefore serves as a measure for cell adhesion and spreading. Compared to cells expressing GIT1 wild type, the cell index of MCF7 cells stably expressing the phosphomimetic mutant S46D was higher, whereas that of cells expressing the GIT1 S46A mutant was lower during the first hour of cell plating (Fig. 19),

indicating that GIT1 phosphorylation on serine 46 positively affects its ability to promote cell adhesion and spreading.

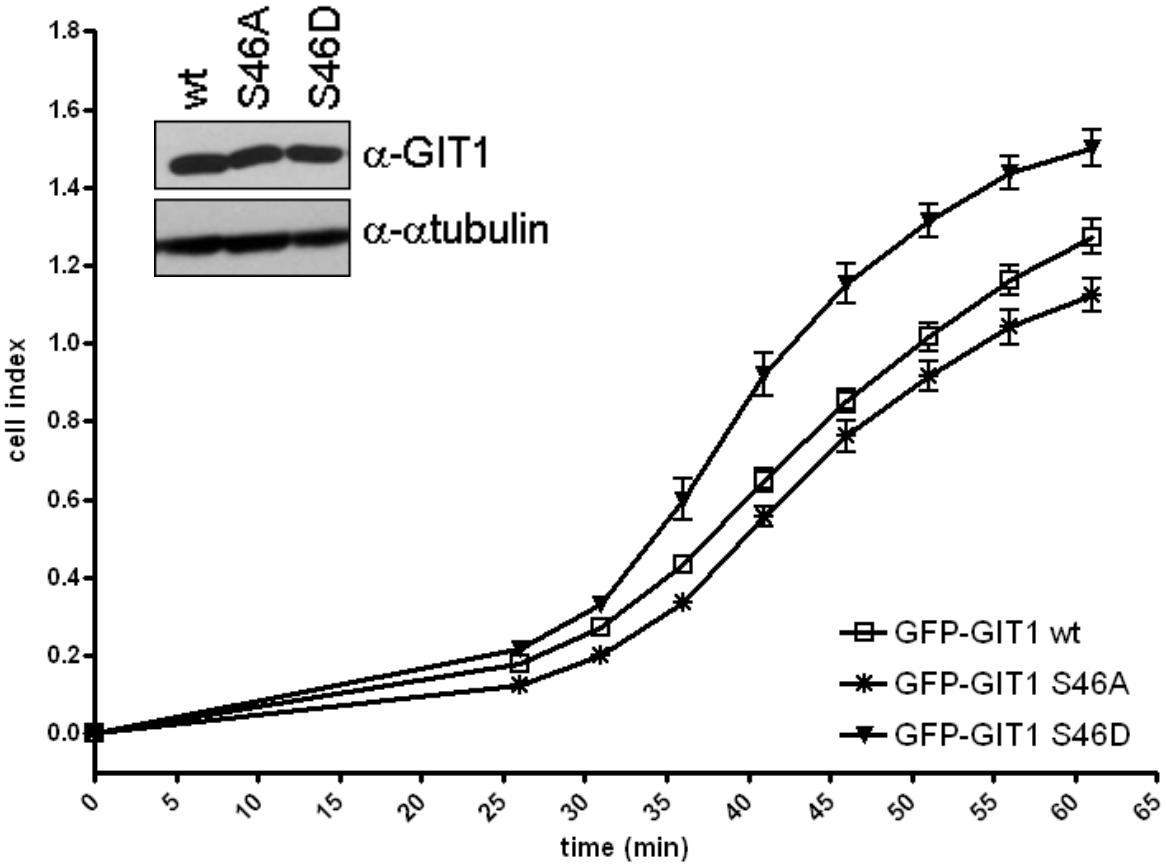


Figure 19: GIT1 phosphorylation on serine 46 increases initial cell spreading

MCF7 cells stably expressing GFP-GIT1 wt, S46A and S46D were plated into collagen-coated E-plates (96-well plate format, 10,000 cells) and the impedance (cell index) of adhering cells was measured every 5 min using a xCELLigence device, starting 25 min after plating. The mean cell index is plotted (n = 4). Error bars represent SD.

We next examined if phosphorylation of GIT1 on serine 46 influences the overall protrusive activity of cells. Live cell images of MCF7 cells plated on collagen-coated glass dishes were taken every 30 minutes and the percentage of change in total cell area after a selected 30 minute interval was determined using Image J software (Fig. 20, outline). Based on this analysis, cells expressing the phosphomimetic S46D mutant showed significantly more protrusive activity than the wild type control cells, whereas cells expressing GIT1 S46A showed less protrusive activity (Fig. 20).

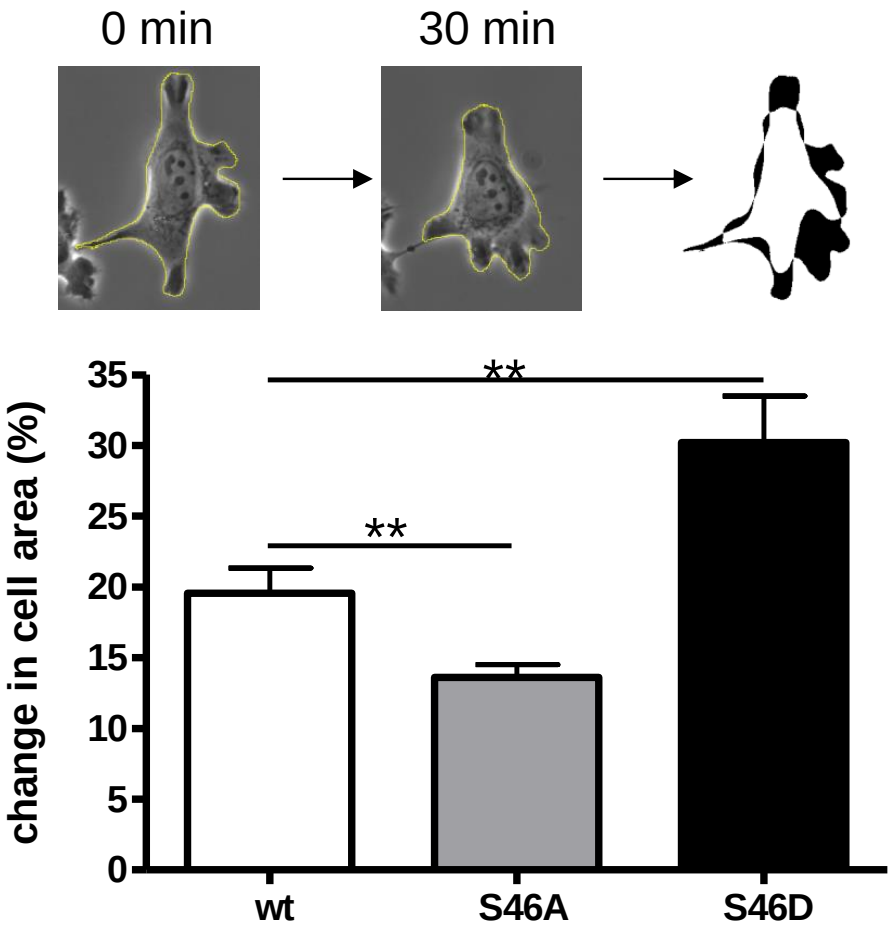


Figure 20: Phosphorylation of GIT1 on S46 increases protrusive activity

MCF7 cells stably expressing GFP-GIT1 wt, S46A and S46D were plated at low density onto collagen-coated glass bottom plates. The next day, single cells were video-imaged by microscopy (GFP and phase contrast) every 30 min. Selected GFP-positive cells were outlined at the beginning and the end of a selected 30 min time interval, the images were overlaid and the change in cell area (in %) was calculated using Image J software. Data show the mean of at least 25 independent single cells. Error bars represent SEM, **p≤0.01.

Finally, in Transwell migration assays, MCF7 cells expressing the GIT1 S46D protein displayed significantly increased cell motility after overnight incubation compared with those expressing the wild type protein, whereas those expressing GIT1 S46A displayed reduced cell motility, in accordance with the results obtained in the other biological assays (Fig. 21A). In the presence of PDBu, the migration of cells expressing GIT1 wt was increased by 1.5-fold, whereas that of cells expressing GIT1 S46D was only increased by 1.2-fold in comparison to the untreated controls (Fig. 21B). This indicates that PDBu-induced phosphorylation of wild type GIT1 contributes to enhanced cell migration, whereas in cells expressing the phosphomimetic GIT1 S46D variant cell migration is already enhanced (Fig. 21A) and cannot be stimulated much further.

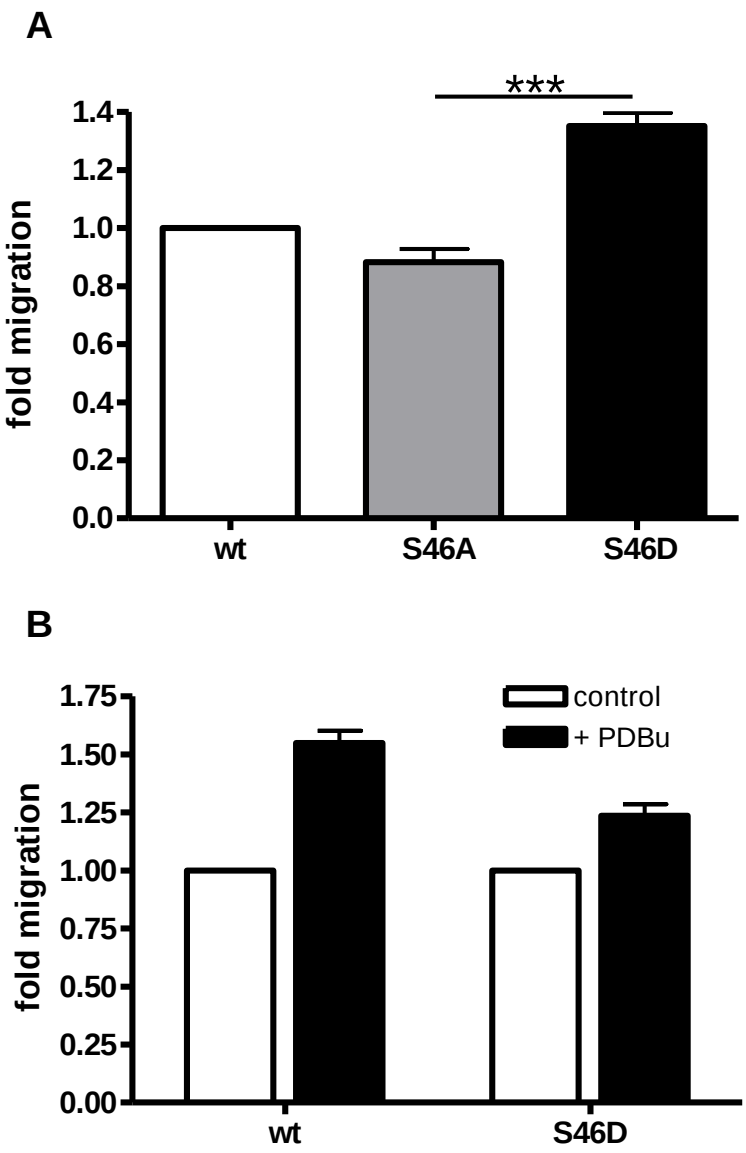


Figure 21: Phosphorylation of GIT1 on S46 increases migration

(A) MCF7 cells stably expressing GFP-GIT1 wt, S46A and S46D were seeded (50.000 cells) in medium containing 0.5% FCS into the upper chamber of a transwell coated with collagen on the underside. The lower well contained medium supplemented with 10 % FCS. Cells that had migrated across the filter after overnight incubation were fixed and stained. The number of migrated cells was determined by counting five independent microscopic fields (20-fold magnification) per filter. Data show the mean of four independent experiments performed with duplicate filters, the wt was set as 1. Error bars represent SEM, ***p≤0.001. (B) Transwell migration was performed and analyzed as described in (A) in the presence of 50 nM PDBu in upper and lower chambers. Data show the mean of two independent experiments performed with duplicate filters; unstimulated cells were set as 1, error bars represent SEM.

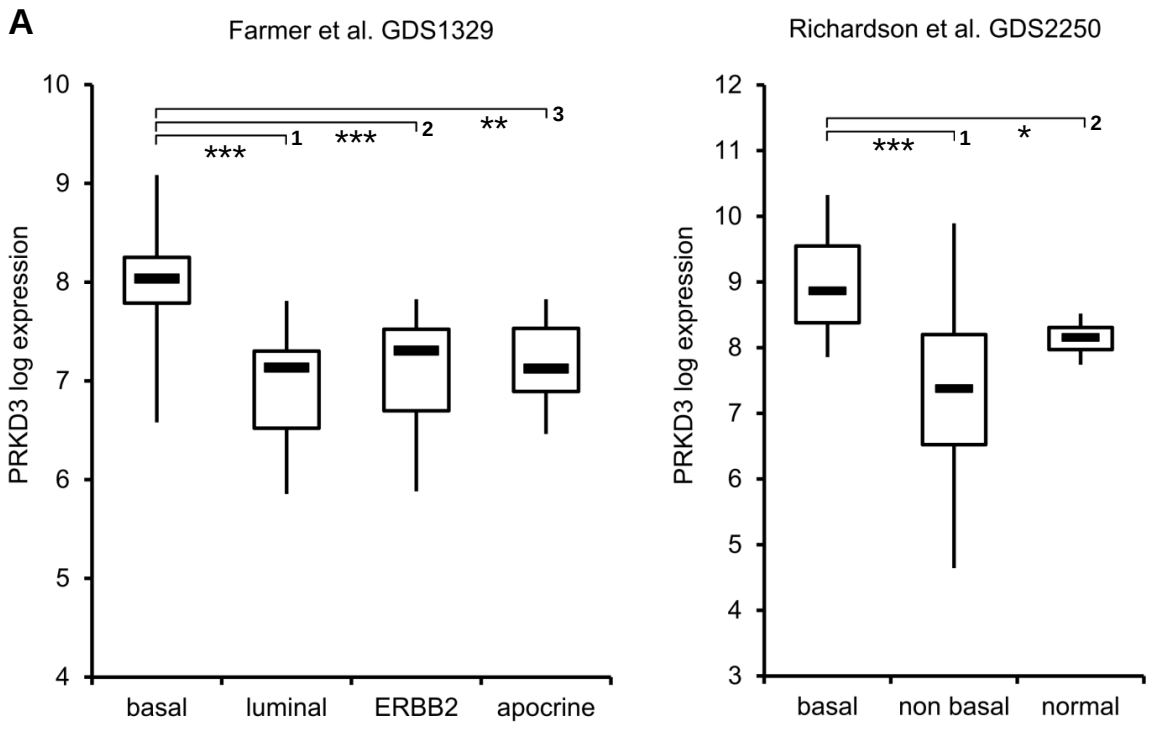
In summary, in this part we identified a novel phosphorylation site in GIT1 on serine 46 that is specifically phosphorylated by PKD3 and is associated with increased cell spreading, cellular protrusive activity and motility of breast cancer cells.

3.2 PKD3 in TNBC

In the first part of the thesis we could show that PKD3 plays a specific role in the regulation of breast cancer cell motility via phosphorylation of GIT1 on serine 46. As enhanced cell motility is an important characteristic of transformed breast cancer cells we were further interested whether PKD3 is also implicated in other processes associated with cellular transformation. So in this part of the thesis, we focused our attention on the potential deregulation of PKD3 expression in breast cancer cells and the identification of associated downstream signaling pathways.

3.2.1 PKD3 expression is elevated in basal-like breast cancer

To gain insight into the expression pattern of PKD3 in different breast cancer subtypes, we analyzed PKD3 transcript levels in two different publicly available microarray gene expression data sets (Farmer, Bonnefoi et al. 2005; Richardson, Wang et al. 2006). As seen in figure 22A, in both data sets, the mRNA level of PKD3 was significantly elevated in basal-like breast cancer compared to the other molecular subtypes. By contrast, mRNA levels of PKD2 were not significantly modulated and those of PKD1 were downregulated in basal-like breast cancer (see Fig. 22B, Richardson et al.), which is in accordance with published observations (Eiseler, Doppler et al. 2009).



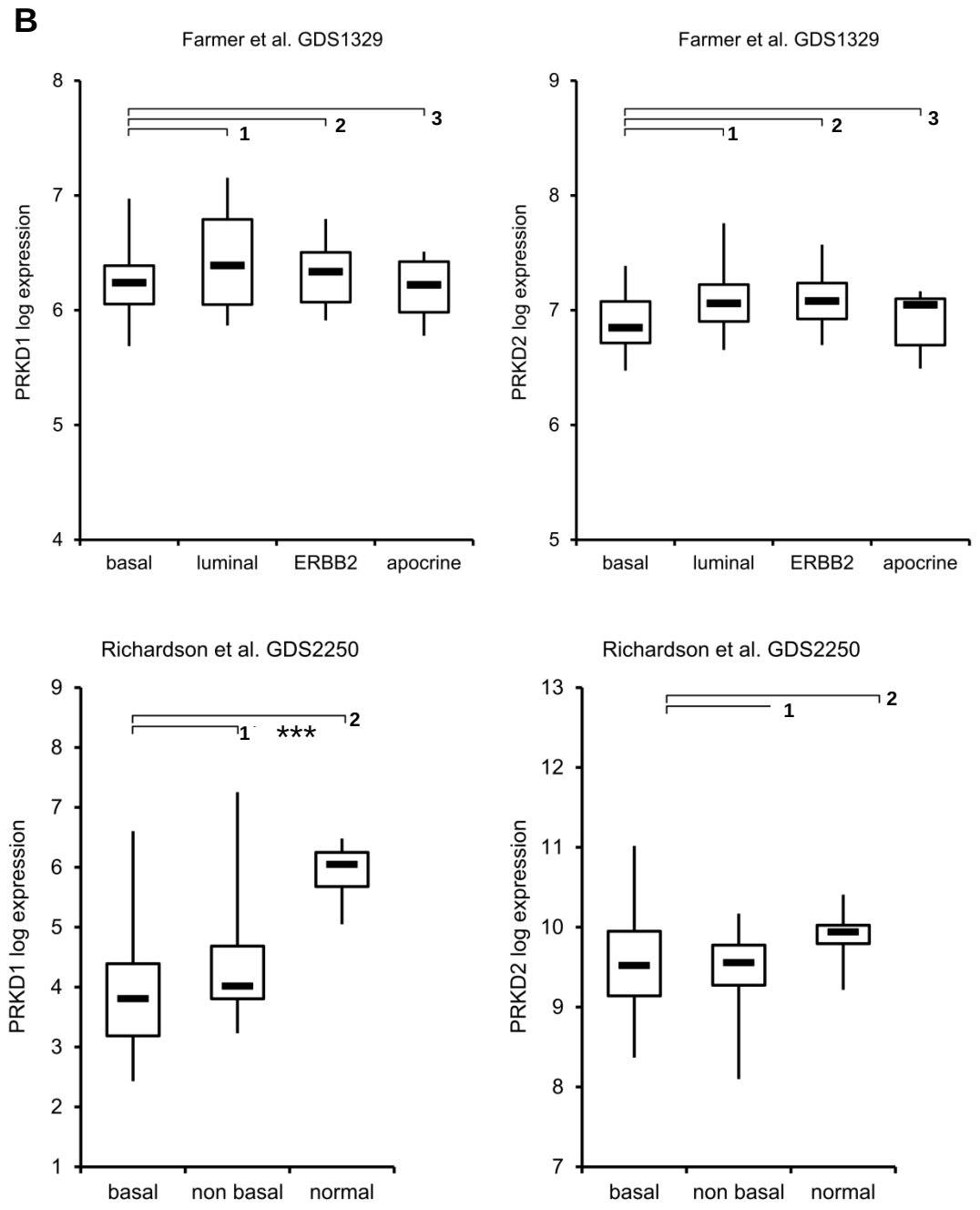


Figure 22: PKD3, PKD1 and PKD2 mRNA transcript levels in breast cancer microarray datasets

(A) PKD3 (also called PRKD3) mRNA expression data was extracted from normalized gene expression microarray datasets. Boxplots with PKD3 mRNA transcript levels for the different breast cancer subtypes described in the studies are shown. Left diagram: Farmer et al. 2005 dataset, [1] p=3.1542E-07; [2] p=0.00072; [3] p=0.00395; right diagram: Richardson et al. 2006 dataset, [1] p=7.7195E-05; [2] p=0.01925. In (B), PKD1 (also named PRKD2) and PKD2 (also named PRKD2) mRNA expression data was extracted from normalized gene expression micro array datasets. Boxplots with PKD1 and PKD2 mRNA transcript levels for the different breast cancer subtypes described in the studies are shown. Upper panel shows Farmer et al. 2005 dataset, PKD1: [1] p=0.13025; [2] p=0.45876; [3] p=0.73425; PKD2: [1] p=0.0673; [2] p=0.4613; [3] p=0.9469; lower panel shows Richardson et al. 2006 dataset, PKD1: [1] p=0.16832; [2] p=0.00015; PKD2: [1] p=0.84445; [2] p=0.19711.

We next investigated by immunoblotting PKD3 protein levels in a panel of breast cancer cell lines using a PKD3 specific antibody (Fig. 23). The antibody detected a single band corresponding to PKD3 with a molecular weight of approximately 110 kDa (Fig. 23). Interestingly, three out of four triple-negative, basal-like breast cancer cell lines (HS-578T, MDA-MB-231 and MDA-MB-468) displayed high levels of PKD3 in comparison to, for example, the luminal breast cancer cell line MCF7. Taken together these data show that PKD3 expression is elevated both, at the transcript and protein level in basal-like TNBC cells.

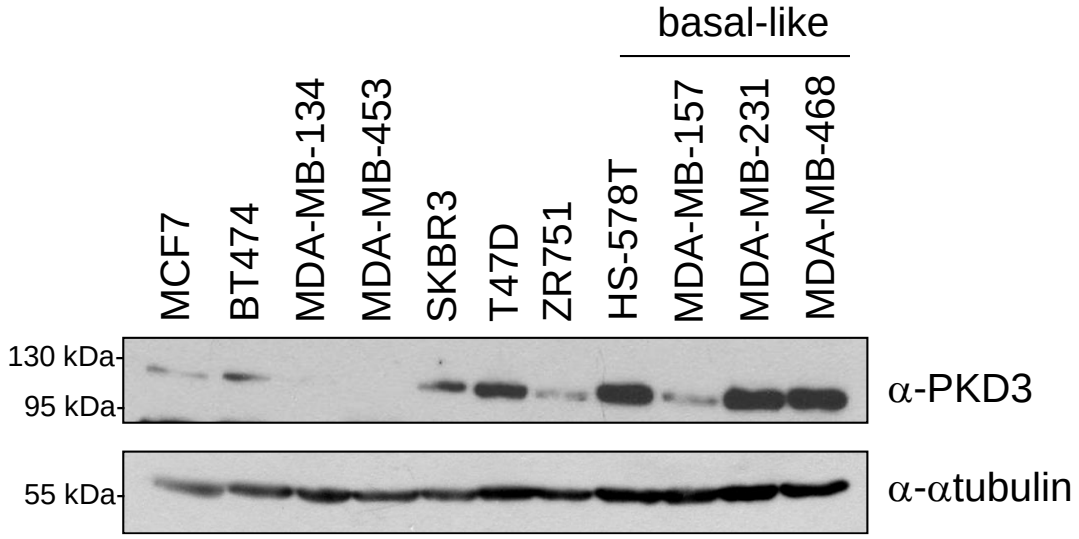


Figure 23: PKD3 expression is elevated in basal-like breast cancer cells

Lysates of breast cancer cell lines were immunoblotted with PKD3- and α–tubulin-specific antibodies.

3.2.2 Identification of S6K1 as a downstream target of PKD3

To identify signaling pathways regulated by PKD3, we overexpressed a catalytically active (ca) GFP-tagged PKD3 variant (S731/735E) in MCF7 cells, which express relatively low levels of PKD3. Whole cell lysates were then analyzed by antibody array comprising mainly phosphospecific antibodies raised against various proteins within key cellular signaling pathways. Compared with arrays incubated with control lysates derived from cells transfected with the empty GFP vector, the intensity of three duplicate spots in the arrays incubated with lysates of PKD3 ca overexpressing cells was upregulated (highlighted in black), all of which corresponded to S6K1 (Fig. 24A). Quantification of spot intensities revealed a 3 fold higher phosphorylation of S6K1 at Thr389 and a 2 fold higher phosphorylation at Thr421/Ser424 and Thr229 in PKD3 ca expressing cells compared with those expressing GFP alone (Fig. 24B).

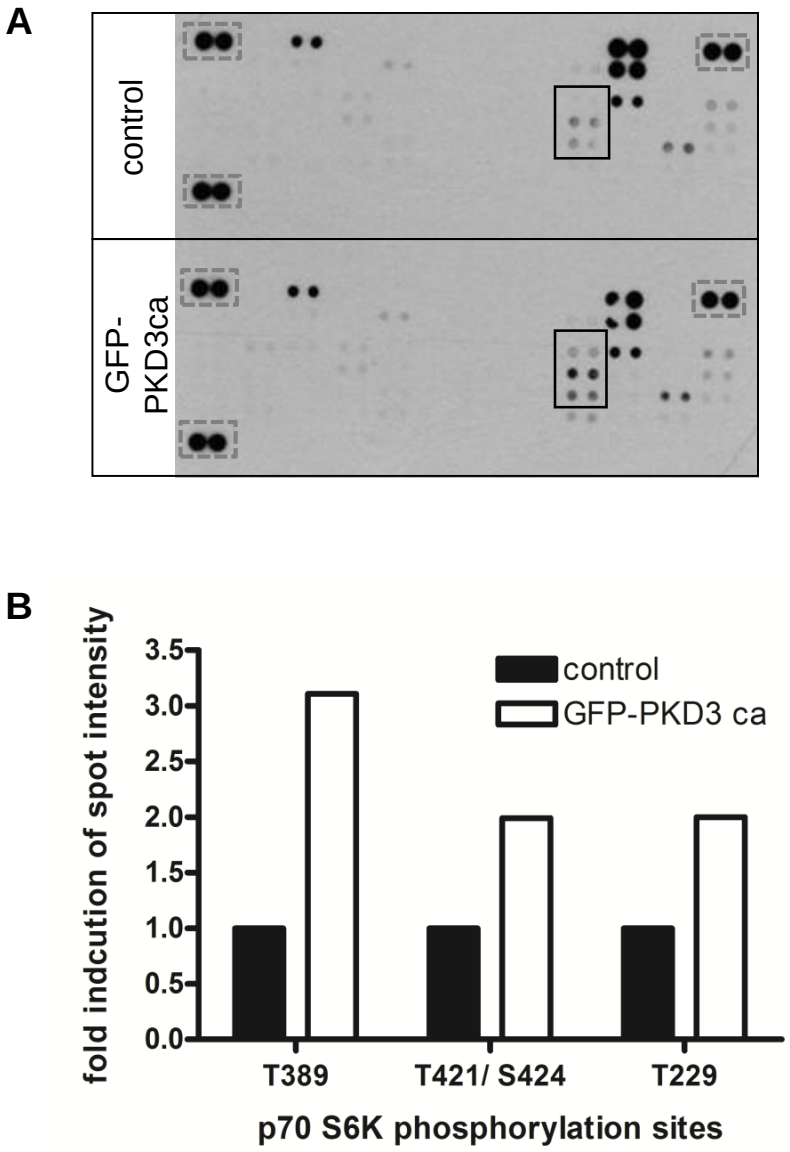


Figure 24: Identification of S6K1 as a potential downstream target of PKD3

(A) Cell lysates of MCF7 cells transiently overexpressing empty vector as a control or GFP-PKD3 ca (catalytically active, S731/735E) were analyzed by antibody array. The internal controls are indicated by dashed grey lines, and the duplicate spots corresponding to the different phosphorylated forms of S6K1 are highlighted in black. In (B), the signal intensity of the highlighted spots was quantified using Image J software. The intensity of control cells was set as 1 and the fold induction of spot intensity for PKD3 ca overexpressing cells was calculated.

To confirm this result, GFP-PKD3 ca was again transiently overexpressed in MCF7 cells and whole cell lysates were analyzed by immunoblotting with an S6K1 Thr389-specific antibody. Indeed, overexpression of PKD3 ca enhanced the recognition of S6K1 and its substrate S6 by phosphospecific antibodies (Fig. 25), which is indicative of increased activity of mTORC1. To determine whether phosphorylation of mTOR substrates in general was upregulated, we also analyzed Akt phosphorylation at Ser473, a main target

of the mTORC2 complex. However, in line with results of the antibody array, neither Akt phosphorylation at Ser473 was modulated by PKD3 ca overexpression, nor was phosphorylation of Erk1/2, suggesting specific activation of the mTORC1-S6K1 pathway. Note that two bands were observed for S6K1 (Fig. 25), second and third panel), corresponding to the p70 and p85 subunits (phosphorylation nomenclature is based on the p70 isoform protein sequence), which are both expressed in most breast cancer cells and show full S6K1 activity (Kim, Akcakanat et al. 2009). These data provide evidence that the mTORC1-S6K1 signaling pathway is a potential downstream target of PKD3.

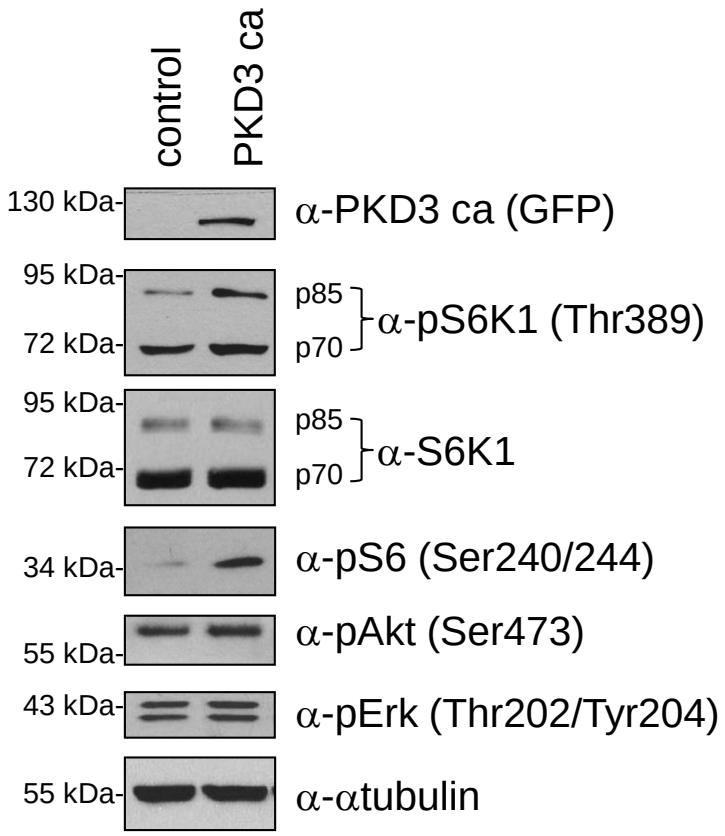


Figure 25: Constitutively active PKD3 induces hyperphosphorylation of S6K1

MCF7 cells were transiently transfected with control vector or a vector encoding GFP-PKD3 ca. Whole cell lysates were analyzed by immunoblotting with the indicated antibodies. Equal loading was confirmed by immunoblotting of whole cell lysates with α-tubulin-specific antibody.

3.2.3 PKD3 depletion compromises S6K1 activation in TNBC cells

We next determined the effect of PKD3 depletion on the mTOR signaling pathway in two basal-like TNBC cell lines (MDA-MB-231 and MDA-MB-468), which express high level of PKD3 (Fig. 23). We used two independent, specific siRNAs to silence PKD3 (siPKD3-1 and siPKD3-2) and as controls, mock as well as control siRNA (non-targeting, siNon)

transfected cells were used (Fig. 26). Cells were starved overnight and then stimulated with serum prior to lysis, as serum is known to activate S6K1 in breast cancer cells (Kim, Akcakanat et al. 2009). As can be seen in figure 26; both siRNAs efficiently knocked down PKD3 in both cell lines (upper panels). Importantly, PKD3 knockdown markedly inhibited the serum-induced phospho-signal at Thr389 of the 85 kDa S6K1 subunit. In contrast to S6K1 phosphorylation, phosphorylation of Akt at Ser473 was not downregulated in PKD3 depleted cells, indicating that mTORC2 activity was not affected by the loss of PKD3. In fact, phosphorylation of Akt in PKD3-depleted MDA-MB-231 cells was slightly increased (Fig. 26, middle panel), which can be explained by the known feedback activation of mTORC2 in response to mTORC1 inhibition (Efeyan and Sabatini 2010). Other pathways such as the MAPK pathway were not modulated by PKD3 depletion based on unchanged Erk1/2 phosphorylation (Fig. 26).

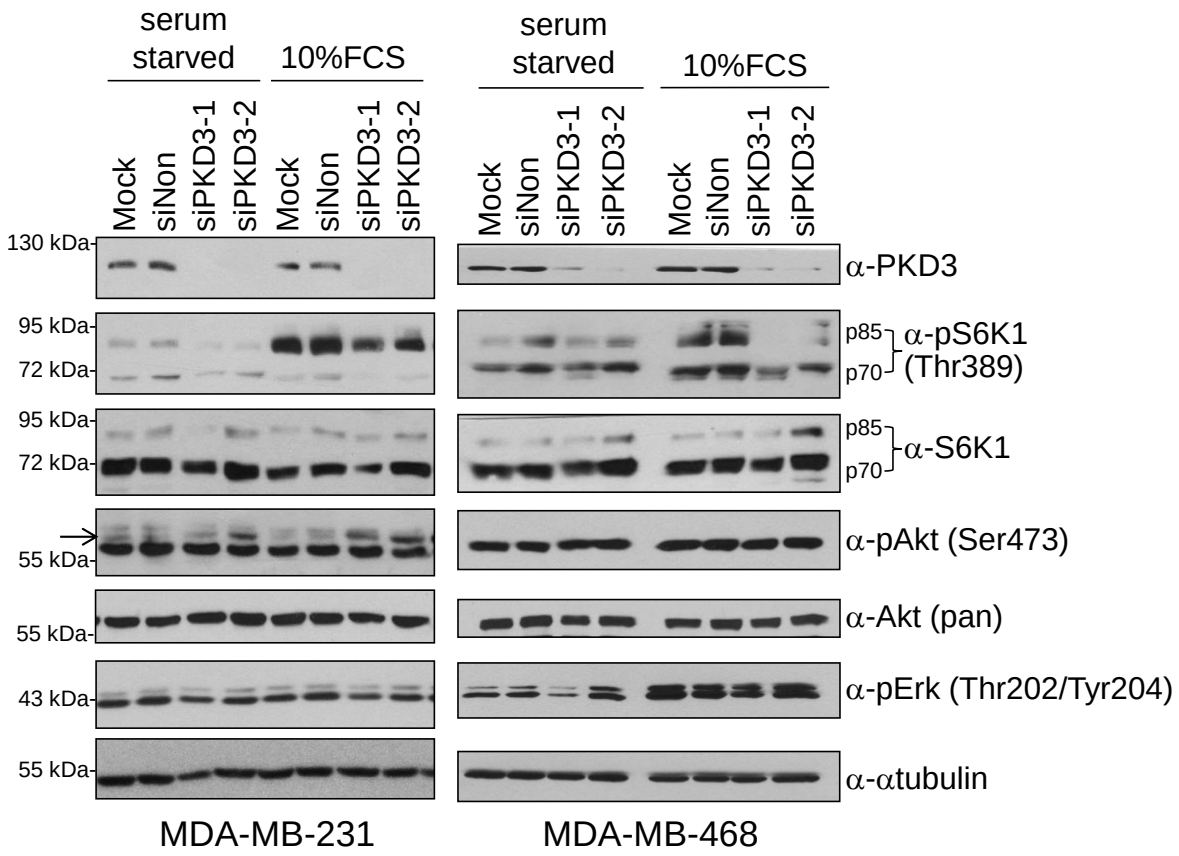


Figure 26: PKD3 is upstream of mTORC1 and S6K1 in TNBC cells

MDA-MB-231 and MDA-MB-468 cells were transiently transfected with two independent, PKD3-specific siRNAs. Control siRNA (siNon) as well as mock-transfected cells were used as controls. 48h after siRNA transfection cells were serum-starved overnight and prior to lysis, cells were either left unstimulated or stimulated with 10% serum for 30 min. Whole cell lysates were analyzed by immunoblotting with the indicated antibodies. Equal loading was confirmed by immunoblotting of whole cell lysates with α-tubulin-specific antibody.

To analyze how acute activation of PKD impacts the mTORC1-S6K1 pathway, we investigated the effect of phorbol-12, 13-dibutyrate (PDBu), an analog of DAG, on the activation of this pathway in the presence and absence of pharmacological PKD inhibitors. PDBu activates novel PKCs, which in turn phosphorylate PKD family members on two specific serines within the activation loop. In addition, PDBu activates PKD directly by high-affinity binding to cysteine-rich motifs within the protein. MDA-MB-231 cells were stimulated with PDBu, followed by immunoblotting of whole cell lysates with the S6K1 phospho-specific antibody (Fig. 27). Figure 27 shows that the S6K1 phospho-specific signal strongly increased upon PDBu stimulation and preincubation of cells with specific pharmacological inhibitors of the PKC/PKD pathway abrogated phospho-S6K1 detection (Fig. 27, top panel). Gö6976 inhibits PKD family members directly, whereas Gö6983 inhibits PKD indirectly by inhibiting novel PKCs. Preincubation of cells with rapamycin, a potent inhibitor of mTORC1, also blocked the PDBu-induced phospho-S6K1 signal, providing evidence that PDBu-induced S6K1 phosphorylation is indeed dependent on mTORC1 and excluding the possibility of an mTORC1 independent pathway. Taken together, these data suggest that PKD3 is upstream of mTORC1 and S6K1 in basal-like TNBC cells.

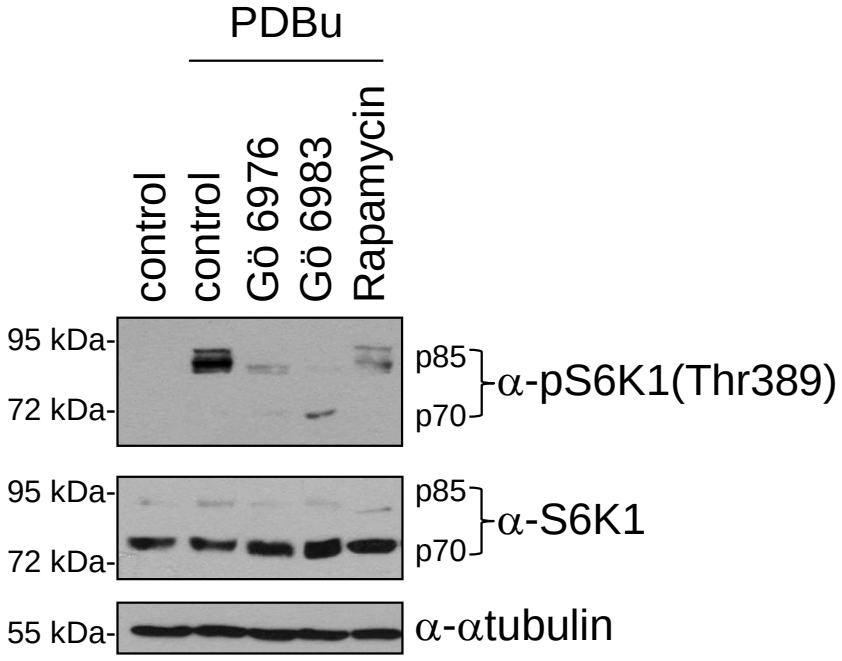


Figure 27: Pharmacological PKD inhibition prevents S6K1 phosphorylation

MDA-MB-231 cells were pretreated with Gö6976 and Gö6983 (5 µM) as well as rapamycin (50 nM) for 3 h, as indicated. DMSO was used as a control. After stimulation with 1 µM PDBu for 15 min, cells were lysed and whole cell lysates were analyzed by immunoblotting with the indicated antibodies. Equal loading was confirmed by immunoblotting of whole cell lysates with α-tubulin-specific antibody.

3.2.4 PKD3 knockdown impairs lysosomal mTOR activation

To shed light onto the cellular interaction between PKD3 and mTOR-S6K1 we performed localization studies in MDA-MB-231 cells. The best characterized localization for the PKD isoforms is at the Golgi complex (Wang 2006; Fu and Rubin 2011), and mTOR has also been described to localize to the Golgi membranes (Drenan, Liu et al. 2004; Liu and Zheng 2007). However, PKD3 was also observed to localize to the nucleus in prostate cancer cells and VAMP2-positive vesicular structures in HEK 293 cells (Lu, Chen et al. 2007; Chen, Deng et al. 2008). Due to the lack of a commercially available antibody suited for the immunocytochemical detection of endogenous PKD3, we ectopically expressed GFP-tagged PKD3 in MDA-MB-231 cells where it localized mainly to the Golgi compartment, as revealed by costaining with the trans Golgi marker p230, with smaller amounts found at the plasma membrane and in the cytosol (Fig. 28A). Endogenous mTOR, however, did not localize to the Golgi complex in MDA-MB-231 cells (Fig. 28B, upper panel), but was instead found to colocalize with the lysosomal marker LAMP1 (Fig. 28B, lower panel). This is in line with findings that mTOR is recruited to and activated at lysosomes in response to amino acids (Sancak, Bar-Peled et al. 2010).

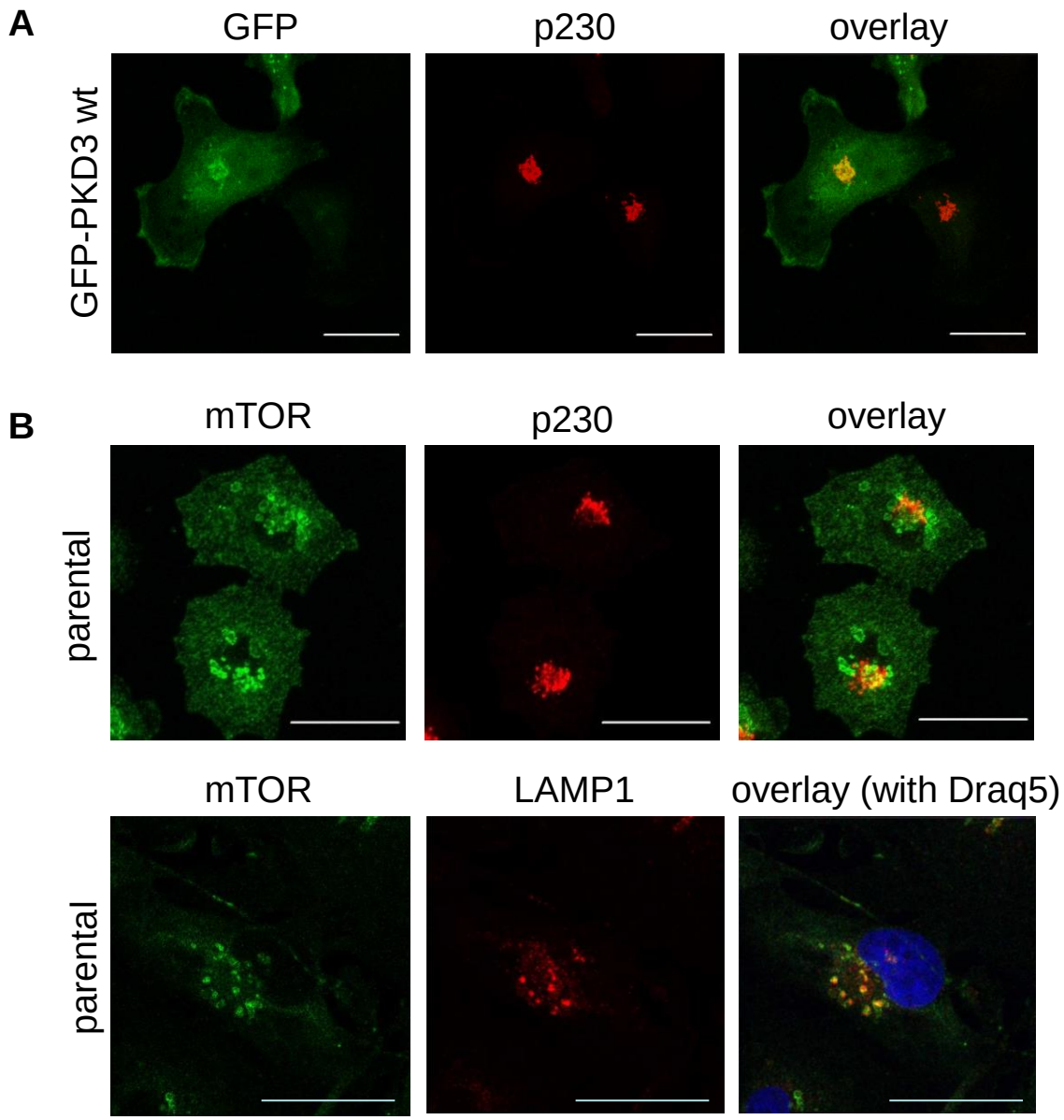
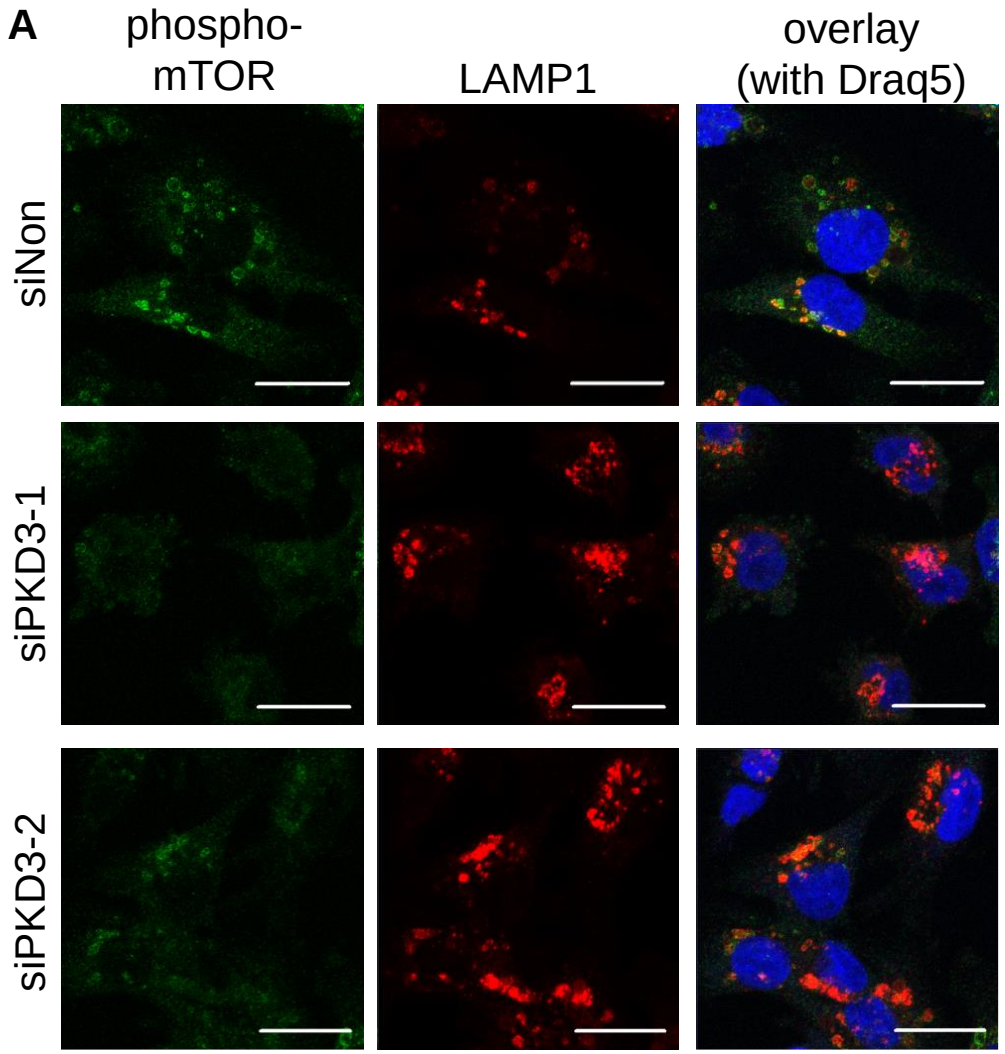


Figure 28: mTOR localizes to lysosomes in MDA-MB-231 cells

(A) MDA-MB-231 cells were plated onto collagen-coated coverslips and transiently transfected with an expression vector encoding GFP-PKD3 wt. The next day, cells were fixed and stained with p230-specific primary and Alexa Fluor 546-conjugated secondary antibodies (red). The images shown are projections of several confocal sections. Scale bar, 20 µm. In (B), MDA-MB-231 cells were plated onto collagen-coated coverslips, fixed and co-stained either with mTOR-specific and p230-specific primary and Alexa Fluor 488- and 546-conjugated secondary antibodies or mTOR-specific and LAMP1-specific primary and Alexa Fluor 488- and 546-conjugated secondary antibodies (green and red as indicated). Nuclei are visualized by Draq 5 staining. The images shown are projections of several confocal sections. Scale bar, 20 µm.

We therefore examined whether the knockdown of PKD3 impacts on mTOR activation at lysosomes by using a phospho-specific antibody that detects active mTOR phosphorylated on serine 2448. In control cells, the phospho-mTOR staining colocalized

with that of LAMP1 (Fig. 29A, upper panel), whereas PKD3 depleted cells presented a weaker, less defined staining pattern, indicating a lower amount of active mTOR at lysosomal membranes (Fig. 29A, middle and lower panel). To investigate this further, we incubated control and PKD3-depleted cells with Lysotracker, which accumulates in acidic organelles. Because mTOR activity is important for the regulation of autophagy (Jung, Ro et al. 2010) we simultaneously analyzed the distribution of LC3. This protein is lipidated by an ubiquitin-like system involving Atg7 and Atg3 that allows it to become associated with autophagic vesicles (Tanida, Ueno et al. 2004). The antibody used in the study mainly detects the lipidated LC3-II form, the presence of which on autophagosomal vesicles serves as an indicator of autophagy. Interestingly, cells lacking PKD3 showed a strong accumulation of Lysotracker containing vesicles, which were further positive for LC3 (Fig. 29B), indicative of increased formation and/or turnover of autophagolysosomes. The loss of PKD3 thus impacts the endolysosomal system, causing a decrease of active mTOR at lysosomes, and as a consequence appears to result in the induction of autophagy.



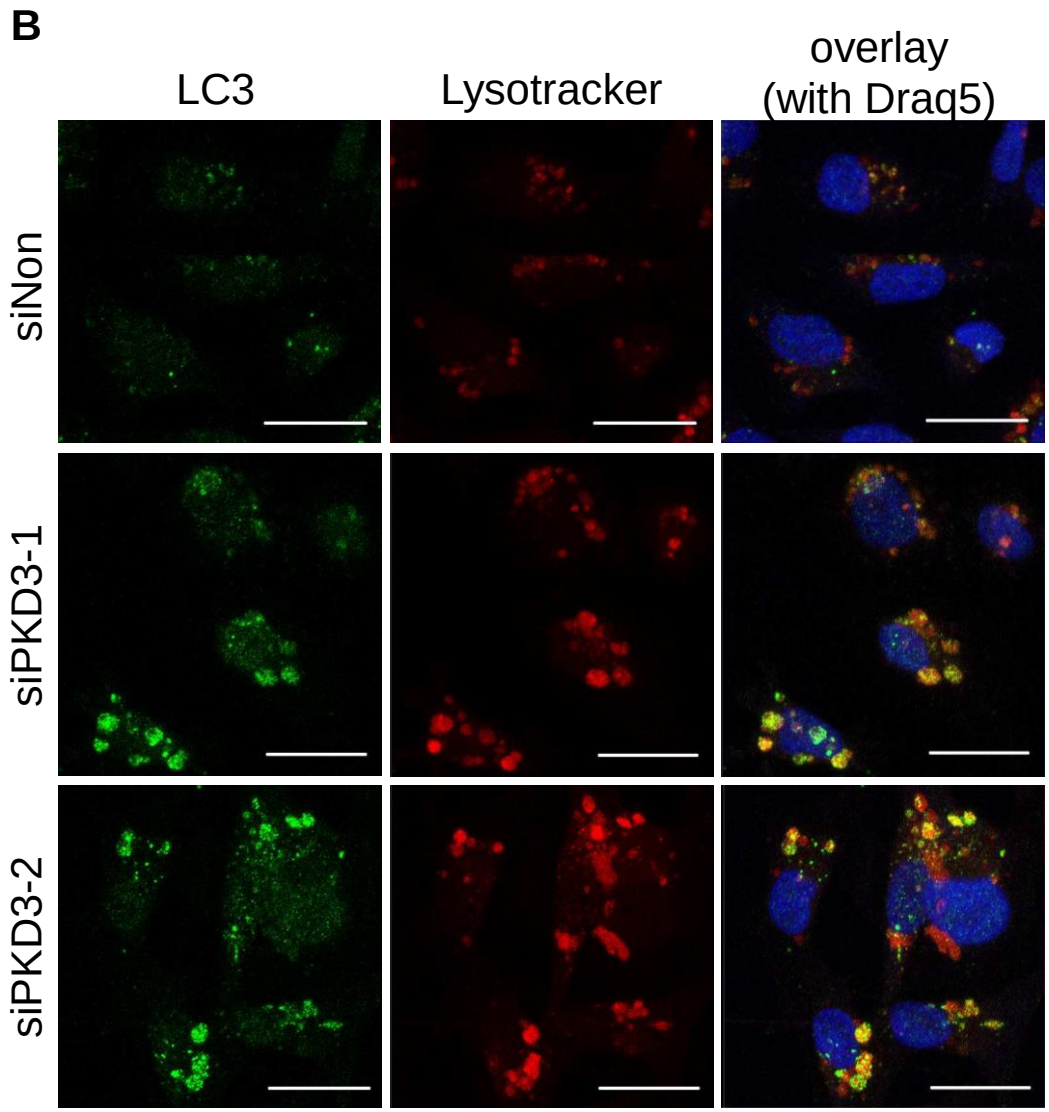


Figure 29: PKD3 knockdown impairs lysosomal mTOR activation

(A) MDA-MB-231 cells were plated onto collagen-coated coverslips and transiently transfected with two independent siRNAs specific for PKD3 or control siRNA (siNon). 72 h post transfection, cells were fixed and co-stained with phospho-mTOR (Ser2448) and LAMP1-specific primary and Alexa Fluor 488- and 546-conjugated secondary antibodies, respectively (green and red as indicated). In (B), MDA-MB-231 cells were transiently transfected as described in (A). Prior fixation, cells were incubated with 75 nM Lysotracker for 2 h (red). Cells were then fixed and stained with LC3-specific primary and Alexa Fluor 488-conjugated secondary antibody (green). In both (A) and (B), nuclei were visualized by Draq5 staining. The images were acquired using identical settings, shown are projections of several confocal sections. Scale bar, 20 µm.

3.2.5 PKD3 knockdown modulates cell morphology and impacts on cell proliferation

The mTORC1-S6K1 signaling pathway is important for the regulation of protein and lipid synthesis, processes which are important for cell proliferation and organismal growth

(Dann, Selvaraj et al. 2007; Ikenoue, Inoki et al. 2008). We therefore investigated related biological effects of PKD3 depletion on MDA-MB-231 and MDA-MB-468 cells. Microscopic analysis of cell morphology revealed that PKD3-depleted cells were less spread than the controls (Fig. 30), but they were not smaller in overall cell size as assessed by FACS analysis (data not shown).

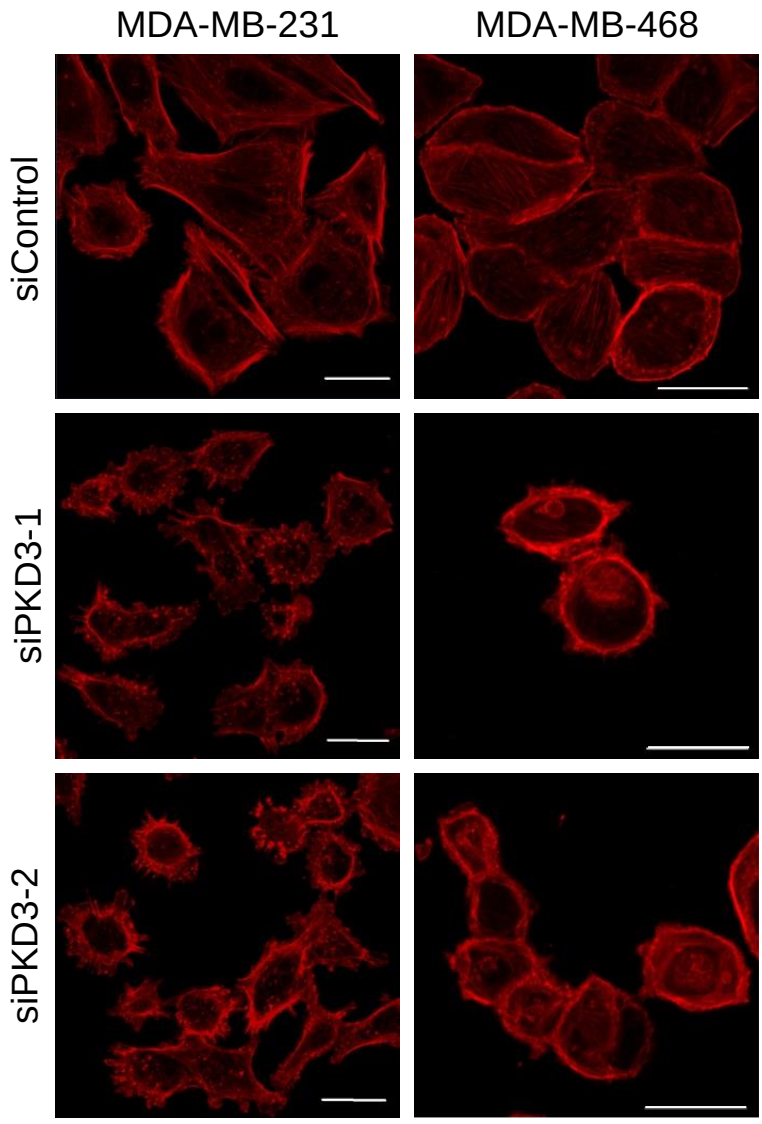


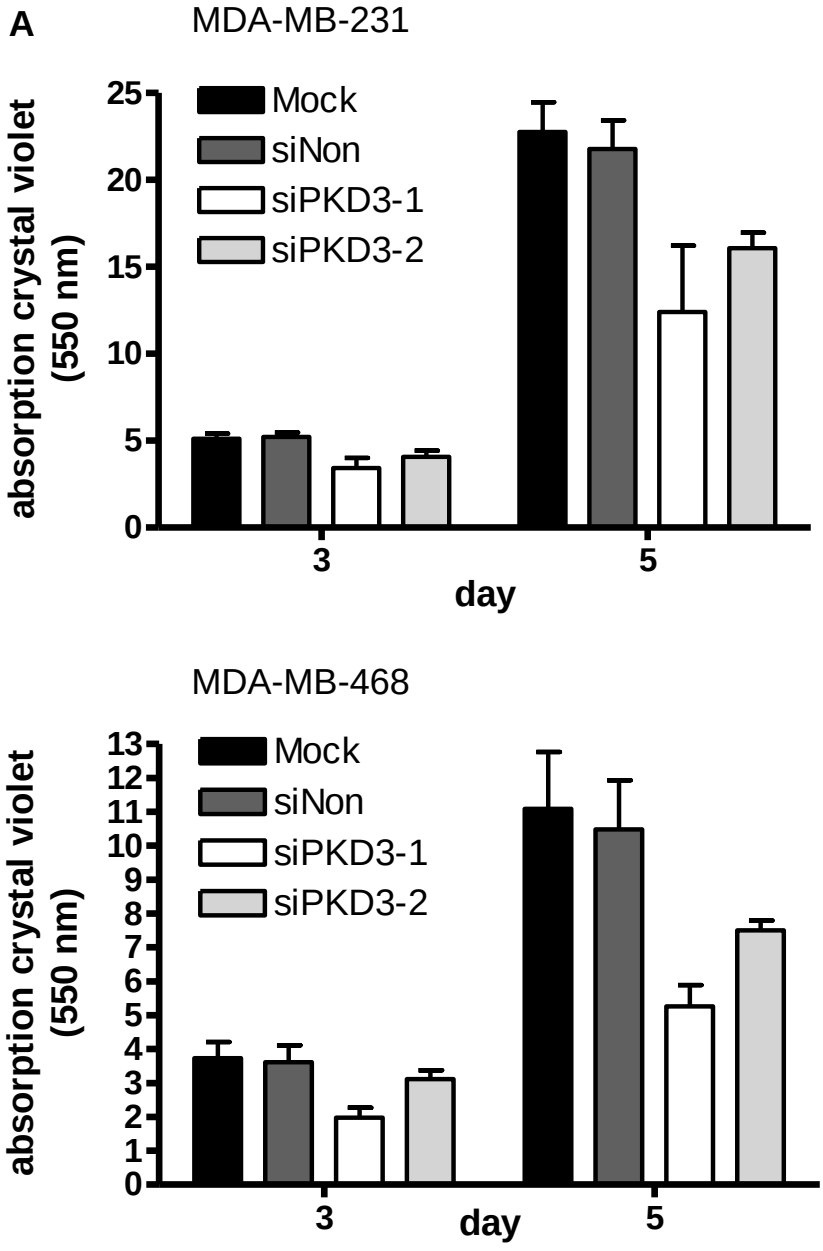
Figure 30: PKD3 knockdown affects cell spreading

MDA-MB-231 and MDA-MB-468 cells were plated onto collagen-coated coverslips and transiently transfected with two independent siRNAs specific for PKD3 or control siRNA (siControl). 72h after transfection, cells were fixed and stained with Alexa Fluor-546 conjugated phalloidin (red). The images shown are projections of several confocal sections. Scale bar, 20 µm.

Next, control and PKD3-depleted MDA-MB-231 and MDA-MB-468 cells were subjected to proliferation assays. Relative viable cell numbers were determined by crystal violet

staining on days 3 and 5 post plating. In both cell lines, PKD3 knockdown moderately decreased the relative viable cell number after 3 days, and markedly decreased the relative viable cell number by almost 50% after 5 days (Fig. 31A). This is in line with results using a pharmacological PKD inhibitor (NB-142-70). Here, a dose-dependent inhibition of cell proliferation with an IC50 of approximately 5 µM was observed for MDA-MB-231 cells (data not shown). At 5 µM, cell proliferation of both, MDA-MB-231 and MDA-MB-468 cells, was reduced by almost 40% after 3 days and 65% after 5 days (Fig. 31B).

In summary, we have shown that the elevated expression of PKD3 in TNBC cells provides a molecular connection between the Golgi and endolysosomal compartments to enhance proliferative mTORC1-S6K1 signaling.



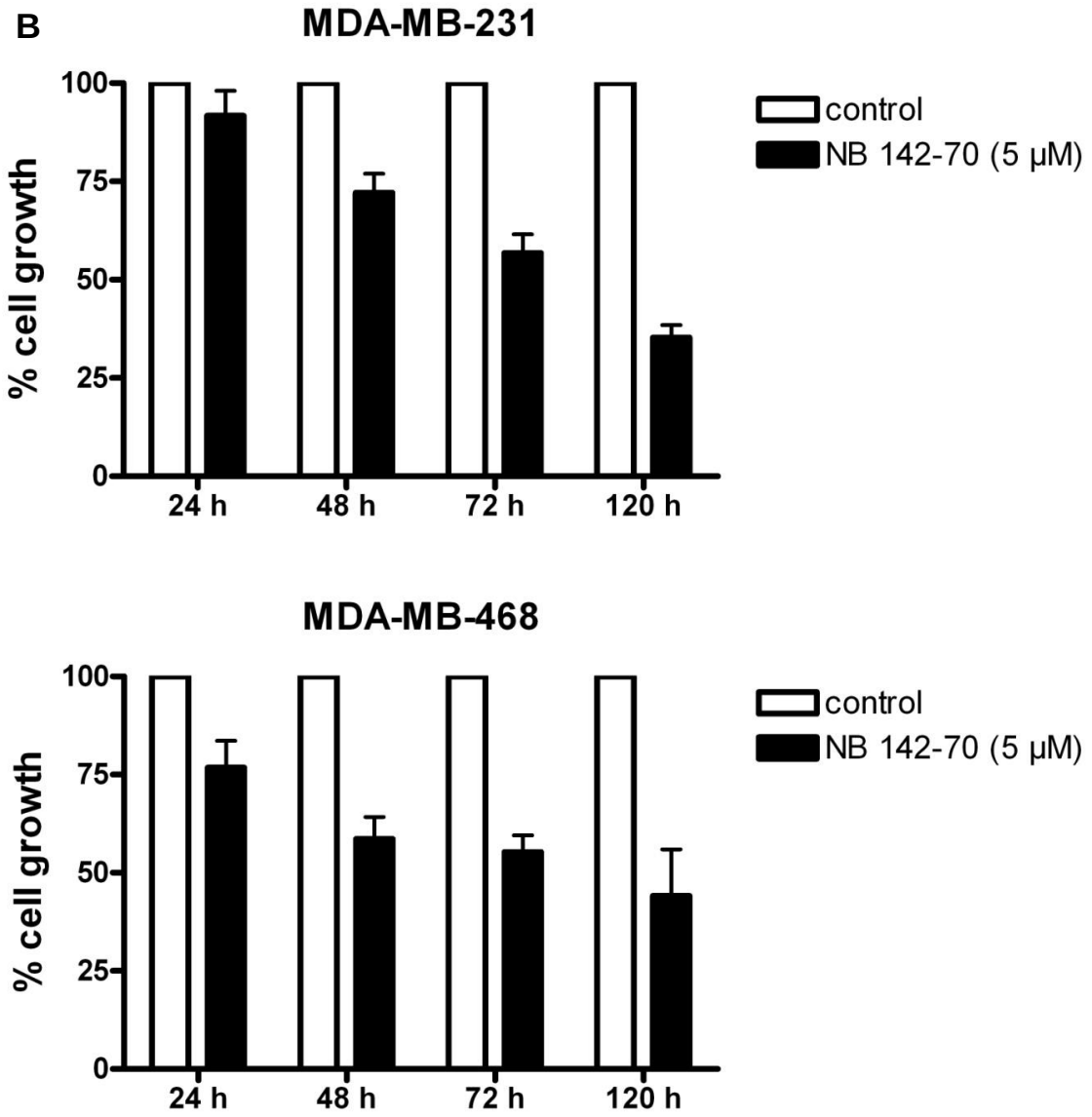


Figure 31: PKD3 knockdown and pharmacological inhibition reduce cell proliferation

(A) MDA-MB-231 and MDA-MB-468 cells were transiently transfected with two siRNAs specific for PKD3. Control siRNA (siNon) as well as mock-transfected cells were used as controls. 24 h after transfection, 2500 cells were seeded in sextublicate into 96well plates and cell density was measured via crystal violet staining after 3 and 5 days. Data show the mean of three independent experiments, all values were normalized to the initial cell density at 6 h after plating. Error bars represent SEM. Proper PKD3 knockdown in both experiments was verified by parallel Western blotting (blots not shown). In (B), MDA-MB-231 and MDA-MB-468 cells were seeded in triplicates into 96-well plates (2.500 cells per well). The next day, cells were treated with 5 µM NB-142-70 and grown for 24, 48, 72 and 120 h. Cell density was measured via crystal violet staining and is presented as percentage of control cells. Data show the mean of two independent experiments, error bars represent SEM.

4 Discussion

The identification of the first PKD isoform was in the early 1990s, since then substantial efforts have been made to unravel the multiple cellular functions of PKD family members. Research over the past two decades revealed that PKDs play an essential role in the regulation of multiple cellular processes such as proliferation, apoptosis, cell migration, immune responses or secretion. Dysfunction of PKD expression and deregulation of PKD activity are associated with a variety of diseases like cardiovascular hypertrophy and several types of cancers (Rozengurt 2011). Most studies about PKD functions have focused on PKD1 and PKD2 and in general, the isoforms are thought to possess overlapping substrate specificities and at least in part, to function redundantly. However, structural differences among the three isoforms imply distinct functional properties and suggest that the isoforms carry out distinct cellular functions. Indeed, functional differences between the three isoforms were demonstrated concerning specific activation pathways, substrate phosphorylation, subcellular localization or their role *in vivo* (Chen, Lu et al. 2005; Hausser, Storz et al. 2005; Doppler and Storz 2007; Lu, Chen et al. 2007; Chen, Deng et al. 2008; Li, Chen et al. 2008; Chen, Li et al. 2009; Ellwanger and Hausser 2013).

The objective of this thesis was to gain further insights into isoform selective functions of the PKD family of proteins with a special focus on PKD3 as this isoform has only been rarely studied so far. In the first part of the thesis I analyzed the regulation of the Arf-GAP protein GIT1 by PKD-mediated phosphorylation in the context of breast cancer cell migration and identified GIT1 as the first PKD3 specific substrate. Phosphorylation of GIT1 on serine 46 by PKD3 modulated GIT1 localization thereby regulating paxillin trafficking and cellular protrusive activity of breast cancer cells. In the second part I focused my attention on a potential role of PKD3 in the progression of TNBC and investigated the associated signaling pathways. Using a phospho-kinase signaling array, S6K1 was identified to be hyperphosphorylated in cells expressing constitutively active PKD3. Based on the reanalysis of transcript profiling studies and my experimental data I could show that PKD3 expression is elevated in TNBC where it contributes to cell proliferation via activation of the mTORC1-S6K signaling pathway.

4.1 GIT1 phosphorylation on serine 46 by PKD3 regulates paxillin trafficking and cellular protrusive activity

In this part of the study I identified and characterized the function of a novel phosphorylation site within GIT1 on serine 46 which is specifically phosphorylated by PKD3. GIT1 is a multidomain protein that is thought to function as an integrator of signaling pathways controlling vesicle trafficking, adhesion and cytoskeletal organization. Due to its multiple binding domains for several different molecules, including Arfs, PIX, paxillin and FAK, GIT1 acts as a scaffolding protein. Despite a growing interest in the GIT family of proteins, their function in integrating signaling pathways that control cell adhesion and migration is not well defined and the molecular mechanisms that control GIT1 activity still remain unclear. GIT1 is phosphorylated on multiple serine, threonine and tyrosine residues as identified by mass spectrometry analysis (Webb, Mayhew et al. 2006) but in most cases the upstream kinases and functional significance of these phosphorylations is unknown. First experiments showed that GIT1 is phosphorylated downstream of PKDs as stimulation of cells with PDBu enhanced the detection of phosphorylated GIT1 (pGIT1) and pharmacological PKD-inhibition prevented GIT1 phosphorylation (Fig. 7A). Overexpression studies with PKD3 wt and ca clearly enhanced phosphorylation of GIT1 (Fig.10) thus identifying GIT1 as a PKD3-specific substrate. More importantly, by using RNA interference studies PKD3 was identified to exclusively phosphorylate endogenous GIT1 as only the knockdown of PKD3 abrogated the detection of pGIT1 (Fig. 9B) whereas the knockdown of PKD1 and PKD2 did not influence GIT1 phosphorylation (Fig. 9A). These findings clearly show that the PKD isoforms also possess non-redundant substrate specificities with PKD3 being the only isoform responsible for endogenous GIT1 phosphorylation.

To identify the PKD3-specific phosphorylation site within GIT1, GIT1 was subjected to mass spectrometry analysis with a specific focus on identifying phosphopeptides that match the PKD consensus motif and serine 46 (S46) was found to be phosphorylated (Fig. 11). The previous analysis of GIT1 by mass spectrometry did not identify serine 46 to be phosphorylated (Webb, Mayhew et al. 2006), which is likely due to the stimulation of cells with PDBu in this study to specifically activate the PKD pathway. In addition to serine 46, five further novel phosphosites were mapped and several others confirmed (see Table 16). Serine 46 was then shown to be directly phosphorylated by PKD3 using an *in vitro* kinase assay and by coexpression experiments (Fig. 12). The external cues that activate PKD3 to trigger GIT1 phosphorylation remain to be identified. The results show that the pharmacological inhibition of novel PKCs by Gö6983 does not block GIT1 phosphorylation on serine 46 (Fig. 7). Phosphorylation of PKD by novel PKCs in the activation loop is generally considered as a prerequisite for activation. However, a PKC-independent role

for PKD3 has been suggested in basal glucose uptake of skeletal muscle cells (Chen, Lu et al. 2005) and more recent studies demonstrated that the rapid PKC-dependent PKD activation is followed by a sustained, PKC-independent phase of catalytic activation by PKD autophosphorylation (Jacamo, Sinnett-Smith et al. 2008; Sinnett-Smith, Jacamo et al. 2009).

My findings further suggest that the phosphorylation state of GIT1 on serine 46 by PKD3 regulates the subcellular localization of GIT1. In MCF7 cells, wild type GIT1 mainly localized to FAs (Fig. 13, upper panel). By contrast, MCF7 cells expressing a GIT1 S46D phosphomimetic mutant contained a high number of cytoplasmic paxillin-positive complexes, which were motile (Fig. 17) and absent in cells expressing GIT1 S46A (Fig. 13, middle and bottom). As the S46D mutant was completely filled with the complexes and showed less GIT1 localized to FAs, it can be concluded that phosphorylation of GIT1 at serine 46 is necessary and sufficient for targeting the protein to cytoplasmic complexes. It was further noticed that the GIT1 S46A mutant was less soluble and could only be efficiently extracted from cells using an SDS-containing lysis buffer, indicating that the dephosphorylated protein associates more strongly with cytoskeletal structures. PBDu stimulation of cells shifted the balance towards a higher number of cytoplasmic complexes, providing further evidence that serine 46 phosphorylation regulates GIT1 subcellular localization (Fig. 15).

Former studies have shown that GIT1 cycles between three distinct subcellular compartments, including adhesion-like structures, the leading edge and cytoplasmic complexes (Manabe, Kovalenko et al. 2002). The cytoplasmic complexes were described as novel, non-vesicular, supramolecular complexes which are motile, contain paxillin, PAK and PIX and function as modules to deliver signaling molecules such as PAK or paxillin to and from adhesions as they form and break down. In the present study I observed localization of the focal adhesion protein paxillin to the cytoplasmic complexes containing GIT1, supporting the idea that GIT1 sequesters paxillin from FAs to cytoplasmic complexes when phosphorylated on serine 46. In the case of the S46D mutant, paxillin was almost entirely relocated to the cytoplasmic complexes. Paxillin is also a multidomain scaffolding protein that functions in the recruitment of both signaling and structural molecules to focal adhesions (Turner 2000). I assume that phosphorylation of GIT1 on serine 46 and the connected localization shift is necessary for enhanced recruitment of paxillin to cytoplasmic complexes, thereby enhancing the overall turnover of paxillin.

GIT1 localization to adhesions and the leading edge was reported to depend on the PBS whereas targeting to cytoplasmic complexes required the central region that contains the ankyrin repeats and PIX binding domain (Manabe, Kovalenko et al. 2002). Based on the microscopic results I suggest that S46 within the GAP domain plays a supplementary role for GIT1 translocation to cytoplasmic complexes. Opposed to the observation by Manabe

and coworkers, in MCF7 cells PAK colocalized with GIT1 only in FAs and was not contained in GIT1 S46D complexes (Fig. 16A) whereas βPIX was contained in both, FAs and cytoplasmic complexes (Fig. 16B). GIT1 phosphorylation at serine 46 may thus alter protein conformation, modulating the interaction with specific protein partners such as PAK and thereby affecting localization. PAK signaling is strongly cross-linked with GIT1 functions and two PAK phosphorylation sites in GIT1 (serines 517 and 709) have been mapped previously (Zhao, Lim et al. 2005; Webb, Kovalenko et al. 2006). PAK is a serine/threonine kinase that binds to the active form of Rac or Cdc42 resulting in a conformational change in PAK, autophosphorylation of the molecule at several sites and subsequent activation (Manser, Leung et al. 1994; Lim, Manser et al. 1996; Lei, Lu et al. 2000; Webb, Mayhew et al. 2006). Besides its role in cytoskeletal dynamics, PAK regulates migration through its effects on the formation of protrusions. PAK interacts with PIX-GIT1 complexes and is activated and recruited by these complexes to adhesions and leading edges (Manabe, Kovalenko et al. 2002). But activation of PAK does not only occur through Rac and Cdc42 activity, GIT1 itself is able to stimulate alphaPAK autophosphorylation through the participation of the GIT1 N-terminal Arf-GAP domain but not Arf-GAP activity and involves phosphorylation of PAK at residues common to Cdc42-mediated activation (Loo, Ng et al. 2004). Thus, the activation of PAK at adhesion complexes involves a complex interplay between the kinase, Rho GTPases and protein partners that provide localization cues. We have to consider that S46 phosphorylation within the Arf-GAP domain of GIT1 could consequently modulate PAK activity in a positive or negative manner and therefore influence migration and cytoskeletal dynamics.

Serine 46 is located within the GAP domain of GIT1 and could thus potentially regulate the GAP function of the protein, but Arf6 activity was not significantly altered in MCF7 cells stably expressing the GIT1 phosphorylation site mutants as judged by GGA3 pulldowns (data not shown). Serine 46 phosphorylation thus appears to decrease the focal adhesion localization of GIT1 independently of its GAP function. The GAP domain is also known to be important for the overall structure of GIT1 (Totaro, Paris et al. 2007) and has been shown to confer GAP-independent functions, e.g. the activation of PAK (Loo, Ng et al. 2004). Furthermore it has recently been shown that GIT1 is regulated by an intramolecular mechanism which composes two distinct intramolecular interactions between the N- and C- terminus of GIT1 (Totaro, Paris et al. 2007). It seems that GIT1 adopts a closed conformation through its Arf-GAP domain and ankyrin repeats, that prevents protein interaction and this may well be regulated by phosphorylation. Serine 46 phosphorylation could thus additionally influence such functions.

Interestingly, further studies revealed that overexpression of activated forms of Rac1 and Cdc42 could promote phosphorylation of GIT1 specifically on serine 46 (data not shown). The intracellular distribution of GIT1 between cytoplasmic complexes and adhesions was shown to be regulated by Rac and Rac is also necessary for the formation of GIT1-

induced protrusions (Manabe, Kovalenko et al. 2002; Nishiya, Kiosses et al. 2005). Conversely, GIT1 can enhance Rac activation through complexing with PIX and PAK, but it can also negatively regulate Rac activation by suppressing Arf6 signaling (Nishiya, Kiosses et al. 2005). There is thus a delicate relationship between GIT1 and Rac signaling that might be regulated by phosphorylation of GIT1 on S46. This could be in line with findings that PKD3 gets activated by signaling through Rac and the alpha subunits of the heterotrimeric G proteins G12 and G13 (Yuan, Rey et al. 2006). So overexpression of activated Rac could activate PKD3 to phosphorylate GIT1 on serine 46.

Cytoplasmic complexes have been implicated in focal adhesion turnover and endocytic trafficking processes that are crucial to directed migration of cells. But how GIT1 controls cell migration is being discussed controversially. GIT1 has both Arf-dependent and Arf-independent activities that affect actin remodeling. Arf-dependent activities comprise the regulation of membrane remodeling that accompanies actin polymerization. Arf-independent functions include acting as upstream regulators of Rho family proteins and providing a scaffold for Rho effectors and exchange factors such as PAK and PIX. Some reports indicate that GAP activity of GIT1 is critical for its function, other reports suggest that GIT1 functions primarily in a signaling complex, as a scaffold or a regulator of PAK with little role for the GAP activity. Taken the findings of this thesis into account, I suggest that the phosphorylation state of GIT1 on serine 46 by PKD3 regulates paxillin trafficking and cellular protrusive activity by modulating GIT1 subcellular localization. I further propose that the enhanced cell spreading, protrusive activity and motility observed in GIT1 S46D expressing cells (Fig. 19, 20 and 21) arises from increased focal adhesion turnover through the continuous trafficking of paxillin to membrane protrusions. This turnover is important for the migratory process as it strongly depends on the coordinated activation and targeting of both structural and signaling molecules to the leading edge of a migrating cell. By contrast, cells expressing GIT1 S46A are less motile due to the stabilization of focal adhesions arising from increased paxillin bound to FAs.

Of note, the PKD phosphorylation site at serine 46 is conserved in GIT2, raising the possibility that GIT2 is regulated in a similar manner, although it should be noted that GIT1 and GIT2 are not exchangeable as they also possess non-redundant functions (Randazzo, Inoue et al. 2007).

While this study focuses on the function of GIT1 in epithelial cells, GIT1 has also been extensively studied in neuronal cells where it regulates neurite extension, dendritic spine morphogenesis and synapse formation. Interestingly, PKD3 is strongly expressed in neuronal cells in the developing embryo (Ellwanger, Pfizenmaier et al. 2008), making it tempting to speculate that PKD3 may also control GIT1-mediated cytoskeletal rearrangements in the central nervous system. Moreover, GIT1 has been recently linked to neurodegenerative disorders since GIT1 was identified as an enhancer of Huntingtin

aggregation in Huntington's disease (Goehler, Lalowski et al. 2004). GIT1 has also been implicated in a wide range of other disorders including cell transformation and oncogenesis (Ahn, Chung et al. 2003), mental retardation (Kutsche, Yntema et al. 2000), vascular development and homeostasis (Stockton, Reutershan et al. 2007), inflammation (Mazaki, Hashimoto et al. 2006) and infectious disease (Baek, Lim et al. 2007). Several reports also suggest GIT1 proteins to have a role in HIV infectivity in an Arf6-dependent manner (Blagoveshchenskaya, Thomas et al. 2002). Thus further attempts towards the understanding of the molecular mechanisms by which GIT1 activity is regulated should also be addressed in the context of neuronal differentiation, cancer and other diseases.

4.2 PKD3 expression is elevated in triple-negative breast cancer cells and contributes to mTOR-S6K pathway activation

In this part I identified PKD3 to be overexpressed in TNBC where it contributes to mTORC1-S6K pathway activation and thereby regulates cell morphology and proliferation. The serine threonine kinase mTOR regulates many major cellular processes such as protein and lipid synthesis, lysosome biogenesis, energy metabolism and cell survival and is implicated in an increasing number of pathological conditions including cancer. In the majority of human tumors, the mTORC1 pathway is aberrantly activated, sustaining the increased proliferation of tumor cells even under nutrient limiting conditions (Dann, Selvaraj et al. 2007; Yecies and Manning 2011). In early-stage TNBC in particular, positive phospho-mTOR staining was correlated with significantly worse overall as well as recurrence-free survival (Ueng, Chen et al. 2012). Here I show that TNBC cells and basal-like breast cancer tissues exhibit increased PKD3 protein and transcript levels, respectively (Fig. 22 and 23). By performing an antibody array detecting phosphorylated proteins of a variety of different signaling pathways I identified S6K1, a member of the mTOR signaling pathway, as a downstream target of PKD3 (Fig. 24). Overexpression of catalytically active PKD3 (Fig. 25) and stimulation of cells with PDBu (Fig. 27) resulted in the hyperactivation of S6K1 whereas PKD3 knockdown reduced S6K1 phosphorylation (Fig. 26) and pharmacological PKD-inhibition prevented S6K1 phosphorylation in a similar fashion as rapamycin, a potent inhibitor of mTORC1 activity (Fig. 27). Interestingly, I did not observe any changes in the phosphorylation of Erk1/2 and Akt, kinases whose activation has previously been implicated in the context of PKD3-positive prostate cancer (Chen, Deng et al. 2008), indicating a selective effect of PKD3 on S6K1 in breast cancer cells (Fig. 25 and 26). Taken together, these data show that PKD3 is upstream of the mTORC1-S6K1 signaling pathway in TNBC.

The two main S6K1 isoforms, p70 and p85, derive from the same gene and are produced by alternative translational initiation (Magnuson, Ekim et al. 2012). In both MDA-MB-231 and MDA-MB-468 cells, the p85 subunit showed a stronger activation in response to serum or phorbol ester compared with that of the more abundant p70 subunit (Fig. 26 and 27). The N-terminal extension of the 85 kDa subunit comprises a nuclear localization signal (Magnuson, Ekim et al. 2012), however, in breast cancer cells, both isoforms were mainly observed in the cytosol (Kim, Akcakanat et al. 2009), and their functional differences are poorly understood. The presented data suggest that p85 S6K1 is the main serum-responsive isoform in TNBC cells that is regulated by PKD3 via mTORC1 signaling.

Further, I focused my attention on the analysis of the molecular link between PKD3 expression and mTOR activity in TNBC. In the cell line MDA-MB-231, PKD3 mainly localized to the Golgi complex (Fig. 28A), raising the question whether PKD3 locally regulates mTOR activity. In the literature it is described that mTOR is recruited to Golgi membranes via a Golgi localization sequence where it is further activated (Drenan, Liu et al. 2004; Liu and Zheng 2007). One possibility for mTOR activation at the Golgi complex by PKD3 could be the Golgi phosphoprotein 3 (GOLPH3), a peripheral membrane protein of the Golgi stacks which gets recruited to Golgi membranes via binding of PI4P (Dippold, Ng et al. 2009) and whose overexpression in cancer cells has been shown to activate the mTORC1 signaling pathway (Scott, Kabbarah et al. 2009). As the function of PKDs at the Golgi complex is mainly the stimulation of PI4P production, elevated PKD3 expression could lead to an increased recruitment of GOLPH3 and thus increased activation of mTORC1 at Golgi membranes. However, knockdown of PKD3 did not decrease GOLPH3 binding to Golgi membranes as revealed by immunofluorescence analysis with a GOLPH3 specific antibody (data not shown) ruling out a direct interaction between PKD3, GOLPH3 and mTORC1. The PLD signaling pathway could also be linked to PKD3-mediated mTORC1 activation as PLD-mediated production of phosphatidic acid (PA) at Golgi membranes is stimulated by PKDs (Malhotra and Campelo 2011) and has been further established to mediate mitogenic activation of mTORC1 (Foster 2009). It was shown that PA directly interacts with mTOR, thereby increasing mTOR's ability to activate downstream effectors (Fang, Vilella-Bach et al. 2001). However, knockdown of PKD3 in TNBC cells or overexpression of PKD3 in MCF7 cells did not significantly modulate PLD activity as measured by a PLD activity assay (data not shown), thus excluding a cooperation of PKD3 and PLD in mTORC1 activation at Golgi membranes via the production of PA.

Interestingly, staining of MDA-MB-231 cells with an mTOR specific antibody revealed no colocalization of mTOR with the Golgi marker p230 but showed that mTOR colocalized with the lysosomal marker LAMP1 (Fig. 28B), indicating that mTOR localizes to lysosomes in TNBC. Moreover, knockdown of PKD3 at the Golgi complex led to a weaker,

less defined staining pattern of phospho-mTOR at the lysosomes, indicating a lower amount of active mTOR at lysosomal membranes (Fig. 29A). This is in line with the literature where it was shown that mTOR gets recruited to and activated at lysosomes by a Ragulator-Rag complex (Sancak, Bar-Peled et al. 2010).

Based on my data I propose that PKD3 provides a molecular link between protein synthesis and sorting at the level of the Golgi complex and the catabolic processes occurring at the lysosomal compartment via a mechanism that remains to be defined. Although PKD kinases are best studied in the context of the constitutive trafficking of cargo proteins to the plasma membrane, there is recent evidence that PKD also affects the delivery of specific proteins to the lysosomes. Kinase-inactive PKD1 and PKD2 inhibited the exit of the lysosomal transmembrane protein mucolipin 1 from the Golgi, suggesting that PKD directly impacts lysosome protein composition (Marks, Holicky et al. 2012). Alternatively, by regulating lipid homeostasis at the TGN membranes, PKD may indirectly affect the function of lysosomes by altering their lipid composition as a consequence of constitutive vesicular transport processes. PA for example is a lipid second messenger that is necessary for mTOR activation (Foster 2009). PI4P is known to participate in the regulation of trafficking from the Golgi to lysosomes during lysosomal biogenesis (Szentpetery, Varnai et al. 2010) and regulates the delivery of the lysosomal membrane protein LIMP2 (Jovic, Kean et al. 2012). As mentioned before, PKDs are involved in the production of both, PA and PI4P at Golgi membranes. Conversely, cholesterol accumulation in the endolysosomal compartment negatively affected the fusion of lysosomes with endocytic and autophagic vesicles (Fraldi, Annunziata et al. 2010) and inhibited mTOR activation in endothelial cells (Xu, Dang et al. 2010). Moreover, depletion of the lipid transfer protein CERT, which shuttles ceramide from the endoplasmic reticulum to Golgi membranes, increased autophagic flux and synergized with paclitaxel in mediating LAMP2-mediated cell death (Lee, Roylance et al. 2012), most likely via a mechanism involving ceramide accumulation within the endoplasmic reticulum. These studies exemplify the various crosstalk mechanisms between the Golgi and endolysosomal system, all of which may potentially impact mTORC1 activation mediated by PKD3. Of note, in some cells, GFP-tagged PKD3 was also observed on vesicle-like structures; however, these did not stain positively for mTOR (data not shown). Nevertheless, I cannot completely rule out that endogenous PKD3 also localizes to lysosomes where it directly acts as an upstream regulator of mTORC1. In line with this, it was shown by Sridhar and colleagues that the lipid kinase PI4KIIIβ associates with lysosomes and regulates lysosomal export and retrieval presumably by producing lysosomal PI4P, which is implicated in the regulation of trafficking and sorting events directly at lysosomal membranes (Sridhar, Patel et al. 2013). PKD3 could thus also regulated PI4P production at lysosomes by phosphorylating local PI4KIIIβ.

Silencing of PKD3 in MDA-MB-231 cells led to the accumulation of LC3 on Lysotracker positive vesicles, which are likely to represent autophagosomes or autophagolysosomes (Fig. 29B). Autophagosomes are induced during autophagy by autophagy-promoting proteins and later fuse with lysosomes to autophagolysosomes in order to promote the degradation of their content. mTOR usually inhibits autophagy by phosphorylating key autophagy-promoting proteins and nutrient starvation induces autophagy through inhibition of mTOR. I assume that the loss of PKD3 in TNBC cells impacts the endolysosomal system, causing a decrease of active mTOR at lysosomes, and as a consequence appears to result in the induction of autophagy. Furthermore, mTOR activity at endomembranes is not only important for autophagy induction, but also for controlling the terminal maturation of lysosomes (Takahashi, Nada et al. 2012) and for the termination of autophagy and reformation of lysosomes (Yu, McPhee et al. 2010). The loss of PKD3 and the associated reduction of mTORC1 activity at lysosomes could thus additionally influence important later steps of autophagy such as reformation of lysosomes or termination of autophagy thereby disturbing the autophagic flux. All in all, it seems that endomembranes such as lysosomes or late endosomes play a growing role in the regulation of mTOR activity and a deregulation in the endolysosomal system could have extensive outcomes (Flinn, Yan et al. 2010). Autophagy is known to have a tumor-suppressive function and constitutive mTORC1 activation may contribute to tumor progression by inhibiting autophagy (Kimmelman 2011; Yecies and Manning 2011). However, in established cancers, autophagy can promote cell survival under a variety of stresses and may even be required for tumor maintenance (Kimmelman 2011). PKD was recently reported to induce autophagy in response to reactive oxygen species by direct phosphorylation and activation of Vps34, a PI3K required for autophagosome formation (Eisenberg-Lerner and Kimchi 2012). These results seem to be in contradiction with the findings presented here; however, Eisenberg-Lerner and Kimchi investigated the induction of autophagy by the PKD1 isoform and under conditions of oxidative stress, which obviously differ from the setting in TNBC cells where PKD3 exerts a protective effect on basal autophagy. Of note, Vps34 has also been described to activate mTOR and induce S6K1 phosphorylation on Thr389 thereby inhibiting autophagy (Byfield, Murray et al. 2005; Nobukuni, Joaquin et al. 2005), making it intriguing to speculate that the different PKD isoforms engage the same substrate to trigger opposing cellular outcomes.

Knockdown of PKD3 in TNBC cells decreased the relative viable cell number by almost 50% after 5 days (Fig. 31A) which I assume to be, at least in part, a consequence of mTORC1 suppression and autophagy in PKD3 depleted cells. However, whether the impact of PKD3 depletion on LC3 accumulation is due to enhanced induction of autophagy or due to a defective autophagy flux was not illuminated in detail in this study. In MDA-MB-231 and MDA-MB-468 cells, the magnitude of pharmacological PKD inhibition on cell proliferation was similar to that upon PKD3 silencing (Fig. 31B). Both cell lines

express PKD2 but no or relatively little PKD1 ((Eiseler, Doppler et al. 2009) and my own observations), indicating that PKD3 is the main PKD isoform in TNBC cells and PKD2 plays a minor contribution to proliferation in these cells. Both, PKD3 knockdown and pharmacological PKD inhibition were more effective with respect to growth inhibition of MDA-MB-231 and MDA-MB-468 cells than rapamycin (data not shown), to which these cells were relatively insensitive. This suggests that regarding the control of cell proliferation in TNBC cells, PKD3 signals through pathways additional to mTORC1. I reported in the first part of the thesis that PKD3 stimulates cell spreading and migration through GIT1 phosphorylation, which is involved in the trafficking of the focal adhesion protein paxillin (Huck, Kemkemer et al. 2012). The rounded cell morphology seen upon PKD3 silencing may thus be explained, at least in part, by impaired trafficking of GIT1-paxillin complexes. Furthermore, it is very likely that PKD3 is required for general Golgi secretory function in TNBC cells which could impact on cell adhesion and proliferation.

For the treatment of TNBC, several targeted agents including mTOR inhibitors such as RAD001 are currently being evaluated in clinical trials in combination with chemotherapy, however, thus far with limited success (Tan and Swain 2008; Crown, O'Shaughnessy et al. 2012). The poor survival outcomes seen with TNBC patients are at least partly due to the lack of therapeutic targets. So the increased understanding of molecular profiles of TNBC subtypes and the identification of deregulated signaling pathways is likely to improve therapeutic strategies with targeted agents. Due to the important functions of PKDs in the cellular homeostasis of different tissues, the therapeutic inhibition of PKD is a delicate issue. Nevertheless, based on the data presented in this thesis, the development of an isoform-specific pharmacological PKD3 inhibitor may hold promise for the targeted treatment of TNBC. In addition, combinatorial therapies could even be more beneficial for the treatment of TNBC. Liu and colleagues for example showed that the combination of EGFR and mTOR inhibition resulted in increased apoptosis in several TNBC (Liu, Yacoub et al. 2011). Based on my results I propose that the combination of PKD3 specific inhibition with EGFR inhibitors could be a promising approach for the treatment of TNBC, which should be addressed in further studies.

4.3 Conclusions and perspectives

The aim of this thesis was to gain deeper knowledge into isoform specific functions of the PKD family of proteins with a special focus on PKD3. Compared to the other two isoforms, PKD3 shows some differences in structure, subcellular localization and activation which favor the opinion that PKD3 also carries out non-redundant functions. This implies that PKD3 regulates a distinct set of substrates to fulfill such functions, which is in accordance with the findings presented in this thesis showing that PKD3 selectively phosphorylates

GIT1 to enhance cell spreading and motility. Furthermore, it is demonstrated that PKD3 is specifically upregulated in TNBC cells where it contributes to mTORC1-S6K pathway activation, thereby enhancing cell proliferation and suggesting an oncogenic role of PKD3 in these cancer cells. Interestingly, those TNBC cell lines which expressed high levels of PKD3 showed a very low level of PKD1 (data not shown). This observation is remarkable as PKD1 expression appears to be required for the maintenance of epithelial characteristics and the loss of PKD1 was shown to correlate with increased breast cancer cell migration, invasion as well as breast tumor progression (Olayioye, Barisic et al. 2013), proposing PKD1 to act as a tumor suppressor in breast cancer cells. This contrast of tumor suppressive versus oncogenic function strongly implies that at least in TNBC cells, PKD1 and PKD3 regulate a different set of substrates in order to fulfill these opposing cellular effects. Oncogenic functions of PKD3 have also been described in lung cancer cells, where PKD3 was identified as part of a bone metastatic signature (Vicent, Luis-Ravelo et al. 2008) and silencing of PKD3 in prostate cancer cells was shown to reduce *in vitro* proliferation, migration, invasion and xenograft growth in nude mice (LaValle, Zhang et al. 2012). Mechanistically, PKD3 promoted the expression and secretion of cytokines and extracellular proteases such matrix metalloproteinase 9 and urokinase-type plasminogen activator via activation of NFkappaB and HDAC signaling pathways, thereby positively contributing to cell migration and invasion (LaValle, Zhang et al. 2012; Zou, Zeng et al. 2012). Another study describes the oncogenic contribution of PKD3 to growth and survival of prostate cancer cells through a PKCε/PKD3 pathway downstream of Akt and ERK 1/2 (Chen, Deng et al. 2008). Taken together, these data provide support that the different PKD isoforms do not only have redundant as well as specific functions, but in some instances may even have opposing functions. This is further in line with observations that silencing of PKD2 in MCF7 cells promoted cell spreading (Peterburs, Heering et al. 2009), whereas PKD3 knockdown in the same cell line (data not shown) and in TNBC cells impaired cell spreading as presented in this study (Fig. 30).

The knockdown of PKD3 in highly invasive MDA-MB-231 cells did not significantly modulate cell migration which might be due to the fact that these cells only express very low amounts of GIT1 (data not shown), suggesting that GIT1 S46 phosphorylation by PKD3 is not crucial for cell migration in TNBC cells. However, as GIT1 is a GAP protein for Arf6, low GIT1 expression levels in MDA-MB-231 cells may lead to aberrant Arf6-GTP levels independently of PKD3 function and Arf6 signaling is correlated with enhanced breast cancer cell adhesion, motility and invasion (Sabe 2003; Hashimoto, Onodera et al. 2004). The clear impact of PKD3 depletion or pharmacological inhibition on cell proliferation in MDA-MB-231 strongly suggests that the role of PKD3 in these cells is rather correlated with the regulation of cell proliferation than with the regulation of cell motility. On the contrary, MCF7 cells express low levels of PKD3 and moderate levels of

GIT1, but here, cell motility strongly depended on GIT1 S46 phosphorylation, thus exemplifying that the function of PKD3 depends on the cell type and cellular context.

In addition, the *in vivo* situation is even more complex, however, over the last few years, a crucial *in vivo* role of the PKD family of proteins in diverse biological processes such as innate immunity, plasticity, cardiac and skeletal muscle remodeling as well as T lymphocyte function has been revealed (Ellwanger and Hausser 2013). In this context, the isoforms are also thought to possess both, overlapping as well as specific physiological functions depending on the activation, localization or substrate specificity of the PKD isoforms in different tissues. For instance, PKD3 was shown to be differentially expressed during organogenesis of the mouse embryo and the strong expression of PKD3 in specific tissues, e.g. cardiac and skeletal muscle, points to an important role for this kinase in the development of these tissues (Ellwanger, Pfizenmaier et al. 2008). However, it would be beneficial to investigate the potential function of PKD3 in these tissues in more detail using transgenic mouse models where the endogenous PKD3 function is diminished by overexpression of a dominant-negative protein or deletion of the PKD3 gene (knockout).

In future studies, it will also be interesting to determine how PKD substrate specificity is achieved as the PKD kinases phosphorylate the same consensus sequence *in vitro*. Indeed, GIT1 was also phosphorylated by PKD1 in an *in vitro* kinase assay (data not shown), indicating that in a cellular context specificity is achieved by different spatial localization, activation and/or the binding of specific protein cofactors. Here, structural differences among the isoforms could be relevant for the formation of distinct protein complexes; the amino terminal region of nonpolar amino acids and the PDZ-binding motif contained in PKD1 and PKD2 are for example absent in PKD3 (Rozengurt, Rey et al. 2005; Wang 2006). Thus, the identification of isoform specific protein interaction partners should be addressed in further studies. In addition, more efforts need to be made to investigate the recruitment and localization of the individual isoforms to different subcellular localizations and the regulation of activity at these sites. Therefore, a convenient tool could be the use of biosensors (e.g. specifically designed fluorescence based kinase activity reporters) to measure and visualize the local and timely restricted activation of PKDs. By designing isoform selective biosensors it might be possible to further distinguish between the different PKD isoforms and to shed light onto isoform specific cellular functions and substrate specificity. Finally, to uncover redundant and non-redundant physiological functions of the three PKD isoforms *in vivo*, more refined transgenic mouse models should be established to allow total or isoform selective, tissue-specific or conditional studies of PKD activity *in vivo*.

Taken together, the results presented in this thesis highlight a unique role of PKD3 in the regulation of breast cancer cell motility and TNBC progression and help us to reconsider our current view of PKD isoform redundancy.

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Publications

Annabell Bischoff, Bettina Huck, Bettina Keller, Michaela Strotbek, Simone Schmid, Melanie Boerries, Hauke Busch, Dafne Müller, and Monilola A. Olayioye. **miR-149 functions as a tumor suppressor by controlling breast epithelial cell migration and invasion.** (Cancer Research, currently under revision)

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