Protein kinase D2 assembles a multiprotein complex at the Trans-Golgi-network to regulate matrix metalloproteinase secretion

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# shared contribution

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**Abstract**

Vesicle formation and fission are tightly regulated at the Trans-Golgi-network (TGN) during constitutive secretion. Two major protein families regulate these processes: members of the Adenosyl-ribosylation-factor-family of small G-proteins (ARFs) and the Protein kinase D (PKD) family of serine/threonine kinases. The functional relationship between these two key regulators of protein transport from the TGN so far is elusive. We here demonstrate the assembly of a novel functional protein complex at the TGN and its key members: Cytosolic PKD2 binds ARL1 and shuttles ARL1 to the TGN. ARL1, in turn, localizes Arfaptin2 to the TGN. At the TGN, where PKD2 interacts with active ARF1, PKD2 and ARL1 are required for the assembly of a complex comprising of ARF1 and Arfaptin2 leading to secretion of MMP2 and -7. In conclusion, our data indicate that PKD2 is a core factor in the formation of this multiprotein complex at the TGN that controls constitutive secretion of MMP cargo.

**Introduction**

The generation of functional transport carriers at the Trans-Golgi-network (TGN) requires the coordinated recruitment of coat proteins, membrane deformation, vesicle formation and vesicle fission (1-3). Various mechanisms guarantee the continuous and timely delivery of specific cargo from the TGN to a particular destination at the plasma membrane. Constitutive trafficking of cargo to apical and basolateral membranes is accomplished by progressive vesicle budding followed by vesicle fission (4). At the molecular level, several proteins control initiation and termination of constitutive secretion. Serine/threonine kinases of the Protein Kinase D (PKD) family are pivotal regulators of vesicle fission at the TGN (1). The PKD family constitutes a subclass of the CAM-kinases (5) and comprises three closely related members, PKD1 (PKCµ), PKD2 and PKD3. Some of the mechanisms by which PKDs are localized to and activated at the TGN have been elucidated. First, PKDs are recruited from the cytoplasm to the TGN via interaction with ARF1 (a GTPase of the ADP-ribosylation-factor family). This interaction is mediated via a proline residue in the PKD second zinc finger domain (6). Subsequently, the kinases are anchored at the TGN by interacting with diacylglycerol (DAG) via their first zinc finger domain (7,8) and activated by specific beta-gamma subunits of small G-proteins as well as PKCeta (9). Various PKD substrates at the TGN are likely to be implicated in the subsequent regulation of shedding of cargo-containing vesicles by PKDs. PKD1 phosphorylates phosphatidylinositol-4-kinase-IIIbeta (10) (PI4KIIIbeta) and ceramide transfer protein (CERT) (11) thereby potentially controlling the release of vesicles by modulating membrane fluidity.

The PKD-ARF1 interaction that we have previously demonstrated (6) physically joins two major regulators of vesicle fission (12,13). Both proteins interact with several other proteins at the TGN. The nature and functional consequences of these interactions are currently unclear. Only class I and II ARFs localize to the Golgi compartment where they have most likely overlapping functions (12,14,15). Class I and II ARFs interact with at least three different types of effectors: COPI (coatamer) (16) or clathrin adaptors (APs) (17,18), lipid-metabolizing enzymes such as PI4K3-beta and Guanosine nucleotide exchange factors (GEF) as well as GTPase activating enzymes (GAPs). Some of these interactions are likely to contribute to the ARF-dependent regulation of vesicle formation, cargo sorting and fission (14). ARF-interacting proteins also include Golgi-localizing gamma-adaptin homology domain proteins (GGAs) (19-21), Bin/Amphiphysin (BAR) domain containing proteins of the Arfaptin subfamily (22-25) was well as PKDs as outlined above (6). Bar-domain proteins/Arfaptins regulate membrane curvature and contribute to budding vesicle formation (26). They have isoform-specific functions in protein transport. Arfaptin1 is a PKD1 substrate. Its phosphorylation at Ser132 by PKD1 releases the protein from the vesicle neck and thereby its inhibitory effect on ARFs allowing granule scission in pancreatic beta cells (22). This is a non-classical function of a BAR-domain protein. PKD1-phosphorylation of Arfaptin1 also regulates its binding to PI(4)-P and trafficking of Chromogranin A in the regulated secretory pathway (27). Arfaptin2, on the other hand, is not a PKD substrate.
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and does not fulfill similar functions (22, 27). It may have more classical Bar-domain protein properties, i.e. stabilizing or actively deforming membranes (28). Arfaptin2 is also localized at the TGN (28) mainly via its interaction with ARF-like GTPases, such as ARL1. Similar to ARFs, ARF-like GTPases control Golgi maintenance and vesicle fission at the TGN (28-31). They also activate ARF1 by recruiting a trans-Golgi specific ARF1-GEF (32).

We are interested in the regulation of constitutive secretion, especially for matrix metalloproteinase (MMP) cargos. Degradation of the extracellular matrix (ECM) by MMPs is a key step during invasion and metastasis of cancer cells (33). MMPs are expressed as inactive pro-enzymes and synthesized with a signal peptide, which is subsequently cleaved during transport through the secretory pathway (34). We have previously shown that constitutive secretion of matripase MMP7 and gelatinase MMP9, which belong to different MMP subfamilies and catalyze proteolysis of different substrates is controlled in a PKD2-dependent manner. Since there are many proteins that regulate constitutive secretion that at least in part interact with either PKD2 and/or ARF1 we here aimed at elucidating the components as well as the formation of a PKD2-ARF1 complex at the TGN in particular for constitutive secretion of MMP cargo.

**Experimental procedures**

**Cell culture**-HEK293T, HeLa, Panc1 and MEFs, PKD2S707A/S711A-MEFs (35,36) were maintained in DMEM media supplemented with 10% FCS and Pen/Strep. HEK293T and HeLa cells were acquired from ATCC. Control MEFs (C57BL/6) and PKD2S707A/S711A-MEFs were generated according to standard protocols (37). Homozygous PKD2S707A/S711A mice (35,36) were kindly provided by D. Cantrell, Dundee, UK. Homozygous PKD2S707A/S711A mice lines were verified by PCR (35). siRNAs were transfected using Oligofectamine or Lipofectamine 2000 (Invitrogen, Darmstadt, Germany). Experiments with ectopically expressed transgenes in HeLa cells were performed using HeLa Monster reagent (Mirus Bio, Madison, USA). HEK293T cells were transfected using PEI (Polysciences Inc., USA).

Plasmids, antibodies and dye-reagents-N-terminal GFP-tagged and non-tagged pcDNA3 expression constructs for PKD1 and PKD2 have been described previously (10,38). Human pcDNA4TO-myc-His-ARL1 was purchased from Biomol (Hamburg, Germany). Human pdEYFP-N1-MMP7 and pdEYFP-N1-Arfaptin2 (NP_001229783 Isoform 1) expression constructs were purchased from Source Bioscience. A siArfaptin2 No1-resistant mutant with silent mutations in the pdEYFP-N1-Arfaptin2 vector was generated by site-directed mutagenesis using the following primers: for: 5'-gtg gcc atc aag ctt aaa ttc ctc gaa gaa aac aag-3' and rev: 5'-ctt gtt ttc ttc gag gaa ttt cag ctt gat ggc cac-3'. Successful mutagenesis was verified by sequencing. Arfaptin2-myc and a bacterial ARF1-His6 expression construct were a gift of Vivek Malhotra (Barcelona, Spain). mRuby, PKD2-mRuby, ARF1-mRuby, PKD2P275G-GFP and pCM6ARF1-myc constructs have been described previously (6). pGEX-4T2-hARL1 and pGEX-6P1-hArfaptin2 was kindly provided by Kazuhisa Nakayama, Kyoto, Japan (28). pGEX-6P1-PKD2 has been described previously (6). Short hairpin RNAs against lacz, PKD1 and PKD2 were described previously (39,40) and purchased from MWG Biotech. Arfaptin2 siRNAs No1 (GCUCAGUUCCUGGAAAGAA) and No2 (GACACGCUCAUGACUGUGA) (27) were also acquired from MWG Biotech (Ebersberg, Germany). ARF1 siRNA has been described in (6) or was purchased from Qiagen (ARF1, SI00299250). ARL1 (SI04282054) siRNA was purchased from Qiagen (Hilden, Germany). Control shRNA and shRNA constructs against PKD2 were purchased from Sigma Aldrich (control shRNA (Mission shRNA, Sigma shc002), PKD2 shRNA (sh PKD2 #1: NM_016457.x-1720s1c1 and sh PKD2 #2:NM_016457.x-294s1c1). TGN46 (AP32690SU-N) antibody was acquired from Acris Antibodies (Herford, Germany). Golgin97 (A-21270) antibody was from Molecular Probes (Invitrogen, Darmstadt, Germany). ARF1 (ab108347), ARL1 (ab76156), MMP14 (ab3644) and Arfaptin2 (ab85106) antibodies were purchased from Abcam. MMP7 antibody (PAB12712) was purchased from Abnova (Taipei City, Taiwan). Anti-Actin AC15 (A5441) and anti-Tubulin (T5168) were from Sigma Aldrich (Sigma Aldrich, St. Louis, USA). Anti-GFP antibody
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PKD2 antibody (ST1042) was obtained from Calbiochem (Merck, Darmstadt, Germany). The MMP2 antibody (#4022) and non-specific normal rabbit IgG control antibody (#2729S) were purchased from Cell Signaling Technology (Frankfurt, Germany). Immunofluorescence secondary antibodies were purchased from Invitrogen (Darmstadt, Germany).

Total cell lysates and co-immunoprecipitation: Total cell lysates and co-immunoprecipitation experiments were performed as described previously (39,41). Following Western transfer quantitative analysis was performed by measuring integrated band density using NIH ImageJ. Values shown represent fold change in respect to controls.

Immunofluorescence confocal microscopy and acceptor-photobleach-FRET: Immunofluorescence and acceptor-photobleach FRET (AB-FRET) stainings were performed as described in (41). HeLa cells were transfected with HeLa Monster on glass cover slips and the next day processed as described in (41). Samples were analyzed by Confocal Laser Scanning Microscopes TCS SP5, SP8 (Leica, Mannheim, Germany) or LSM710 (Zeiss, Jena, Germany) equipped with respective 40x, 63x or 100x Plan Apo oil immersion or water objectives. Images were acquired in sequential scan mode. Gain and offset during scanning were set in a way that all cellular structures were imaged within the linear range of detectors having no saturated pixels and black-level background values. Processing as well as intensity correlation analyses were performed using NIH ImageJ. Quantitative co-localization analysis calculating Pearson’s correlation coefficients was done on black-level background images of cropped Golgi areas as shown in figures, using clearly localized Golgi markers such as TGN46 or ARF1-mRuby as masks for analysis. FRET experiments were performed with transiently transfected HeLa cells fixed, processed and stained as stated previously (41,42). In order to measure the direct interaction of proteins over a population of cells at the time of fixation we have utilized quantitative AB-FRET analysis. This method allows the determination of direct protein-protein interactions indicated by an apparent molecular proximity below 10nm (41,43-49). The FRET acceptor molecules in a region of interest (ROI) are bleached by an intensive laser line and a respective increase in donor fluorescence, indicating FRET, is measured following acceptor depletion. The AB-FRET measurements were carried out by acquiring pre- and post-bleach images of donor and acceptor using an automated time series. As an internal threshold 8-bit images (256 gray values) were used for recording raw data in order to only register robust changes in fluorescence intensities as respective gray value changes in images. FRET experiments were acquired from at least three independent transfections/sources. Image analysis was performed using NIH ImageJ. Quantitative FRET analysis was executed by calculating mean FRET efficiency and SEM of non-thresholded raw data in Sub-ROIs randomly placed as indicated in figure legends [%FRET=((Donor_{post}-Donor_{pre})/Donor_{post})x100]. Statistical significance was calculated using two-tailed unpaired students t-test.

Arfaptin2-YFP fluorescence recovery after photobleaching (FRAP) experiments at the TGN: FRAP experiments with Arfaptin2-YFP at the TGN were acquired using a Leica TCS SP8-HCS confocal microscope equipped with a HCX PL APO 40x/1.10 W motCORR CS2 water immersion objective at 37°C in buffered L15 media without FCS to match secretion conditions. HeLa cells were seeded in glass-bottom dishes (Matek Cooperation, USA) and co-transfected with pcDNA3-PKD1, -PKD2, pcDNA4TO-ARL1-myc as well as pcDNA3-ARF1-myc constructs together with Arfaptin2-YFP. FRAP time-bleach series of TGN-accumulated Arfaptin2-YFP were recorded in Phenol-Red-free media using 90% open pinholes to compensate for dynamic movements of Golgi structures for 30 cells and three independent transfections. Statistical significance was calculated for the mean and SEM of the "relative recovery values" after 60, 120 and 180 seconds. All cells...
were recorded utilizing identical FRAP settings.

**MMP14 surface FACS staining:** Panc1 cells were transduced with lentiviruses expressing two PKD2 shRNAs and a non-specific sh scramble control. Cells were subjected to Puromycin selection (6µg/ml) for 3 weeks. Semi-stable cells were tested for the PKD2 knockdown and used for FACS experiments. Cells (8 x 10^5 cells/6well) were harvested by trypsinization and placed in suspension in buffered L15 full media (10% FCS, 1% PenStrep). In order to retain cargo at the TGN, cells were incubated at 18°C for 4 hours. Subsequently, cells were washed in serum-free L15 media two times and shifted to 37°C to release TGN-retained cargo under serum-free conditions. At T0h and T2h cells were placed on ice and surface MMP14 protein was stained using the following protocol: Cells were centrifuged at 2000rpm for 3' at 4°C and incubated for 30' in 1ml Blocking Buffer on ice (PBS + 10% FCS + 0.1% sodium azide). Antibodies were prepared as master mixes diluted in blocking buffer (MMP14, Isotype control: 1µg antibody/50µl). Cells were incubated in 50µl primary antibody on ice for 1h, washed 2 times in blocking buffer and incubated with secondary antibody (anti-Alexa-488 antibody, 1:400) as well as DAPI for 30' on ice in the dark. Cells were washed two times, filtered through a polyamid membrane (50µm pores) and transferred into FACS tubes. Samples were analyzed on a BD Biosciences LSRII flow cytometer (Laser setup: 405 nm viol et laser, 488 nm). Isotype controls and unstained parental cells were used to set gates for the respective antibodies. Data analysis was performed using FCS Express and Flowing Software 2.5.1 (Perttu Terho, Turku Center for Biotechnology, University of Turku, Finland)

**Purification of ARF1-His6, ARL1-GST, Arfaptin2-GST, GST proteins and GST-pulldown experiments:** Fusion proteins (ARF1-His6, ARL1-GST, Arfaptin2-GST, GST) were expressed in BL21 bacteria (NEB, Frankfurt, Germany) by diluting growth cultures 1:10 followed by induction at OD 600=0.6 with 1mM IPTG (Thermo Scientific, Rockford, USA) for 5h at room temperature. PKD2-GST bacteria were induced at 18°C overnight to reduce formation of inclusion bodies. GST-fusion proteins were purified as described previously (6). Samples were purified either by Glutathione-Sepharose (GE Health Care, Freiburg, Germany) or HisPur™-Ni-NTA purification as stated by the manufacturer (Thermo Scientific, Rockford, USA). After column elution proteins were concentrated while buffer was exchanged against assay buffer using Vivasin2 concentrators (10,000 or 3000 MWCO, Satoriaus Stedim Biotech). Pulldown experiments were performed from HEK293T cell lysates (1mg/assay) using ARL1-GST on beads and respective controls. Lysates were prepared in assay buffer (25mM Tris-HCl, pH 7.5, 150mM Sodium chloride, 5mM Magnesium chloride, 1% NP-40) supplemented with Complete and PhosStop inhibitors (Roche, Munich, Germany). Assays were incubated for 4h and then washed three times with assay buffer. Pulldown assays were resolved on 4-20% Tris-glycine gradient gels (Thermo Scientific, Rockford, USA). Associated proteins were probed in Western blots.

**ARF1-His6 and ARL1-GST in-vitro GTP- and GDP-loading:** ARF1-His6/ARL1-GST (10µg/assay, Fig. 4) and ARL1-GST (20µl ARL1-GST beads/assay, Fig. 6) in assay buffer (25mM Tris-HCl, pH 7.5, 150mM Sodium chloride, 5mM magnesium chloride, 1% NP-40) were batch-loaded for 15 min at 30°C with fresh GTP/GTPgammaS (0.1mM) or GDP (1mM) (Sigma Aldrich, St. Louis, USA) under EDTA-induced low-affinity binding conditions (10mM). Loading was terminated by addition of magnesium chloride (60mM). Loading conditions were verified by ARF1-activity pulldown assays as described below.

**ARF1-activity pulldown assays:** GTP-bound ARF1-His6 was precipitated from different in-vitro loading conditions using an ARF1 activity pull-down and detection kit with 100µg of purified GST-GGA3-PBD protein according to the manufacturer’s description. (Thermo Scientific, Rockford, USA). Pulldowns were resolved on 10% SDS gels and probed with an ARF1-specific antibody.

**Statistical analyses:** Statistical analysis was performed using Prism software version 6.00 for Windows, GraphPad Software, San Diego California USA. Graphs shown depict mean and SEM for all conditions. Statistical significance in
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graphs is indicated by asterisks (ns, non-specific; *, p=0.05 to 0.01; **, p=0.01 to 0.001; ***, p<0.001; ****, p<0.0001).

Results

Role of PKD isoforms in constitutive secretion of MMP-7-PKD isoforms have been identified as critical regulators of the vesicle fission process at the TGN controlling both, constitutive and stimulated secretion processes in cells (6,7,9). Many polarized and non-polarized cells express both PKD1 and PKD2. However, there is data that protein transport may be differentially regulated by these two isoforms (3,48). Therefore, we first examined MMP7-YFP release from HEK293T cells upon depletion of PKD1 or PKD2, respectively (Fig. 1A-C). MMP7-YFP is partially localized at TGN46-/Golgin97-positive TGN structures (Fig. 1A) that are also utilized by PKDs (48). Our data show that MMP7 release was not affected by selective depletion of PKD1, but was significantly inhibited by knockdown of PKD2 (Fig. 1B and C), in line with our previous data for MMP9 (48).

Since constitutive secretion of MMPs from the TGN were predominantly affected by PKD2, we focused on this isoform in our subsequent experiments.

In order to further substantiate the role of active PKD2 in MMP secretion we employed a mouse model with a knock-in mutation of activation loop serines 707/711 to alanines in PKD2 (PKD2SSAA), preventing activation of the endogenous PKD2 isoform (35,36). MMP2 is strongly expressed in fibroblasts (50). We therefore generated MEFs from PKD2SSAA knock-in and genetically unaltered control animals to investigate constitutive MMP2 secretion. Our data revealed that MMP2 secretion by PKD2SSAA-knock-in and genetically unaltered control animals to investigate constitutive MMP2 secretion. Our data revealed that MMP2 secretion by PKD2SSAA-knock-in MEFs was significantly reduced (Fig. 1D and E). The PKD2SSAA modification did not affect the intracellular protein expression of MMP2. Thus, activation of endogenous PKD2 is indeed required for constitutive secretion of endogenous MMP2 cargo.

PKD2 recruits ARL1 to the TGN-PKD2-GFP partially co-localizes with ARF1 and ARL1 at the TGN in HeLa cells. Endogenous PKD2 also partially co-localized with Arfaptin2-YFP at the TGN. As expected, Arfaptin2-YFP co-localized with ARF1 at the TGN (Fig. 2A). Quantitative Foerster energy transfer (FRET) demonstrated a direct interaction of PKD2 and ARF1 (Fig. 2B). We also detected a novel, direct interaction of PKD2 with ARL1. Both interactions occurred in the cytoplasm, but more pronounced at the TGN (Fig. 2B). The interaction of ARF1 with PKD2 at the TGN required the proline residue at position 275 in PKD2, as described previously (8). The novel interaction between ARL1 and PKD2 was independent of the Pro275 residue in PKD2 (Fig. 2C). Interactions between PKD2, ARF1 and ARL1, respectively, were verified by co-immunoprecipitations in HEK293T cells (Fig. 2D and E). Arfaptin2 co-immunoprecipitated with both, ARL1 (Fig. 2F) (28,29) and ARF1 (Fig. 2G) (51) thereby providing another link between PKD2 and ARF1. The specificity of FRET interactions for PKD2 and ARL1 at the TGN was also examined by employing ectopically expressed proteins with fluorescence tags (Fig. 2H). We confirmed the direct interaction of ARL1-myc (anti-myc-Alexa-488) with PKD2-mRuby. There was no binding of mRuby protein to ARL1 (negative control). There was also no direct interaction of ectopically expressed ARL1-myc (anti-myc-Alexa-488) with ARF1-mRuby or of Arfaptin2-myc (anti-myc-Alexa-488) with PKD2-mRuby, respectively. FRET studies with Arfaptin2-myc and ARF1-mRuby as well as Arfaptin2-myc with mRuby protein served as positive and negative controls, respectively (Fig. 2H). In summary, our FRET experiments and biochemical studies suggest the following protein interactions in a novel complex at the TGN: PKD2 interacts with ARL1 and ARF1. The interaction of ARL1 with PKD2 was not affected by the P275G-mutation in PKD2. Therefore, binding of ARF1 and ARL1 are likely to be mediated by different parts of the protein. Moreover, our data show that Arfaptin2 can interact with both ARL1 and ARF1 but there was no detectable direct interaction of ARF1 and ARL1 or of PKD2 and Arfaptin2 at the TGN. Since PKD2 interacts with ARL1 already in the cytoplasm (Fig. 2B) and ARF1 recruits PKD2 to the TGN (6), PKD2 is likely to act as a shuttle for ARL1 from the cytoplasm to the TGN and provides a link between ARL1 and ARF1.
Arfaptin2 is recruited to the TGN by the ARL1-PKD2 complex-ARL1 directly interacts with Arfaptin2 and is thought to be responsible for its Golgi localization (28,29). Therefore, Arfaptin2 could be part of the ARL1-PKD2-ARF1 complex. To investigate the role of ARL1 and PKD2 in Arfaptin2-TGN-localization we performed fluorescence recovery after photobleaching (FRAP) experiments in HeLa cells to monitor Arfaptin2-YFP recovery after its depletion at the TGN in the presence of different interaction partners (Fig. 3A-C). Co-expression of Arfaptin2 with ARL1 or PKD2, respectively, enhanced Arfaptin2-YFP recovery at the TGN significantly and to a similar extent (Fig. 3A and B). The PKD2-P275G-mutant, which is unable to bind ARF1 (Fig. 2C) and does not efficiently localize to the TGN, did not enhance fluorescence recovery. To mimic the formation of the entire complex we co-expressed all previously described members: Arfaptin2-YFP with ARF1, ARL1 and PKD2. Here Arfaptin2 recovery was markedly accelerated compared to all other samples and even significantly enhanced beyond the ARL1 or PKD2 conditions (Fig. 3A and B). A fluorescence-loss in photobleaching (FLIP) analysis demonstrated that concomitantly with the increase in TGN-localized recovery, Arfaptin2 fluorescence intensity was lost in the peripheral areas of cells (Fig. 3C), suggesting directed recruitment to the TGN. In addition to the FRAP experiments, we performed quantitative co-localization studies with Arfaptin2-YFP in HeLa cells at the TGN following depletion of ARL1 or PKD2, respectively. For these experiments we used TGN46 as the most efficient TGN marker, as judged by quantitative intensity correlation analysis with PKD2-GFP (data not shown). Localization of Arfaptin2 at the TGN was significantly reduced upon knockdown of either ARL1 or PKD2 (Fig. 3D and E). We also investigated the interaction of endogenous ARL1 with Arfaptin2-myc at the TGN upon depletion of PKD2 by FRET (at Furin-GFP-positive TGN-structures). In line with our previous experiments, knockdown of PKD2 significantly decreased this interaction (Fig. 3F). Again, there was no direct interaction of PKD2 and Arfaptin2 detectable by FRET (Fig. 3F). Consequently, the cytoplasm/Golgi ratio of Arfaptin2-YFP was significantly increased in the absence of ARL1 (Fig. 3G and H). Knockdown of PKD2 furthermore significantly prevented localization of endogenous ARF1 to the TGN in cytoplasm/Golgi ratio experiments (Fig. 3I and J). Taken together, our data indicate that Arfaptin2 interacts with ARL1 and is recruited to the TGN by an ARL1/ PKD2 complex.

Arfaptin2 is recruited into ARF1 complexes via interaction with ARL1 and PKD2-ARF1 interacts with Arfaptin2 in artificial liposomes and this interaction increases with membrane curvature (51). Since PKD2 indirectly recruits Arfaptin2 to the TGN, we went on to investigate whether the interaction of ARF1 with Arfaptin2 (51) required PKD2 and/or ARL1. In co-immunoprecipitation experiments endogenous Arfaptin2 was present in ARF1-myc complexes (Fig. 4A and B). This interaction was impaired upon knockdown of ARL1 or PKD2, respectively (Fig. 4A and B). These data establish PKD2 as a novel, critical linker between ARL1/Arfaptin2 and ARF1 since its presence was crucial for the assembly of the ARF1-ARL1-Arfaptin2 complex at the TGN. PKD2 is activated at the TGN (9). This raised the question whether catalytic activity of PKD2 would play a role in the recruitment of Arfaptin2 into ARF1 complexes. Constitutively active PKD2-SSEE (6) significantly reduced the interaction of Arfaptin2-YFP with ARF1 in co-immunoprecipitation assays. In contrast, kinase-inactive PKD2-KD (K580W) slightly enhanced Arfaptin2-binding to ARF1 compared to vector controls (Fig. 4C and data not shown). Thus, inactive, rather than active PKD2 is likely to recruit Arfaptin2 into ARF1 complexes at the TGN.

**Arfaptin2, ARL1, PKD2 and ARF1 form a GTP-dependent complex in-vitro** - In order to assess a complex formation by the above described interaction partners, we performed in-vitro binding studies with purified proteins (Fig. 4D-F): ARF1-His6, ARL1-GST, PKD2-GST and Arfaptin2-GST. Active GTPases at the TGN were mimicked by GTP-loading of samples containing ARF1 and ARL1 proteins. Purified proteins were incubated overnight to allow for complex formation. ARF1-His6 was precipitated from samples by an ARF1 antibody and associated complex proteins were probed in Western blots. Fig. 4D indicates that binding of PKD2 to ARF1 immunoprecipitates was...
strongly dependent on GTP-loading. These results are supported by our published data demonstrating enhanced binding of PKD2 to GTP-loaded ARF1 (6). In line with the results described above, the presence of PKD2 in GTP-loaded samples increased the amount of Arfaptin2 in ARF1 immunoprecipitates beyond the level of all other conditions. Since Arfaptin2 preferentially binds to GTP-loaded ARL1 and ARF1, binding was strongly reduced in GDP-loaded samples containing PKD2. In line with these data as well as preferential binding of PKD2 to active ARF1-GTP (6), we also detected ARL1 more prominently in PKD2-containing ARF1-immunoprecipitates loaded with GTP in respect to the GDP-state.

However, ARL1 also co-precipitated in the GTP-loaded control samples omitting PKD2. This may be explained by different binding preferences of complex proteins when PKD2 is not present and a formation of Arfaptin2 dimers that are capable of binding both active ARF1- and ARL1-GTPases. Yet, since Arfaptin2 was most prominently present in GTP-loaded immunoprecipitates containing PKD2, our data suggest that PKD2 indeed is capable of bringing complex proteins together and thereby fosters Arfaptin2 recruitment into ARF1-immunocomplexes (Fig. 4D).

Arfaptin2, ARL1, PKD2 and ARF1 control constitutive secretion of endogenous MMP cargo—Next we examined the functional role of this complex in the secretion of different MMP cargos (48). Arfaptin2 has previously been reported not to play a role in the regulation of constitutive secretion as determined by ssHRP secretion assays (27). Surprisingly, we found that upon knockdown of Arfaptin2 by two different siRNAs MMP7-YFP secretion was significantly inhibited by 62.3% and 66.4%, respectively (Fig. 5A and B). Likewise, knockdown of ARL1, the Arfaptin2 transporter protein, impaired MMP7-YFP secretion by about 54% compared to controls (Fig. 5A and B). The effect on MMP7 secretion was also confirmed by examining endogenous MMP7 secretion. Knockdown of all respective complex proteins: Arfaptin2, ARL1, ARF1 and PKD2 similarly and significantly impaired the secretion of endogenous MMP7 from Panc1 cells by about 65-75% (Fig. 5E and F).

These data demonstrate a functional regulation of constitutive MMP secretion by the above described molecular complex. To substantiate the unexpected and novel function of Arfaptin2 during constitutive secretion of MMPs we further performed knockdown and rescue experiments for another MMP-cargo: MMP2. Like, MMP7, MMP2 also colocalizes with the TGN-markers TGN46 and Golgin97 (Fig. 5G). Upon depletion of Arfaptin2 in Panc1 cells, secretion of endogenous MMP2 was markedly impaired by 75%, whereas a rescue experiment by re-expression of a siRNA-resistant Arfaptin2-YFP construct at endogenous levels almost completely restored MMP2 secretion (Fig. 5H and I). Taken together, Arfaptin2 appears to be a regulator of constitutive MMP secretion from the TGN. Of note, depletion of the respective molecular complex proteins at the TGN equally affected constitutive secretion of the endogenous MMP cargos.

Since PKDs are known regulators of TGN integrity (3) we further wanted to exclude that knockdown of PKD2 would cause a general impairment in all secretory processes from the TGN due to a breakdown in Golgi integrity. In order to verify specificity of phenotypes for constitutive secretion of MMP cargo from cells after PKD2-depletion, we evaluated the abundance of membrane-type matrix-metalloproteinase 14 (MMP14), a cargo that is released from the TGN and transported to the plasma membrane (52), on the surface of Panc1 cells by flow cytometry. Our data indicate that knockdown of PKD2 in Panc1 cells did not change MMP14 protein expression, nor was MMP14 surface staining impaired by PKD2 depletion with two specific shRNAs 2 hours after the release of an 18°C temperature block (Fig. 5J). Thus, depletion of PKD2 strongly affects release of constitutive secretory MMP 2/7/9 cargos from the TGN, whereas is does not impair release of MMP-cargo destined for the plasma membrane, e.g. MMP14.

Regulation of the ARF1-ARL1-Arfaptin2 complex by PKDs is isoform-selective—PKD isoforms differentially regulate secretion of MMP cargo with PKD1 playing only a minor role (Fig. 1B and C) (48). Having established the formation of a protein complex at the TGN by PKD2 required for efficient constitutive secretion we speculated that the PKD isoforms may differ with respect to their complex formation capacity. We performed FRET
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Interaction studies of PKD1 and PKD2 isoforms with ARF1 and ARL1, respectively. These experiments showed that the interaction of PKD1-GFP with ARF1 was significantly weaker (reduced by 85.17%) compared to PKD2-GFP, both at the TGN and in the cytoplasm (Fig. 6A and B). Interestingly, there was no difference in co-localization of PKD1 or PKD2 with both ARF1-mRuby and TGN46, indicating that this reduced interaction of PKD1-GFP with endogenous ARF1 was not due to differences in the subcellular localization (data not shown). The interaction of PKD1-GFP with ARF1 was also weaker (by 36%) compared to the interaction of ARL1 with PKD2-GFP (Fig. 6A and B). Thus, the interaction of ARF1 and ARL1 with PKDs seems to be isoform-selective.

We therefore first performed co-localization studies with PKD1 and -2 isoforms which both co-localized with ARL1 TGN-domains (Fig. 6C). Then, we examined the interaction of ARF1 with PKD1 and -2 using in-vitro GST-pulldown experiments. Purified ARL1-GST was loaded with GTP or GDP nucleotides and subsequently incubated with HEK293T cell lysates equally expressing PKD1 and -2. Indeed, we could detect an isoform-selective preferential interaction with PKD2, whereas binding of PKD1 was significantly impaired by about 55% (Fig. 6D-F). Interaction of ARL1 with PKDs was independent of the GTP-loading state, since there was no difference in binding for GDP- and GTP- conditions (Fig. 6D-F). The GTP-loading state of ARL1 was additionally tested by pulldown experiments using Arfaptin2-YFP expressed in cell lysates (Fig. 6G and H). As expected, Arfaptin2 was precipitated more prominently by ARL1-GTP, although ARL1-GDP also showed residual Arfaptin2 binding (Fig. 6G and H). This is in line with ARL1 pulldown experiments for Arfaptin2 performed by Man et al. 2011 (29), suggesting that Arfaptin2 may also bind to some extent to ARL1 in its GDP-bound state.

If these data were right, PKD1 in comparison to PKD2 should be less able to indirectly recruit Arfaptin2, via ARL1 to the TGN. To test whether PKD2 would more efficiently foster Arfaptin2 recruitment to the TGN, we then performed FRAP experiments. Arfaptin2-YFP recovery at the TGN was only very weakly affected by PKD1 and not significantly different from the vector controls. However, Arfaptin2-YFP recovery was significantly enhanced by PKD2 (Fig. 6I and J). Thus, the complex comprising of ARF1, ARL1 and Arfaptin2 at the TGN is mainly formed when PKD2 is present. The interaction studies with PKD1 and PKD2 isoforms (Fig. 6A-E) further suggest that differences in constitutive secretion of MMP cargo by PKD1 and -2 may be controlled by their respective ability to bind to ARF1 and ARL1 GTPases. Only PKD2 was able to perform all required functions: Recruitment of ARL1-Arfaptin2 to the TGN and into ARF1 complexes. PKD2 is therefore crucial for the coordinated formation of mature transport carriers by this multiprotein complex at the TGN.

In summary, our data show that PKD2 plays a selective and novel dual role during the regulation of constitutive secretion from the TGN. Inactive PKD2 is critical for the formation of a complex comprising of ARF1, ARL1 and Arfaptin2. PKD2 thereby recruits Arfaptin2 to the TGN and into ARF1 complexes via its direct interactions with ARF1 and ARL1 (Fig. 7). Upon its own activation at the TGN, active PKD2 then is involved in processes resulting in enhanced constitutive secretion of cargo, likely by regulating vesicle fission from TGN (1,2,6,10,11).

Discussion

The coordinated regulation of vesicle biogenesis and transport from the TGN to the plasma membrane determines cellular integrity and function and requires considerable fine-tuning. Two major protein families have been identified as regulators of vesicle biogenesis and fission controlling secretion at the TGN: The PKD family and ARF-GTPases. We have demonstrated a physical interaction between PKDs and ARF1. ARF1 recruits PKDs from the cytoplasm to the TGN (6). Here we identify the components of a larger functional protein complex that controls constitutive secretion at the TGN and comprises of PKD2, ARF1, ARL1 and the Arfaptin2 isoform. In this complex, PKD2 constitutes a novel and crucial link between the GTPases ARL1 and ARF1 at the TGN. Via ARL1, PKD2 indirectly recruits Arfaptin2 to the TGN and into ARF1 complexes (Figs. 2-4). Whether Arfaptin2 recruitment is
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facilitated by residual binding of Arfaptin2 to ARL1-GDP in the cytoplasm and shuttling to the TGN, still is not fully understood. However, our in-vitro data (Fig. 6G, H) indicate, in line with pulldown experiments performed by Man et al. (29), that in contrast to Arfaptin1 (29), a considerable amount of Arfaptin2 is also bound to non-active ARL1, and thus could be shuttled by the PKD2-ARL1-GDP complex to the TGN. Such a scenario is also supported by our FRAP and FLIP studies (Fig. 3C and D) demonstrating loss of cytoplasmic fluorescence concomitant with Arfaptin2-YFP recruitment to the TGN.

Furthermore, the precise role of Arfaptin-family Bar-domain proteins in the different secretory pathways and in particular during constitutive secretion is still not completely understood. In Drosophila, Arfaptin null-mutants are viable (53) and Drosophila Arfaptin also does not seem to play a major role the regulation of constitutive secretion (27). On the other hand, Arfaptin2 was shown to actively stabilize and induce membrane curvature (28) and its binding to ARF1 at vesicle budding sites requires ARF1 activity (22,29,51). Thus, Arfaptin2 could have a function for secretion in mammalian cells, but an important role for Arfaptin2 in constitutive secretion has been challenged due to only minor effects in modulating secretion of ectopically expressed constitutive ssHRP and regulated Chromogranin A (CgA) cargos (27). In this study we demonstrate that Arfaptin2 is relevant for constitutive MMP secretion from the TGN. Knockdown of Arfaptin2 significantly and markedly impaired endogenous MMP7 and MMP2 secretion, which could be rescued by re-expression of Arfaptin2 at endogenous levels (Fig. 5). This suggests that Arfaptin2 plays a role in membrane sculpturing during formation of MMP2 and MMP7 containing vesicles. We propose that Arfaptin2, in the complex assembled by PKD2 at the TGN, may have critical functions during vesicle biogenesis at certain cargo subdomains which could be predominantly utilized by secreted MMPs, such as MMP7 and MMP2. The regulation of the ARF1-ARL1-Arfaptin2 complex by PKDs was highly selective for PKD2. PKD1, another PKD isoform that has been shown to control cargo transport, had only a minor effect on MMP7 secretion (Fig. 1A and B) (48) and exhibited only a weak interaction with ARF1 (Fig. 6A and B) as well as ARL1 (Fig. 6A-F). In turn, PKD1 was also not significantly implicated in recruiting Arfaptin2 at the TGN (Fig. 6I and J). Thus, this could explain its limited effects on MMP7 secretion.

We therefore propose a model for the PKD2 assembled complex at the TGN, in which non-active PKD2 binds to ARL1, recruits ARL1 to the TGN and thereby indirectly determines Arfaptin2 recruitment. PKD2-ARL1 also control positioning of Arfaptin2 in ARF1 complexes at the TGN, whereby Arfaptin2 and ARF1 interact at budding sites. Arfaptin2 dimers may then stabilize or enhance membrane deformation (28) to control cargo loading and vesicle formation (Fig. 7). PKD2 is subsequently activated at the TGN by interacting with DAG and/or Gbeta/gamma subunits as well as PKCeta (9). Active PKD2 then generates a suitable lipid environment and facilitates vesicle scission by phosphorylation of substrates, such as PI4K3beta (10).

These data therefore attribute PKD2 a novel role in the assembly of a functional protein complex at the TGN and substantially extend its known functions during vesicle scission. Remarkably, these processes are mediated by direct binding of PKD2 to ARF1 and ARL1 and not by phosphorylation of substrates. Data obtained in MEFs from mice deficient in PKD2 enzymatic activity (PKD2S707A/S711A knock-in mutation (35,36)) further confirmed that the active kinase is indeed required for proper secretion of endogenous MMP2 cargo (Fig. 1F and G), a function that has been also recently challenged (54).

Thus, the PKD2-ARF1 protein complex is a critical dual regulator of vesicle formation and scission at the TGN during constitutive secretory processes.

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Competing Interest
The authors declare no conflict of interest.

Author contributions
TE and TS conceived the study. TE, CW, CK and AI performed experiments and evaluated data. TE and TS wrote the manuscript.

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Figure legends

Figure 1: Constitutive secretion of MMP cargo is controlled by PKD2 in an isoform-selective manner. A, Panels A'–F', Co-localization of ectopically expressed MMP7-YFP with TGN46- and Golgin97-positive structures in HeLa cells. Images depict single confocal sections. Arrowheads indicate areas of co-localization. B, Knockdown of PKD2 significantly impairs secretion of MMP7-YFP from HEK293T cells after 24 hours, whereas PKD1 shows no effect. Cells were treated with siRNAs. After 24h cells were transfected with MMP7-YFP cargo. Secreted MMP7-YFP was probed in Western blots of equal amounts of FCS-free cell culture supernatants after 24h. The graph depicts relative fold integrated band density for MMP7-YFP levels in supernatants from three independent experiments. Integrated band density was quantified using NIH ImageJ. Statistical significance was calculated using two-tailed paired student's t-test. C, Control blots for the experiment shown in B. The MMP7-YFP levels in supernatants were probed with an anti-MMP7 antibody. PKD1 and PKD2 knockdowns were verified by specific antibodies. MMP7-YFP expression was probed by anti-GFP. D, Role of PKD2 during constitutive secretion of endogenous MMP2 cargo. Genetic inactivation of endogenous PKD2 in MEFS generated from homozygous PRKD2<sup>5707A/S711A</sup> knock-in mice impairs secretion of endogenous MMP2. Control- and PKDSSAA-MEFS were seeded in 6-well plates at a density of 400.000 cells/well. Secreted MMP2 was probed in Western blots of equal amounts of FCS-free cell culture supernatants after 24h. The graph depicts the statistical analysis of relative fold integrated band density for endogenous MMP2 in supernatants of respective experimental conditions from 28 experiments. Integrated band density was quantified using NIH ImageJ. Statistical significance was calculated from 25 experiments using two-tailed paired student's t-test following elimination of outliers by the method of Rout (Graph Pad Prism 6.0; Q=5%). E, One experiment quantified in D. MMP2 levels in MEF supernatants and total cell lysates were probed by anti-MMP2 antibody. Genetic inactivation of PKD2 was verified by specific antibodies against the PKDS916-autophosphorylation site. Actin was used as a loading control.

Figure 2: PKD2 co-localizes and interacts with ARF1 and ARL1. A, Panels A'–D', partial co-localization of PKD2-GFP with endogenous ARF1 and the trans-Golgi marker Golgin97. Panels E'–H', partial co-localization of PKD2-GFP with endogenous ARL1 and TGN46. Panels I'–L', partial co-localization of Arfaptin2-YFP with endogenous PKD2 and Golgin97. Panels M'–P', partial co-localization of Arfaptin2-YFP with ARF1-mRuby
PKD2 assembles a secretory complex at the TGN

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and Golgin97. Images depict single confocal airy sections. Scale bar: 10 µm. Arrowheads indicate areas of co-localization. B, Interaction of PKD2-GFP with endogenous ARF1 and ARL1. Quantitative AB-FRET analysis for the interaction of PKD2-GFP with ARL1 and ARF1 probed by anti-ARF1/ARL1 and Alexa-568 secondary antibodies. Specificity control: Interaction of GFP with ARF1/ARL1-Alexa-568. The % FRET values were calculated from one equally sized Sub-ROI placed at the TGN and in the perinuclear cytoplasm within the bleached area for each cell. The scatter graph depicts single FRET and mean percent FRET values as well as SEM for N=15 cells and three independent experiments. Cells displaying no FRET in the bleach ROI were set to 0% during analysis. Statistical significance test during FRET studies: Two-tailed unpaired student’s t-test. Values on scatter graphs indicate mean % FRET values and SEM for all ROIs evaluated. C, The P275G mutation abrogates the interaction with ARF1 but not ARL1. Quantitative AB-FRET analysis for the interaction of PKD2- and PKD2P275G-GFP with endogenous ARF1/ARL1 probed with Alexa-546 secondary antibodies. The scatter graph depicts single FRET and mean % FRET values as well as SEM for N=15 cells and three independent experiments. Cells displaying no FRET in the bleach ROI were set to 0% during analysis. D, PKD2 and ARL1 interact. Co-immunoprecipitation experiment (CoIP) of ARL1- myc with PKD2. HEK293T cells were co-transfected with ARL1-myc and pCDNA3-PKD2. ARL1-myc was precipitated from cell lysates and immunocomplexes were probed for the presence of PKD2 using an anti-PKD2 antibody. Immunocomplexes were re-probed for ARL1-myc expression. E, PKD2 and ARF1 interact. Co-immunoprecipitation experiment of ARF1-myc with pcDNA3-PKD2. HEK293T cells were co-transfected with ARF1-myc and pcDNA3-PKD2. ARF1-myc was precipitated from cell lysates and immunocomplexes were probed for the presence of PKD2 using an anti-PKD2 antibody. Immunocomplexes were re-probed for ARF1-myc expression. F, ARL1 and Arfaptin2 interact. Co-immunoprecipitation experiment of ARL1-myc with Arfaptin2-YFP. HEK293T cells were co-transfected with ARL1-myc and Arfaptin2-YFP. ARL1-myc was precipitated from cell lysates and immunocomplexes were probed for the presence of Arfaptin2-YFP. G, ARF1 and Arfaptin2 interact. Co-immunoprecipitation experiment of ARF1-HA with Arfaptin2-myc. HEK293T cells were co-transfected with Arfaptin2-myc and ARF1-HA. Arfaptin2-myc was precipitated from cell lysates and immunocomplexes were probed for the presence of ARF1-HA using an anti-HA antibody. Immunocomplexes were re-probed for Arfaptin2 expression by an anti-myc antibody. One representative experiment of four is shown for all CoIP studies in D-G. H, Interaction of ectopically expressed PKD2 and ARL1 at the TGN to verify specificity of the FRET approach. Quantitative AB-FRET analysis for the interaction of ARL1-myc probed by anti-myc and Alexa-488 secondary antibodies with PKD2-mRuby. Specificity controls: Interaction of ARL1-myc-Alexa-488 with mRuby. Interaction of ARL1-myc-Alexa-488 with ARF1-mRuby. Additional binding studies: Interaction of Arfaptin2-myc-Alexa-488 with PKD2-mRuby as well as mRuby (negative control) or ARF-1-mRuby (positive controls). The % FRET values were calculated from one equally sized Sub-ROI placed at the TGN within the bleached area for each cell. The scatter graph depicts single FRET and mean % FRET values as well as SEM for N=15 cells and three independent experiments. Cells displaying no FRET in the bleach ROI were set to 0% during analysis. Statistical significance test during FRET studies: Two-tailed unpaired student’s t-test. Values on scatter graphs indicate mean % FRET values and SEM for all ROIs evaluated.

Figure 3: PKD2 and ARL1 recruit Arfaptin2 to the TGN. A, Arfaptin2 TGN-localization is determined by ARL1, PKD2 and ARF1. Fluorescence recovery after photobleaching (FRAP) experiments with Arfaptin2-YFP at the TGN of HeLa cells to study Arfaptin2 recruitment dynamics. Cells were additionally co-transfected with ARL1, PKD2, PKD2P275G and a combination of ARF1, ARL1 as well as PKD2. The graph displays the mean relative recovery with SEM of Arfaptin2-YFP fluorescence at the TGN over time after bleaching. Analysis was performed for 30 cells and three independent experiments per condition. B, Statistical analysis of the differences in fluorescence recovery in different conditions at time points T60, T120, T180 seconds post bleaching.
Recovery kinetics of 30 cells from three independent transfections shown in A were used for analysis. Statistical significance was calculated using two-tailed unpaired student's t-test. The graph depicts FRAP and fluorescence-recovery in photobleaching (FLIP) kinetics for one cell from A. FLIP ROIs were placed in peripheral regions of the respective cells. D, PKD2 and ARL1 determine TGN-localization of Arfaptin2. Example images for a co-localization of Arfaptin2-YFP with TGN46 following treatment of cells with lacz control (A'-C'), PKD2 (D'-F') and ARL1 (G'-I') siRNAs. Cropped images used for quantitative intensity correlation analysis are shown in white frames. Arrowheads indicate areas of co-localization. E, Quantitative analysis of co-localizations from D for N=30 cells and three independent experiments. The graph depicts the mean Pearson's coefficient and SEM for co-localization of Arfaptin2-YFP with TGN46. Intensity correlation analysis was performed using NIH ImageJ ICA-Plugin. Specific knockdown of ARL1 and PKD2 was verified in Western blots on the right-hand side. F, Interaction of ARL1 and Arfaptin2 at the TGN is dependent on PKD2. Quantitative AB-FRET analysis for the interaction of Arfaptin2-myc-Alexa-568 with endogenous ARL1-Alexa-647 following knockdown of PKD2. The scatter graph depicts single FRET and mean % FRET values as well as SEM for N=25 cells and three independent experiments at the TGN. Furin-GFP was used as TGN-marker for placement of two Golgi-Sub-ROIs. Cells displaying no FRET in the bleach ROI were set to 0% during analysis. As an additional control FRET analysis was also performed for PKD2-GFP and Arfaptin2-myc-Alexa-568 in 25 cells. No interaction was detectable. G, ARL1 determines Arfaptin2 TGN-localization. Quantitative analysis of Arfaptin2-YFP cytoplasm/Golgi distribution in HeLa cells. TGN46 was used as additional marker for Arfaptin2-YFP-positive TGN structures. Panel A'-D', exemplary cells used for quantitative analysis following treatment with lacz control (A', B') and ARL1 (C', D') siRNAs. Four equally sized and randomly chosen ROIs for cytoplasm and Golgi structures used for mean of ROI analyses are shown in images. H, Statistical analysis of Arfaptin2-YFP cytoplasm/Golgi distribution. The graph depicts mean cytoplasm/Golgi ratios and SEM for N=15 cells and two independent experiments. Ratios were calculated from the mean of four cytoplasm and Golgi ROIs. I, PKD2 determines TGN-localization of ARL1. Quantitative analysis of the endogenous ARL1 cytoplasm/Golgi distribution in HeLa cells following knockdown of PKD2. Panels A'-D', cytoplasm/Golgi distribution of ARL1 following treatment with lacz control siRNA. Panels E'-H', cytoplasm/Golgi distribution of ARL1 following treatment with PKD2 siRNA. Four equally sized and randomly chosen ROIs (images C', D', G' and H') for cytoplasm and ARL1-positive TGN-structures as indicated by the additional marker BIG1 were used for mean of ROI analyses. J, Statistical analysis of endogenous ARL1 cytoplasm/Golgi distribution from I. The graph depicts mean cytoplasm/Golgi ratios and SEM for N=15 cells and 2 independent slides. All images shown represent single confocal sections. Scale bar: 10µm. Statistical significance during co-localization and FRET analysis was calculated by two-tailed unpaired student’s t-test.
following expression of vector, constitutively active PKD2SSEE and kinase-inactive PKD2KD (K580W) in HEK293T cells. Co-precipitated Arfaptin2-YFP was quantified by calculating the integrated density from blots using NIH ImageJ. Statistical significance was calculated using two-tailed paired student's t-test. D, ARF1, ARL1, PKD2 and Arfaptin2 form complexes in-vitro. Purified ARF1-His6, ARL1-GST, PKD2-GST, Arfaptin2-GST and GST proteins, 10µg each, were mixed as indicated and GTPases were loaded with GTP or GDP in a final volume of 100µl as described in the experimental procedures section. Proteins were incubated overnight to allow for complex formation and ARF1 was immunoprecipitated from samples. Associated complex proteins in different conditions were probed with specific antibodies in Western blots. One of three experiments is shown. E, Purified input proteins were verified by probing GST-fusion proteins in Western blots. A Coomassie-stained 4-20% Tris-glycine gradient gel demonstrating the input of proteins used in D, E is displayed on the right-hand side. Relevant bands are marked by red asterisks. F, Verification of GTP/GDP-loading conditions by ARF1-GTP pulldown assays. ARF1-His6 (2µg) was loaded with GTP, GTPgammaS and GDP as indicated in experimental procedures and GTP-bound ARF1-His6 was precipitated using an ARF1 activity pull-down and detection kit by 100µg of purified GST-GGA3-PBD protein. Pulldowns were resolved on 10% SDS gels and probed in Western blots with an ARF1-specific antibody.

Figure 5: Arfaptin2 controls constitutive secretion of endogenous MMP7 and MMP2 cargo. A, Knockdown of Arfaptin2 with two different siRNAs impairs secretion of MMP7-YFP from HEK293T cells. Cells were treated with siRNAs. After 24h cells were transfected with MMP7-YFP cargo. Secreted MMP7-YFP was probed in Western blots of equal amounts of FCS-free cell culture supernatants after 24h. The graph depicts the statistical analysis of relative fold integrated band density for MMP7-YFP levels in supernatants of respective experimental conditions from three independent experiments. An example is shown on top of the graph. MMP7-YFP levels in supernatants were probed with an anti-MMP7 antibody. B, Representative transgene expression and knockdown controls for the MMP7-YFP secretion assay displayed in A. C, Knockdown of ARL1 impairs secretion of MMP7-YFP from HEK293T cells. The cells were treated with siRNAs, supernatants were collected and subsequently analyzed as indicated in A. The graph depicts the statistical analysis of relative fold integrated band density for MMP7-YFP levels in supernatants of respective experimental conditions from three independent experiments. An example is shown on top of the graph. D, Transgene expression and knockdown controls for the MMP7-YFP secretion assays displayed in C. E, Knockdown of Arfaptin2, PKD2, ARF1 and ARL1 significantly impairs secretion of endogenous MMP7 from Panc1 cells after 24 hours. Panc1 cells were seeded at a density of 300.000 cells/well and transfected with the indicated siRNAs. After 36h standard growth media was replaced with FCS-free media and supernatants were harvested after 24h. The graph depicts relative fold integrated band density for endogenous MMP7 in supernatants from four independent experiments. A blot depicting MMP7 secretion into supernatants is placed on top of the graph. F, Control blots for the experiment depicted in E. blots display Arfaptin2, PKD2, ARF1 and ARL1 knockdowns as well as MMP7 expression in total cell lysates. G, Panels A’-D’, Co-localization of ectopically expressed MMP2-GFP with TGN46- and Golgin97-positive structures. Images depict single confocal sections. Arrowheads indicate areas of co-localization. The scale bars represent 10µm. H, Knockdown of Arfaptin2 significantly impair secretion of endogenous MMP2 cargo from Panc1 cells. Ectopic expression of Arfaptin2-YFP rescues impaired secretion of MMP2 following depletion of endogenous Arfaptin2. Panc1 were seeded at a density of 200.000 cells/well and transfected with the indicated siRNAs. After 24h silacz (controls) and Arfaptin2 knockdown samples were transfected with vector or siRNA-resistant Arfaptin2-YFP to rescue depletion of Arfaptin2 with nearly endogenous ectopic expression levels. After 36h standard growth media was replaced with FCS-free media and supernatants were harvested after 14h. A blot depicting MMP2 secretion into supernatants is placed on top of the graph. The graph depicts relative fold integrated band density for endogenous MMP2 in supernatants of four independent experiments. I, Knockdown and re-expression
controls for Arfaptin2 as well as MMP2 expression in lysates shown in H. Integrated band densities in all Western blot experiments were quantified using NIH ImageJ. Statistical significance was calculated using two-tailed paired student’s t-test. J, Membrane-destined MMP14 cargo release from the TGN following depletion of PKD2. Membrane-type surface MMP14 was probed on stable Panc1 cells transduced with lentiviruses expressing two PKD2 shRNAs and a non-specific sh scramble control. Panc1 cells were harvested by trypsination and incubated at 18°C for 4h in buffered L15 media (10% FCS, 1% PenStrep) to retain cargo at the TGN. Subsequently, cells were switched to 37°C (in serum-free L15 media) to release the Golgi block and surface MMP14 was detected at time points T0 and T2 hours after release by flow cytometry with a suitable MMP14 antibody on living cells. Flow cytometry was performed using a BD Biosciences LSRII cytometer (Laser setup: 405 nm, 488 nm) after gating for singlets and living DAPI-negative cell populations. Unstained parental cells and isotype controls for the MMP14 antibody were used to set gates accordingly. Overlay histograms indicate unstained parental cells (grey) as well as MMP14 and isotype controls for the different conditions. Corrected percent values indicate percent MMP14-positive cells minus percent isotype for the indicated conditions. One of three experiments (10,000 cells/condition) is shown for T0 and T2 hour time points. Data analysis was performed and FACS-blots were generated using Flowing Software 2.5.1.

**Figure 6:** Regulation of the ARF1-ARL1-Arfaptin2 complex by PKDs is isoform selective. A, PKD2 interacts with ARF1 and ARL1 whereas interaction of PKD1 with ARF1 is strongly impaired. Quantitative AB-FRET analysis for the interaction of PKD1- and PKD2-GFP with endogenous ARF1 and ARL1 stained with Alexa-546 antibodies. The scatter graph depicts single FRET and mean % FRET values for N=15 cells and three independent experiments. Data for the PKD2 interaction with ARF1 and ARL1 is partially shown in Fig. 2C. B, Sub-ROI analysis (TGN/cytoplasmic area) of FRET experiments in A. The scatter graph depicts single FRET and mean % FRET values of a Sub-ROI analysis at the TGN and in perinuclear cytoplasmic areas within the bleached ROI. Cells displaying no FRET in the bleach-ROI were set to 0% during analysis. C, PKD1 and -2 partially colocalize with endogenous ARL1 at the TGN46-positive structures. Arrowheads indicate colocalization. Scale: 10µm. D, PKD2 preferentially interacts with ARL1. Pulldown assays with ARL1-GST loaded with GTP or GDP as indicated in experimental procedures. Loaded ARL1-GST on beads was incubated for 4h at 4°C with HEK293T cell lysate (1mg/assay) equally expressing PKD1 or PKD2. Pulldown assays were resolved by Western blots and co-precipitated PKDs were detected using an anti-PKD antibody. The graph depicts the statistical analysis of three ARL1-GST pulldown experiments. The amount of co-precipitated PKDs was quantified by calculating integrated density from Western blot bands using NIH ImageJ. Numbers on bar graphs indicate normalized mean and SEM values. Intensities were normalized to the ARL1-GDP-PKD2 pulldown condition. Statistical significance test: two-tailed paired student’s t-test. E, Representative pulldown experiment from D. GST-beads and empty Glutathione-Sepharose beads were used as controls. A Ponceau stain of the blot membrane shows ARL1-GST levels in pulldown experiments. F, Expression of PKD1 and 2 isoforms in HEK293T lysates used in ARL1-GST pulldown experiments in E. G, Control for GDP-/GTP-loading in D. Loaded ARL1 was used to precipitate Arfaptin2-YFP from HEK293T cell lysates. GDP- and GTP-dependent binding of Arfaptin2-YFP to ARL1-GST was probed in Western blots. GST-beads and empty Glutathione-Sepharose beads were used as controls. Ns, non-specific antibody bands. A Ponceau stain of the blot membrane shows ARL1-GST levels in pulldown experiments. H, Expression of Arfaptin2-YFP and YFP (control) in HEK293T lysates used in ARL1-GST pulldown experiments in G. I, PKD2 and not PKD1 efficiently determines Arfaptin2 recruitment to the TGN. Fluorescence recovery after photobleaching (FRAP) experiments with Arfaptin2-YFP at the TGN of HeLa cells. Cells were also co-transfected with pcDNA3-PKD2 or -PKD1. The graph display the mean relative recovery with SEM of Arfaptin2-YFP fluorescence at TGN over time after bleaching. Analysis was performed for 30 cells and three independent experiments per condition. Data for vector and PKD2 co-transfected samples is also shown in Fig.
3A and B. J, The graph depicts the statistical analysis of Arfaptin2-YFP recovery calculated for the 120 second time point. Statistical significance test: Two-tailed unpaired student’s t-test.

**Figure 7: Model for ARF1-PKD2-ARL1 and Arfaptin2 complex interactions controlling constitutive secretion.** A. Proposed complex formation. Non-active PKD2 binds to ARL1, recruits ARL1 to the TGN and thereby indirectly determines Arfaptin2 recruitment via ARL1. ARF1 shuttles PKD2 to the TGN (6). B. TGN complex: PKD2-ARL1 also control positioning of Arfaptin2 in ARF1 complexes at the TGN, whereby Arfaptin2 and ARF1 interact at budding sites. Arfaptin2 dimers may then stabilize or enhance membrane deformation (28) to facilitate cargo loading and vesicle formation. PKD2 is subsequently activated at the TGN by interacting with DAG and/or Gbeta/gamma subunits as well as PKCeta (9) to generate a suitable lipid environment for vesicle scission by phosphorylation of substrates, such as PI4K3beta (10).
A

MMP7-YFP

TGN46

Golgin07

MMP7-YFP

B

MMP7-YFP

Supernatant: anti-MMP7

Control blots:

anti-MMP7

anti-GFP

C

MMP7-YFP

Control blots:

anti-PKD1

anti-PKD2

anti-GFP

anti-Actin

D

Relative fold integrated density MMP7-YFP (Supernatant)

N = 25

-0.6195 ± 0.06117

****

N = 25

E

Control blots:

anti-MMP2

anti-Actin

anti-pPKDS916 (autophosphorylation)

anti-Actin
**Figure 2**

- **Panel A**
  - Images A' to D' showing fluorescent markers and their respective merges.
  - Markers include PKD2-GFP, ARF1-568, Golgin97-647, and Merge.

- **Panel B**
  - Graph with data points and error bars indicating statistical analysis.
  - Data points labeled with significance levels (***, **, *, *) and sample sizes (N = 15).
  - Percent FRET values: 1.334 ± 0.5557 (N = 15) and 2.624 ± 0.7865 (N = 15).

- **Legend**
  - GFP/ARF1-568 (Cytoplasm)
  - GFP/ARF1-568 (Golgi)
  - PKD2-GFP/ARL1-568 (Cytoplasm)
  - PKD2-GFP/ARL1-568 (Golgi)
Figure 2

C

\[
\begin{align*}
N & = 15 \\
\frac{1.014 \pm 0.3647}{N = 15} & = \text{NS}
\end{align*}
\]

D

\[
\begin{align*}
105 \text{ kDa} & \quad \text{PKD2} \\
20 \text{ kDa} & \quad \text{ARL1-myc}
\end{align*}
\]

Control blots:

\[
\begin{align*}
\text{PKD2} & \quad 105 \text{ kDa} \\
\text{Actin} & \quad 43 \text{ kDa}
\end{align*}
\]

E

\[
\begin{align*}
105 \text{ kDa} & \quad \text{PKD2} \\
19 \text{ kDa} & \quad \text{ARF1-HA}
\end{align*}
\]

Control blots:

\[
\begin{align*}
\text{PKD2} & \quad 105 \text{ kDa} \\
\text{Actin} & \quad 43 \text{ kDa}
\end{align*}
\]

F

\[
\begin{align*}
75 \text{ kDa} & \quad \text{Arfaptin2-YFP} \\
20 \text{ kDa} & \quad \text{IgG}
\end{align*}
\]

Control blots:

\[
\begin{align*}
\text{Arfaptin2-YFP} & \quad 75 \text{ kDa} \\
\text{anti-PKD2} & \quad 43 \text{ kDa}
\end{align*}
\]

G

\[
\begin{align*}
19 \text{ kDa} & \quad \text{ARF1-HA} \\
46 \text{ kDa} & \quad \text{IgG}
\end{align*}
\]

Control blots:

\[
\begin{align*}
\text{ARF1-HA} & \quad 19 \text{ kDa} \\
\text{anti-HA} & \quad 43 \text{ kDa}
\end{align*}
\]

H

\[
\begin{align*}
1.644 \pm 0.5085 & \quad \text{NS} \\
1.041 \pm 0.3647 & \quad \text{NS}
\end{align*}
\]
Figure 3

A

![Graph showing the relative normalized Arfapin2-YFP intensity during recovery over time for different conditions.]

- **FLIP**: ARF1/ARL1/PKD2
- **ARL1**: ARF1/ARL1/PKD2
- **PKD2**: PKD2
- **Vector**: Vector
- **PKD2P275G**: PKD2P275G

Time [s]

0 25 50 75 100 125 150 175 200 225

N=30 cells

B

![Bar graph showing the relative normalized Arfapin2-YFP intensity during recovery at different time points for different conditions.]

- **T60s**: T60s
- **T120s**: T120s
- **T180s**: T180s

C

![Graph showing the relative normalized Arfapin2-YFP intensity during recovery over time for different conditions.]

- **FLIP**: ARF1/ARL1/PKD2
- **ARL1**: ARF1/ARL1/PKD2
- **PKD2**: PKD2
- **Vector**: Vector
- **PKD2P275G**: PKD2P275G

Time [s]

0 25 50 75 100 125 150 175 200 225

by guest on March 24, 2020
http://www.jbc.org/Downloaded from
Figure 3

D

A' silacz Arfaptin2-YFP TGN46
B' Arfaptin2-YFP TGN46
C' Merge

D' siPKD2 Arfaptin2-YFP TGN46
E' Merge

F

TGN marker: Furin-GFP

G

A' Ratio ROIs
Arfaptin2-YFP silacz
B' TGN46 silacz
C' Ratio ROIs
Arfaptin2-YFP siARL1
D' TGN46 siARL1

H

Ratio mean of ROI
Cytosol/Golgi of Arfaptin2

silacz 0.2119 ± 0.01307 N=15
siARL1 0.2769 ± 0.02283 N=15

%FRET

silacz/ Arfaptin2-myc-568/ARL1-647 (TGN)
1.950 ± 0.3510 N=50

siPKD2/ Arfaptin2-myc-568/ARL1-647 (TGN)
3.083 ± 0.3327 N=50
Figure 3

**I**

A' Big1

B' silacZ

C' Merge Ratio ROIs

D' Ratio ROIs ARL1

**J**

E' Big1

F' siPKD2

G' Merge Ratio ROIs

H' Ratio ROIs ARL1

### J

|               | Cyto/Golgi |
|---------------|------------|
| silacZ        | 0.3729 ± 0.02098 (N = 15) |
| siPKD2        | 0.5181 ± 0.02167 (N = 15) |

**J**

Significance: ****

0.3729 ± 0.02098

0.5181 ± 0.02167

N = 15
Figure 4

A

| ARF1-myc | siBaz | siBaz, siPKD2 | siPKD2 | siARL1 |
|----------|-------|---------------|--------|--------|
| ~40 kDa  | -     | +             | +      | +      |
| 19 kDa   | -     | +             | -      | +      |
| IP: anti-myc, probe: anti-Arfaptin2 |
| Arfaptin2 |

B

Fold integrated density of Arfaptin2 CoIP band

Control blots:

105 kDa anti-PKD2
19 kDa anti-ARL1
43 kDa anti-Arfaptin2

19 kDa anti-Actin

C

Fold integrated density of Arfaptin2 CoIP band

D

GST  +  -  -  -
ARF1-His6  +  +  +  +
ARL1-GST  +  +  +  +
Arfaptin2-GST  +  +  +  +
PKD2-GST  -  +  +  +

IP: ARF1 Beads

130 kDa anti-PKD2
67 kDa anti-Arfaptin2
47 kDa anti-ARL1
19 kDa anti-ARF1

E

Loading control:

19 kDa ARF1-His6
55 kDa GST-GGA3 PBD

Fold integrated density of Arfaptin2 CoIP band

0.6093 ± 0.08107 N = 4
0.610 ± 0.05773 N = 4

0.5318 ± 0.1081 N = 6
1.251 ± 0.3471 N = 6

ns

* **
Figure 5

A

Supernatant: anti-MMP7

55 kDa

N = 3

0.3771 ± 0.1367

N = 3 0.3360 ± 0.07407

* -62.3%

-66.4%

secreted MMP7-YFP

MMP7-YFP

B

Control blots:

MMP7-YFP

40 kDa Arfaptin2

anti-Arfaptin2

55 kDa MMP7-YFP

43 kDa anti-GFP

anti-Arl

Actin

C

Supernatant: anti-MMP7

55 kDa

N = 3

0.4627 ± 0.09691

N = 3 0.4627 ± 0.09691

* -53.7%

-53.7%

secreted MMP7-YFP

MMP7-YFP

D

Control blots:

MMP7-YFP

19 kDa Arl1

anti-Arl1

55 kDa MMP7-YFP

43 kDa anti-GFP

anti-Actin

Actin
Figure 5

**E**

![Graph showing relative fold integrated density of endogenous MMP7 in supernatant](image)

**F**

![Control blots with various antibodies](image)

**G**

![Immunofluorescence images](image)

**H**

![Bar graph showing relative fold integrated density of endogenous MMP2 in supernatant](image)

**I**

![Control blots with various antibodies](image)
Figure 5

J

Panc1 sh scramble

Panc1 sh PKD2 No1

Panc1 sh PKD2 No2

Panc1 sh scramble

Panc1 sh PKD2 No1

Panc1 sh PKD2 No2

T0 h

after release

counts

0.16% 0.22%

parental isotype MMP14

T2 h

after release

counts

9.48% 52.68%

corrected: 43.2%

0.38% 0.25%

corrected: 47.94%

0.22% 0.19%

corrected: 52.19%

105 kDa

anti-PKD2

PKD2

58 kDa

anti-MMP14

MMP14

43 kDa

anti-Actin

Actin

Panc1 sh scramble

Panc1 sh PKD2 No1

Panc1 sh PKD2 No2

Alexa Fluor 488-A

(surface MMP14)

Alexa Fluor 488-A

(surface MMP14)

Alexa Fluor 488-A

(surface MMP14)
Figure 6

A

C

B

D

-85.17%

PKD2-GFP/ARL1-546
PKD1-GFP/ARL1-546
PKD2-GFP/ARL1-546
PKD1-GFP/ARL1-546

2.243 ± 0.4547
N = 15

0.6067 ± 0.3473
N = 15

4.090 ± 0.9258
N = 15

0.6067 ± 0.3473
N = 15

1.428 ± 0.4474
N = 15

1.424 ± 0.6105
N = 15

% FRET

8.68 ± 2.418
N = 15

1.841 ± 0.7916
N = 15

6.725 ± 2.040
N = 15

1.424 ± 0.6105
N = 15

% FRET

8.68 ± 2.418
N = 15

1.841 ± 0.7916
N = 15

6.725 ± 2.040
N = 15

1.424 ± 0.6105
N = 15

PKD2-GFP/ARL1-588 (Golgi)
PKD1-GFP/ARL1-588 (Golgi)
PKD2-GFP/ARL1-588 (Golgi)
PKD1-GFP/ARL1-588 (Golgi)

1 N=3

1.136 ± 0.1704
N = 3

1 N=3

0.4556 ± 0.0221
N = 3

0.442 ± 0.0993
N = 3

fold integrated density of PKD1 and PKD2 in ARL1-651 pulldown assays (N=3)

PKD2 (ARL1-GTP)
PKD2 (ARL1-GDP)
PKD1 (ARL1-GDP)
PKD1 (ARL1-GTP)

PKD2 (ARL1-GTP)
PKD2 (ARL1-GDP)
PKD1 (ARL1-GDP)
PKD1 (ARL1-GTP)
Figure 6
Figure 7

(A) Complex formation

\[ \text{ARL1-PKD2 determine Arfaptin2 localization at the TGN and into active ARF1 complexes} \]

complex TGN-localization (ARF1-PKD2, Ref. 6)

(B) TGN complex

constitutive secretion of MMPs

Putative function of Arfaptin2: curvature sensing and stabilization during carrier formation (Ref. 28, 50)

Functional complex protein interactions at the TGN

interaction facilitates interaction
Protein kinase D2 assembles a multiprotein complex at the Trans-Golgi-network to regulate matrix metalloproteinase secretion
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