Protein Kinase D controls mitotic Golgi complex fragmentation through a Raf-MEK1 pathway

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Abbreviations

AC	acidic domain
ADF	actin-depolymerizing factor
APS	ammonium persulfate
AR	androgen receptor
Arf	ADP ribosylation factor
Arl	Arf-like
Arp2/3	Actin-related proteins 2/3
ATP	Adenosine-5'-triphosphate
°C	degree celsius
ca	constitutive active mutant
Cdc2	cell division cycle 2
CERT	ceramide transfer protein
COP	coatomer protein
conc.	concentration
CtBP/BARS	C-terminal binding protein/brefeldin A adenosine diphosphate–ribosylated substrate
CRD	cysteine rich domain
DAG	diacyl-glycerol
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
DTT	dithiothreitol
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
EGTA	ethylene glycol tetraacetic acid
ER	endoplasmic reticulum
ERGIC	ER-Golgi intermediate compartment
ERK1/2/1c	extracellular signal-regulated kinase 1/2/1c
EtOH	ethanol
FAPP1/2	four-phosphate-adaptor protein 1/2
FBS	fetal bovine serum
FRAP	fluorescence recovery after photobleaching
GFP	green fluorescent protein
GM130	Golgi matrix protein 130
GOLPH3	Golgi phosphoprotein 3
GPCR	G protein-coupled receptor
GRASP-65/55	Golgi reassembly-stacking protein of 65/55kD
GST	Glutathion-S-Transferase
HCl	hydrogen chloride
HDAC	histone deacetylase
HEPES	N-2-Hydroxyethylpiperazine-N′-2-ethanesulfonic Acid
hr	hour
HRP	horseradish peroxidase
Hsp27	heat shock protein 27
IF	immunofluorescence
IP	immunoprecipitation
IP_3	inositol 1, 4, 5-trisphosphate
IQ	ilimaquinone
JNK	c-Jun N-terminal kinases

KAB	kinase assay buffer
KCl	potassium chloride
KHM	potassium acetate, HEPES, magnesium acetate
KOH	potassium hydroxide
LPA	lysophosphatidic acid
M	mitosis
mM	millimolar
mg	milligram
mA	milliampere
ManII	Mannosidase II
MAPK	mitogen- activated protein kinase
max	maximal
MEB	mitosis extract buffer
MEK1/1b	mitogen-activated protein kinase kinase 1/1b
MPF	maturation promoting factor
Mg^{2+}	magnesium
$MgCl_2$	magnesium chloride
min	minutes
ml	milliliter
MOPS	3-(N-morpholino) propanesulfonic acid
MT	microtubule
MTOC	microtubule organizing center
NaCl	sodium chloride
NaDOC	sodium deoxycholate
Na_2HPO_4	disodium hydrogen phosphate
Na_3VO_4	sodium orthovanadate
NaF	natrium fluoride
NCS-1	neuronal calcium sensor
NES	nuclear export signal
NF κB	nuclear factor kappa-light-chain-enhancer of activated B cells
ng	nanogram
NLS	nuclear localization sequence
N-terminus	amino-terminus
O/N	over night
OSBP	oxysterol-binding protein
PA	phosphatidic acid
PAK	p21 activating kinase
PBS	phosphate buffered saline
PFA	paraformaldehyde
Plk1/3	polo-like kinase 1/3
PDGF	platelet derived growth factor
PDZ	postsynaptic density-95/discs large/zonula occludens-1-binding motif
PFA	paraformaldehyde
pH3	phospho-Histone H3
PH	pleckstrin homology
PI	propidium iodide
PIPES	piperazine-N,N'-bis(2-ethanesulfonic acid)
PIP_2	phosphatidylinositol 4,5-bisphosphate
PI4P	phosphatidylinositol 4-phosphate
PI4K	phosphatidylinositol-4 kinase
PI4KIIα	phosphatidylinositol-4 kinase IIα
PI4KIIIβ	phosphatidylinositol-4 kinase III beta

PKC	protein kinase C
PKD	protein kinase D
PLC	phospholipase C
POD	peroxidase
Rab11	Rab-protein 11
RIN1	Ras-interacting protein
RT	room temperature
rpm	rounds per minute
S phase	synthesis phase
Ser	serine
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
siRNA	small interfering RNA
SSH1L	slingshot-1-like
TEMED	N,N,N,N-tetramethylethylenediamine
TGN	trans-Golgi network
Tris	tris-hydroxymethyl-aminomethane
UTP	Uridine-5'-triphosphate
VEGF	vascular endothelial growth factor
Vol	volume
v/v	volume/volume
V	volt

Summary

The process of Golgi inheritance in mammalian cells involves multiple signaling pathways and proteins to ensure correct partitioning of Golgi membranes among dividing cells. As the Golgi apparatus is a single-copy organelle, the mechanism of separation is rather complex. First Golgi stacks become separated by the action of several key proteins such as BARS, MEK1 or GRASP-65. During cell cycle progression, Golgi stacks further break down into small vesicles that become dispersed throughout the cytoplasm. This fragmentation process is a prerequisite to allow equal distribution of Golgi membranes between daughter cells.

Essential for mitotic entry is the cleavage of Golgi inter stack connections. Since the blocking of this particular event causes an arrest of cells in G2, it defines the Golgi mitotic checkpoint.

In this work PKD was identified as a novel regulator in Golgi mitotic checkpoint control. The PKD family of protein kinases has a well characterized role at the *trans*-Golgi network regulating fission of cargo-containing vesicles en route to the plasma membrane. However, by now only few publications proposed a role for PKD in cell division.

In this study we provide evidence that siRNA-mediated depletion of PKD 1 and 2 delays the passage of synchronized HeLa cells into M phase. Furthermore, a semi-intact assay approach identified PKD as a regulator of Golgi fragmentation, since PKD inhibition abolished dispersion of Golgi stacks. Detailed microscopic analyses such as mitotic index determinations and Golgi integrity measurements, respectively, demonstrate that PKD acts on the level of Golgi ribbon cleavage during G2.

Finally, evidence is provided that PKD acts through a Raf-1-MEK pathway to exert its function during mitosis; however, Raf-1 appeared to be not a direct PKD substrate.

Taken together, this study demonstrates a novel role of PKD in Golgi mitotic checkpoint control by acting upstream of Raf-1/MEK1. The data further emphasize the importance of PKD in the maintenance of the structural integrity of the Golgi complex.

Zusammenfassung

Die Vererbung des Golgi-Komplexes in Säugerzellen erfordert das Zusammenspiel zahlreicher Signalwege und Proteine um sicherzustellen, dass gleich viele Golgi-Membranen zwischen den Tochterzellen verteilt werden. Durch das Einwirken verschiedener Schlüsselproteine, wie BARS, MEK1 oder GRASP-65 werden die Golgi Stapel in einem ersten Schritt voneinander getrennt. Während des folgenden Zellzyklusverlaufs werden die Stapel weiter aufgesplittet und als kleine Bläschen im Zytoplasma verteilt. Dieser Fragmentierungsprozess ist die Voraussetzung für eine gleichmäßige Aufteilung von Golgi-Membranen zwischen den Tochterzellen. Für den Eintritt in die Mitose ist das Abtrennen der Golgi-Stapel-Verbindungen erforderlich, da anderenfalls die Zellen in der G2 Phase arretieren. Dieser Schritt wird deshalb als „mitotischer Golgi Kontrollpunkt“ definiert.

In dieser Arbeit konnte PKD als neuer Regulator dieses Kontrollpunkts bestimmt werden. Am Trans-Golgi-Netzwerk sind die Mitglieder der PKD-Familie für das Abschnüren von Fracht-gefüllten Vesikeln, die für die Plasma Membran bestimmt sind, verantwortlich. Bis jetzt gibt es nur wenige Publikationen die PKD eine Rolle während der Zellteilung zuschreiben.

In dieser Studie wird gezeigt, dass der Verlust von PKD1 und 2 die Durchlaufzeit von HeLa Zellen in der Mitose erheblich verzögert. Experimente mit halbintakten Zellen ergaben, dass PKD die mitotische Fragmentierung des Golgi-Komplexes reguliert, da eine PKD-Hemmung die Teilung der Golgi Stapel verhinderte. Mit mikroskopischen Analysen, wie beispielsweise die Bestimmung des mitotischen Indexes oder Messungen der Golgi Integrität, konnten wir darüber hinaus zeigen, dass PKD das Abtrennen der Golgi Stapel in der G2 Phase vermittelt. Zudem konnten wir nachweisen, dass PKD in der Mitose durch einen Raf-1-MEK Signalweg agiert. Allerdings scheint Raf-1 kein direktes PKD Substrat zu sein.

Diese Arbeit leistet einen wichtigen Beitrag für die Aufklärung der Regulation des mitotischen Golgi Kontrollpunkts.

1 Introduction

1.1 The Protein Kinase D family

The Protein Kinase D (PKD) family of serine/threonine protein kinases consists of three isoforms in eukaryotic cells. All three members exhibit high structure homologies not only among each other and with isoforms of the Protein Kinase C (PKC) family.

The first and most well-known member was identified in 1994, initially termed PKCμ, together with its homolog in mouse designated as mouse PKD (Johannes et al. 1994; Valverde et al. 1994). Subsequently, another kinase within the PKC family was found in mammals in 1999 originally termed as PKCv (Hayashi et al. 1999). This kinase was also distinct in structure compared to PKC family members and therefore PKCμ and PKCv were assigned to a new subgroup within the PKC family. Finally, an additional member of the new sub-family has been discovered in 2001 and was termed as PKD2 (Sturany et al. 2001). All three members share an overall similar structure and are today referred as to PKD1 (PKCμ), PKD2 and PKD3 (PKCv).

PKD1 occupies an unique position within the PKD family being the most extensively characterized isoform with homologs in mice, rats, worms, yeast and flies (Sundram et al. 2011).

1.1.1 Structural characteristics of PKD

The PKD protein is composed of a catalytic domain at the C-terminus, responsible for the catalytic action of the kinase, and a regulatory domain at the N-terminus (Figure 1).

The regulatory domain comprises a cysteine-rich domain (CRD) including two cystein-rich zinc-finger like motifs, termed C1a and C1b, followed by a pleckstrin homology module (PH) (Rykx 2003). The CRD binds diacyl-glycerol (DAG) and DAG analogs with high affinity, thereby determining the localization and cellular responses of the protein (Iglesias et al. 1998; Auer et al. 2005). DAG is an important second messenger involved in a variety of signaling pathways and it is proposed to induce negative membrane curvature (Zimmerberg and Kozlov 2006).

The C1a region is necessary for binding to DAG at intracellular membranes, such as the Golgi complex, and to promote nuclear export. The C1b domain manages nuclear import and mediates translocation to the nucleus and the plasma membrane (Maeda et al. 2001; Rey and Rozengurt 2001; Rozengurt et al. 2005; Auer et al. 2005).

The PH domain of PKD is located between the CRD and the catalytic domain and is important for the interaction with other proteins. During nuclear translocation the PH domain is required for nuclear exit along with the C1a domain (Auer et al. 2005). Moreover, the PH domain exerts a negative regulatory effect on the catalytic activity of PKD, since deletion of the PH domain renders the protein constitutively active (Iglesias and Rozengurt 1998). Similarly, deletion of the CRD also leads to full activation of PKD; however only C1b exerts an inhibitory effect on kinase activity (Iglesias et al. 1998). Thus, the regulatory domain maintains the protein in an inactive state. Also present in the regulatory part of the protein is an acidic domain (AC), which is inserted between the CRD and the PH domain and rich in negatively charged amino acids (Figure 1).

Solely, PKD1 and PKD2 possess a region of nonpolar amino acids (rich in alanine and/or proline residues) near the amino-terminus, which is absent in PKD3. 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. For instance, it is required for an appropriate surface localization of Kidins220, the first identified PKD substrate in brain and neurons (Sánchez-Ruiloba et al. 2006).

Additionally, PKD2 exhibits a serine-rich stretch in the linker region between C1a and C1b.

Overall, high homology between PKD isoforms exists mainly in terms of the catalytic region and the first cysteine-rich domain (Figure 1; LaValle et al. 2010). The N-terminus and regions between CRD and PH domain exhibit larger differences among PKD1, PKD2 and PKD3 (Wang 2006). Thus, isoform specific differences determine substrate and signaling specificities within different tissues. This allows PKD to interact with a multitude of proteins and modulate their functions in a variety of pathways.

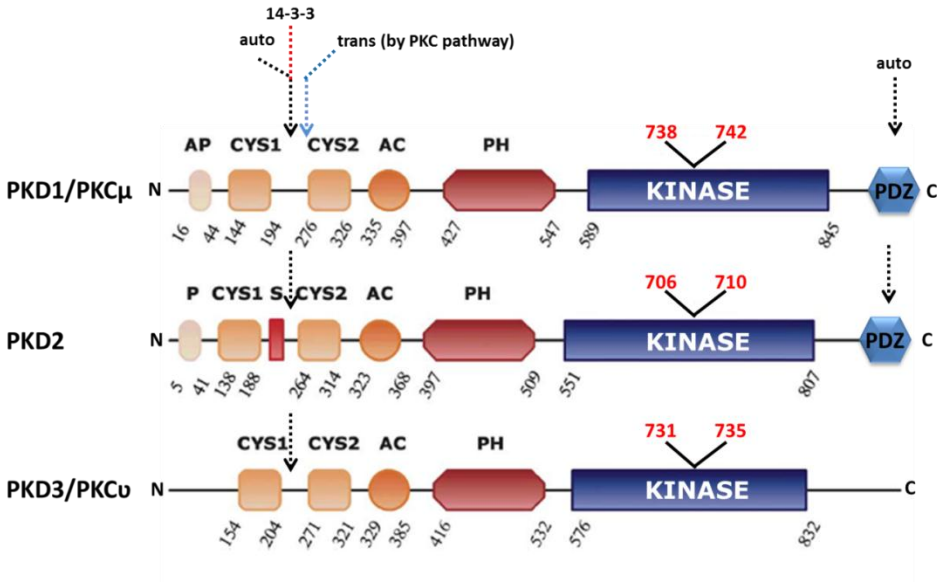


Figure 1- Molecular structure of PKD isoforms.
Numbers correspond to amino acid positions. Serines within the activation loop that become phosphorylated are highlighted in red. Arrows indicate autophosphorylation sites, the 14-3-3 binding site and a transphosphorylation site. AP, alanine/proline regions; CYS1/CYS2, cysteine-rich domain; AC, acidic-rich domain; PH, pleckstrin homology domain; PDZ, postsynaptic density-95/discs large/zonula occludens motif; N, amino-terminus; C, carboxy-terminus; S, serine-rich region. Modified from Rykx et al. 2003.

1.1.2 PKD classification

The structural features of PKD isoforms specified in 1.1.1 separate PKD isoforms from their former family of PKC kinases.

Similarities between PKD and PKC occur only in the CRD, which is highly homologous (Chen et al. 2008). The catalytic domain at the C-terminus of PKD, however, is more similar to myosin light chain kinase and Ca^{2+}-calmodulin kinase superfamily (van Lint et al. 2002; Chen et al. 2008). Additionally, the amino-terminus of PKD exhibits a PH domain which is absent in PKCs, but lacks an autoinhibitory pseudosubstrate motif present in PKCs (Valverde et al. 1994; Rykx 2003).

Hence, PKD and PKC isoforms diverge in substrate specificity and signaling mechanisms within different tissues (Nishikawa et al. 1997).

1.1.3 PKD activation

PKD proteins are ubiquitously expressed in multiple cell types regulating different signaling pathways. In a basal state, PKD displays low catalytic activity. To exert their function, PKD isoforms have to be activated. Activation is carried out

by different mechanisms, the most prominent among those is the PKC-PKD signaling cascade (Rozengurt et al. 2005).

Upon ligand binding to G-protein coupled receptors (GPCR) or through B-cell or T-cell receptor activation, Phospholipase C (PLC) becomes activated and mediates the hydrolysis of phosphatidylinositol-4,5-bisphosphate (PIP_2) into DAG and inositol-1,4,5-triphosphate (IP_3) (Waldron et al. 1999; Matthews et al. 2000; Yuan et al. 2000). DAG recruits PKD and PKC to membranes, subsequently activates PKC, which in turn transphosphorylates PKD at two serine residues within the activation loop (Waldron et al. 2001, Figure 1). Activation loop phosphorylation of PKD triggers the release of auto-inhibition of the PH domain leading to full activation of kinase activity (Waldron and Rozengurt 2003). Mutation studies for PKD1 confirm the significance of these phosphorylation sites, since substitution of the serine residues for alanine inhibits, and substitution for glutamatic acid enhances PKD1 activation (Iglesias et al. 1998; Van Lint et al. 2002; Rykx 2003).

Novel PKC isoforms such as PKCδ, ε, θ and η are strong PKD activators; however, classical PKCs like PKCα can also activate PKD (Li et al. 2004). Atypical PKCs fail to phosphorylate PKD (Zugaza et al. 1996). PKC isoforms are differentially expressed in diverse cell types and tissues, thereby determining substrate specificity towards PKD (Mellor and Parker 1998; Rozengurt et al. 2005).

Apart from the activation loop phosphorylation sites, PKD1 also exhibits autophosphorylation motifs (Figure 1). One is located in the PDZ domain and is often phosphorylated following activation loop phosphorylation (Rozengurt et al. 2005). This site is crucial for conformational changes of the protein and for kinase activity duration (Vertommen et al. 2000; Rybin et al. 2009; Figure 1). Another autophosphorylation site lies within the regulatory domain, required for binding to 14-3-3 proteins (Hausser et al. 1999; Figure 1). 14-3-3 binding attenuates PKD1 activity by altering its subcellular localization (Van Lint et al. 2002).

PKD2 and PKD3 become also autophosphorylated within the regulatory domain; however, there is no evidence for 14-3-3 binding. As described above, PKD3 lacks the PDZ and thereby the autophosphorylation site within the C-terminus (Rykx 2003; Sánchez-Ruiloba et al. 2006).

At the Golgi compartment, PKD binds to Golgi membranes via DAG and gets activated through PKCη (Díaz Añel and Malhotra 2005). Once activated, PKD

regulates fission of cargo vesicles from the trans-Golgi network (TGN) (Liljedahl et al. 2001; 1.3.1).

Besides this, PKD activity is also induced by a src-Abl-dependent pathway through phosphorylation within the PH domain. The mode of activation is also dependent on PKCδ-mediated phosphorylation (Storz et al. 2004).

1.1.4 Subcellular localization of PKD

PKD can be activated by a number of different stimuli such as pharmacological agents, like phorbol esters, or physiological stimuli (e.g. neuropeptides, angiotensin, growth factors, GPCR agonists or hormones) (Matthews et al. 1999; Rey et al. 2001). In unstimulated cells, PKD1 is localized mainly in the cytoplasm; however, other fractions can be found in the nucleus, at the Golgi complex and mitochondria (Rey et al. 2001; Liljedahl et al. 2001; Hausser et al. 2002; Rozengurt et al. 2005).

In response to receptor stimulation, PKD1 is rapidly recruited from the cytosol to the plasma membrane where it binds to locally produced DAG via its cysteine-rich domain (Matthews et al. 1999; Rozengurt et al. 2005). PKCs are also recruited to the plasma membrane and activate PKD1 within the activation loop. PKC-mediated activation is required for translocation back into the cytosol and for redistribution to other organelles (Rey et al. 2001).

Following translocation from the plasma membrane, PKD1 subsequently enters the nucleus, since the protein exhibits a nuclear localization sequence (NLS) and a nuclear export signal (NES) within the regulatory domain (see 1.1.1; Rey, Sinnett-Smith, et al. 2001; Auer et al. 2005). Similar activation and localization mechanisms exist for PKD2 and PKD3 (Rozengurt et al. 2005). Comparable to PKD1, PKD2 is mainly localized in the cytosol. Following cell membrane recruitment and activation, it accumulates in the nucleus (Rey et al. 2003; Auer et al. 2005). PKD3 is found in the nucleus and the cytoplasm, both upon plasma membrane activation and under resting conditions (Rey et al. 2003).

As described above, PKD is also found at Golgi membranes where it binds to the local pool of DAG via its C1a domain (Bard and Malhotra 2006). The Golgi-associated PKCη is activated by DAG and in turn phosphorylates PKD (Waldron et al. 1999; Bard and Malhotra 2006). PKD localization to the Golgi is essential for regulating fission of transport vesicles at the TGN (Yeaman et al. 2004).

Of note, PKD recruitment to the TGN is independent of kinase activity, since kinase dead PKD mutants are also recruited to the TGN (Liljedahl et al. 2001; Rey and Rozengurt 2001). Reducing the local pool of DAG or deletion of the CRD, completely abrogates PKD recruitment to Golgi membranes (Maeda et al. 2001; Baron and Malhotra 2002).

1.2 PKD signaling

PKDs modulate multiple signaling pathways and thereby regulate various biological functions such as proliferation, differentiation, apoptosis, immune responses, oxidative stress responses or secretion.

- Proliferation, apoptosis and cell survival

PKDs have been linked to several pathways, which are known to control cell proliferation. By this means, PKD turned out to possess anti- as well as pro-proliferative properties, respectively.

For instance, PKD antagonizes proliferation by inhibiting the proto-oncogene c-jun through attenuation of EGF (epidermal growth factor)-induced activation of JNK (c-Jun N-terminal kinase) (Bagowski et al. 1999; Hurd and Rozengurt 2001; Hurd et al. 2002; Rozengurt et al. 2005).

On the other hand, PKD modulates the Ras–Raf–MEK–ERK pathway and promotes proliferation through direct phosphorylation of its *in vivo* substrate RIN1 (RAS-interacting protein). Phosphorylated RIN binds to 14-3-3 proteins, thus causing RIN1-dissociation from Ras and thereby activating the Ras-Raf-MEK-ERK pathway (Wang et al. 2002). Additionally, PKD can also directly activate the MAPK (mitogen-activated protein kinase) pathway through activation of Raf-1 (Hausser et al. 2001).

Furthermore, PKD overexpression promotes Swiss 3T3 cell proliferation in response to bombesin, vasopressin or phorbol esters (Zhukova et al. 2001). PKD is highly expressed in dividing, but low in differentiating mouse keratinocytes (Rennecke et al. 1996).

PKD also possesses substrate specificities towards class II HDACs (histone deacetylases). HDACs are repressors of gene transcription. In endothelial cells, PKD phosphorylation of HDAC5 in response to VEGF (vesicular endothelial growth factor) induces gene expression, through translocation of HDAC5 from the nucleus to the cytoplasm, leading to proliferation and angiogenesis (Ha et al. 2008).

Moreover, it has been reported that PKD is involved in processes, which cause cell death or contrariwise promote cell survival. In response to oxidative stress, PKD mediates cell survival by activation of NF-κB and JNK pathways (Storz et al. 2005; Zhang et al. 2005). Oppositely, PKD phosphorylation of Bit1 (Bcl-2 inhibitor of transcription) induces apoptosis (Biliran et al. 2008), similar to PKD-mediated repression of androgen receptor (AR) activity, presumably through phosphorylation of heat shock protein 27 (Hsp27) (Hassan et al. 2009). PKD-mediated inhibition of AR function plays a major role in prostate cancer development (Jaggi et al. 2003).

- Cell migration, invasion and adhesion

PKD's implication in several pathways regulating cell motility is closely related to cancer progression and metastasis.

Upon PDGF (platelet derived growth factor) stimulation, PKD mediates αvβ3 integrin recycling from early endosomes to the plasma membrane, which is indispensable for adhesion during cell spreading and migration (Woods et al. 2004). In addition, PKD interacts also with E-Cadherin, which leads to increased cell-cell adhesion (Jaggi et al. 2005; Syed et al. 2008).

It is also known that PKD acts as negative regulator of cell migration through phosphorylation of cortactin and slingshot 1 (SSH1L) (Storz 2009).

Actin polymerization is obligatory for cell migration, since it drives the formation of cell protrusions that are used to adhere to the extracellular matrix and thus navigates the cell in a certain direction (DesMarais 2002).

In invadopodia of breast cancer cells, cortactin induces actin polymerization through F-actin binding and interaction with Arp2/3 (Actin-related protein). PKD phosphorylation of cortactin reduces its ability to induce actin polymerization resulting in a decreased invasive potential of the cells (De Kimpe et al. 2009; Eiseler et al. 2007, 2010).

Another protein crucial in terms of cell motility is the actin-depolymerizing factor ADF/cofilin. It is responsible for actin severing at the leading edge creating free actin filament barbed ends, which promotes cell migration (DesMarais et al. 2005). Cofilin activity is regulated by LIM kinase 1 and SSH1L phosphatase, respectively (San Martín et al. 2008). LIM kinase 1 phosphorylates and thereby inactivates cofilin, whereas SSH1L phosphatase dephosphorylates and in that way activates cofilin (Nagata-Ohashi et al. 2004; Bernard 2007; Horita et al.

2008). SSH1L binds to F-actin and co-localizes with PKD1. Upon RhoA activation, PKD-mediated phosphorylation of SSH1L causes dissociation from the actin cytoskeleton by sequestering SSH1L into the cytoplasm through the action of 14-3-3 proteins. This leads to inhibition of cell motility owing to persistent cofilin inactivation (Eiseler et al. 2009; Storz 2009; Peterburs et al. 2009; Barišić et al. 2011). Further data of Eiseler et al. corroborate the role of PKD in this context, since PKD suppresses matrix metalloproteinase expression, which inhibits breast cancer cell invasion (Eiseler et al. 2009).

Having listed the different roles for PKD in signaling one can imagine that its deregulation results in a variety of diseases such as cancer, diabetes and cardiovascular hypertrophy.

1.3 PKD signaling at the Golgi complex

In this work, we focused on PKD function at the Golgi complex. In epithelial cells, PKD is found at the Golgi compartment modulating the organization of this organelle in terms of secretion.

1.3.1 The Golgi apparatus

The Golgi apparatus is the only organelle within the cell, named after its discoverer Camillo Golgi in 1898 (Munro 2011). It is a single-copy organelle located around the microtubule organization center (MTOC) in the perinuclear area of the cell (Zaal et al. 2011; Figure 2). The Golgi is composed of multiple stacks of flat membranes, called “cisternae”, ordered in a polarized fashion from *cis*- to *trans* (Jamora 1999; Figure 2). In eukaryotic cells, stacks are laterally linked via tubular bridges also referred as to “non-compact zones”, which confers the Golgi a ribbon-like shape. Microtubules (MTs) hold Golgi stacks in juxtanuclear position (Thyberg and Moskalewski 1999). However, the Golgi complex in plants, invertebrates, fungi, yeast (*Saccharomyces cerevisiae*) or mammalian cells such as skeletal, muscle and cardiac cells lacks the interconnections between adjacent stacks, thus appearing as isolated units scattered throughout the cytoplasm (Preuss et al. 1992; Latijnhouwers et al. 2005; Marra et al. 2007; Zaal et al. 2011). The Golgi complex is, besides of stacked cisternae, made up of Golgi proteins, such as several golgins and GTPases of the Arf (ADP-ribosylation factor) and Arl (Arf-like) families, holding the stacks together to retain the overall structure (Short et al. 2005).

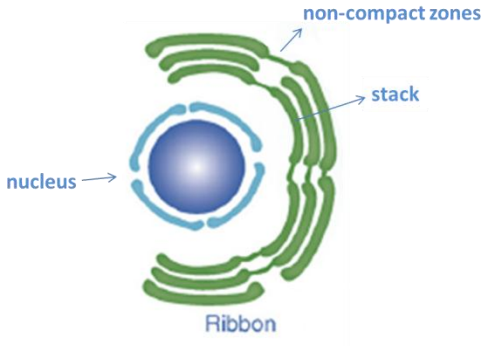


Figure 2- The Golgi ribbon.
The Golgi ribbon is comprised of stacks interconnected via tubular bridges termed as "non-compact zones" and located near the nucleus in interphase cells. The organelle is made up of proteins that form a proteinaceous network required for structure maintenance. Modified from Corda and Colanzi 2007.

- Protein sorting and secretion

The Golgi apparatus plays a crucial role in protein processing and sorting. It is part of the endomembrane system, which processes newly synthesized proteins and navigates them to their final destinations (Vitale and Galili 2001; Figure 3). Polypeptides, generated at rough ER (endoplasmic reticulum) sites, are either destined to stay within the ER, as resident enzymes, or to be further processed in the Golgi compartment. Determination is carried out by coat proteins or receptors, which recognize specific sorting motifs of cargo proteins (Pfeffer 2007). The *cis*-Golgi receives proteins emerging from ER exit sites within COPII (coatomer protein II) – coated vesicles. As proteins move through Golgi stacks they are modified and tagged for delivery to their target membranes. Finally, the cargo is enveloped into newly formed vesicles at the *trans*-side (De Matteis and Luini 2008; Figure 3).

Protein modifications are initiated in the ER and further accomplished within the Golgi complex. Once entered the proximal side of the Golgi, proteins serve as substrates for glycosylation and oligosaccharide-chain processing, since the cisternae of Golgi stacks are heterogenous in composition (Dunphy et al. 1981). The *trans* face of the Golgi apparatus is termed *trans*-Golgi network (TGN). The TGN almost regulates the entire secretory machinery. Cargo is finally modified, packaged into vesicles and released for intra-or extracellular destinations. This anterograde ER-Golgi pathway is called "conventional secretion" (De Matteis and Luini 2008; Nickel and Rabouille 2009).

The anterograde protein transport from the ER to the Golgi in COPII-coated vesicles is indispensable for vesicle budding (Zanetti et al. 2011). However, continuous movement of vesicles from ER exit sites to Golgi membranes requires a mechanism by which proteins and lipids can be recycled. This is accomplished by retrograde transport of cargo within COPI-coated vesicles (Lee et al. 2004). In between ER exit sites and the *cis*-Golgi exists a network of vesicular tubular clusters, known as ER-Golgi intermediate compartment (ERGIC). This network presumably separates cargo-filled COPI and COPII-coated vesicles for retrograde and anterograde transport, respectively (Marra et al. 2001; Appenzeller-Herzog and Hauri 2006; Figure 3). Cargo vesicles exiting the TGN are directed either to the apical or basolateral surface to endosomes or to the ER. Depending on cell type, the TGN also produces secretory granules which serve as storage for enzymes and hormones. These granules release their interior through extracellular stimuli (De Matteis and Luini 2008, Figure 3).

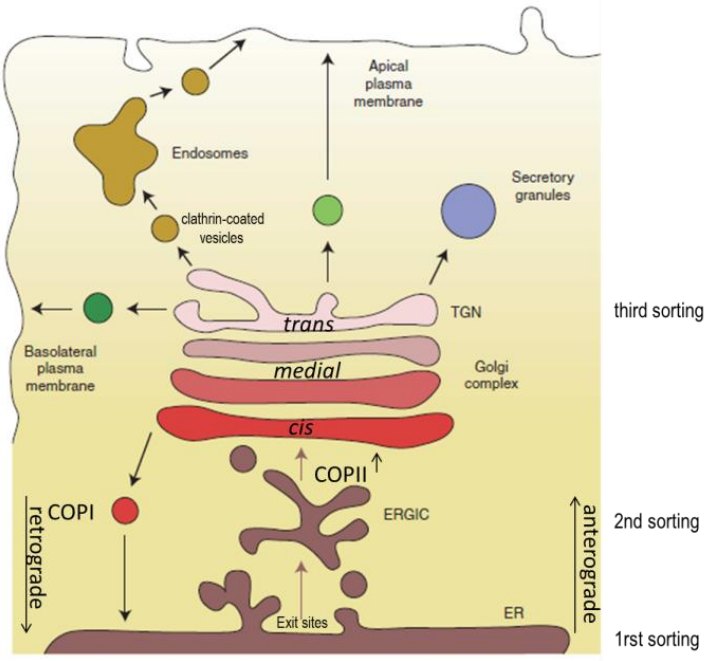


Figure 3- The Golgi complex and its function in conventional secretion.
Mammalian Golgi stacks are typically made up of several layers of membrane structures known as cisternae. The Golgi stack is divided in three compartments: *cis*, *medial* and *trans* Golgi. Each compartment contains different enzymes, which control several modification functions. Conventional secretion is carried out by incoming COPII-coated vesicles emerging from the ER, which fuse with *cis*-Golgi membranes and are further processed as they move through the Golgi stack. Outgoing transport vesicles exit the TGN en route to the plasma membrane (green circles), to endosomes in clathrin-coated vesicles (brown circles), or to the ER in COPI-coated vesicles (red circle). In some cell types (e. g endocrine cells) secretory granules are produced to store the cargo (blue circle). TGN indicates *trans*-Golgi network; ER, endoplasmic reticulum; ERGIC, ER-Golgi intermediate compartment; COP, coatomer protein. Modified from Malhotra and Campelo 2011.

- Golgi integrity

In mammalian interphase cells, the Golgi complex is located near the MTOC and the nucleus (Figure 4). This close proximity is due to the interdependence of these organelles, since MTs are required for the integrity of Golgi membranes and for its intracellular distribution (Veit et al. 1993; Burkhardt 1998). The MTOC is comprised of the centrosome with its centrioles, the outgrowth starting point of MTs. Thus, the centrosome is the major MT nucleation and anchorage center within the cell (Bettencourt-Dias and Glover 2007; Miller et al. 2009).

Disruption of the Golgi ribbon through agents like nocodazole is due to the depolymerization of MTs (Thyberg and Moskalewski 1999). Interestingly, MT-mediated Golgi fragmentation does not switch off secretion generally (Cole et al. 1996).

During mitosis, MTs rearrange to form the mitotic spindle, which is needed for a proper segregation of chromosomes (Thyberg and Moskalewski 1999; Blagden and Glover 2003; Bettencourt-Dias and Glover 2007). Thus, Golgi positioning is disrupted by the rearrangement of MTs en route to form the mitotic spindle (Jesch et al. 2001). Furthermore, there is evidence that the disassembly of Golgi membranes implicates microtubules to segregate Golgi fragments between daughter cells, similarly to chromosome separation by the mitotic spindle (Shima et al. 1998). Thus, a mechanism would be provided for precise and accurate partitioning of Golgi vesicles during cytokinesis.

Additionally, it is believed that MT are involved in membrane trafficking to and from the Golgi (Mizuno and Singer 1994; Lippincott-Schwartz et al. 1995; Zanetti et al. 2011).

Of note, the actin cytoskeleton appears to be required for Golgi maintenance, too. It is proposed that it maintains the continuity of cisternae, since treatment with toxins that depolymerize actin filaments leads to swelling of cisternae. Conversely, agents that stabilize actin filaments promote fragmentation of Golgi stacks (Egea et al. 2006; Lázaro-Diéguez et al. 2006).

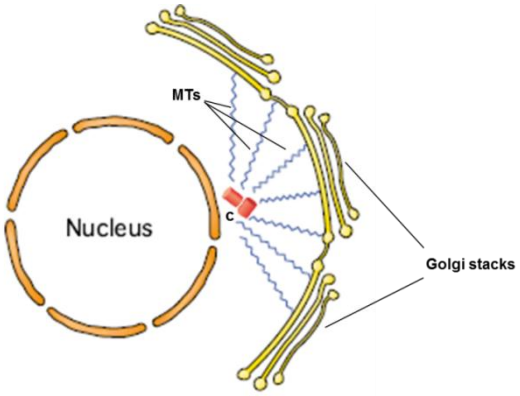


Figure 4- Golgi and microtubules.
Microtubules, emanating out of centrioles, are connected with Golgi stacks. Thus, Golgi localization near the nucleus and Golgi structure maintenance is accomplished. C indicates centrioles; MTs, microtubules. Modified from Colanzi et al., 2003.

- Lipid metabolism

In addition to protein processing and sorting, the Golgi apparatus also plays an important role in sphingolipid biosynthesis and lipid homeostasis.

Membrane biogenesis and vesicle trafficking at the TGN is mainly dependent on phosphatidylinositol 4-phosphate (PI4P) metabolism. PI4P is particularly found at Golgi membranes, and in lower concentrations at the ER and endosomes (Hammond et al. 2009). PI4P is a crucial signaling lipid, regulated by phosphatidylinositol 4-kinases (PI4K) and effectors triggering the vesicle budding machinery (Graham and Burd 2011). PI4 kinases generate PI4P by phosphorylation of PI (phosphatidylinositol) on the D-4 position. The two major PI4 kinases at the Golgi complex are PI4KIIα and PI4KIIIβ (Balla and Balla 2006). PI4KIIIβ is recruited to the TGN through binding to the GTPase ARF1 and gets activated by PKD. In addition, PI4KIIIβ also binds to NCS-1 (neuronal calcium sensor), which in turn is associates with ARF-1 (Zhao et al. 2001). PKD phosphorylation enables PI4KIIIβ to bind to 14-3-3 proteins, which protects PI4KIIIβ from dephosphorylation. Thus, PI4KIIIβ lipid kinase activity is stimulated, which triggers PI4P production at Golgi membranes and cargo transport to the plasma membrane (Godi et al. 1999; Hausser et al. 2005, 2006).

At the TGN, PI4P binds to several effectors, such as GOLPH3 (Golgi phosphoprotein 3), Rab11 (Rab-protein 11), the ceramide transfer protein (CERT), oxysterol-binding protein (OSBP) or four-phosphate-adaptor protein 1 and 2 (FAPP1 and 2), respectively (Levine and Munro 2002; Hanada et al. 2003; Godi et al.

2004; Wood et al. 2009; Graham and Burd 2011). Some of these proteins occupy central positions in the sphingolipid metabolism. Sphingolipids are components of cell membranes and their metabolism generates important second messengers, needed for lipid homeostasis and vesicle trafficking from the TGN (Ohanian and Ohanian 2001). Lipids are transported from the ER to the Golgi either through vesicle trafficking, diffusion along membranes that lie in close apposition, or through active transport by lipid transfer proteins (D'Angelo et al. 2008).

Ceramide, the main precursor of sphingolipids, is synthesized in the ER and subsequently shuttled to the TGN in a nonvesicular fashion through the lipid transfer protein CERT (Kok et al. 1998). CERT is targeted to Golgi membranes via its PH domain and binds to PI4P at the TGN. After binding CERT delivers ceramide, which is then converted into sphingomyelin (SM) and DAG by the *trans* Golgi SM synthase-1 (Hanada et al. 2003; Fugmann et al. 2007; Rao et al. 2007; Hanada et al. 2009).

OSBP is proposed to act as a sterol sensor and is also targeted to the TGN via PI4P. OSBP mediates cholesterol transport from the ER to the TGN, which leads to an accumulation of cholesterol in TGN membranes. Furthermore, it was shown that OSBP is involved in SM synthesis and regulation of CERT activity (Perry and Ridgway 2006; Ngo and Ridgway 2009). Interestingly, inhibition of Golgi localization of OSBP leads to Golgi fragmentation (Nhek et al. 2010). Therefore, OSBP appears to play an important role in Golgi maintenance. These results are in line with observations concerning expression of constitutively active PKD, which is responsible for OSBP dissociation from the TGN, since CERT and OSBP are TGN substrates of PKD (Fugmann et al. 2007; Nhek et al. 2010). PKD negatively regulates CERT and OSBP activities, since PKD-mediated phosphorylation leads to dissociation of both proteins from the TGN, thus reducing SM and DAG levels at Golgi membranes. Dephosphorylation of CERT is executed by the ER-resident protein phosphatase PP2Cϵ, hence CERT is able to shuttle again to the TGN for another round of ceramide delivery (Saito et al. 2008).

PKD is a crucial modulator in this complex metabolism in a negative and a positive fashion. PKD-mediated PI4KIIIβ phosphorylation induces PI4P production at the TGN (Hausser et al. 2006), thus enables CERT and OSBP proteins to

bind to TGN membranes (Hanada et al. 2003; Perry and Ridgway 2006; Balla and Balla 2006). CERT relieves ceramide which is subsequently converted into SM and DAG, whereupon more PKD molecules are recruited to and activated at the TGN (Fugmann et al. 2007). Vice versa, PKD phosphorylates CERT and OSBP negatively, leading to dissociation from the TGN resulting in lower SM, DAG and cholesterol levels (Olayioye and Hausser 2011).

Overall, it is conceivable that through the tight interaction of these lipids and kinases the complex process of vesicle fission at the TGN is accomplished.

Of note, only PKD1 and 2 isoforms appear to exhibit substrate specificities towards PI4KIIIβ and CERT (Hausser et al. 2005; Fugmann et al. 2007).

1.3.2 PKD and secretion

PKD's role at the Golgi complex was first identified through studies with the sponge metabolite ilimaquinone (IQ). Upon IQ treatment, Golgi membranes are completely vesiculated and reassemble to functional Golgi ribbons after IQ removal (Takizawa et al. 1993; Figure 5). Obviously, IQ treatment forces Golgi fragmentation through overactivation of effector proteins responsible for this event. G-protein subunits βγ and PKD turned out to be the effectors inducing Golgi fragmentation after hyperactivation (Jamora et al. 1997, 1999). Specifically subunits β1γ2 and β3γ2 are required for the disruption of Golgi membranes (Díaz Añel and Malhotra 2005).

Besides IQ-mediated fragmentation of Golgi membranes, overexpression of a constitutively active form of PKD also converts the Golgi into small vesicles, whereas PKD inhibition or the expression of a kinase dead mutant abolishes budding of cargo-containing vesicles from the TGN (Liljedahl et al. 2001; Bossard et al. 2007; Figure 5). Of note, inactivation of PKCη leads to accumulation of cargo at the TGN as well, pointing to a PKCη-PKD pathway in terms of vesicle fission (Bard and Malhotra 2006).

In addition, treatment with nocodazole also leads to Golgi fragmentation through depolymerization of MTs (Cole et al. 1996; 1.3.1). Interestingly, this nocodazole-induced disruption appears also to be dependent on local PKD activation (Fuchs et al. 2009). Of note, the mechanisms why Golgi membranes appear to break down are diverse, for instance IQ-mediated Golgi complex breakdown is MT independent, whereas Golgi dispersal after treatment with nocodazole involves MTs (Takizawa et al. 1993; Veit et al. 1993).

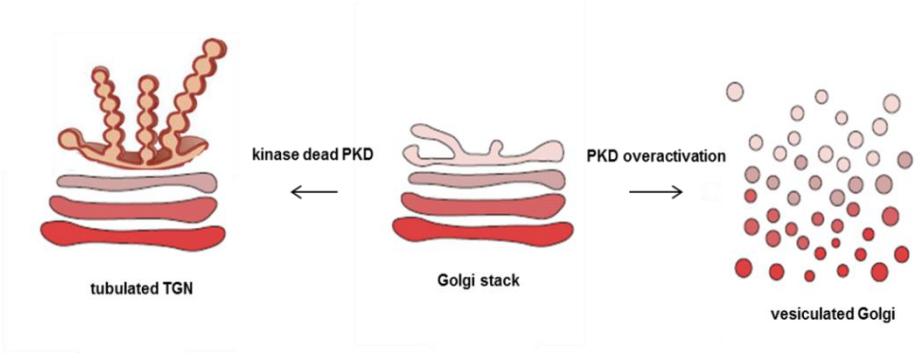


Figure 5- Golgi organization after PKD activation or inhibition.
Golgi membranes completely vesiculate after PKD overactivation, either induced through IQ treatment or through overexpression of a constitutively active form of PKD (right side). Nocodazole treatment leads also to disruption of Golgi membranes involving depolymerization of MTs. Cells transfected with a kinase dead mutant of PKD show a tubulated TGN. Cargo-filled vesicles fail to cut off from the TGN and appear as long tubules (left side). TGN indicates *trans*-Golgi network. Modified from Malhotra and Campelo 2011.

PKD is recruited to the Golgi via the local DAG pool and binds to DAG via its C1a domain (Maeda et al. 2001; Baron and Malhotra 2002; 1.1.1). Recently, Pusapati et al. showed that also the C1b domain is able to bind to the Golgi through interaction with ARF1. Nevertheless, binding to DAG is sufficient to keep PKD at the TGN (Pusapati et al. 2010; Malhotra and Campelo 2011).

PKD activation at the TGN is proposed to be mediated by the tight interaction of Gβγ subunits, DAG and PKCη (Díaz Añel and Malhotra 2005; Irannejad and Wedegaertner 2010). Jamora et al. proposed in 1999 that PKD gets activated through Gβγ subunits by direct binding to the PH domain (Jamora et al. 1999). In 2005, Díaz Añel and Malhotra assumed that Gβγ subunits induce the production of DAG, presumably through action of phospholipases, whereupon PKCη and PKD are recruited and activated at the TGN (Park et al. 1993; Rhee 2001; Díaz Añel and Malhotra 2005; Bard and Malhotra 2006). Finally, Malhotra and Campelo published in 2011 that binding of Gβγ to the PH domain of PKD relieves the negative effect on kinase activity. Independently, PKCη phosphorylates PKD within the activation loop (Malhotra and Campelo 2011; Figure 6). Alleviation of kinase inhibition of Gβγ subunits is probably due to inhibition of MAPK p38δ, since MAPK p38δ is able to phosphorylate PKD within the PH domain thereby leading to PKD inhibition. Thus, insulin secretion from pancreatic β cells is noticeable increased when p38δ is deleted (Sumara et al. 2009; Malhotra and Campelo 2011).

In summary, PKD is recruited to the TGN via DAG. Gβγ subunits bind to PKD within the PH domain this alleviates the negative function of the PH domain towards PKD kinase activity. In addition, inhibitory MAPK p38δ phosphorylation is abolished, and PKCη subsequently activates PKD within the activation loop (Figure 6). The local DAG pool is provided by a PLC-Gβγ pathway through activation of phospholipase D or through conversion of ceramide, respectively (Díaz Añel and Malhotra 2005; Bard and Malhotra 2006; Sonoda et al. 2007; Hanada et al. 2009).

The PKD-mediated fission pathway is specialized for basolateral transport from the TGN, since after inhibition of PKD kinase activity only transport from the TGN to the basolateral cell surface is altered (Liljedahl et al. 2001; Yeaman et al. 2004; Rémillard-Labrosse and Lippé 2009). Of note, all three PKD isoforms are specifically involved in this transport (Yeaman et al. 2004). However, recent data from Marks et al. provide evidence that PKD1 and 2 are also involved in vesicle transport to lysosomes, since expression of a kinase-dead PKD mutant abrogated Mucolipin-1 transport from the TGN to the lysosomes (Marks et al., 2012).

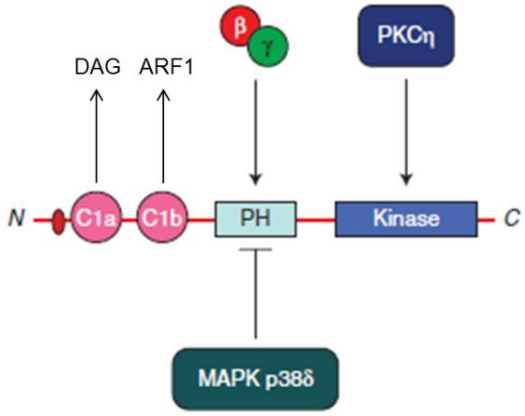


Figure 6- PKD activation at the TGN.
PKD is recruited to the TGN via its C1a domain that binds to DAG. C1b is also proposed to mediate TGN binding via ARF1. PKD activation is carried out by Gβγ binding to the PH domain, which avoids MAPK p38δ phosphorylation of PKD and inhibition of kinase activity is reversed in parallel. Subsequently, PKD is phosphorylated and thereby activated through PKCη. DAG indicates diacyl-glycerol; ARF1, ADP-ribosylation factor; βγ, G-protein beta gamma subunits; N, amino-terminus; C, carboxy-terminus; PH, pleckstrin homology domain; C1a, first cysteine-rich domain; C1b, second cysteine-rich domain; MAPK, mitogen-activated protein kinase p38 delta. Modified from Malhotra and Campelo 2011.

1.3.3 Golgi fragmentation during mitosis

Cell division in mammalian cells needs successful duplication and segregation of cellular contents. This includes organelles and the genome, similarly (Imoto et al. 2011). The inheritance of the Golgi apparatus as major organelle in terms of secretion is of particular importance to sustain cellular function. The heredity of such low copy number organelles is challenging, thus the regulation of Golgi division implicates a series of sequential events leading to an accurate reproduction of Golgi membranes between daughter cells (Shorter and Warren 2002; Colanzi and Corda 2007). As the Golgi complex is built up of interconnected stacks of flattened cisternae, the mechanism of partitioning is accomplished through disruption of the Golgi ribbon into small vesicles to ensure correct partitioning among dividing cells (Lucocq and Warren 1987; Nelson 2000).

The dispersal of Golgi membranes is separated in two sequential steps. First, non-compact zones between Golgi stacks have to be cleaved in G2 phase, thus generating tubulo-reticular elements so called “Golgi blobs” (Nelson 2000; Colanzi et al. 2003). These “blobs” are separated from each other; however, they are still localized to the perinuclear area (Colanzi and Corda 2007; Figure 7). During meta- and anaphase, coincident with nuclear envelope breakdown, Golgi blobs undergo further disassembly into small vesicles that become dispersed throughout the cytoplasm appearing as the so-called “Golgi haze”. Once entered telophase, fragments fuse with each other and reassemble to new Golgi ribbons within maturing cells (Colanzi et al. 2003; Axelsson and Warren 2004; Figure 7).

This Golgi fragmentation process turned out to be ER-independent and thus equal amounts of Golgi material are separated between dividing cells directed by the mitotic spindle (Shima et al. 1998; Jesch et al. 2001; Jokitalo 2001; Axelsson and Warren 2004; Pecot and Malhotra 2004; 2006). However, the mechanism of Golgi inheritance is controversial discussed, since other publications provide evidence that Golgi division is dependent on ER activities (Zaal et al. 1999; Prescott et al. 2001; Altan-Bonnet et al. 2006).

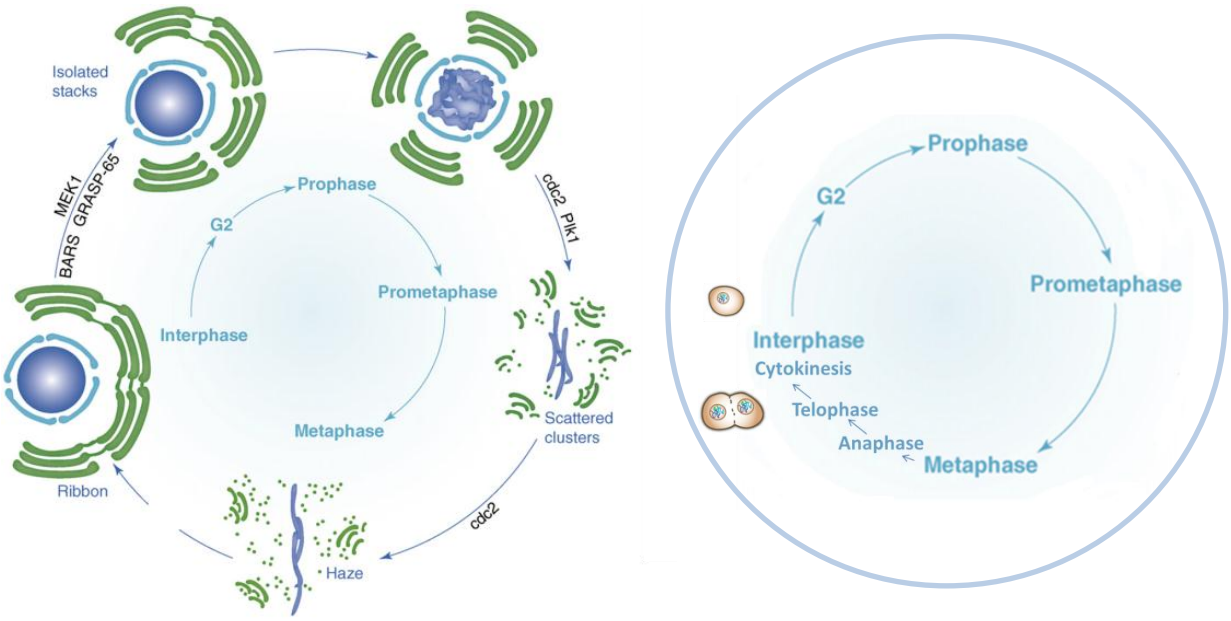


Figure 7- Golgi complex inheritance during cell division.
The Golgi complex is located in the perinuclear region within the cell. During G2, connections between adjacent stacks are cleaved and thus separated. During subsequent mitotic phases, "Golgi blobs" become further fragmented into small vesicles appearing as Golgi haze throughout the cytoplasm. At the end of mitosis, vesicles fuse with each other to equally split into the nascent daughter cells (left image). Different mitotic steps (right image). Modified from Corda and Colanzi 2007.

- The Golgi mitotic checkpoint

The two-step fragmentation process is required for proper segregation of the Golgi complex during mitosis. Most notably, the cleavage step in G2 appears to be indispensable for mitotic transition at all, thus defining the "Golgi mitotic checkpoint" (Sütterlin et al. 2002; Hidalgo Carcedo et al. 2004). A lot of work was done concerning this topic and several proteins were identified being involved in ribbon severing during G2 (Colanzi and Corda 2007; Rabouille and Kondylis 2007; Wei and Seemann 2009).

Major key players required for the first fragmentation step are GRASP-65 (Golgi reassembly-stacking protein of 65kD), GM130 (Golgi matrix protein 130) and CtBP/BARS (C-terminal binding protein/brefeldin A adenosine diphosphate–ribosylated substrate) (Sütterlin et al. 2002, 2005; Puthenveedu et al. 2006; Colanzi et al. 2007).

CtBP/BARS (BARS) promotes Golgi disruption, since its depletion reduced Golgi fragmentation up to 80 % in normal rat kidney cells, whereas re-

expression of recombinant BARS rescued fission activity in the same cells (Hidalgo Carcedo et al. 2004).

Colanzi et al. investigated BARS' contribution in G2/M transition in more detail and it has become clear that BARS severs non-compact zones during G2, a process indispensable for subsequent Golgi complex breakdown and thus for entry mitosis (Sütterlin et al. 2002; Colanzi et al. 2007). Of note, the second step of Golgi fragmentation turned out to be independent of BARS (Colanzi et al. 2007), which clearly demonstrates that interfering with BARS activity causes a cell cycle arrest in G2 owing to cleavage inhibition of non-compact zones. In cells possessing already separated Golgi stacks, G2/M transition is not affected upon BARS inhibition (Colanzi et al. 2007).

GRASP-65 is known to mediate Golgi post-mitotic reassembly through linking adjacent stacks via trans-oligomerization (Wang et al. 2005; Xiang and Wang 2010). Oligomerization is disrupted by mitotic phosphorylation, thus the oligomerization process is negatively regulated by phosphorylation (Wang et al. 2003). GRASP-65 is hyperphosphorylated during mitosis by mitotic kinases such as Cdc2 (cell division cycle 2) and polo-like kinase1 (Plk 1), a process which is associated with Golgi unstacking (Lin et al. 2000; Sütterlin et al. 2001; Wang et al. 2003; Preisinger et al. 2005; Yoshimura et al. 2005).

Moreover, GM130 complexes with GRASP-65 and vesicle tethering factor p115 for lateral linking of Golgi stacks and for facilitating vesicle fusion at the *cis*-Golgi, respectively (Barr et al. 1997; Marra et al. 2001; Puthenveedu et al. 2006; Nakamura 2010). During mitosis, GM130 gets also phosphorylated by Cdc2 kinase, which prevents its binding to p115 and thus vesicle fusion at the *cis*-Golgi. This event is related with fission of Golgi membranes (Lowe et al. 1998, 2000; Preisinger et al. 2005). However, it is proposed that GM130 untethering interferes with the second step of Golgi membrane dispersal, mediated by Cdc2 kinase (Lowe et al. 2000; Sundaramoorthy et al. 2010; Figure 7). This is also in line with the finding that Cdc2 kinase activity is not required for dispersal of pericentriolar Golgi stacks (Acharya et al. 1998; Colanzi et al. 2000; Figure 7).

Overall, depletion of GRASP-65 or GM130 results in disassembly of Golgi stacks, but also to aberrant spindle formation, suggesting a potential role for these proteins in cell division, but also, particularly in Golgi maintenance (Sütterlin et al. 2005; Puthenveedu et al. 2006; Feinstein and Linstedt 2008;

Xiang and Wang 2010). In addition, it has been reported that an additional GRASP protein, GRASP-55, acts similarly to GRASP-65 in terms of Golgi dis- and reassembly (Xiang and Wang 2010).

Another crucial molecular component in terms of Golgi partitioning in G2 is mitogen-activated protein kinase kinase 1 (MEK1) (Wright et al. 1999; Colanzi et al. 2000; Colanzi et al. 2003). MEK1 gets phosphorylated at the onset of mitosis, which leads to conformational changes within the protein structure, thus enables MEK1 to bind Golgi membranes (Colanzi et al. 2000). In agreement, mitotically activated MEK1 is proposed to be different to interphase MEK1 (Colanzi et al. 2000; Shaul et al. 2009).

The only known downstream substrates of MEK1 so far, ERK1 and 2 (extracellular signal-regulated kinase 1 and 2), are not required for the process of mitotic Golgi dispersal (Acharya et al. 1998). Thus, it is conceivable that mitotically modified MEK1 changes its substrate specificity (Colanzi et al. 2000). Hence, other MEK1 substrates must be activated to exert fission of non-compact zones. Shaul and Seger provide evidence that an alternative splice form of ERK, ERK1c drives Golgi fragmentation during mitosis through a MEK pathway (Aebersold et al. 2004; Shaul and Seger 2006). Upon phosphorylation, ERK1c localizes to the Golgi complex at late G2 phase, and inhibition or depletion attenuates Golgi fragmentation (Shaul and Seger 2007). Interestingly, more recent work of Shaul et al. revealed that an alternative splice form of MEK1, termed MEK1b, phosphorylates ERK1c during mitosis. These data suggest that a MEK1b/ERK1c pathway regulates Golgi ribbon cleavage at the G2/M boundary (Shaul et al. 2009).

In summary, activated MEK1 converts the Golgi ribbon in tubulo-reticular elements (“Golgi blobs”) and in turn, MEK1 inhibition abolishes Golgi fragmentation, thus entry in mitosis is delayed (Colanzi et al. 2000). Therefore, specific MEK1 phosphorylation at the onset of mitosis is needed for cleaving non-compact zones, thus to overcome the Golgi checkpoint (Colanzi et al. 2000; Colanzi et al. 2003; Feinstein and Linstedt 2007). Furthermore, this initial cleavage process is accomplished independently of the mitosis initiation kinase Cdc2 (Colanzi et al. 2000). Possibly, MEK1 unlinking of non-compact zones may alleviate Cdc2 activation needed for further disassembly of Golgi blobs, since MEK1 is required for the first, and Cdc2 kinase for the second fragmentation

step (Kano et al. 2000; Shorter and Warren 2002; Feinstein and Linstedt 2007; Figure 7). In addition, GRASP-65 depletion prior to mitosis abrogated MEK1 requirement for mitotic entry, suggesting a pivotal role for MEK1 unlinking Golgi stacks in G2 (Feinstein and Linstedt 2007).

Mitotic activation of MEK1 is through Raf-1 and not MEK1 kinase1, since inhibiting of Raf-1 avoids mitotic entry (Colanzi et al. 2003). Nevertheless, it is not clear, which kinase acts upstream of Raf-1 in mitosis, because it turned out to be independently from the initial MAPK pathway kinase Ras (Ziogas et al. 1998; Laird et al. 1999).

However, downstream signaling of MEK1 in mitosis and the implication of MEK1 in mitotic entry at all is discussed controversial, since other groups proposed that MEK1 activation of ERK2 induces GRASP-55 phosphorylation and thus Golgi unstacking, providing a role for known MEK1 downstream targets in the severing event (Jesch et al. 2001; Feinstein and Linstedt 2008). Further work from Lowe et al. promotes the finding that MEK1 activity is not required for mitotic ingression, supporting Cdc2 and GM130 being the most prominent candidates (Lowe et al. 1998; Draviam et al. 2001). In addition, it exists evidence that MEK1 activates Plk3 and that appears to be important in Golgi fragmentation as well (Xie et al. 2004). It is known that Plk3 interacts with MEK1 and ERK2 and it also affects microtubule structure (Ruan et al. 2004).

Obviously, more than one fission mechanism might be involved in mitotic Golgi fragmentation. Thus, it is conceivable that several mitotic kinases and components act consecutively at different levels to accomplish the complex process of Golgi mitotic inheritance.

1.4 Goals

The role of PKD at the Golgi complex in terms of secretion is well-established. Hyperactivation of PKD, through overexpression of a constitutive active mutant or drug treatment such as ilimaquinone, induces breakdown of Golgi membranes. Prior to entering mitosis, the stacks of the Golgi cisternae are separated from each other and inhibiting this process delays entry of mammalian cells into mitosis.

The aim of this work was to unravel whether PKD interferes with Golgi complex dispersal during mitosis, most notably, as initiating kinase during the first fragmentation step in G2.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

Chemicals	Company
Acrylamide, Rotiphorese Gel 30	Carl Roth GmbH & Co, Karlsruhe
Ammonium persulfate (APS)	Carl Roth GmbH & Co, Karlsruhe
Adenosine-5‘-triphosphate (ATP)	Sigma-Aldrich, Deisenhofen
β-Glycerophosphate	Sigma-Aldrich, Deisenhofen
β-Mercaptoethanol	Sigma-Aldrich, Deisenhofen
Blocking reagent	Roche Diagnostics, Mannheim
Bovine Serum Albumin (BSA)	Sigma-Aldrich, Deisenhofen
Bradford assay	Carl Roth GmbH & Co, Karlsruhe
Bromphenol blue	Serva, Heidelberg
Complete Protease Inhibitor Cocktail (EDTA free)	Roche Diagnostics, Mannheim
Creatine kinase	Roche Diagnostics, Mannheim
Creatine phosphate	Roche Diagnostics, Mannheim
Digitonin	Sigma-Aldrich, Deisenhofen
Dimethyl sulfoxide (DMSO)	Carl Roth GmbH & Co, Karlsruhe
Dithiothreitol (DTT)	Carl Roth GmbH & Co, Karlsruhe
DRAQ5™	Biostatus Limited, Leicestershire, United Kingdom
Ethylene glycol tetraacetic acid (EGTA)	Carl Roth GmbH & Co, Karlsruhe
Ethylene diamine tetraacetic acid (EDTA)	Carl Roth GmbH & Co, Karlsruhe
Fluoromount-G	Southern Biotech, Birmingham, USA
Glycerol	Carl Roth GmbH & Co, Karlsruhe
Glycine	Carl Roth GmbH & Co, Karlsruhe
GST-MEK1 (inactive)	Millipore, Dundee, Scotland
HEPES (4-(2-hydroxyethyl)-1- piperazineethanesulfonic acid)	Carl Roth GmbH & Co, Karlsruhe
Hoechst 33258 (bisbenzimide)	Sigma-Aldrich, Deisenhofen
Hydrochloric acid (HCl)	Carl Roth GmbH & Co, Karlsruhe
Magnesium acetate	Sigma-Aldrich, Deisenhofen
Magnesium chloride ($MgCl_2$)	Sigma-Aldrich, Deisenhofen
Methanol (MeOH)	Carl Roth GmbH & Co, Karlsruhe
Mowiol® 4-88 (immunofluorescence mounting media)	Polysciences Europe GmbH, Eppelheim
N,N,N,N-Tetramethylethyldiamine (TEMED)	Carl Roth GmbH & Co, Karlsruhe
Paraformaldehyde (PFA)	Carl Roth GmbH & Co, Karlsruhe
Phosphatase Inhibitor Cocktail	Roche Diagnostics, Mannheim
PIPES (piperazine-N,N’-bis(2-ethanesulfonic acid))	Sigma-Aldrich, Deisenhofen
Potassium acetate	Sigma-Aldrich, Deisenhofen

Potassium chloride (KCl)	Sigma-Aldrich, Deisenhofen
Potassium hydroxide (KOH)	Sigma-Aldrich, Deisenhofen
Prestained Protein Ladder	MBI Fermentas, St. Leon-Rot
Propidium iodide (PI)	Invitrogen, Karlsruhe
Protein G Agarose beads	KPL, Gaithersburg, USA
RNase A stock solution (20 mg/ml)	Invitrogen, Karlsruhe
Sodium chloride (NaCl)	Carl Roth GmbH & Co, Karlsruhe
Sodium dodecyl sulfate (SDS)	Carl Roth GmbH & Co, Karlsruhe
Sodium orthovanadate (Na_3VO_4)	Sigma-Aldrich, Deisenhofen
Spermidine	Sigma-Aldrich, Deisenhofen
Spermine	Sigma-Aldrich, Deisenhofen
Thimerosal	Carl Roth GmbH & Co, Karlsruhe
Thymidine	Sigma-Aldrich, Deisenhofen
Tris-hydroxymethyl-aminomethane (Tris)	Carl Roth GmbH & Co, Karlsruhe
Trypan blue	Sigma-Aldrich, Deisenhofen
Triton X-100	Carl Roth GmbH & Co, Karlsruhe
Tween 20	Carl Roth GmbH & Co, Karlsruhe
Uridine-5'-triphosphate(UTP)	GE Healthcare, München

2.1.2 short interfering RNAs

Short interfering RNAs (siRNA) were obtained from Eurofins MWG Operon, Ebersberg, Germany.

siRNA	**Sequence 5'-3'**
siLacZ	5'-GCGGCUGCCGGAAUUUACC-3'
siPKD1 #1	5'-GUCGAGAGAAGAGGUCAAA-3'
siPKD1 #2	5'-GGAAGAGAUGUAGCUAUUAA-3'
siPKD2 #1	5'-GCAAAGACUGCAAGUUUAATT-3'
siPKD2 #2	5'-GGACAUCAAUGACCAGAUC-3'

2.1.3 smartpool siRNAs

Smartpools were purchased from Thermo Scientific, Karlsruhe, Germany.

smartpool siRNA	**Target sequences in mRNA**
ON-TARGETplus SMARTpool control	unknown
ON-TARGETplus SMARTpool PRKD1 (Gene ID: 5587)	GAAGAGAUGUAGCUAUUAA
	GAAAGAGUGUUUGUUGUUA
	GAAUGCAGCUUUCAUGUAU
	UCGAAAUCACUACGGCAAA

ON-TARGETplus SMARTpool PRKD2 (Gene ID: 25865)	CAAUGGAGAUGUGCCGAUG
	GGAAGAUGGGAGAGCGAUA
	CGACCAACAGAUACUAUAA
	GCUGAAGAGCUGAGCCGUA

2.1.4 Human cell lines

Cell line	Source
HeLa S3 (CCL-2.2)	ATCC-LGC, Wesel
HeLa Man II-GFP	V. Malhotra, Barcelona, Spain

2.1.5 Cell culture reagents

Cell culture reagent	Company
CID 755673 (PKD inhibitor)	Tocris Bioscience, Bristol, UK
Collagen R solution	Serva, Heidelberg
DMEM	Invitrogen, Karlsruhe
FBS (HyClone)	Thermo Scientific, Karlsruhe
Fibronectin-like Engineered Protein Polymer	Sigma-Aldrich, Deisenhofen
Oligofectamine	Invitrogen, Karlsruhe
PD 98059 (MEK inhibitor)	Cell Signaling, Frankfurt (Main)
RPMI 1640	Invitrogen, Karlsruhe
*Trans*IT-HeLaMONSTER®	Mirus Bio, Madison, USA
Trypsin-EDTA (10 x stock)	Invitrogen, Karlsruhe

2.1.6 Primary antibodies used for Western Blot analysis

Antibody	Species	Dilution	Company
anti- B-Raf (F-7): sc-5284	mouse	1:200	Santa Cruz Biotechnology, Santa Cruz, USA
anti-Cdk1/Cdc2 (pY15)	mouse	1:250	BD, Heidelberg
anti-Cdk1 (p34)	rabbit	1:1000	BD, Heidelberg
anti-pMEK1/2 (S221)	rabbit	1:1000	Cell Signaling, Frankfurt (Main)
anti-phospho-Histone H3 (pSer10)	rabbit	1:1000	Sigma-Aldrich, Deisenhofen
anti-PKD1 (C-20)	rabbit	1:2000	Santa Cruz Biotechnology, Santa Cruz, USA
anti-PKD2	rabbit	1:2000	Calbiochem, S. Francisco, USA
anti-PKD3	rabbit	1:2000	V. Malhotra, Barcelona, Spain

anti-Raf-1 (C-12): sc-133	rabbit	1:1000	Santa Cruz Biotechnology, Santa Cruz, USA
anti-tubulin-α Ab-2 (Clone DM1A)	mouse	1:2000	Thermo Scientific, Karlsruhe

2.1.7 Primary antibodies used for indirect immunofluorescence

Antibody	Species	Dilution	Company
anti-p230	mouse	1:300	BD,Heidelberg
anti-GM130	mouse	1:200	BD, Heidelberg
anti-phospho-Histone H3 (pSer10)	rabbit	1:1000	Sigma-Aldrich, Deisenhofen

2.1.8 Secondary antibodies

Antibody	Species	Dilution	Company
Alexa Fluor® 546 anti-rabbit	goat	1:500 (IF)	Invitrogen, Karlsruhe
Alexa Fluor® 633 anti-mouse	goat	1:500 (IF)	Invitrogen, Karlsruhe
Alexa Fluor® 488 anti-mouse	goat	1:500 (IF)	Invitrogen, Karlsruhe
POD-anti-mouse IgG (heavy & light chain)	goat	1:10000	Dianova, Hamburg
POD-anti-rabbit IgG (heavy & light chain)	goat	1:10000	Dianova, Hamburg

2.1.9 Plasmids

Plasmid	Source
caMEKK1-mCherry	Addgene (Plasmid 31880, Cambridge, USA) (Covassin et al. 2009)
peGFPc3-GFP-PKD1 mouse	kindly provided by Sharon Matthews (University of Dundee, United Kingdom)

2.1.10 Buffers and solutions

10% APS solution	10% (w/v) APS in H_2O
ATP regenerating system	100 mM ATP 100 mM UTP 200 mM creatine phosphate 2.76 mg/ml creatine kinase in KHM buffer
Blocking solution (IF)	5 % (v/v) FBS in PBS
Blocking solution (WB)	0.5 % (v/v) blocking reagent 0.05 % (v/v) Tween 20 0.01 % (v/v) thimerosal in PBS
Blotting buffer	25 mM Tris-HCl, pH 8.3 192 mM glycine 20% (v/v) methanol
Digitonin solution	digitonin (in DMSO) 30 µg/ml in KHM
ECL homemade	solution A: 250 µg/ml in 0.1M Tris-HCl, pH 8.6 solution B: 1.1 mg/ml p-coumaric acid in DMSO working solution: solution A + 1:10 solution B + 30% (v/v) H_2O_2
KAB	20 mM MOPS, pH 7.2 5mM EGTA 25 mM β- glycerophosphate 1mM Na_3VO_4 1mM DTT
Kinase assay mix	per reaction: 1 µg GST-MEK1, 10 µl Mg^{2+}/ATP solution (75mM $MgCl_2$; 20 mM ATP dissolved in KAB), KAB added to a final volume of 40 µl

KHM	25mM HEPES, pH 7.2 125 mM potassium acetate 2.5 mM magnesium acetate
KHM-KCl	1 M KCl in KHM
Lysis buffer	1 % (v/v) Triton X-100 20 mM Tris, pH 7.5 150 mM NaCl 1 mM EDTA 1 mM EGTA 10 mM NaF 20 mM β-glycerophosphate 1 mM sodium orthovanadate plus complete protease inhibitor cocktail (EDTA-free, 1:25)
MEB	15 mM PIPES, pH 7.2 50 mM KCl 10 mM $MgCl_2$ 20 mM ß-Mercapto-EtOH 20 mM β-glycerophosphate 15 mM EGTA 0.5 mM spermidine 0.2 mM spermine 1 mM DTT plus complete protease inhibitor cocktail (EDTA-free, 1:25) in H_2O
PBS	140 mM NaCl 2.7 mM KCl 8 mM Na_2HPO4 1.5 mM KH_2PO_4
PBS-Tween	0.05 % (v/v) Tween 20 in PBS
PFA	4 % (w/v) in PBS
PI staining solution	PI (50 µg/µl) RNase A (20pg/µl) in PBS
Ponceau S	0,1% (w/v) Ponceau S 5% (v/v) acetic acid

Protein sample buffer (5x stock)	312,5 mM Tris-HCl, pH 6.8 25% (v/v) β-mercaptoethanol 25% (v/v) glycerol 10% (v/v) SDS 0,05% (w/v) bromphenole blue
RIPA lysis buffer	50 mM HEPES, pH 7.4 1 % (v/v) Triton X-100 0.5 % (v/v) NaDOC 0.1 % (v/v) SDS 50 mM NaF 5mM EDTA plus complete protease inhibitor cocktail (EDTA-free, 1:25)
SDS running buffer	25mM Tris pH 8.8 192mM glycine 0.1 % SDS
Separating gel solution	10% (v/v) acrylamide 380 mM Tris, pH 8.8 0.1 % SDS 0.1 % APS 0.06 % TEMED in H_2O
Stacking gel solution	4 % acrylamide 0.1 % SDS 125 mM Tris, pH 6.8 in H_2O
Stripping solution	2 % (v/v) SDS 6.25 mM Tris, pH 6.8 in H_2O add 0.7 % β-mercaptoethanol prior to use

2.1.11 Consumables

Consumables	Company
0.2 µm filter	Sarstedt, Nümbrecht
24-gauge needle Sterican	Braun, Wertheim
blotting paper, type Whatman	A. Hartenstein, Würzburg
cell culture dishes	Greiner, Frickenhausen
cell culture flasks	Greiner, Frickenhausen
cell culture plates	Greiner, Frickenhausen
Cryo vials 1ml	Greiner, Frickenhausen
falcon tubes	Greiner, Frickenhausen
glass coverslips 18 mm x 18 mm	Carl Roth GmbH & Co, Karlsruhe
Nitrocellulose blotting membrane	Pall Corporation, Pensacola, USA
pipette tips	Greiner, Frickenhausen
reaction tubes	Eppendorf, Hamburg
serological pipettes	costar®, Corning Incorporated, New York, USA
replica dishes	Sterilin Limited, Newport, UK
syringe	BRAUN, Wertheim
universal fit filter tips	Corning Incorporated, New York, USA
X-ray films	CEA, Strangnas, Sweden

2.1.12 Equipment

Equipment	Company
Cell culture incubator	Binder, Tuttlingen
CK2 (standard light microscope)	Olympus, Hamburg
Curix 60 processor	Agfa, Düsseldorf
Cytomics FC 500 (FACS)	Beckman Coulter, Krefeld
Eppendorf centrifuge 5415 R	Eppendorf, Hamburg
Laminar flow	NuAire, Plymouth, UK
LSM 710 (confocal laser scanning microscope)	Zeiss, Jena
NanoDrop® ND-1000 (Spectrophotometer)	peQLab, Erlangen
Neubauer counting chamber	Multimed, Kirchheim
pipettes (1 - 20 µl / 20 - 200 µl / 200 - 1000 µl)	Eppendorf, Hamburg
Rotanta 460R (low speed centrifuge)	Multimed (Hettich), Kirchheim
Sonopuls HD 200 (sonyfier)	Bandelin, Berlin
Vortex Genie 2	Scientific Industries, Bohemia, USA

2.2 Methods

2.2.1 Cell culture

HeLa cells were obtained from ATCC-LGC and HeLa cells stably transfected with Mannosidase II fused to GFP were obtained from Prof. Dr. Vivek Malhotra (Centre of Genomic Regulation, Barcelona, Spain). Cells were maintained in RPMI 1640 or DMEM supplemented with 10 % fetal calf serum under sterile conditions at 37°C with 5 % CO_2 humidified atmosphere. Confluent cultures were passaged using trypsin/EDTA. Long-time storage of $1x10^6$ cells in liquid nitrogen was performed using the respective culture medium containing 10 % DMSO. Cells were used until they reached passage 40.

2.2.2 siRNA transfection using Oligofectamine

Transient single or double knockdowns of PKD were performed using small interfering RNAs. A siRNA targeting the bacterial gene LacZ (siLacZ) served as non-targeted control. Transfection was carried out using Oligofectamine™ reagent according to the manufacturer's protocol. For transfection in 100 x 20mm dishes, the growth medium was replaced with serum-free medium and cells were transfected at 40 % confluency. 32 µl Oligofectamine were diluted in 88 µl OptiMEM® and incubated for 5 min at RT. 80 µl (for co-transfection 40 µl, respectively) of each siRNA (20 µM stock conc.) were diluted in 1400 µl OptiMEM® and combined with the diluted Oligofectamine for 20 min, to allow the mixture to form complexes. After 20 min, the transfection solution was added to the cells and incubated at 37°C with 5 % CO_2. 4 hrs post transfection 2 ml growth medium supplemented with 30 % FBS was added, yielding a final concentration of 10 % FBS.

SiRNA experiments performed in culture vessels with different surface areas were scaled up or down according to the surface area.

To ensure specificity of the siRNAs, smartpool siRNAs (see materials) were used to exclude off-target effects. Smartpools are mixtures of siRNAs directed against different sequences of the same target mRNA.

2.2.3 Plasmid transfection with *Trans*IT-HeLaMONSTER®

Transfections of HeLa cells with expression plasmids were performed using *Trans*IT-HeLaMONSTER®. Cells should be 60 % confluent prior to transfection.

The following protocol corresponds to the manufacturer's alternate transient DNA transfection protocol for 100 x 20 mm dishes. To prepare *Trans*IT-HeLa-DNA complexes, 22.5 µl *Trans*IT-HeLa Reagent were diluted in 375 µl serum-free medium and incubated for 10 min. 7.5 µg plasmid -DNA were added and the mixture was again incubated for 10 min. During incubation 15 µl MONSTER Reagent were diluted in 135 µl H_2O. First, *Trans*IT-DNA complexes were added to the cells followed by the diluted MONSTER Reagent. Cells were incubated at least 24 hrs at 37°C with 5 % CO_2.

2.2.4 Cell cycle synchronization using a double thymidine block

To analyze cell-cycle-specific proteins, cells should reside in the same phase of the cell cycle. To this end, HeLa cells were treated twice with thymidine. The protocol is designed to synchronize HeLa cells at the G1/S border by inhibiting the DNA synthesis machinery. First of all, cells were seeded into appropriate culture dishes and on coverslips, respectively. The following day, the growth medium was replaced by growth medium containing thymidine in a final concentration of 2 mM, and cells were incubated for 19 hrs. Thereafter, cells where released from the first block by 3x washing with serum free medium and were re-fed with growth medium for 9 hrs. Subsequently, cells were subjected to the second thymidine block for another 16 hrs. Incubation steps were performed at 37°C with 5 % CO_2. After the second release, cells were harvested at distinct time points for Western Blot and FACS analyses, respectively.

2.2.5 Cell cycle synchronization of knockdown cells

In order to investigate the function of a target protein during specific cell cycle phases, HeLa cells were seeded into 100 x 20mm dishes and transfected with siRNA the next day (see 2.2.2). 4 hrs post transfection, cells were synchronized using the double thymidine block (see 2.2.4). After the second release, dishes with control and knockdown cells were trypsinized at various time points. Cells of each dish were used for Western Blot and FACS analyses, respectively. For immunoblotting, cells were lysed with hot lysis procedure (see 2.2.17).

2.2.6 PKD inhibitor treatment of synchronized cells

To confirm results obtained from knockdown experiments, we treated synchronized HeLa cells with different reagents, which inhibit PKD function. CID 755673, a specific PKD inhibitor, and Gö6976 known as PKC and PKD inhibitor

were added in single approaches. Treatment was started simultaneously with the second release from thymidine (see 2.2.4), and cells were further processed for Western Blot analysis (see 2.2.19).

2.2.7 Rescue experiment

HeLa cells grown on 100 x 20 mm culture dishes were transfected with siRNAs (see 2.2.2) and arrested at the G1/S border by thymidine treatment (see 2.2.4). During the first release from thymidine, one half of the cells were additional transfected with plasmid-DNA using *Trans*IT-HeLaMONSTER® (see 2.2.3). Cells were fixed to distinct time points post release and stained with PI for flow cytometry analysis (see 2.2.8), or subjected to immunofluorescence (see 2.2.14).

2.2.8 PI staining for flow cytometry analysis

The following protocol was used to determine the DNA content of ~ $1x10^6$ synchronized HeLa cells by flow cytometry. First, trypsinized cells were pelleted and washed in 1 ml 1x cold PBS and resuspended in additional 400 µl. EtOH (-20°C) was added dropwise while vortexing and cells were incubated O/N at 4°C. The next day cells were centrifuged (1500 rpm, RT) to remove the EtOH, washed in 3 ml 1x cold PBS, resuspended in 500 µl of PI solution (see materials) and incubated at 37°C with 5 % CO_2 for 30 min in the dark. Subsequently, samples were analyzed using flow cytometry. Cell cycle distribution was measured by fluorescence intensity in the FL2 channel of the cytometer since PI intercalates between the bases of the DNA. Therefore the measured intensity is proportional to the DNA content of the cells. FACS profiles display the cell cycle in three parts: G1, S-phase and G2/M. Cells in G1 phase display half of the fluorescence intensity than G2/M cells, since cells in G2/M have a duplicated chromosome set.

2.2.9 Mitotic index determination

HeLa cells were seeded into six well plates and transfected with siRNA the next day (see 2.2.2). 24 hrs post transfection cells were re-plated onto collagen-coated coverslips (see 2.2.13) and fixed for IF the next day (see 2.2.14). Using the mitosis marker pH3 the distribution of the different mitotic stages of each sample was determined. The quantification was carried out with Image J.

2.2.10 Preparation of mitotic and interphase extracts

HeLa cells grown on 145 x 20mm plates to ~ 70 % confluency were treated with thymidine (2mM) for 10-12 hrs thereby causing the arrest of cells in S phase. Subsequently, cells were washed 3x with PBS and incubated with nocodazole (500 ng/ml) O/N at 37°C with 5 % CO_2 to arrest them in mitosis. Cells in mitosis are rounded up and thereby become loosely attached to the culture vessel. Therefore, cells were detached from the dishes by a “mitotic shake off” procedure, leaving the non-mitotic cells still attached. Two washing steps with ice cold PBS and one with MEB (see materials) were performed. Cells were then pelleted and resuspended in 2x volume of MEB. After swelling on ice for 10 min, cells were homogenized using a 24 gauge needle. To yield the supernatant termed “mitotic extract”, cells were centrifuged in a table top ultracentrifuge using a TLS55 rotor for 45 min at 48000 rpm. The supernatant was divided in 100 µl aliquots, immediately frozen in liquid nitrogen and stored at -80°C.

For preparation of interphase extracts, confluent HeLa cells grown on 145 x 20mm plates were washed and harvested with a cell scraper. Subsequently procedures were similar to those described above for mitotic extract preparation. Both extracts contained protein concentrations in the range of 5-10 mg/ml.

2.2.11 Semi-intact assay

HeLa cells, stably transfected with Mannosidase II-GFP (ManII-GFP), were grown on fibronectin-coated coverslips (see 2.2.13) to ~ 90 % confluency. To accumulate the cells in S Phase of the cell cycle, cells were treated with 2 mM thymidine at 37°C with 5 % CO_2 for 8 hrs. The cells were washed with KHM buffer at RT, shifted to ice and washed again with cold KHM buffer. For permeabilization, cells were treated with 30 mg/ml digitonin in KHM buffer for 90 seconds at RT. Digitonin is a cholesterol-specific reagent that renders the plasma membrane porous owing to the high amounts of cholesterol found in the plasma membrane, while maintaining the overall integrity of the cells. After digitonin treatment, semi-intact cells were washed with 1 M KCl-KHM buffer to remove cytosolic proteins followed by a final washing step using KHM. Coverslips with semi-intact cells were inverted on a 50 µl drop of a reaction mixture on a sheet of parafilm and incubated in a 32°C water bath for 1 hr. The reaction mixture

contained mitotic or interphase extract together with an ATP regenerating system (see materials).
After incubation, cells were fixed with 4 % PFA and sealed to the slide with Fluoromount-G. Golgi state was monitored using a confocal laser scanning microscope.

2.2.12 Golgi FRAP

To investigate membrane continuity, HeLa-Man II-GFP cells were seeded onto glass-bottom dishes and transiently transfected with siRNAs (see 2.2.2) the next day. 48 hrs post transfection, half of the cells were incubated with 40 µg/ml bisbenzimide at 37°C with 5 % CO_2 for 18 hrs whereas the other half has been left untreated. Bisbenzimide is a topoisomerase-I inhibitor, which induces the accumulation of cells in G2 due to activation of the DNA damage checkpoint. Subsequently, the diffusion mobility of the Man II-GFP chimera in living cells was imaged at RT using a confocal laser scanning microscope. An initial pre-bleach image was taken followed by bleaching the Golgi region of interest 5 times with high-intensity laser light (488 nm line, 80 % laser power). Recovery of fluorescence in the bleached area was measured over time by scanning every 2 seconds.

2.2.13 Coating of glass coverslips for immunofluorescence

To perform immunofluorescence, cells were seeded onto glass coverslips. For better attachment, glass coverslips were coated either with collagen R (1µg/ml) for 1 hr at 37°C, or with fibronectin (50µg/ml) for 5 min at 37°C. Prior to use, coverslips were rinsed with PBS.

2.2.14 Immunofluorescence microscopy

Cells grown on coated coverslips (see 2.2.13) were fixed with 4% PFA for 15 min followed by Glycin (1M) treatment for 10 min. Cell membranes were then permeabilized using 0.1 % Triton-X-100 (diluted in PBS) for 2 min and subsequently blocked with 5 % FBS diluted in PBS for 30 min. All steps were performed at RT and between each step, cells were washed with PBS. After blocking unspecific binding sites, cells were incubated with a specific primary antibody (diluted in 5 % blocking) for 2 hrs, washed 3x with PBS and finally incubated with a secondary fluorescent-coupled antibody for 1.5 hrs. If DNA staining

was needed, cells were incubated with 2.5 µM DRAQ 5 in PBS for 15 min prior to mounting with Fluoromount–G. Hoechst staining was performed by mounting the slides with Mowiol® 4-88 supplemented with Hoechst 33258 (bisbenzimide). Cells were analyzed with a confocal laser scanning microscope using 405, 488, 546, and 633 nm excitation.

2.2.15 Raf kinase Assay

HeLa cells seeded into 100 x 20mm culture dishes-were transfected with siRNA (see 2.2.2) and synchronized using thymidine (see 2.2.5). 0 and 8 hrs post release, cells were lysed in RIPA buffer (see materials) and further subjected to immunoprecipitation (see 2.2.16). Precipitates were washed 4x with KAB (see materials) and subsequently mixed with the kinase assay mix (see materials), containing the purified substrate and ATP in KAB for 30 min at 30°C. Samples were boiled for 3 min at 95°C and separated by SDS-PAGE.

2.2.16 Immunoprecipitation

Equal amounts of cell lysates were incubated with specific antibodies O/N at 4°C. The protein-antibody complexes were captured with protein G-Agarose beads for 3 hrs at 4°C and washed afterwards with an appropriate lysis buffer. Precipitated proteins were further processed to kinase assay experiments.

2.2.17 Protein extraction for immunoblotting

Cells were lysed in an appropriate lysis buffer supplemented with 1x Complete (see materials) to obtain total cell lysates. Cells were therefore incubated on ice for 10 min followed by centrifugation (16,000 x g/15 min/4°C, Eppendorf Centrifuge 5415 R), to remove the cell debris. Supernatants were mixed with protein sample buffer (see materials) and boiled at 95°C for 3 min. Samples were separated by SDS-PAGE (10%) (see 2.2.18).

Synchronized knockdown cells were trypsinized and pelleted. For protein extraction, cells were processed by hot lysis. To this end, pellets were washed with 1x PBS, hot protein sample buffer (95°C) was added and pellets were immediately mixed. After 10 min incubation at 95°C, extracts were shifted to ice and centrifuged (16,000 x g/15 min/4°C, Eppendorf Centrifuge 5415 R). Samples were subjected to NuPAGE® Novex® Bis-Tris Mini gels (see materials).

Protein concentrations were determined utilizing Roti®-Quant (materials) corresponding to the manufacturer's protocol. The reaction is based on the Bradford method.

2.2.18 SDS- PAGE

For protein identification, equal amounts of extracted proteins were separated according to their molecular weight. To this end, a separating gel (pH 8.8) containing 10% acrylamide was prepared, followed by a stacking gel consisting of 4% acrylamide (pH 6.8). Proteins were negatively charged by adding protein sample buffer (see materials) and thus were migrating towards the anode when an electric field was applied. 1x SDS running buffer was added in the gel chamber and electrophoresis was performed at 50 mA/ V max for 70 min. To identify proteins from small to large size in one sample, NuPAGE® Bis-Tris gradient gels (4-12%) were used according to the manufacturer's protocol.

2.2.19 Western Blotting

Proteins separated by SDS-PAGE (see 2.2.18), were immobilized on nitrocellulose membranes (see materials) by semi-dry blotting. Protein transfer was carried out at 160 mA/max V for 2 hrs. NuPAGE® gels were blotted onto nitrocellulose membranes using the wet blot module system XCell II™ at 200 V/mA max for 1.5 hrs. To prevent unspecific binding, membranes were incubated in 0.5 % blocking solution (see materials) for at least 30 min at RT. Specific primary antibodies (diluted according to the manufacturer's instructions in blocking solution) raised against the target proteins were added O/N at 4°C. The next day, membranes were washed in PBS-Tween (materials) 3x for 10 min followed by incubation with HRP-conjugated secondary antibodies for 1 hr. Prior to visualization of the proteins, membranes were again washed with PBS-Tween and incubated with homemade ECL solution (see materials) for 2 min. Different exposure times to X-ray films were performed after incubation with ECL.

3 Results

3.1 The Protein kinase D family in epithelial cells

In epithelial cells, PKD isoforms are predominantly localized at the Golgi complex, specifically at the TGN, to regulate the fission of vesicles (Prestle et al. 1996; Hausser et al. 2002). In this work the cervical cancer epithelial cell line "HeLa" was used to elucidate the role of PKD at the Golgi at the onset of mitosis.

3.1.1 PKD isoform expression and siRNA-mediated depletion in HeLa cells

First of all, PKD isoform expression levels in HeLa cells were analyzed (Figure 8A). Western Blot analyses using specific PKD antibodies revealed that all three isoforms are expressed in this cell line. In order to investigate the influence of PKD proteins on Golgi function during different mitotic steps, we caused a transient loss of the proteins using specifically designed siRNAs directed against PKD1, PKD2 and PKD3, respectively. As control, a siRNA directed against LacZ, which targets the bacterial enzyme β-galactosidase, was used. Efficient silencing of the individual isoforms was achieved already 48 hrs post transfection (Figure 8B-D).

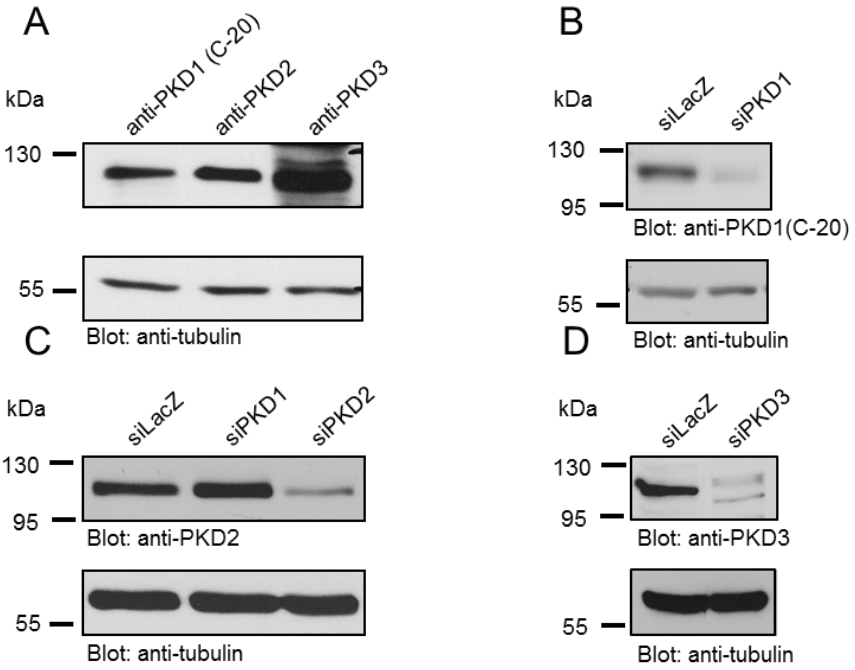


Figure 8- Detection of PKD isoform expression in HeLa cells.
(A) 1x 10^6 HeLa cells were lysed and expression of PKD isoforms analyzed by Western blotting (B-D) HeLa cells were transfected with siRNAs specific for PKD1 (B), PKD2 (C) and PKD3 (D) and the control siRNA siLacZ. Detection was performed using PKD isoform specific antibodies. Equal protein amounts were verified with a tubulin-α-specific antibody.

To investigate if PKD depletion impacts on the cell cycle, we stained PKD1, 2 and 3 knockdown cells, respectively, with PI to visualize the DNA content of cells in G1, S- and G2/M- phase of the cell cycle (see 2.2.8; Figure 9) by flow cytometry. Control cells (siLacZ) exhibited a characteristic PI-staining pattern. Specifically, most of the cells were in G1 phase either resting or providing for S-phase. In addition some cells were currently duplicating their DNA during S-phase and a minor population was dividing in G2/M-phase. Of note, PKD1 and PKD2 knockdown cells showed a reduction of cells in G1, and an accumulation in G2/M-phase, when compared with siLacZ control cells. The PI-staining pattern of PKD3-depleted cells was comparable to control cells. Thus flow cytometry analyses demonstrated that the loss of PKD1 and 2 caused aberrant cell cycle progression whereas the loss of PKD3 failed to do so. This result led to the assumption that PKD3 is less implicated in cell cycle progression and we therefore focused on PKD1 and 2 in further experiments.

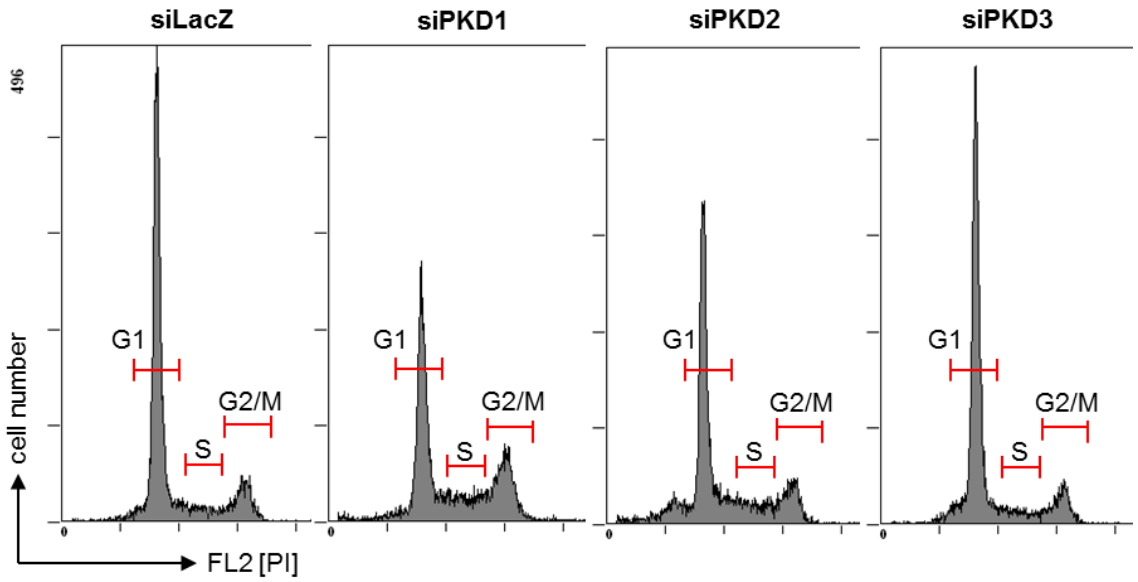


Figure 9- FACS profiles of HeLa cells after PKD knockdown.
HeLa cells were transfected with siRNA specific for LacZ and the three PKD isoforms, respectively. 72 hours post transfection cells were stained with PI to determine the DNA content by flow cytometry.

To exclude off-target effects of the PKD1 and 2 siRNAs we used a second set of siRNAs directed against different target sequences within human PKD1 and PKD2. In case of PKD1 we made use of ‘smartpool siRNAs’. Smartpool siRNAs are mixtures of five different siRNAs directed against different target sequences

of the same mRNA to gain more efficient protein knockdowns and to diminish off-target effects.

Compared with the first set of siRNAs we observed again an efficient and selective knockdown of PKD1 and PKD2 using the PKD1 specific smartpool siRNAs and the second siRNA specific for PKD2, respectively (Figure 10A, B).

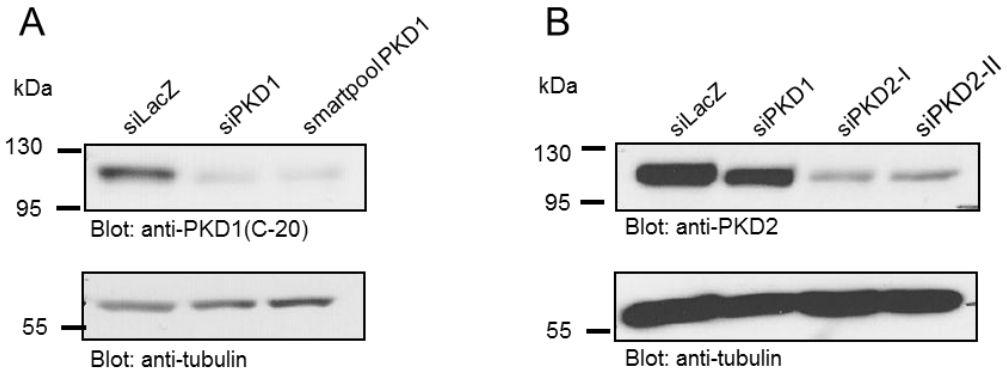


Figure 10– PKD knockdown in HeLa cells with an independent set of siRNAs specific for PKD1 and 2.
HeLa cells were transfected with an independent set of siRNAs specific for PKD1 and PKD2 to exclude off-target effects. (A) Knockdown of PKD1 using a smartpool of siRNAs specific for human PKD1 and (B) silencing of PKD2 with an alternative siRNA. Silencing efficiency was verified with PKD-specific antibodies. Detection of tubulin-α served as loading control.

3.2 Cell cycle studies of PKD depleted cells

3.2.1 Cell cycle synchronization

To study the division cycle in mammalian cells, in particular to investigate a certain protein, it is useful to accumulate the cells of interest in the same stage of the cell cycle (Ma and Poon 2011). To this end we made use of a double thymidine block to synchronize HeLa cells at the G1/S border (see 2.2.4, Figure 11A). High amounts of thymidine interfere with the deoxynucleotide metabolism, thus inhibiting DNA synthesis machinery (Prescott et al. 1971). Blocking the cells at the G1/S- border is a reversible process because after thymidine washout, cells pass the cell cycle synchronously (Ma and Poon 2011). To assure the G1/S block, PI-stained synchronized cells were compared with control cells using flow cytometry (Figure 11B). Control cells displayed the typical PI-staining profile with the majority of cells being in G1 phase and a small portion in S and G2 stage, respectively. Thymidine treated cells, however, displayed a single peak demonstrating that cells arrest at the G1/S border.

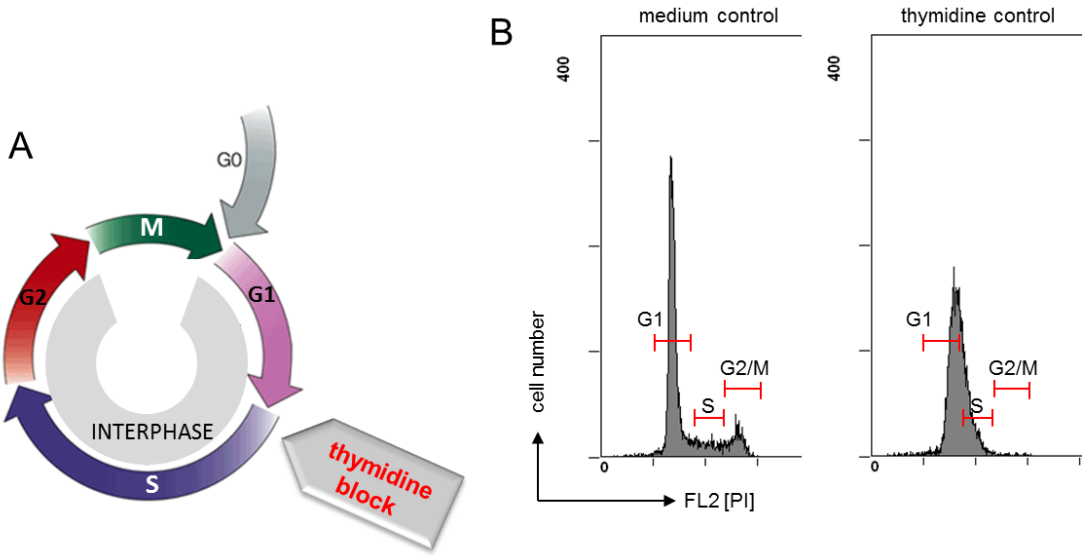


Figure 11- Synchronization of HeLa cells using thymidine.
In order to investigate PKD function in a particular step during cell cycle, HeLa cells were treated with thymidine which causes an arrest at the G1/S- border (A). After release from thymidine, synchronized cells pass through the cell cycle. Thymidine treatment was performed twice to achieve a better G1/S-block. (B) DNA content was analyzed in PI-stained cells either left untreated (left graph) or thymidine-treated (right graph). Representative controls for all FACS analyses.

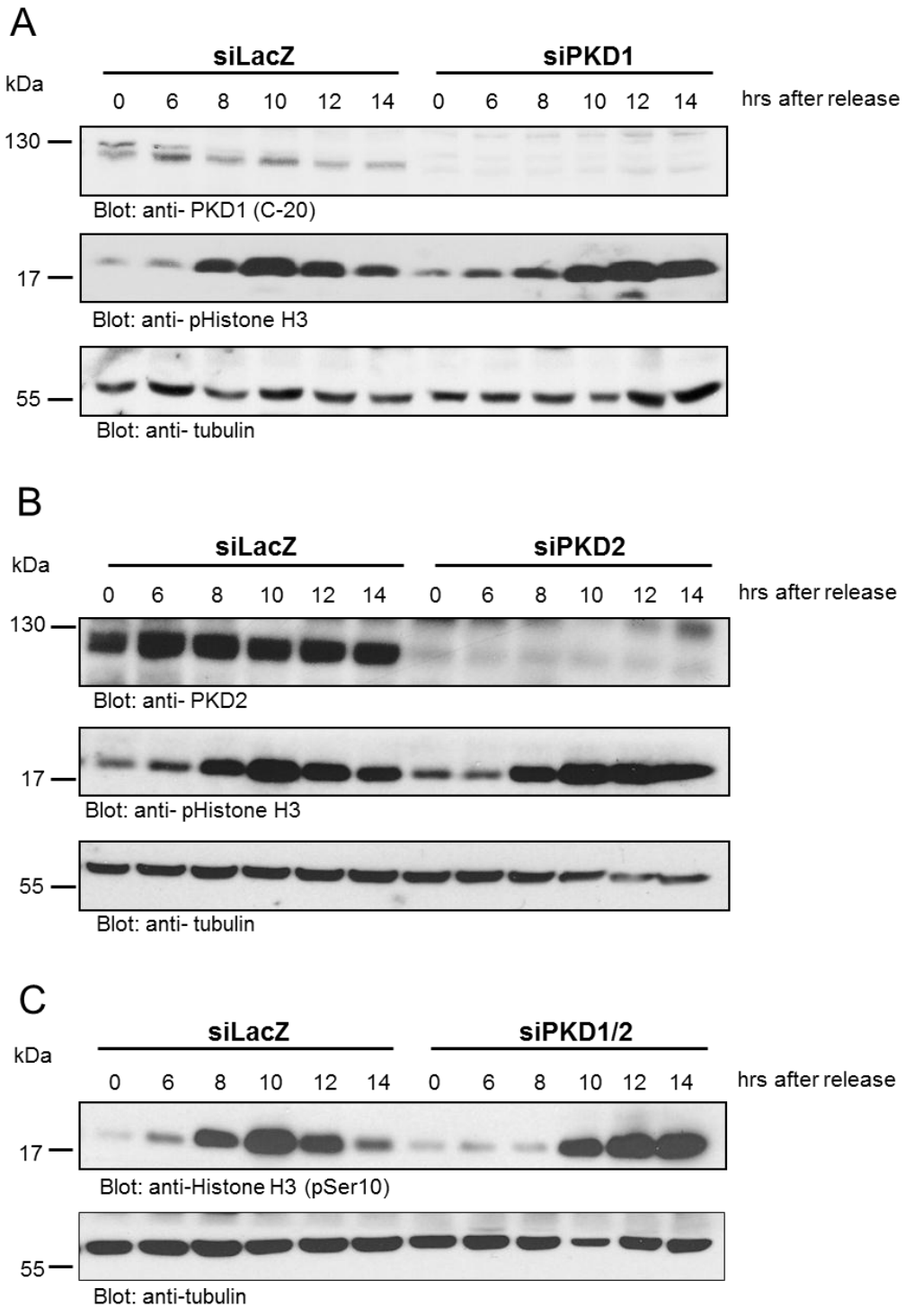
3.2.2 PKD knockdown leads to an aberrant cell cycle progression

Former studies revealed, that PKD isoforms are active during mitosis and associated with mitotic structures (Papazyan et al. 2008). Fuchs et al. showed in 2009, that PKD is active at the Golgi compartment and crucial for nocodazole-induced Golgi dispersal (Fuchs et al., 2009). In line with this, PKD overactivation induces fragmentation of Golgi membranes (Díaz Añel and Malhotra 2005). Additionally, Jamora et al. published in 1999 that IQ-induced Golgi fragmentation is dependent on PKD. It is thus conceivable that PKD activation may be important for one or more steps of Golgi fragmentation during mitosis and thus cell cycle progression.

To shed light on this process, we monitored synchronized control cells, and in parallel, synchronized cells depleted for PKD1, PKD2 or both isoforms together during G2/M transition (Figure 12). Cells were treated with siRNA prior to synchronization (see 2.2.5, and scheme next page).

As mitosis marker we utilized phosphorylated Histone 3 (H3). Phosphorylation of H3 at serine 10 within the N-terminus arises in G2 and is correlated with initial chromosome condensation (Hendzel et al. 1997). Dephosphorylation at this residue occurs just before telophase (Hendzel et al. 1997). By the use of a phospho-specific Histone H3 we thus can monitor onset and end of mitosis in control and knockdown cell lysates by Western blot analysis. In control cells, the pH3 signal was weak 0 and 6 hrs after the release, increased dramatically after 8 hrs, (Figure 12, siLacZ panels) and diminished 14 hrs after release. Of note, the pH3 signal peaks 10 hrs after thymidine release, which was already described elsewhere (Van Horn et al. 2010).

In PKD1-depleted cells the pH3 signal was slightly delayed compared to control cells. Of note, the signal was still at a high level 14 hrs after release (Figure 12A). This was also observed in PKD2 knockdown cells, albeit initial phosphorylation of H3 started 8hrs after release (Figure 12B). The most striking difference between control and knockdown cells was observed upon depletion of PKD1 and 2 (Figure 12C). Here we detected a delayed phosphorylation of Histone 3 starting 10 hrs after release. Control and double knockdown cells of each time point were also processed for determination of DNA content by flow cytometry analyses (Figure 12D). PKD–depleted cells showed a delay in mitosis already 8 hrs after release. This delay continued 10 hrs post release. While about 70 % of control cells already passed G1 twice, 70 % of knockdown cells still resided in G2/M. This is even more evident 12 and 14 hrs after release. Here, almost all control cells were in G1 while about 30 % of PKD-depleted cells still resided in G2/M. However, PKD-depleted cells were still able to progress mitosis.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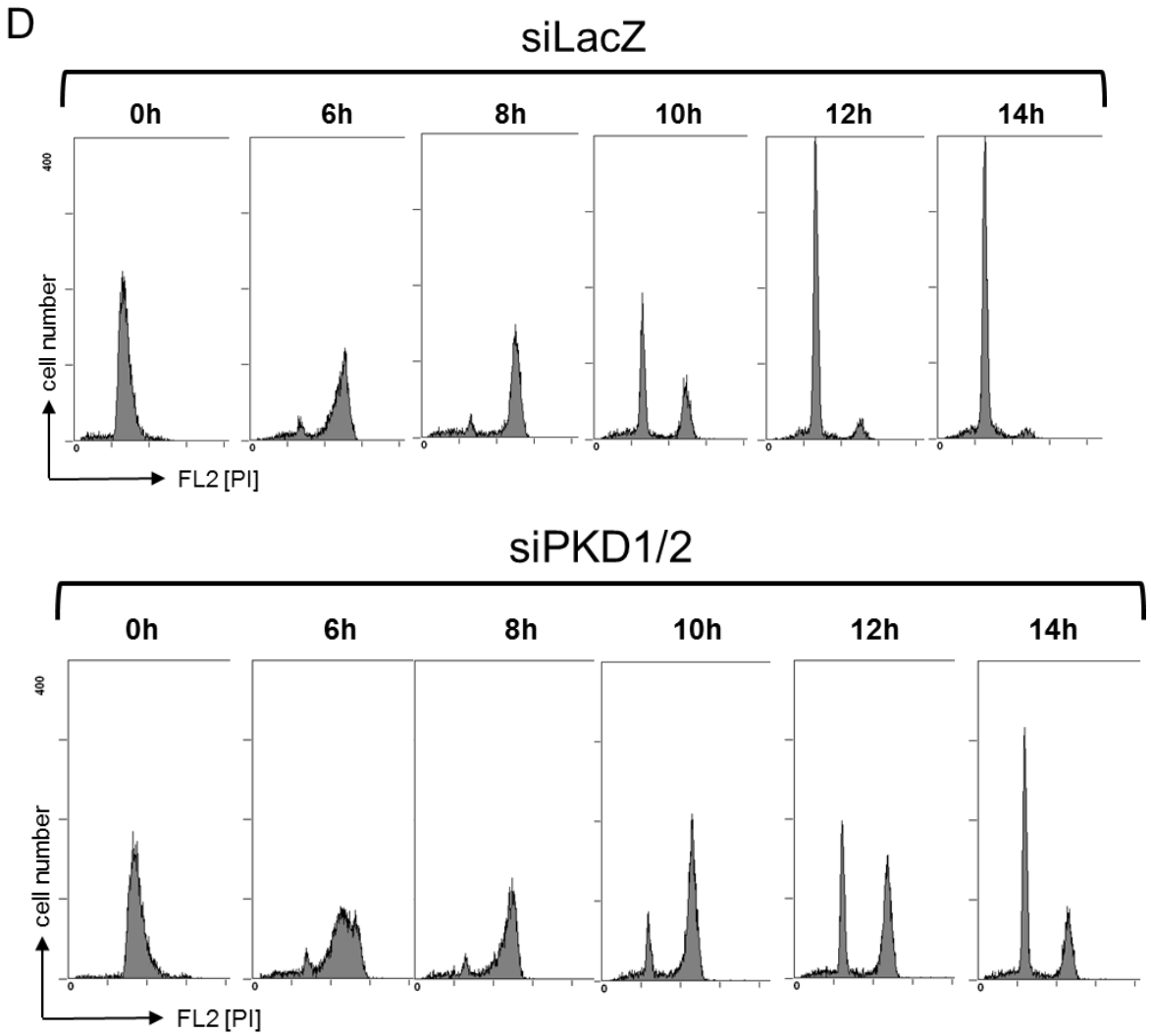
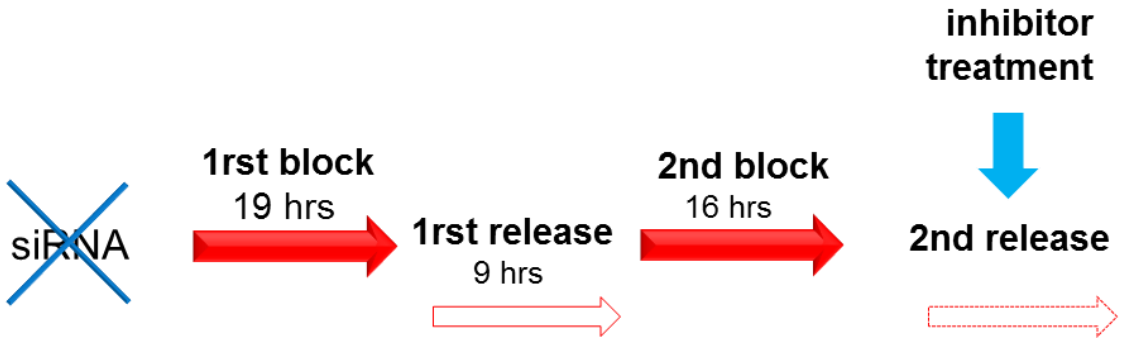


Figure 12- Effect of PKD depletion on mitosis.
HeLa cells were transfected with siRNAs specific for PKD1 (A), PKD2 (B), PKD1 and 2 (C) and with LacZ-specific control siRNA. Subsequently, cells were synchronized using a double thymidine block. After thymidine release, cells were either lysed and analyzed by Western blotting (A-C) or cells were stained with PI to determine the DNA content by flow cytometry (D) at indicated time points (0-14h). (A-C) Expression of PKD1 and 2 was assessed using PKD-specific antibodies, detection of tubulin-α served as loading control, mitosis was monitored by the use of a pH3 specific antibody.

3.2.3 PKD inhibition retains cells in mitosis

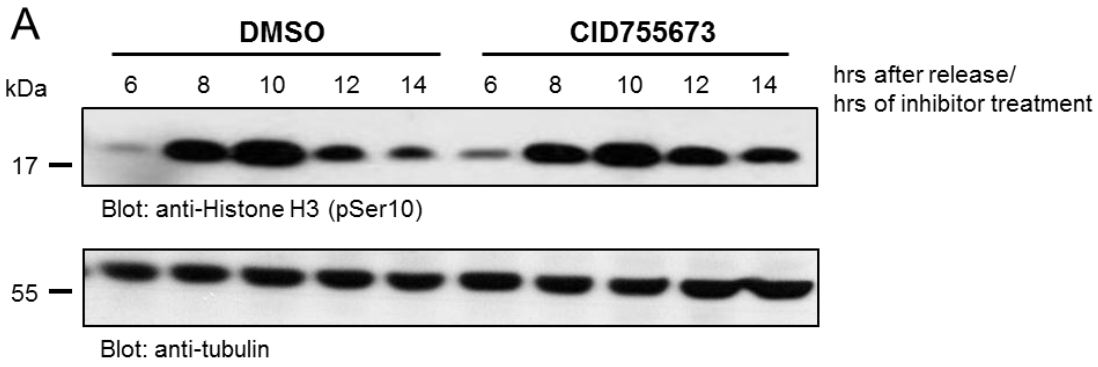
So far, we observed that the signal for pH3 arises with a delay of approximately 2 hours, and is still detectable 14 hrs post release in cells depleted for PKD1 and 2. To confirm that this time shift is due to the lack of PKD, we carried out an alternative approach to impede PKD activity. To do so, we treated the cells with two different PKD inhibitors after the second thymidine block (see scheme next page).



In a first experiment we assessed the effect of CID 755673 on the phosphorylation of H3. CID 755673 is a selective PKD inhibitor, which suppresses enzyme activity of all three PKD isoforms without affecting the action of other related protein kinases (Sharlow et al. 2008).

Upon treatment with this inhibitor, onset of mitosis was not altered in both settings (Figure 13A), since the pH3 signal increased 8 hrs and peaks 10 hrs post release. However, 12 and 14 hrs after release the differences became more obvious, in that the pH3 signal of controls was almost abrogated while in CID 755673 treated cells it was still detectable.

In an additional experiment we used Gö 6976 to investigate mitotic progression. Gö 6976 is an ATP-competitive inhibitor of several cPKCs and in addition inhibits PKD activity with an IC_{50} of 20nM (Gschwendt et al. 1996). By the use of this inhibitor, pH3 signals were already detectable 6 hrs post release and still elevated 14 hrs after thymidine release (Figure 13B), while the signal in controls was only detectable 8, 10 and to a lesser extent 12 hrs post release.



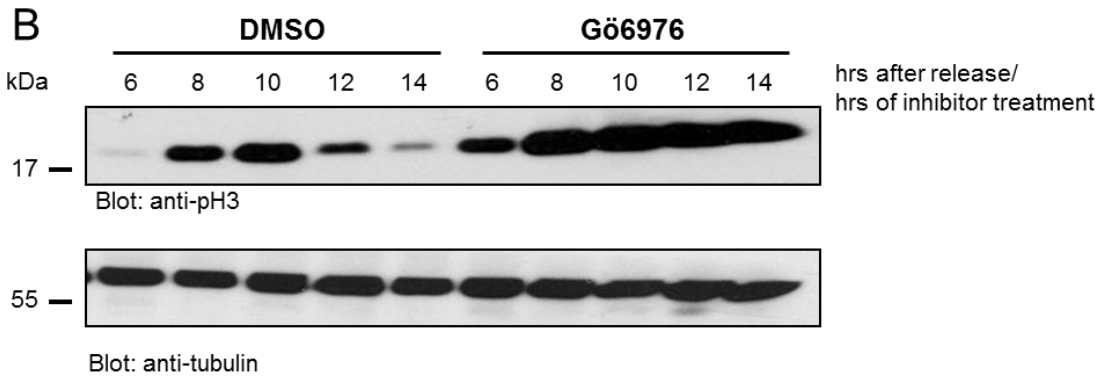


Figure 13- pH3 levels after PKD inhibition.
Synchronized HeLa cells were treated with DMSO, CID755673 (25µM) (A) or Gö6976 (1µM) (B). Cells were lysed at indicated time points and analyzed by Western Blotting. Onset of mitosis was monitored using a pH3 specific antibody and equal loading was verified by probing with a tubulin-specific antibody.

3.2.4 The loss of PKD influences other cell cycle kinases

To check whether the activity of other kinases is impaired by PKD depletion, we investigated Cdc2 levels in lysates obtained from synchronized PKD knockdown cells (Figure 14). Cdc2 is a prominent cell cycle-dependent kinase which controls entry into mitosis at the G2/M boundary (Timofeev et al. 2010). Cdc2 is the driving force for G2 progression; however phosphorylation at tyrosine 15 (Y15) by Wee1 kinase inhibits G2/M transition (Parker and Piwnica-Worms 1992; McGowan and Russell 1993).

In control cells, tyrosine phosphorylation of Cdc2 kinase is decreased 10 hrs post release, since dephosphorylation comes along with mitotic progression. On the other hand, cells lacking PKD isoforms 1 and 2 displayed only a slightly reduced Y15 phosphorylation 10 hrs after release; and this phosphorylation was still elevated 14 hrs after thymidine washout (Figure 14).

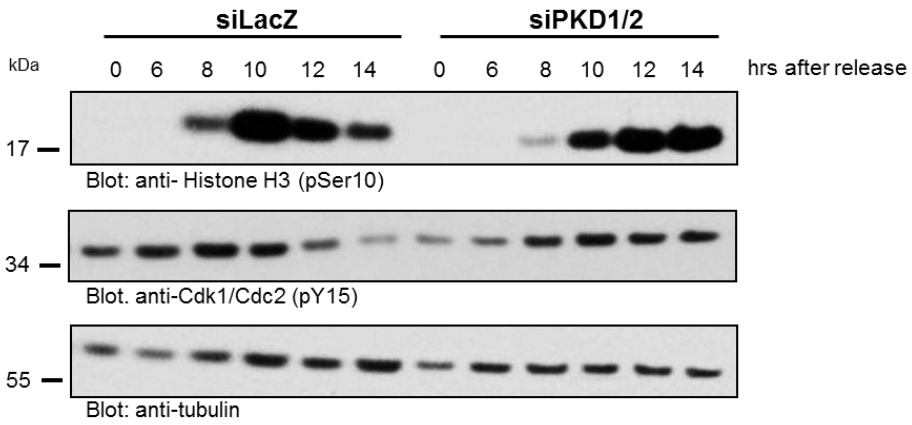


Figure 14- Phosphorylation of Cdc2 in PKD-depleted cells.
Synchronized HeLa cells co-transfected with PKD1 and PKD2 and a siLacZ-specific siRNA, respectively, were lysed and cell lysates analyzed for phosphorylation levels of Cdk1/Cdc2 and pH3 by Western blotting using a pY15-Cdk1/Cdc2 and a pH3 (pS10) specific antibody, respectively Protein levels were verified with an antibody specific for tubulin

3.3 Golgi complex dispersal during mitosis

Mammalian cells undergo extensive morphological changes during mitosis owing to several events such as condensation of DNA, depolymerization of microtubules as well as fragmentation of the Golgi complex and nuclear envelope, respectively (Hartwell and Weinert 1989). To ensure correct partitioning between daughter cells, Golgi membranes have to be dispersed during a two-step fragmentation process (Colanzi et al. 2003). First of all, tubular bridges connecting adjacent stacks have to be cleaved, otherwise cells arrest in G2 (Persico et al. 2009). Thus, fragmentation of the Golgi complex turned out to be a requirement for mitotic entry not a consequence of mitosis. Therefore the G2 step is also called “The Golgi checkpoint” (Sütterlin et al. 2002; Colanzi and Corda 2007; 1.3.3).

3.3.1 PKD is a crucial kinase for Golgi complex break up during mitosis

Several methods have emerged during the last decades to investigate Golgi dispersal at the onset of mitosis in more detail and to elucidate key players at the Golgi apparatus, which are responsible for entry into mitosis; however one assay revealed to be a successful tool to determine the impact of specific kinases in Golgi dispersal during mitosis. This “semi-intact assay” was established by the Malhotra lab in 1998.

The idea was to monitor Golgi membrane organization of permeabilized interphase normal rat kidney cells upon incubation with previously prepared mitotic extract of the same cells. Interphase Golgi stacks appeared to be fragmented up to 80 % just by addition of mitotic cytosol (Acharya et al. 1998). By the use of this method target kinases involved in Golgi fragmentation can be identified by pretreating mitotic extracts with appropriate kinase inhibitors (see 2.2.11 ; Acharya et al. 1998).

To do so, we prepared mitotic and interphase extracts of HeLa cells (see 2.2.11) and carried out the semi-intact assay with HeLa cells stably transfected with Mannosidase II, a Golgi resident enzyme, fused to GFP (Figure 15). To control if

the prepared extracts have interphase and mitotic properties, respectively, we carried out Western blot analysis using Cyclin B2 as mitotic marker. Cyclin B2 is one of the regulatory subunits of the maturation promoting factor (MPF), which initiates mitosis. Cyclin B2 synthesis is required to activate MPF and is degraded at the end of mitosis, thus inactivating MPF (Murray and Kirschner 1989; Park et al. 2007). Expression of Cyclin B2 was strongly increased in mitotic extracts compared to interphase extracts as seen in Figure 15E. Prior to inhibitor treatment, we monitored Golgi fragmentation in permeabilized cells upon incubation with respective extracts. Microscopy images showed that in 80 % of the cells the interphase Golgi stacks stayed intact when incubated with interphase extract (Figure 15A, F). However, upon incubation with mitotic extracts Golgi membranes became fragmented in almost 90 % of permeabilized cells. After having these experimental conditions established, we analyzed the effect of inhibitor treatment of mitotic extracts on permeabilized cells.

Studies of the Malhotra lab revealed that MEK1 is crucial for Golgi complex break up, since using the MEK1 inhibitor PD 98059 leads to a strongly reduced fragmentation of Golgi stacks in this assay (Acharya et al., 1998). To this end, we treated permeabilized cells with PD 98059 and the specific PKD inhibitor CID 755673 (Figure 15C, D, G). The extent of Golgi fragmentation was quantified using confocal microscopy. As expected, inhibition of MEK1 decreased the amount of cells harboring a fragmented Golgi from 90% down to 40% compared to control cells without inhibitor treatment (Figure 15A, C, G). Of significance is the finding that PKD inhibitor treatment even exceeds the PD 98059 effect.

Compared to the control, almost 50 % of permeabilized cells lost their activity to vesiculate Golgi membranes upon PKD inhibition. These data show clearly that PKD is sufficient and necessary for Golgi complex breakdown in Hela cells during mitosis.

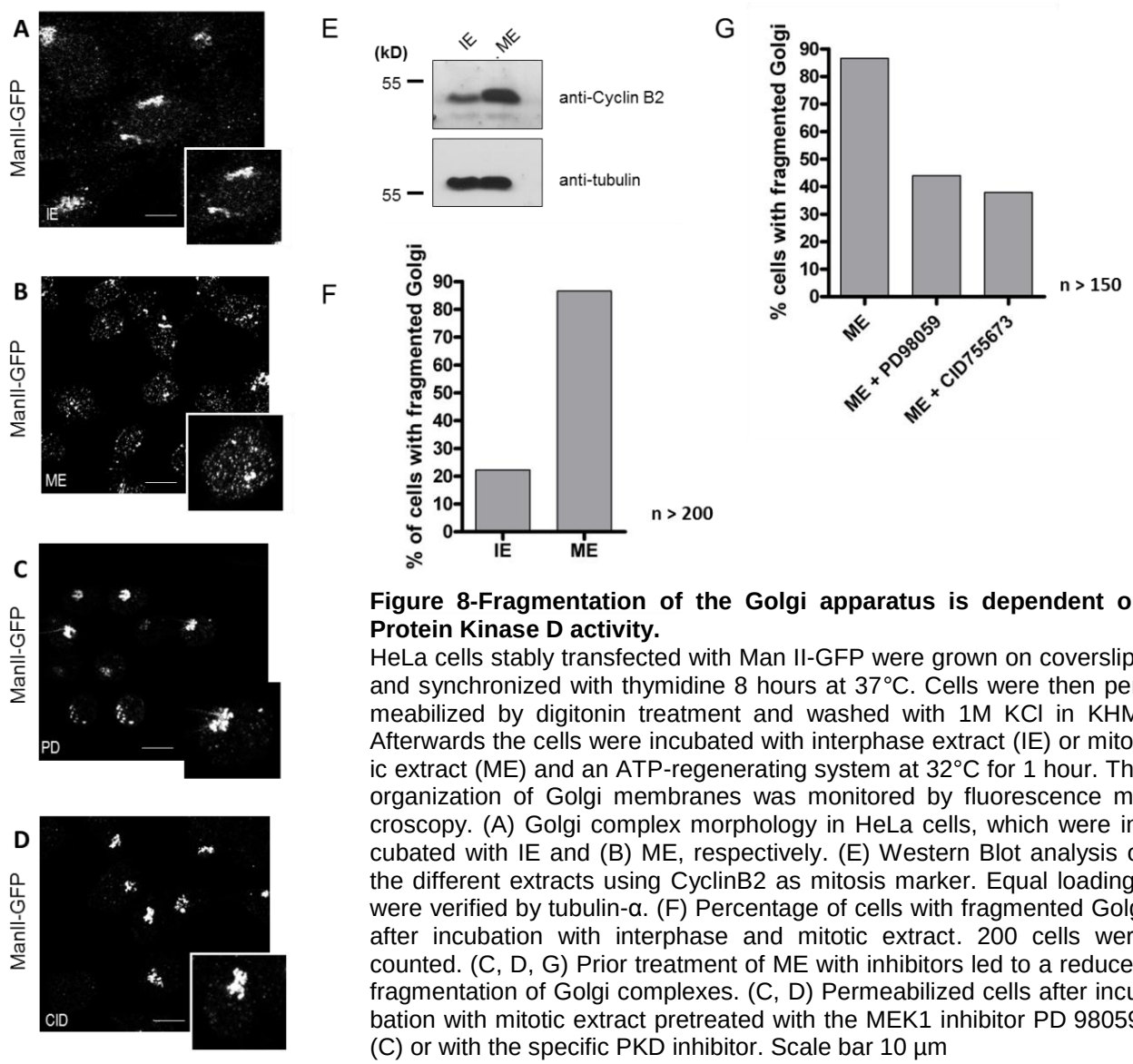


Figure 8-Fragmentation of the Golgi apparatus is dependent on Protein Kinase D activity.
HeLa cells stably transfected with Man II-GFP were grown on coverslips and synchronized with thymidine 8 hours at 37°C. Cells were then permeabilized by digitonin treatment and washed with 1M KCl in KHM. Afterwards the cells were incubated with interphase extract (IE) or mitotic extract (ME) and an ATP-regenerating system at 32°C for 1 hour. The organization of Golgi membranes was monitored by fluorescence microscopy. (A) Golgi complex morphology in HeLa cells, which were incubated with IE and (B) ME, respectively. (E) Western Blot analysis of the different extracts using CyclinB2 as mitosis marker. Equal loadings were verified by tubulin-α. (F) Percentage of cells with fragmented Golgi after incubation with interphase and mitotic extract. 200 cells were counted. (C, D, G) Prior treatment of ME with inhibitors led to a reduced fragmentation of Golgi complexes. (C, D) Permeabilized cells after incubation with mitotic extract pretreated with the MEK1 inhibitor PD 98059, (C) or with the specific PKD inhibitor. Scale bar 10 µm

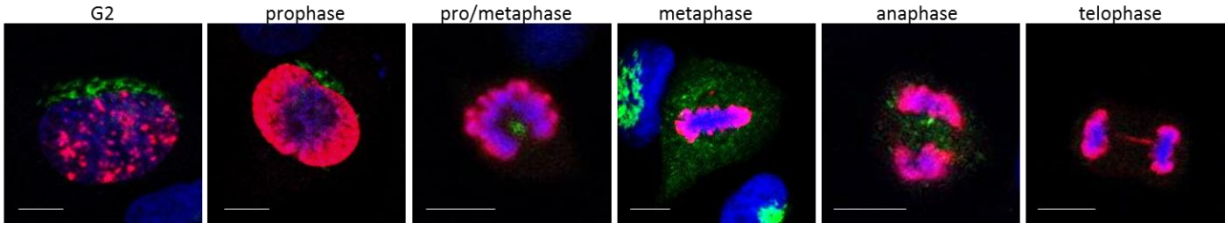
3.3.2 PKD depletion leads to a cell cycle arrest in G2

The first step of Golgi fragmentation occurs in G2, when perinuclear Golgi stacks are converted into tubulo-reticular elements (“Golgi blobs”). During the following

mitotic phases, these elements become further fragmented into small vesicles appearing as a “Golgi haze” throughout the cytoplasm (Colanzi et al. 2003; Altan-Bonnet et al. 2006). In telophase these vesicles refuse to form new functional Golgi ribbons in the dividing cells (Colanzi and Corda, 2007).

Having shown that PKD is a crucial kinase in terms of Golgi fragmentation during mitosis, we were further interested in which mitotic stage PKD interferes with the vesiculation process. To this end, we analyzed HeLa cells depleted of PKD1 or PKD2 or PKD1 and 2 on a single cell level. Therefore, control and knockdown cells were stained for pH3 to visualize different mitotic stages (Figure 16A). Whole cell numbers were determined and the percentage of mitotic cells in different mitotic phases was calculated (Figure 16B-E). Upon PKD knockdown, cells accumulated predominantly in G2 phase of the cell cycle (Figure 16B-E). These results are in line with previous data, since PKD-deleted cells failed to progress the cell cycle in a normal fashion (see Figure 12). Specifically, PKD1 silencing led to an arrest in G2 phase and to a greater extent in prophase (Figure 16B). Conversely, the loss of PKD2 caused an accumulation mainly in G2 stage, and to a lesser degree, in prophase (Figure 16C). Regarding silencing of PKD1 and PKD2 together, almost 90 % of cells were enriched in G2 phase, whereas only a minimal proportion of about 8 % of cells were retained in prophase (Figure 16D). Knockdown of PKD1 and 2 using smartpool siRNAs exhibited similarly patterns (Figure 16E). These data indicate that PKD2 is possibly the major isoform determining the outcome of cells at the Golgi checkpoint during G2 phase.

A



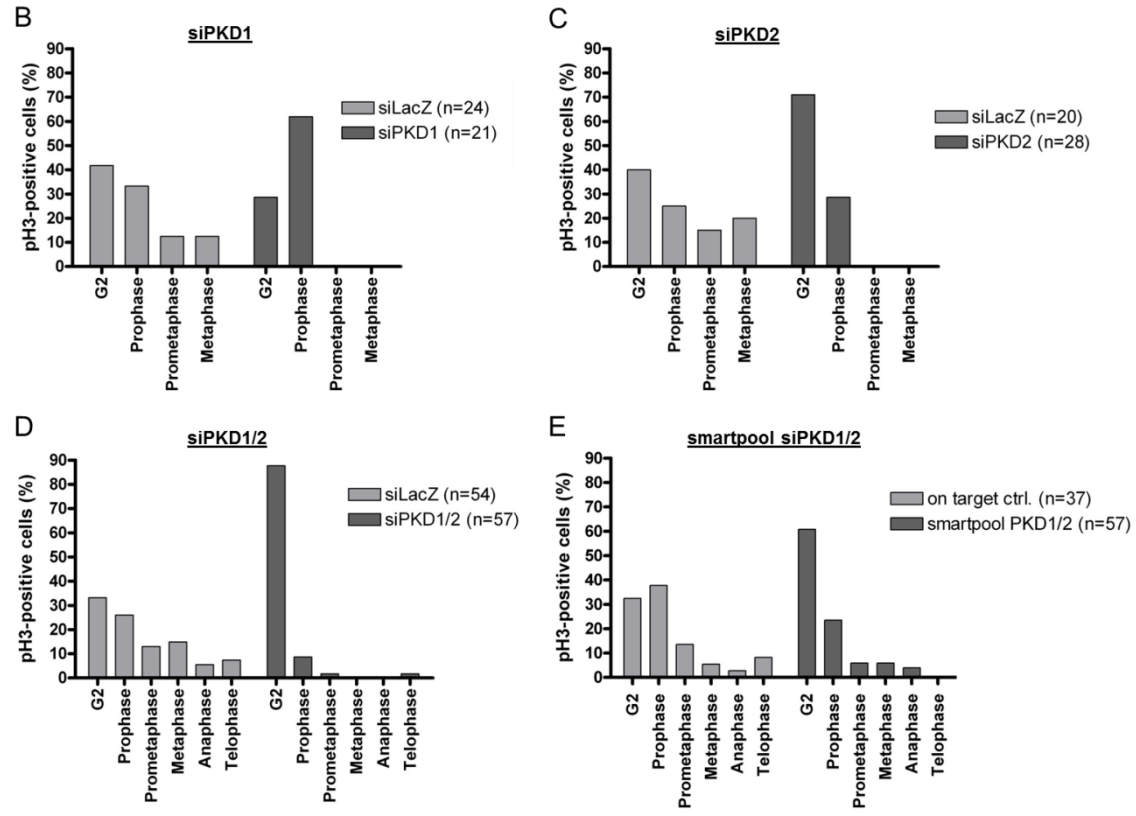


Figure 16- Mitotic index of Hela cells.
24 hours after siRNA transfection HeLa cells were replated onto collagen-coated coverslips, allowed to attach overnight and were fixed with 4% paraformaldehyde. (A) Staining was performed with Alexa546-coupled pH3 specific antibody to identify different mitotic stages (purple signal), Alexa488-coupled GM130 specific antibody to identify cis-Golgi structures (green signal) and Draq5 to visualize DNA (blue signal). Scale bar 10 µm. (B-E) Statistical analyses of mitotic cells. Cells were counted and classified according to their cell cycle status.

3.3.3 Cells lose their Golgi fragmentation ability when depleted of PKD

Since our results indicate that cells cannot exit G2/prophase of the cell cycle when PKD is absent, we further investigated the Golgi status during G2 phase. To this end, we stained synchronized, PKD1/2-depleted cells 0 h and 10 hrs after thymidine release for pH3 and GM130 to visualize mitotic phases and the Golgi complex, respectively (Figure 17). As expected, control cells showed ribbon cleavage of tubular bridges during G2, whereas PKD knockdown cells failed to do so (Figure 17A-C). More than 30 % of depleted cells had lost their ability to fragment the Golgi complex in G2 (Figure 17C). These results are in line with previous data and confirm the assumption that PKD is participating in this particular step.

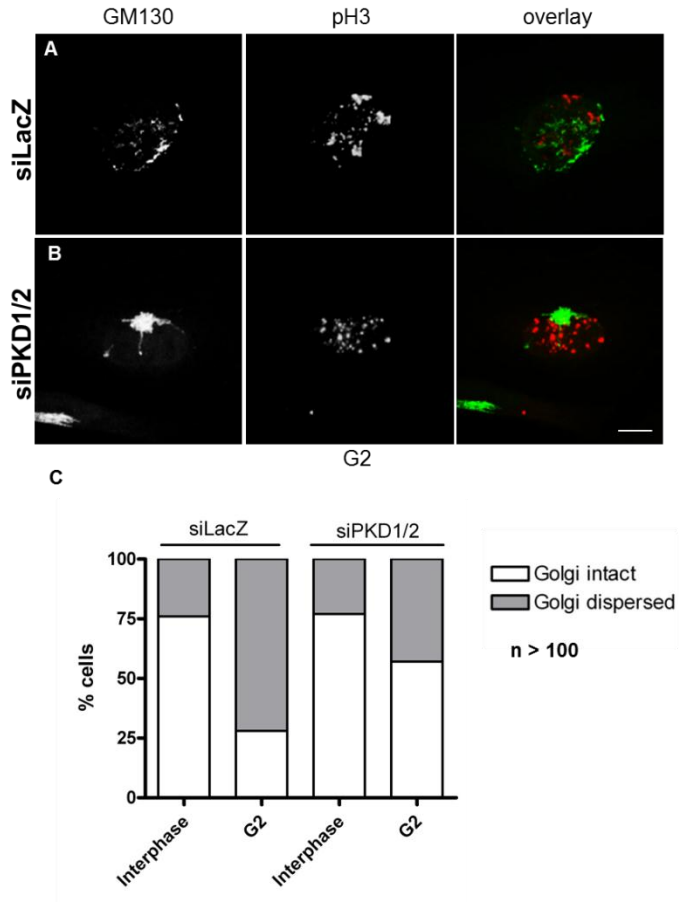


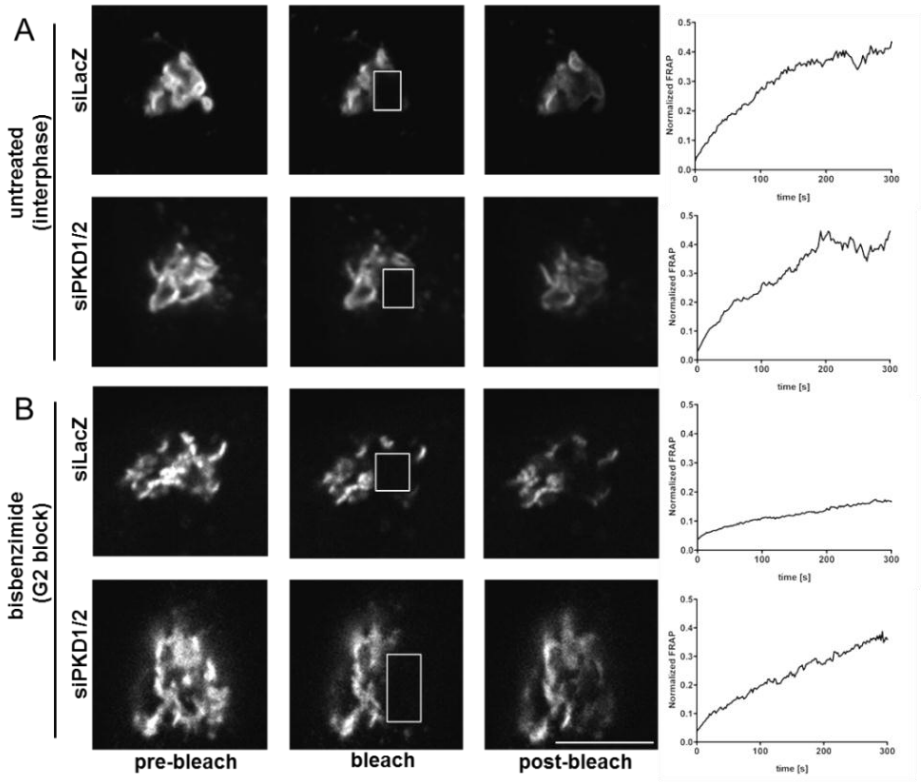
Figure 17-The Golgi complex stays intact in G2 after PKD knockdown.
Synchronized HeLa cells co-transfected with siPKD1 and siPKD2 were fixed. Cells were stained with antibodies specific for GM130 and pH3 to visualize the Golgi apparatus and mitotic structures, respectively. (A) Scale bar 10 µm. (C) Quantification of cells with an intact/dispersed Golgi during S-Phase and G2, respectively.100 cells were counted per sample.

3.3.4 PKD is required for ribbon cleavage during G2

To analyze whether PKD is crucial for the cleavage of the non-compact zones of the Golgi ribbon in G2 we used a quantitative approach based on fluorescence recovery after photo bleaching (FRAP) of Golgi resident enzymes (Colanzi et al. 2007; 2.2.12). These enzymes diffuse along the length of the Golgi ribbon, as revealed by their fast FRAP. Hence, severing of tubular connections between stacks impedes diffusion of resident enzymes and thus impairs FRAP. HeLa cells stably transfected with Man II-GFP, were either left untreated or accumulated in G2 stage with the topoisomerase-I inhibitor bisbenzimide (Colanzi et al. 2007). A region corresponding to half of the Golgi complex

was bleached by repeated laser illumination at high intensity and the FRAP of ManII-GFP was examined over

time. In untreated control and PKD1/2 knockdown cells, recovery of ManII-GFP fluorescence was rapid, consistent with an intact Golgi ribbon (Figure 18A). In bisbenzimide-treated control cells, however, FRAP of Man II-GFP was slower and did not reach the plateau that was seen in untreated cells, indicating that the non-compact zones were cleaved and thus continuous diffusion of enzymes was interrupted (Figure 18B). By contrast, PKD1/2-depleted cells treated with bisbenzimide demonstrated fast FRAP comparable to that seen in untreated cells. Quantitative analysis confirmed that bisbenzimide treated PKD1/2 depleted cells show a significantly increased FRAP compared to control cells (Figure 18C), proving that PKD1/2 knockdown cells harbor an intact Golgi ribbon in G2 stage. Our results thus clearly show that PKD is specifically required for severing of the non-compact zones of the Golgi complex in G2, which is a prerequisite for cells to pass the G2/M Golgi checkpoint.



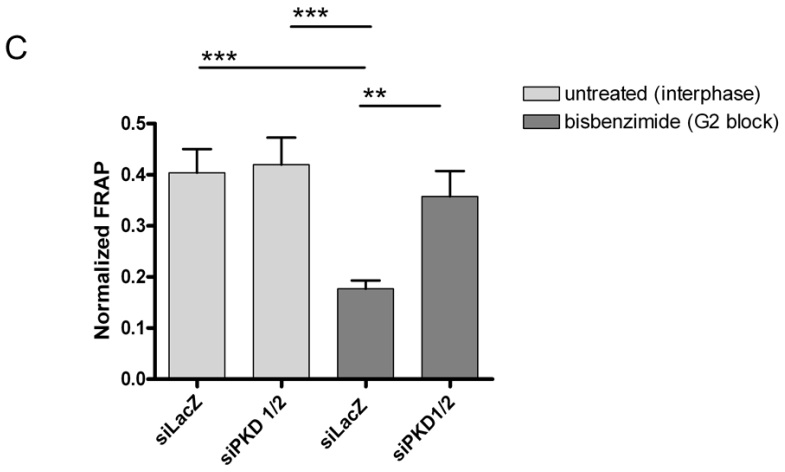


Figure 18- PKD mediates cleavage of the Golgi ribbon during G2.
HeLa cells stably transfected with Man II-GFP and transiently transfected with siLacZ or co-transfected with siPKD1 and siPKD2 were left untreated (Panel A, interphase) or treated with bisbenzimide (Panel B, G2). 50% of the Golgi area was photobleached and FRAP was measured. (A, B) Representative images and FRAP curves. Images are illustrating the Golgi complex before (pre-bleach), directly after (bleach) and at end of bleaching (post-bleach). Scale bar 10µm. FRAP kinetics of interphase cells (A) and G2-accumulated cells (B). Fluorescence recovery in bleached areas was monitored every 2s. Curves are normalized to non-bleached areas. (A, C) Images exhibit a recovery after photobleaching, or (B, C) display no recovery after bleaching. (C) Quantification of fluorescence recovery in the photobleached region. Data are from analysis of 10 cells for each condition, and from two independent experiments. Statistical significances were evaluated using the Kruskal-Wallis test for non-parametric distributions followed by Dunn's Multiple Comparison test. ($p<0.001$=***; $p<0.01$=**).

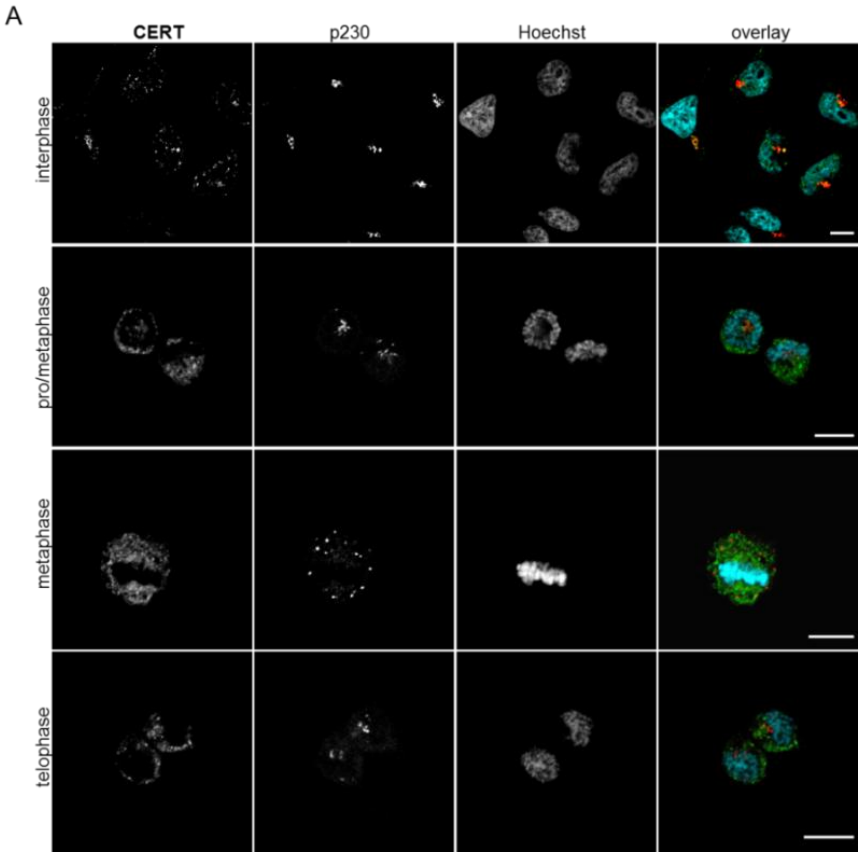
3.4 Common Golgi substrates of PKD are not involved in the fragmentation pathway

Since our results indicate that PKD is crucial in Golgi fragmentation during G2, we further wanted to know if known PKD downstream targets are involved in this process. PI4KIIIβ, OSBP and CERT are common PKD substrates at the Golgi compartment, indispensable for lipid metabolism and biogenesis (Hausser et al. 2006; Hanada et al. 2007; Graham and Burd 2011; 1.3.1).

PKD activates PI4KIIIβ through phosphorylation and thus stimulates PI4KIIIβ lipid kinase activity, resulting in increased PI4P production at the TGN (Hausser et al. 2006; Graham and Burd 2011). High PI4P levels lead to OSBP and CERT recruitment, since both proteins bind via PI4P to Golgi membranes (De Matteis et al. 2005; Nhek et al. 2010; Subathra et al. 2011). Of note, PKD-mediated phosphorylation inhibits Golgi complex localization of OSBP and CERT (Fugmann et al. 2007; Nhek et al. 2010), thereby creating a negative feedback loop on DAG-dependent PKD activation, since CERT is necessary for *de novo*

DAG synthesis at the TGN (De Matteis and Godi 2004; Olayioye and Hausser 2011).

Secretion is proposed to be turned off during mitosis (Kreiner and Moore 1990), thus local PI4P pools are abolished, presumably leading to dissociation of OSBP and CERT from the TGN (Graham and Burd 2011). To investigate whether OSBP and CERT are implicated in mitotic Golgi complex fragmentation we analyzed their subcellular localization during mitosis (Figure 19). As microscopy images clearly show, OSBP and CERT are co-localized with Golgi structures only in interphase cells (Figure 19, interphase panels). When cells enter mitosis, PKD substrates were dissociating from the Golgi compartment (Figure 19, lower panels). Expectably, these results provide strong evidence that known PKD substrates at the Golgi complex, such as OSBP and CERT, are not involved in PKD-mediated dispersal of the Golgi complex during mitosis.



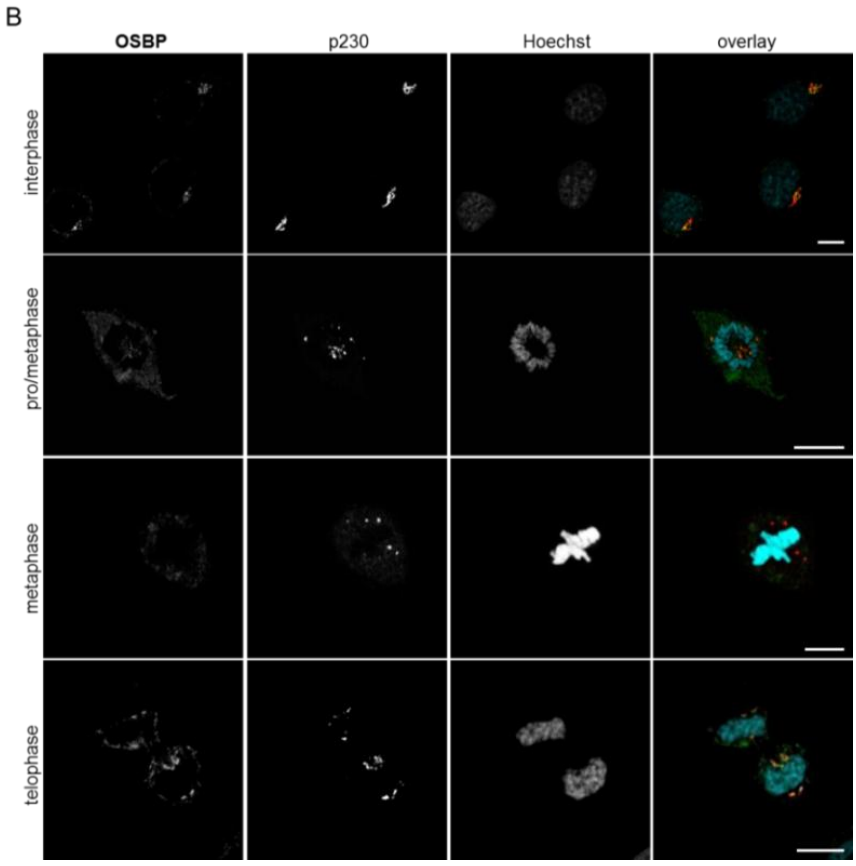


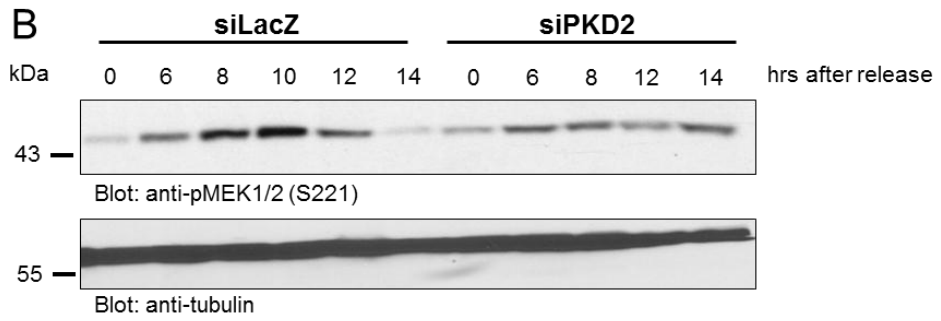
Figure 19– PKD substrates CERT and OSBP dissociate from the Golgi during Mitosis. Unsynchronous HeLa cells were seeded onto collagen-coated coverslips and stained for immunofluorescence analysis. Localization of CERT (A) and OSBP (B) was visualized using specific antibodies (green signal). Golgi staining was performed using the p230 specific antibody (red signal) and the DNA was stained using Hoechst dye (cyan signal). (A, B) (interphase panels A, B). (prophase-telophase panels, A, B). Scale bars are representing 10 µm.

3.5 The Raf-MEK cascade in Golgi fragmentation during mitosis

To date, the Raf-MEK pathway is the best characterized pathway involved in mitotic Golgi fragmentation (Colanzi et al. 2000; Colanzi et al. 2003; Chambard et al. 2007; Shaul et al. 2009). MEK1 is proposed to be the key kinase, which is phosphorylated by RAF-1, and itself is activating a downstream kinase, different from ERK1/2, which is finally responsible to fragment the Golgi complex in G2/M (Acharya et al. 1998; Shaul and Seger 2006). However, little is known about upstream kinases activating Raf-1 since Ras is recommended to play only a minor role in this process (Shaul et al. 2009).

3.5.1 PKD depletion decreases MEK phosphorylation

We investigated whether PKD is upstream or downstream of active MEK1. MEK1 is specifically activated during mitosis in a Raf-1 dependent manner (Colanzi et al. 2003). We therefore synchronized siLacZ, siPKD1, siPKD2 and siPKD1/2- transfected HeLa cells at the G1/S border using a double thymidine block. Cells were harvested at distinct time points after the second thymidine wash out and MEK1 phosphorylation was visualized using Western Blot analysis with a pS221-MEK1 specific antibody, which reports Raf-induced activation of MEK (Alessi et al. 1994). In control cells MEK1 was phosphorylated at 6 hours after release (Figure 20A-C) and stayed elevated until 12 (Figure 20B) or 14 hours (Figure 20A,C), respectively. Strikingly, singly or combined depletion of PKD1 and PKD2 strongly reduced MEK1 phosphorylation during mitosis (Figure 20A-C).



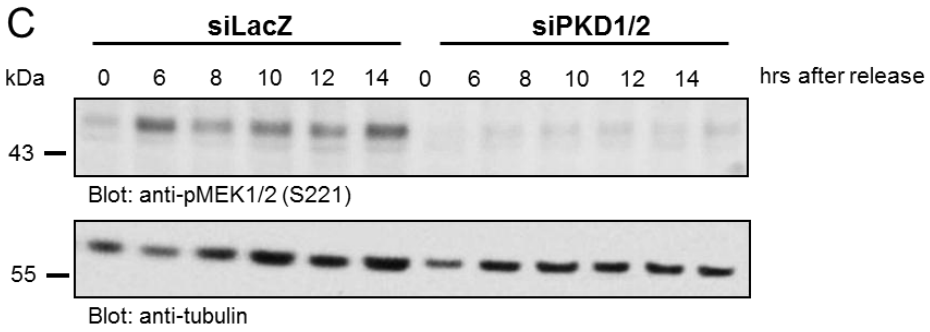


Figure 20- MEK phosphorylation levels upon PKD knockdown.
HeLa cells after transfection with a control siRNA (siLacZ), siPKD1 (A), siPKD2 (B) and siPKD1/2 (C) were synchronized at the G1/S border by a double thymidine treatment and released for the indicated times. Cells were lysed and phosphorylation of MEK1 was detected by Western Blot analysis using an antibody specific for phosphorylated serine 221. Detection of tubulin served as a loading control.

3.5.2 PKD influences Raf-1 activity

To confirm that PKD is upstream of Raf-1, we carried out a Raf kinase assay using lysates obtained from synchronized control cells and cells depleted for PKD1/2 (see 2.2.15, 2.2.16). Cells were harvested in interphase, right after the second release from thymidine (0h), and at the onset of mitosis (8h). As described previously, MEK phosphorylation should increase at the onset of mitosis (Colanzi et al., 2003). Raf-1 and B-Raf were immunoprecipitated, mixed with ATP and purified MEK as a substrate and phospho-MEK levels were examined in Western blot analyses (Figure 21).

MEK phosphorylation levels in B-Raf precipitates were weak and did not show any changes between the different set ups (Figure 21, left side). However, in case of Raf-1, we observed a strong MEK phosphorylation in siLacZ controls eight hours after thymidine release compared with interphase lysates (0h). In PKD knockdown cells, however, MEK1 phosphorylation was strongly diminished 8 hours after thymidine release (Figure 21, right side). These data indicate that PKD controls mitotic Raf-1 activity. However, PKD in vitro kinase assays revealed that neither Raf-1 nor B-Raf is directly phosphorylated by PKD (data not shown).

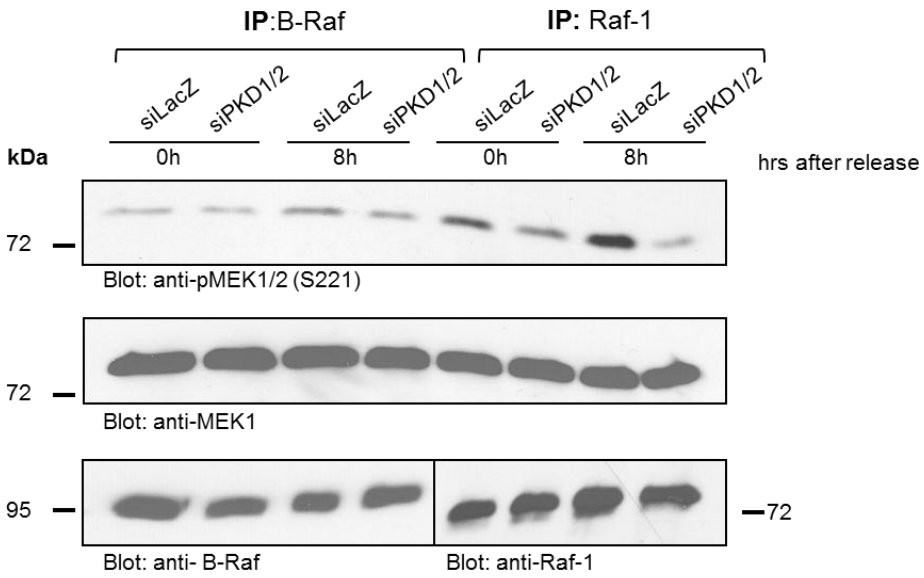


Figure 21- Raf-1- activity after PKD depletion.
HeLa cells transfected with a control siRNA (siLacZ) or PKD1 and 2-specific siRNAs were synchronized at the G1/S border by a double thymidine treatment, immediately harvested (0 hours) or released for 8 hours prior to analysis. Cells were lysed and C-Raf kinase activity was determined as described in the materials section. MEK1 phosphorylation was monitored using the pS221 antibody and MEK1 and Raf-1 levels were visualized using specific antibodies as indicated.

3.5.3 A hyperphosphorylated MEK1 rescues PKD depleted cells

Since our results indicate that PKD is upstream of the Raf-MEK pathway in terms of mitotic Golgi fragmentation, we further investigated if a constitutive active form of MEK1 is able to rescue cleavage of non-compact zones during G2 in PKD-depleted cells.

To this end, we monitored synchronized control versus PKD-depleted cells, with and without overexpression of a constitutive active form of MEK1 fused to mCherry (see 2.2.7; Figure 22). SiLacZ control cells showed a dispersed Golgi complex during G2, whereas PKD-depleted cells failed to do so (Figure 22A, left side, B). Upon caMEK1 overexpression, Golgi fragmentation was restored to the control level in PKD-depleted cells (Figure 22A, right side; B). In line with previous results, quantification revealed that PKD knockdown cells accumulate in mitosis (Figure 22C), predominantly in G2 (Figure 22D). Of note, expression of caMEK1 rescued PKD depleted cells from the G2 arrest allowing them to exit G2/prophase (Figure 22D).

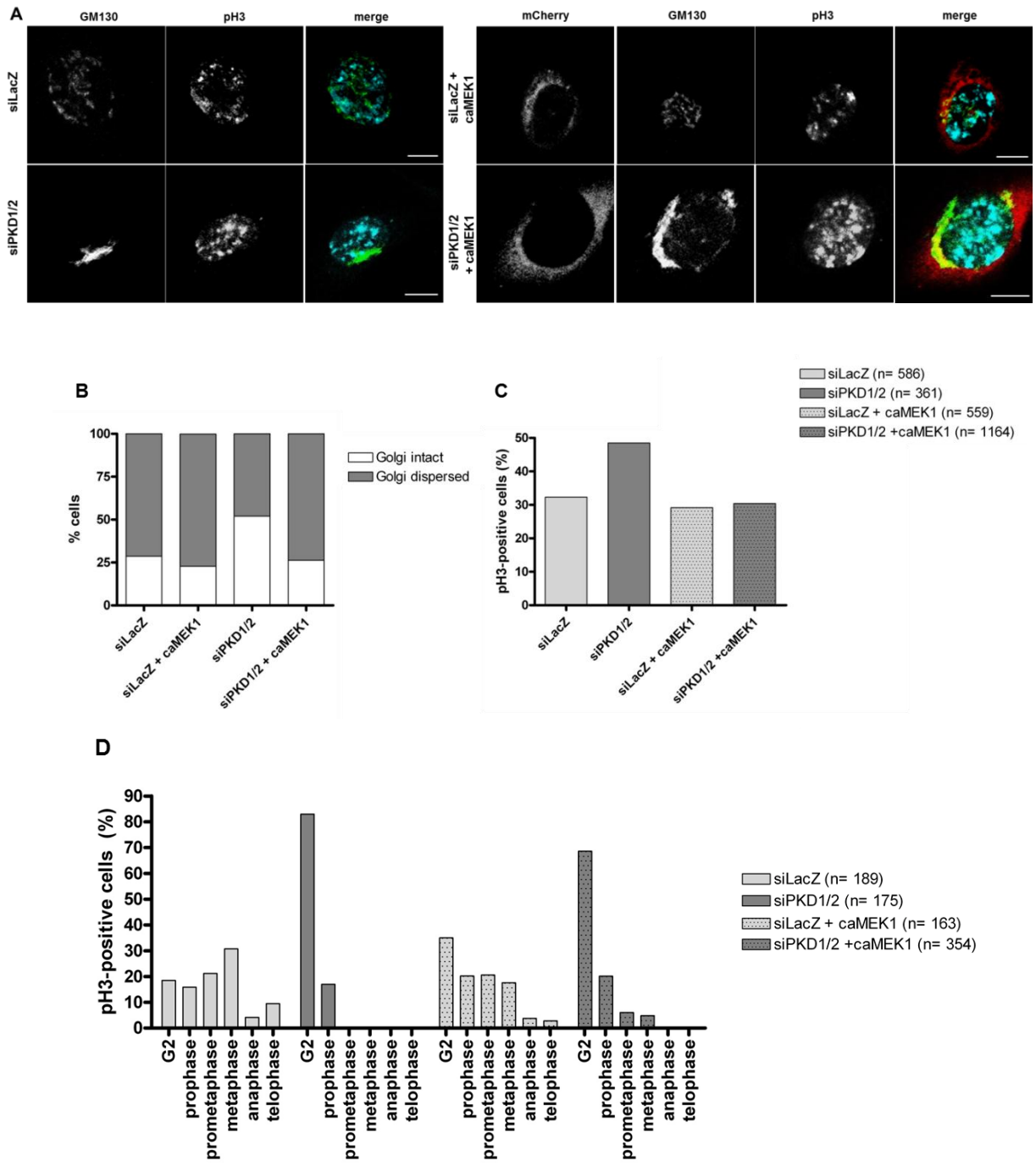


Figure 22- caMEK1 rescue of PKD knockdown cells.
HeLa cells cultured on collagen-coated coverslips were synchronized at the G1/S border and transfected with siLacZ or siPKD1/2. One sample of each set up was additionally transfected with a caMEK1- plasmid fused to mCherry. Cells were released for 10, 12, and 14 hours, fixed, and stained with antibodies specific for GM130 and pH3. (A) Representative images showing Golgi complex morphology of control cells (siLacZ), PKD1/2 depleted cells (siPKD1/2) and siRNA-transfected cells expressing caMEK1-mCherry (siLacZ + caMEK1 and siPKD1/2 + caMEK1) in G2 stage. Color code: GM130 (green), pH3 (cyan), caMEK1-mCherry (red). Scale bar 10 µm. (B) The graph displays the results of the quantification of cells in interphase and G2 stage harboring an intact or a dispersed Golgi complex. At least 35 cells per sample were analyzed. (C) The percentage of pH3-positive cells was quantified. n = total number of cells analyzed. (D) Classification of the different cell cycle stages stages was determined as described in figure 9. N indicates the total number of cells (C) or the number of pH3-positive cells analyzed (D).

3.6 PKD overexpression rescues phenotype in part

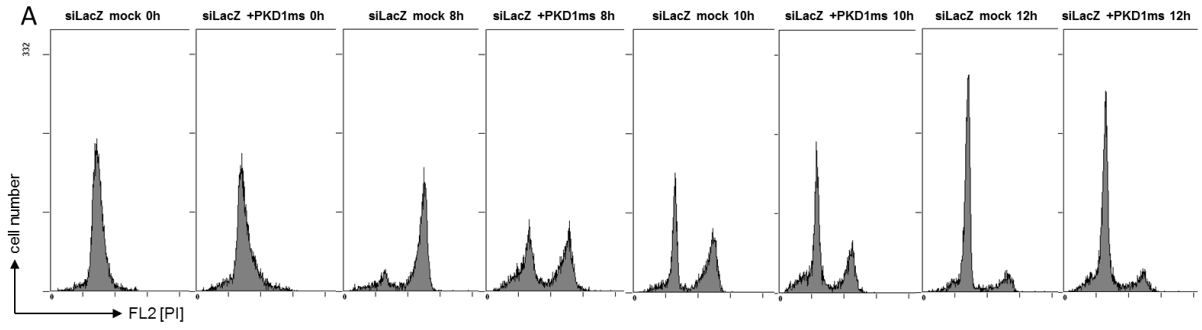
To further confirm the role of PKD in mitotic Golgi fragmentation, we employed a PKD rescue experiment.

To do so, we utilized synchronized HeLa cells, transfected with siRNAs specific for LacZ and PKD1/2, respectively. One set of control and knockdown cells was additionally transfected with a siRNA-resistant mouse-PKD1 expression plasmid. Cells were harvested at distinct time points after thymidine removal and stained with PI to determine the DNA content by flow cytometry (see.2.2.7; Figure 23).

Unexpectedly was the finding we observed for siLacZ controls. Upon overexpression of PKD1, the cell cycle of control cells was greatly accelerated already 8 hrs post release (Figure 23, panel A). About 80 % of control cells were arranged in G2/M phase, the remaining 20 % of cells stayed in G1. In contrast, upon overexpression, 50 % of control cells were already in G1, suggesting that an enhanced PKD1 expression could force up mitotic duration. This increase in speed was observed for subsequent time points, too (10, 12, 14 hrs post release).

PKD-depleted cells showed a delay in mitosis, which is in line with previous experiments (see Figure 12D). However, as seen for 8 hrs post release, 40 % of cells, with additional PKD1 expression, were already in G1, compared with only about 15-20 % of control cells without overexpression. This difference was detectable 10, 12 and 14 hrs after release, too (Figure 23; panel B).

Overall, PKD knockdown cells co-transfected with PKD1-mouse were able to pass mitosis faster; however the overexpression of PKD could not rescue the control phenotype completely. This outcome is possibly due to the lack of PKD2 overexpression.



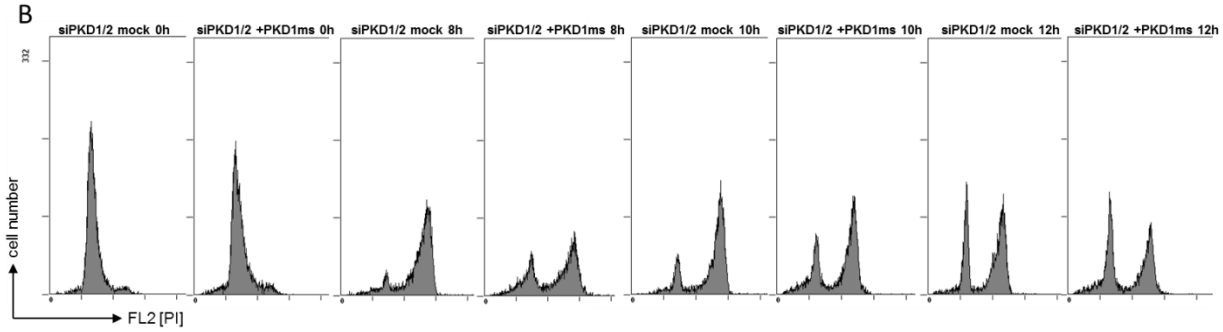


Figure 23- Expression of mouse PKD1 rescues mitotic delay in PKD1/2 depleted HeLa cells.
Synchronized HeLa cells were transiently transfected with siRNAs specific for PKD1 and PKD2. 24 hours later cells were treated with transfection reagent (mock) or transfected with a siRNA-resistant mouse-PKD1 plasmid fused to GFP. 72 hours after siRNA transfection, cells were fixed at the indicated time points and stained with PI for DNA-content measurements using flow cytometry.

To omit the possibility that the FACS pattern shown in Figure 23 is because the mPKD1-transfected cells cannot pass mitosis at all, we analyzed the pH3 localization pattern by fluorescence microscopy (Figure 24). Additionally, the Golgi complex was visualized using a GM130-specific antibody.
Microscopy images show clearly that cells expressing mPKD1-GFP are positive for pH3 and thus are able to pass mitosis.

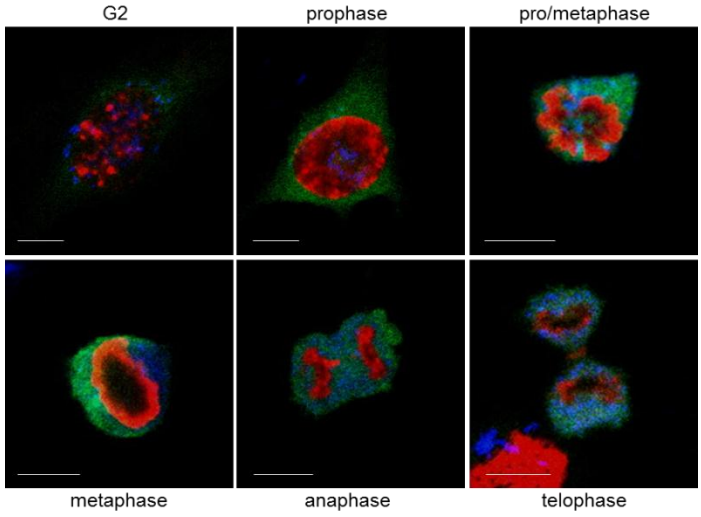


Figure 24– mouse-PKD1-GFP expression during mitosis.
HeLa cells transfected with mouse-PKD1-GFP (green) were fixed and stained for pH3 (red) and GM130 (blue) to visualize cells in G2 and mitosis and the Golgi complex, respectively. Images shown are projections of several confocal sections. Scale bar 10 µm.

4 Discussion

The PKD family of protein kinases is involved in multiple cellular processes such as proliferation, apoptosis, cell migration, immune responses or secretion. Dysfunction of PKD expression and misregulation are associated with a variety of diseases like cardiovascular hypertrophy and several types of cancers.

PKD's role in the formation of transport carriers at the TGN is well- investigated, however, there are still unanswered questions that need to be addressed.

In this work the role of PKD in Golgi complex breakdown during mitosis, an event that ensures correct partitioning of Golgi membranes into dividing cells, was examined. As a result, it was found that the cleavage of the non-compact zones in G2 phase is PKD dependent.

4.1 The role of PKD in cell cycle progression

As shown in the results (Figure 8), the HeLa cells used in our lab, express all three PKD family members. This is controversial to Malhotra's and colleagues finding that PKD1 is lacking in this cell line (Bossard et al. 2007). However, HeLa cell lines are very heterogenous in nature, since this cell line is being used over many decades. Therefore, it is conceivable that differences occur within the same cell line.

In order to elucidate the role of PKD isoforms in cell cycle behavior it appears that only PKD1 and PKD2 participate in the event of mitosis (Figure 9). Furthermore, our analyses suggest that PKD1 and PKD2 display redundant functions, since single knockdown experiments revealed similar results for both isoforms.

PKD depletion using siRNA and treatment with PKD inhibitors led to a delay of mitotic progression. This was assessed by monitoring Histone H3-phosphorylation in Western Blot analysis or through DNA content measurements (Figure 12, 13).

Furthermore, a semi-intact assay was applied to verify PKD as a mitotic kinase regulating Golgi complex dispersal (Figure 15). Upon PKD inhibition, Golgi stacks failed to break down, confirming the assumption that PKD is a crucial kinase mediating Golgi fragmentation.

More detailed analyses using immunofluorescence approaches uncovered that PKD is necessary for severing non-compact zones in G2 (Figures 16-18).

Therefore, PKD was identified to interfere with the Golgi mitotic checkpoint, hence to enable mitotic entry. This is in agreement with previous experiments, which revealed that PKD depletion causes a delay in mitotic progression.

How PKD exerts its function is still elusive. PKD-specific substrates responsible for vesicle fission such as OSBP and CERT (Fugmann et al., 2007; Nhek et al., 2010) were not localized at the Golgi complex in mitotic cells (Figure 19). This is supported by a study showing that CERT shows reduced Golgi complex localization in nocodazole stimulated cells (Chandran and Machamer 2008). Of note, it is known that secretion is shut down during mitosis (Kreiner and Moore 1990) suggesting that PKD signals via a different set of proteins in mitotic cells. Indeed, PI4KIIIβ shows reduced localization to Golgi membranes in mitotic cells (Godi et al., 1999) further supporting this hypothesis.

Western Blot analyses of mitotic lysates revealed that PKD depletion comes along with decreasing phosphorylation of MEK, pointing to a connection between PKD and the MAPK pathway. Indeed, Raf-1 activity turned out to be dependent on PKD, which was assessed by an indirect kinase assay (Figure 21). Of note, Raf-1 but not B-Raf gets activated via PKD.

Unfortunately, PKD is not phosphorylating Raf-1 directly (data not shown), thus the questions remains how the signal is transferred from PKD to Raf-1.

4.2 Putative upstream mechanisms activating Raf-1 in mitosis

MEK1 gets activated in early mitosis through Raf-1 phosphorylation (Colanzi et al. 2003); however, the mechanism leading to Raf-1 activation is still elusive. It appears to be different to the growth factor activated MAPK pathway and therefore not dependent on Ras signaling (Ziogas et al. 1998; Laird et al. 1999).

In this work we show that PKD acts upstream of Raf-1 determining its activation (Figure 21) and thereby influencing mitotic entry (Figure 12); nonetheless Raf-1 turned out to be not a direct PKD substrate (Hausser et al., 2001; data not shown).

Earlier studies provide evidence, that Src kinases as well as several PKCs phosphorylate Raf-1 in mitosis and are therefore required for G2/M transition (Kolch et al. 1993; Roche et al. 1995; Laird and Shalloway 1997). However, phosphorylation by Src and PKCs turned out to be not sufficient to fully activate Raf-1 in mitosis, pointing to other kinases involved in Raf-1 activation (Laird et

al. 1999). Thompson and Fields proposed in 1996 that PKC β_{II} is required for G2/M transition by phosphorylating lamin B, which comes along with nuclear lamina disassembly. Interestingly, inhibitor treatment arrested cells in G2, which is similar to our observations. Thus, it is conceivable that the observed arrest is also attributed to inhibition of PKD in parallel, which is known to get activated upon PKC phosphorylation (Thompson and Fields 1996; 1.1.3).

Recently, Valente and co-workers proposed that PKD is part of a multiprotein complex, together with several other proteins such as BARS, PI4KIIIβ, 14-3-3γ or PAK, to regulate vesicle fission at the TGN (Valente et al. 2012). PKD is suggested to stabilize this complex by phosphorylating PI4KIIIβ. These findings are not unexpected, since PKD, PI4KIIIβ and BARS are well-established proteins in terms of vesicle fission at the TGN (Weigert et al. 1999; Liljedahl et al. 2001; Baron and Malhotra 2002; Yeaman et al. 2004; Hausser et al. 2005; Bossard et al. 2007; 1.3.2). Moreover, BARS is also known to be a major regulator inducing fission of Golgi tubular bridges in G2 phase (Colanzi et al. 2007; 1.3.3).

However, how BARS exerts its function is still elusive. Several studies provide evidence that BARS alone is neither sufficient for ribbon cleavage in G2, nor for fission of vesicles at the TGN. Upon addition of mitotic or interphase cytosol, respectively, BARS is able to exert its fission activities pointing to additional components required for the severing process of BARS (Weigert et al. 1999; Hidalgo Carcedo et al. 2004; Colanzi et al. 2007).

Interestingly, Weigert et al. provide evidence that BARS acts as acyltransferase to generate PA (phosphatidic acid) from LPA (lysophosphatidic acid) (also described as LPAAT activity) and this drives fission by changing the membrane lipid composition (Weigert et al. 1999). However, Gallop et al demonstrated that the LPAAT activity associated with CtBP/BARS is a co-purification artifact (Gallop et al., 2005). Thus, the question remains whether BARS is involved in regulation of lipid metabolism. Despite of this, several publications propose that LPA and PA are crucial components in membrane fission by modulating the lipid metabolism and influencing membrane curvature (Kooijman et al. 2003, 2005; Malhotra and Campelo 2011).

Remarkably, data about MAPK signaling revealed that PA activates the MAPK kinase cascade by interacting with Raf-1 (Ghosh et al. 1996, 2003; Rizzo et al. 1999), and this process seems to be Ras-independent (Rizzo et al. 2000).

These data suggest that PKD acts through a BARS-PA-dependent mechanism, which activates Raf-1 at least in terms of secretion.

However, Valente et al. admitted that BARS fission activity interferes also with other trafficking steps besides secretion, such as mitosis (Hidalgo Carcedo et al. 2004; Colanzi et al. 2007; Valente et al. 2012). Thus, it is conceivable that prior to mitosis, signals are initiating a cascade involving the multiprotein complex consisting of BARS, PKD, PI4KIIIβ, 14-3-3γ, and PAK leading to ribbon cleavage in G2. Hidalgo Carcedo and co-workers further explain these findings with the hypothesis that fission activity of BARS in interphase is presumably enhanced through phosphorylation in mitosis (Hidalgo Carcedo et al. 2004).

Given that this complex assembles in mitosis, PKD acts probably indirectly through BARS, activated BARS changes the lipid composition in support of PA, which in turn activates the Raf-1 cascade and facilitates fission of non-compact zones in G2 (Figure 25).

However, fission activity of BARS was still induced although with lower efficiency, when BARS LPAAT activity was abolished (Hidalgo Carcedo et al. 2004). This suggests rather a facilitating role in terms of fission than an obligatory one, which offers a dual role for BARS in terms of Golgi ribbon cleavage in G2 (Hidalgo Carcedo et al. 2004; Corda et al. 2006). If Raf-MEK indeed gets activated through BARS-provided PA, inhibition of BARS acyltransferase activity and in turn of the Raf-MEK pathway would impede mitotic entry of cells. Actually, depletion of MEK1 in G2 delays the passage of cells in mitosis but does not block mitotic entry totally (Wright et al. 1999; Feinstein and Linstedt 2007; 3.2.2). In contrast, inhibition of BARS in contrast completely blocks cell cycle in G2 (Colanzi et al., 2007; Hidalgo Carcedo et al., 2004). Thus, BARS activity is indispensable for the transition, whereas MEK1 just alleviates it. It is therefore possible that both activities of BARS are required to overcome the Golgi mitotic checkpoint on time. This was also speculated by Colanzi et al. in 2007, proposing connected functions for MEK1 and BARS in terms of Golgi mitotic checkpoint control (Colanzi et al. 2007).

In summary, once activated by mitotic signals, the multiprotein complex consisting of BARS, PKD, PI4KIIIβ, 14-3-3γ, PAK and others assembles at the Golgi complex leading to BARS activation. Subsequently, BARS catalyzes acylation of PA from LPA whereupon the MAPK cascade is started, facilitating Golgi rib-

bon cleavage in G2, in concert with BARS mediated severing-activities alone (Figure 25).

Of note, disruption of the multiprotein complex results in failure of vesicle detachment from the TGN, a common process also monitored in cells expressing a kinase dead mutant of PKD (Liljedahl et al. 2001; Bossard et al. 2007; Valente et al. 2012). PKD's function in this process is to phosphorylate PI4KIIIβ, which in turn facilitates and stabilizes binding to 14-3-3γ an event indispensable for the formation of the protein complex, hence for fission activity (Valente et al. 2012). It thus seems to be reasonable that only PKD1 and PKD2 are found to interfere with cell cycle events (Figure 9), since these two isoforms are known to phosphorylate PI4KIIIβ at the TGN, whereas PKD3 failed to do so (Hausser et al. 2005).

Furthermore, Valente et al. demonstrated that PAK phosphorylates BARS to stabilize the complex. However, Zang and colleagues provide evidence that during mitosis PAK gets phosphorylated and subsequently activates Raf-1 (Zang et al. 2001; 2002). It is possible that PAK stabilizes BARS through phosphorylation, thereby indirectly promoting the activation of Raf-1, and in parallel, PAK phosphorylates Raf-1 directly. This provides a dual role for PAK in mitosis whereby the Raf-MEK pathway is activated (Figure 25).

In addition, data from Spratley et al. revealed that PKD phosphorylates PAK4, thus interfering indirectly with cell migration (Spratley et al. 2011). Since PKD is in close proximity to PAK in the multiprotein complex, PKD might phosphorylate PAK and PI4KIIIβ in parallel, thereby initiating the RAF-MEK cascade which triggers Golgi fragmentation in mitosis (Figure 25).

It is tempting to speculate that PKD as well as PAK exert dual functions within the protein complex in order to activate the Raf-MEK cascade regulating mitotic entry (Figure 25). However, it is also possible that BARS is severing tubular bridges independently of PKD whereas Raf-1 gets activated by a PKD-PAK pathway. Thus the Golgi ribbon gets cleaved through the synergistic action of both pathways.

Of note, cells are able to progress mitosis independently of BARS and MEK in case inter stack connections are cleaved prior to G2 upon treatment with brefeldin A, nocodazole or GRASP-65 depletion (Hidalgo Carcedo et al. 2004; Feinstein Linstedt 2007). This demonstrates that both proteins become super-

fluous in terms of mitotic entrance in the same cells. Hence, BARS and MEK are operating on the same level, and this comes along with mitotic entry owing to cleavage of non-compact zones of the Golgi ribbon.

Besides BARS and MEK1, the third key player required for this initial cleavage event is GRASP-65 (Sütterlin et al. 2001; Colanzi and Corda 2007; 1.3.3). Foremost known to mediate Golgi reassembly, it gets mitotically phosphorylated by Plk and Cdc2 (Lin et al. 2000; Sütterlin et al. 2001; Wang et al. 2003, 2005; 1.3.3). Presumably, due to these phosphorylations Golgi stacks become destabilized, which alleviates mitotic entry (Sütterlin et al. 2001). Interestingly, Maroto et al. proposed in 2008 that PAK phosphorylates Plk in early stages of mitosis. Moreover, inhibition of PAK delayed G2/M transition in HeLa cells (Maroto et al. 2008).

Considering the established model (Figure 25), it is conceivable that PAK, possibly activated by PKD, exerts influence on GRASP-65 via Plk, thus contributing to ribbon cleavage in G2 in an additional way. This is rounding up the circuit of the three known key players acting in G2 through the action of distinct kinases (Figure 7).

The situation *in vivo* is definitely more complicated, thus it needs further investigation to prove whether these kinases act in concert to accomplish the intricately process of mitotic entry. Nevertheless, on the basis of this work we could clearly show that PKD is a regulator of the Golgi mitotic checkpoint.

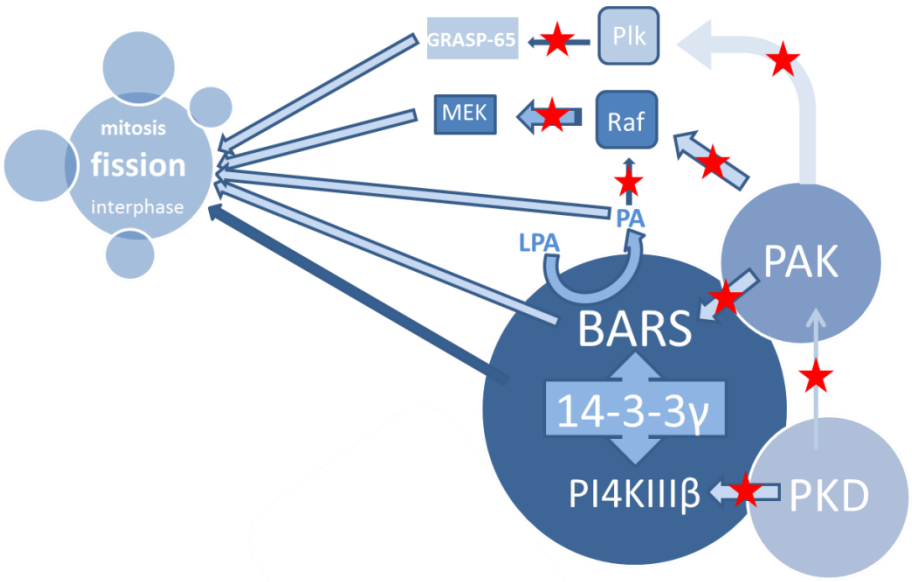


Figure 25 – Current model of PKD-mediated activation of the Raf-MEK pathway in severing tubular bridges in G2.
PKD is part of a multiprotein complex consisting of a 14-3-3γ-dimer, BARS, PI4KIIIβ, PAK and other components. PKD phosphorylates PI4KIIIβ, which facilitates binding to 14-3-3γ. 14-3-3γ binds BARS in parallel, forming the core of the multiprotein complex. PAK stabilizes the core by phosphorylation of BARS (Valente et al. 2012). PKD is able to phosphorylate PAK (Spratley et al. 2011) suggesting a dual role for PKD in this complex. PAK is phosphorylating Raf-1 when trapped in mitosis using nocodazole (Zang et al. 2001; Zang et al. 2002). PAK also activates Plk, which in turn compromises GRASP-65 stabilizing activities leading to ribbon severing in G2 (Sütterlin et al. 2001). BARS induces fission in G2 and in interphase either directly (light blue arrow), by the help of the complex (dark blue arrow) or through subsequent acylation of LPA to PA (rounded arrow), whereupon the Raf-MEK cascade is initiated. PAK indicates p21-activated kinase; BARS, brefeldin A adenosine diphosphate–ribosylated substrate; LPA, lysophatidic acid; PA, phosphatidic acid; PI4KIIIβ, phosphatidylinositol-4 kinase III beta; Plk, polo-like kinase; GRASP-65, Golgi reassembly-stacking protein of 65 kDa. Stars indicate phosphorylations.

4.3 MEK1 in mitosis

MEK1 signaling in mitosis, initiated upon Raf-1 activation, has been discussed a lot in recent years. It turned out to be independent of the common MEK1 substrates ERK1/2 (Acharya et al. 1998); however, this has been disproved by studies providing a MEK-ERK pathway in mitosis, leading to GRASP-55 phosphorylation and hence Golgi fragmentation (Jesch et al. 2001; Duran et al. 2008; Feinstein and Linstedt 2008). Colanzi, Malhotra and colleagues discussed a role for an ERK-like protein and a different MEK1 being involved in MAPK signaling in terms of mitosis (Acharya et al. 1998; Colanzi et al. 2000; Colanzi et al. 2003; Duran et al. 2008).

Remarkable studies of Shaul and Seger confirmed this hypothesis. They suggest a role for an ERK splice variant, termed ERK1c, being the target of MEK1 in mitosis and responsible for ribbon cleavage in G2 (Shaul and Seger 2006). More recently they also introduced a different MEK, MEK1b, being the upstream kinase of ERK1c in mitosis facilitating Golgi fragmentation (Shaul et al. 2009). It is conceivable that downstream signaling of Raf-1 happens through MEK1b-ERK1c and in turn GRASP-55, to exert fission of non-compact zones. Possibly, publications proposing a role for ERK1/2 in mitosis detected ERK1c instead of ERK1/2, which is recognized by common ERK antibodies as well, not knowing dealing with a splice variant of ERK1/2.

Of note, the involvement of MEK in mitotic entry at all is disputed by several publications, providing evidence that Golgi fragmentation is MEK-independent (Lowe et al. 1998; Draviam et al. 2001). In this study, however, MEK was confirmed as crucial kinase in severing non-compact zones in G2 downstream of PKD (Figure 22, 12). These data fit well to those publications proposing that MEK depletion leads to a mitotic delay (Wright et al. 1999; Feinstein and Linstedt 2007). This observed delay is maybe attributed to the lack of several pathways, initiated by PKD, acting synergistically to accomplish mitotic fragmentation together with BARS severing activity alone (Figure 25). Thus cells are rather delayed than blocked in the particular step.

A totally different approach how MEK1 could be activated during G2 involves the centrosome. It is known that the Golgi complex and the centrosome have a dependent relationship in mitosis, since the fragmentation step in G2 comes along with centrosome separation (Sütterlin and Colanzi 2010; Persico et al. 2010). Therefore, MEK1 possibly gets activated through signals generated during centrosome doubling and mediates centrosome separation, a prerequisite for Golgi fragmentation and mitotic entry. PKD is known to be activated during mitosis and located at the centrosome (Papazyan et al. 2008), probably modulating MEK1 activation.

4.4 PKD activation in G2 phase

In this work we identified PKD as a mitotic kinase, regulating Golgi inter stack connections responsible for mitotic entry. However, the mechanism of PKD activation could not be resolved.

As described previously, the Golgi complex and the MT cytoskeleton comprise a tight relationship in terms of Golgi complex localization and integrity (Veit et al. 1993; Burkhardt 1998; 1.3.1). Nocodazole treatment of cells converts the Golgi complex into mini-stacks, owing to MT depolymerization (Cole et al. 1996; Drecktrah and Brown 1999). Moreover, it has been reported that PKD is active upon this nocodazole-induced Golgi complex break-up, pointing to a connection between PKD and the MT cytoskeleton (Fuchs et al. 2009). Given that Golgi disassembly is dependent on PKD activation, one can figure that MT depolymerization generates a signal to accomplish PKD activation. Additionally, it is proposed that mitotic MEK1b activation is stimulated upon nocodazole treatment (Shaul et al. 2009).

Interestingly, it has been reported that an increase in Rho activity promotes the dispersion of the Golgi complex into mini-stacks as well (Zilberman et al. 2011).

Rho GTPases are known to regulate reorganization of the actin cytoskeleton and are involved in tubulin dynamics, respectively (Raftopoulou and Hall 2004). Foremost known to act in cytokinesis, more recent studies provide evidence that Rho signaling is also required in mitosis (Chevrier et al. 2002; Bakal et al. 2005; Narumiya and Yasuda 2006). Interestingly, inhibition of Rho GTPases in synchronized HeLa cells perturbed G2/M progression but did not completely arrest cells in G2 (Ando et al. 2007). In addition, a study from the Storz lab revealed that Rho A activates PKD as a consequence of cell-cell contact disruption, which in turn induces NF-kB activation (Cowell et al. 2009).

Taken together, it is conceivable that MT and actin cytoskeleton rearrangements that occur prior to mitosis lead to PKD activation via a Rho signaling pathway. Once PKD is activated, it marks the start for a Raf-MEK pathway to accomplish Golgi ribbon cleavage in G2 via different signaling cascades (Figure 25).

4.5 Conclusions and perspectives

Cell division is a complex process involving multiple proteins and kinases participating in several signaling pathways working in parallel as well as synergistically to accomplish correct partitioning of cell contents. The focus of this work was to clarify the role of PKD in terms of separation of the Golgi complex during mitosis. Foremost known to be involved in secretion at the TGN, we identified PKD as a regulator of mitotic entry.

PKD acts in G2 in severing non-compact zones, which connect adjacent stacks, a process indispensable for mitotic entry.

In order to fragment the Golgi ribbon, PKD appears to initiate a cascade involving Raf-1 and MEK, however, it is most likely that PKD acts synergistically with other mitotic proteins such as BARS and GRASP-65 to overcome the Golgi checkpoint in G2.

For the future, further investigations are needed to identify the entire signaling network regulating the Golgi mitotic checkpoint, and its impact on mitosis.

Moreover, *in vitro* and *in vivo* studies are required to deepen our knowledge in this field, particularly with regard to physiological and pharmacological consequences.

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I hereby assure that I performed this work independently without further help or other materials than stated.

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