Elevated Protein Kinase D3 (PKD3) Expression Supports Proliferation of Triple-negative Breast Cancer Cells and Contributes to mTORC1-S6K1 Pathway Activation*

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Bettina Huck1, Stephan Duss5, Angelika Hauser3, and Monilola A. Olayioye1,2

From the 1University of Stuttgart, Institute of Cell Biology and Immunology, Allmandring 31, 70569 Stuttgart, Germany and 2Friedrich Miescher Institute of Medical Research, Maulbeerstrasse 66, 4058 Basel, Switzerland

Here, we show that the expression of the Golgi-localized serine-threonine kinase protein kinase D3 (PKD3) is elevated in triple-negative breast cancer (TNBC). Using an antibody array, we identified PKD3 to trigger the activation of S6 kinase 1 (S6K1), a main downstream target of the mammalian target of rapamycin complex 1 (mTORC1) signaling pathway. Accordingly, PKD3 knockdown in TNBC cells led to reduced S6K1 phosphorylation, which was associated with impaired activation of mTORC1 at endolysosomal membranes, the accumulation of the mannose 6-phosphate receptor in and the recruitment of the autophagy marker light chain 3 to enlarged acidic vesicles. We further show that PKD3 depletion strongly inhibited cell spreading and proliferation of TNBC cells, identifying this kinase as a potential novel molecular therapeutic target in TNBC. Together, our data suggest that PKD3 in TNBC cells provides a molecular connection between the Golgi and endolysosomal compartments to enhance proliferative mTORC1-S6K1 signaling.

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To whom correspondence should be addressed: University of Stuttgart, Institute of Cell Biology and Immunology, Allmandring 31, 70569 Stuttgart, Germany. Tel.: 49-711-685-69301; Fax: 49-711-685-66774; E-mail: monilola.olayioye@izi.uni-stuttgart.de.

The abbreviations used are: PKD, protein kinase D; TNBC, triple-negative breast cancer; S6K1, S6 kinase 1; mTOR, mammalian target of rapamycin; mTORC1, mTORC1 complex 1; 4E-BP1, eIF4E-binding protein 1; Akt/PKB, protein kinase B; ca, catalytically active; PDBu, phorbol-12,13-dibutyrate; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; LC3, microtubule-associated protein light chain 3; pAb, polyclonal antibody.

Background: TNBC is an aggressive breast cancer subtype for which effective targeted therapies are still lacking.

Results: PKD3 is increased in TNBC tissues and cells and contributes to mTORC1-S6K1 activation at endolysosomal membranes.

Conclusion: PKD3 in TNBC cells is required for endolysosomal homeostasis and cell proliferation.

Significance: PKD3 may represent a potential drug target in TNBC.

Protein kinase D (PKD)3 is a family of serine/threonine kinases comprising three family members, PKD1/PKCµ, PKD2, and PKD3/PKCβv. All isoforms comprise a tandem repeat of zinc finger-like cysteine-rich motifs at the N terminus (C1 domain), which is responsible for diacylglycerol or phorbol ester binding, a pleckstrin homology domain, and a catalytic domain at the C terminus that shares homology with the calmodulin-dependent kinases. High homology between the three isoforms exists, particularly in the catalytic and C1 domains, but they also show some structural differences in the N-terminal region and in the regions flanked by the C1 and pleckstrin homology domains, which may confer isoform-specific functions (1, 2). The PKD isoforms are known to localize to the Golgi complex and the plasma membrane, and they have also been reported to shuttle to the nucleus (3). At the Golgi complex, PKD regulates vesicular traffic to the plasma membrane, whereas at the plasma membrane, PKD is involved in the regulation of cell shape, movement, and invasion (4, 5). Most studies have focused on PKD1 and/or PKD2, whereas relatively little is known about PKD3. Due to their similarity, the PKD isoforms have been thought to possess overlapping substrate specificity and function redundantly. However, emerging evidence suggests that the isoforms vary regarding their expression, subcellular localization, and regulation and that the activation of the different PKD isoforms might have distinct cellular outcomes (4, 5). For example, PKD1/2-mediated phosphorylation of substrates such as slingshot homolog 1 and Ras and Rab interactor 1 leads to the inhibition of cell migration (6–8), and reduced PKD1 expression levels were observed by immunohistological analyses of invasive breast cancer samples (9), suggesting that the loss of PKD1 may be associated with breast tumor progression. By contrast, Chen et al. (10) showed that PKD3 is up-regulated in primary prostate cancers and prostate cancer cell lines and stimulates prosurvival pathways, indicating a positive correlation between PKD3 expression and tumorigenesis. This implies that PKD3 regulates a distinct set of substrates to fulfill such functions, which is in accordance with our recent findings that PKD3 selectively phosphorylates the multidomain protein GIT1 (G-protein coupled receptor kinase-interacting protein 1) to enhance cell spreading and motility (11). Here, we focused our attention on the potential deregulation of PKD3 expression in breast cancer and the identification of associated downstream signaling pathways.

Breast cancer is a heterogeneous disease including clinical, morphological, and molecular very distinct entities. It can be classified into several distinct subtypes according to different parameters such as histological grade, tumor size, lymph node
involvement, receptor status, or affected signaling pathways (12). Basal-like breast cancers frequently lack expression of the estrogen, progesterone, and HER2/ErbB receptors, and these cancers are referred to as triple negative. This subtype accounts for 10–20% of all breast carcinomas and is correlated with poor prognosis, survival rate, and a high metastatic potential (13). Due to the negative hormone receptor and HER2/ErbB2 status of TNBC, treatment options are limited, and thus, efforts are being made to identify TNBC-associated deregulated signaling pathways for the development of improved targeted therapies.

The mammalian target of rapamycin (mTOR) is an important serine/threonine protein kinase of the P13K-related kinase family, which functions as an environmental sensor and regulates organismal growth, cell physiology, and homeostasis. Due to its important role in coupling energy and nutrient abundance to the execution of cell growth, division, and homeostasis, deregulation of the mTOR signaling pathway is implicated in an increasing number of pathological conditions including obesity, type 2 diabetes, aging, neurodegeneration, and cancer (14, 15). mTOR is the catalytic subunit of two distinct complexes, mTOR complex 1 and mTOR complex 2 (mTORC1 and mTORC2), which consist of several additional regulatory proteins. The subunit composition of each mTORC dictates its substrate specificity. Main substrates of mTORC1 are S6 kinase 1 (S6K1) and eukaryotic initiation factor 4E-binding protein 1 (4E-BP1), both implicated in the regulation of mRNA and protein synthesis. S6K1 belongs to the AGC kinase family, exists in four isoforms (the main isoforms being p70 and p85 kDa, but p60 and p31 kDa isoforms have also been described) and is regulated by complex multi-site phosphorylation. Maximal S6K1 activity requires T-loop phosphorylation by 3-phosphoinositide-dependent protein kinase 1 at threonine 229 (Thr229) and more importantly hydrophobic motif site phosphorylation by mTORC1 at Thr389 (16). Emerging evidence suggests that aberrant mTORC1-S6K1 signaling contributes to cancer (15). Besides the mTORC1-S6K1 axis, mTORC1 also controls the synthesis of lipids, regulates cellular metabolism and ATP production, inhibits autophagy, and negatively regulates the biogenesis of lysosomes (14). mTORC2 controls several members of the AGC subfamily of kinases, including Akt, serum and glucocorticoid-induced protein kinase 1, and PKCδ isoforms have also been described, and is regulated by complex multi-site phosphorylation. Maximal S6K1 activity requires T-loop phosphorylation by 3-phosphoinositide-dependent protein kinase 1 at threonine 229 (Thr229) and more importantly hydrophobic motif site phosphorylation by mTORC1 at Thr389 (16). Emerging evidence suggests that aberrant mTORC1-S6K1 signaling contributes to cancer (15).

Using a phosphokinase signaling array, we identified S6K1 to be hyperphosphorylated in cells expressing constitutively active PKD3. Based on the reanalysis of transcript profiling studies and our experimental data, we propose that PKD3 expression is elevated in TNBC where it contributes to cell proliferation via activation of the mTORC1-S6K signaling pathway.

**EXPERIMENTAL PROCEDURES**

**Antibodies and Reagents**—Antibodies used in this study were as follows: rabbit anti-PKD3 pAb, mouse anti-GFP mAb (Roche Biosciences), mouse anti-phospho-p70 S6 kinase (Thr389) mAb, rabbit anti-p70 S6 kinase pAb, rabbit phospho-S6 ribosomal protein (Ser473) pAb, rabbit anti-phospho-Akt (Ser473) mAb, rabbit anti-phospho-mTOR (Ser2448) mAb, rabbit anti-phospho-mTOR (Ser2481) pAb, rabbit anti-mTOR mAb, rabbit anti-phospho-4E-BP1 (Thr37/46) mAb and rabbit anti-LC3B mAb (all from Cell Signaling), mouse anti-α-tubulin mAb (Sigma-Aldrich), mouse anti-LAMP1 and anti-mannose 6-phosphate receptor mAbs (Developmental Studies Hybridoma Bank), and mouse anti-p230 trans-Golgi and anti-cathepsin D mAbs (BD Biosciences). HRP-labeled secondary anti-mouse and anti-rabbit IgG antibodies were from GE Healthcare, Alexa Fluor 488- and 546-labeled secondary anti-mouse and anti-rabbit IgG antibodies, Alexa Fluor 546 phalloidin, and LysoTracker Red DND-99 were from Invitrogen, Gø6976 and Gø6983 were from Calbiochem, phorbol-12, 13-dibutyrate (PDBu) was from Enzo Life Sciences, Draq5 was from NEB, and NB 142–70 was from Tocris Bioscience.

**Cell Culture and Transfection**—MDA-MB-231 cells were obtained from CLS Cell Lines Services (Heidelberg, Germany). MCF7, T47D, MDA-MB-157, and MDA-MB-134 cells were obtained from Cornelius Knabbe (Institute of Clinical Pharmacology, Stuttgart, Germany), BT474 and SKBR3 cells were from Nancy Hynes (Friedrich Miescher Institute, Basel, Switzerland), MDA-MB 453 cells were from Jane Visvader (The Walter and Eliza Hall Institute, Melbourne, Australia), and HS-578T, ZR751, and MDA-MB-468 cells were from Bernhard Lüscher (Rheinisch-Westfaelische Technische Hochschule Aachen University, Aachen, Germany). MDA-MB-231 cells were cultivated in DMEM (Invitrogen); all other cell lines were cultured in RPMI 1640 (Invitrogen), supplemented with 10% FCS (PAA Laboratories) and incubated in a humidified atmosphere of 5% CO2 at 37°C. MCF7 cells were transiently transfected by nuclease according to the manufacturer’s instructions (protocol P020, Kit V; Amaxa). MDA-MB-231 cells were transiently transfected with Lipofectamine LTX with Plus reagent (Invitrogen) according to the manufacturer’s instructions. For RNAi, cells were transfected with siRNA using Oligofectamine or Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer’s instructions. Knockdown of all siRNA experiments was verified by parallel Western blot analysis. The siRNAs used were as follows: 5’-GCA GAG UGA AAG UUC CAC ACA CAU U-3’ (siPKD3–1); 5’-GCU GCU UCU CCG UGU UCA AGU CCU A-3’ (siPKD3–2); and 5’-GGGCGCGCCCGGAAUUAACCTT-3’ (siLacZ). PKD3-specific siRNAs were from Invitrogen, siLacZ was synthesized by MWG Biotech, and the On-Target Plus control SMARTpool (siNon) was from Dharmacon. Expression vectors encoding WT and catalytically active PKD3 in TNBC was described previously (11).

**Microarray Data Analysis**—Normalized gene expression microarray data from datasets GSE3744/GDS2250 (17) and GSE1561/GDS1329 (18) was retrieved from NCBI gene expression omnibus (GEO). Expression values for PKD3, PKD1, and PKD2 (also called PRKD3, PRKD1, and PRKD2) were extracted, and box plots were generated in concordance with published subgroup classifications of each data set. Student t test analysis was performed to assess significance of PKD expression between subgroups.

**Cell Lysis, SDS-PAGE, and Western Blotting**—Cells were lysed in TEB buffer (50 mtr Trits (pH 7.5), 150 mNl NaCl, 1 Triton-X-100, 1 mm sodium orthovanadate, 10 mm sodium flu...
oride, and 20 mM β-glycerophosphate plus Complete protease inhibitors (Roche Applied Science), and lysates were clarified by centrifugation at 16,000 × g for 10 min. Protein concentration was determined with Bio-Rad DC protein assay, and equal amounts of proteins were separated by SDS-PAGE and transferred to PVDF membrane (Roth). The membrane was blocked with a 0.5% blocking reagent (Roche Applied Science) in PBS containing 0.1% Tween 20 and then incubated with primary antibodies, followed by HRP-conjugated secondary antibodies. Visualization was with the ECL detection system (Pierce).

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

**Immunofluorescence Microscopy**—Cells were grown on glass coverslips coated with 25 μg/ml collagen (Serva) and fixed with 4% PFA for 10 min, permeabilized with PBS containing 0.2% Triton X-100 for 5 min, and blocked with 5% goat serum (Invitrogen) in PBS containing 0.1% Tween 20 for 30 min. Cells were then incubated for 2 h with primary antibodies in blocking buffer, followed by washing steps with PBS and incubation with secondary antibody in blocking buffer for 1 h. Nuclei were counterstained with Draq5 (1:1000) for 15 min in blocking buffer and washed twice with PBS containing 0.1% Tween 20. Coverslips were mounted in Fluoromount G (Southern Biotechnology) and analyzed on a confocal laser-scanning microscope (Zeiss LSM 700) using 488, 561, and 633 nm excitation and the oil objective lens plan-Apochromat 63×/1.40 differential interference contrast M27. Images were processed with the ZEN software.

**Proliferation Assays**—Cells were transiently transfected with PKD3-specific siRNAs. Cells transfected with control siRNA as well as mock-transfected cells were used as controls. 24 h after transfection, 2500 cells were seeded in sextuple into 96-well plates and then fixed with Roti®-Histofix (4%) and stained with crystal violet (0.2% in double-distilled H2O) after 6 h, or 3 and 5 days later. After rinsing and drying, the dye was dissolved in 200 μl of methanol, and absorption was measured at 550 nm using a microplate reader (Tecan Infinite 200 Reader). The absorption on days 3 and 5 was normalized to the absorption measured 6 h after plating.

**RESULTS**

**PKD3 Expression Is Elevated in Basal-like Breast Cancer**—To gain insight into the expression pattern of PKD3 in different breast cancer subtypes, we analyzed PKD3 transcript levels in two different publicly available microarray gene expression data sets (17, 18). As seen in Fig. 1A, in both data sets, the mRNA level of PKD3 was significantly elevated in basal-like breast cancer compared with the other molecular subtypes. By contrast, mRNA levels of PKD2 were not significantly modulated, and those of PKD1 were down-regulated in basal-like breast cancer (data not shown), which is in accordance with...
published observations (9). We next investigated by immunoblotting PKD3 protein levels in a panel of breast cancer cell lines using a PKD3-specific antibody (Fig. 1B, upper panel). The antibody detected a single band corresponding to PKD3 with a molecular mass of \(110\) kDa (Fig. 1B, upper panel). Interestingly, three of four triple-negative, basal-like breast cancer cell lines (HS-578T, MDA-MB-231, and MDA-MB-468) displayed high levels of PKD3 in comparison with, for example, the luminal breast cancer cell line MCF7. Taken together, these data show that PKD3 expression is elevated both at the transcript and protein level in basal-like TNBC cells.

**Identification of S6K1 as a Downstream PKD3 Target**—To identify signaling pathways regulated by PKD3, we overexpressed a catalytically active GFP-tagged PKD3 variant (ca, S731E/S735E) in MCF7 cells, which express relatively low levels of PKD3. Whole cell lysates were then analyzed by an antibody array comprising mainly phosphospecific antibodies raised against various proteins within key cellular signaling pathways. Compared with arrays incubated with control lysates derived from cells transfected with the empty GFP vector, the intensity of three duplicate spots in the arrays incubated with lysates of PKD3 ca overexpressing cells was up-regulated (highlighted in black), all of which corresponded to S6K1 (Fig. 2A). Quantification of spot intensities revealed a 3-fold higher phosphorylation of S6K1 at Thr\(389\) and a 2-fold higher phosphorylation at Thr\(229\) in PKD3 ca expressing cells compared with those expressing GFP alone (Fig. 2B).

To confirm this result, GFP-PKD3 ca was again transiently overexpressed in MCF7 cells, and whole cell lysates were analyzed by immunoblotting with an S6K1 Thr\(389\)-specific antibody. Indeed, overexpression of PKD3 ca enhanced the recognition of S6K1 and its substrates S6, but not that of 4E-BP1, by phosphospecific antibodies (Fig. 2C), which is indicative of increased activation of the mTORC1-S6K1 pathway. This is supported by the slightly enhanced mTOR phosphorylation at Ser\(2448\), which is specific for mTORC1, whereas Ser\(2481\) phosphorylation, which is associated with mTORC2 (19), was not up-regulated in PKD3 ca overexpressing cells (Fig. 2C). Note that two bands were observed for S6K1 (Fig. 2C, middle panels), corresponding to the p70 and p85 isoforms (phosphorylation nomenclature is based on the p70 isoform protein sequence), which are both expressed in most breast cancer cells (20). In contrast to the ubiquitous phosphorylation at Ser\(2481\), mTOR phosphorylation at Ser\(2448\) was also more prominent in the PKD3-positive TNBC cell lines (Fig. 1B). In line with the specific activation of mTORC1-S6K1, neither Akt phosphorylation at Ser\(473\), a main target of mTORC2, nor Erk1/2 phosphorylation were modulated by PKD3 ca overexpression (Fig. 2C). Together, these data provide evidence that the mTORC1-S6K1 signaling pathway is a potential downstream target of PKD3.

**PKD3 Depletion Compromises S6K1 Activation in TNBC cells**—We next determined the influence of PKD3 on the mTOR signaling pathway at the endogenous level, by acute activation and knockdown of the kinase respectively. In MDA-MB-231 cells, which express high PKD3 levels (Fig. 1B), we first investigated the effect of PDBu, an analog of diacylglycerol, on mTOR phosphorylation at Ser\(2448\), which is specific for mTORC1, whereas Ser\(2481\) phosphorylation, which is associated with mTORC2 (19), was not up-regulated in PKD3 ca overexpressing cells (Fig. 2C). Note that two bands were observed for S6K1 (Fig. 2C, middle panels), corresponding to the p70 and p85 isoforms (phosphorylation nomenclature is based on the p70 isoform protein sequence), which are both expressed in most breast cancer cells (20). In contrast to the ubiquitous phosphorylation at Ser\(2481\), mTOR phosphorylation at Ser\(2448\) was also more prominent in the PKD3-positive TNBC cell lines (Fig. 1B). In line with the specific activation of mTORC1-S6K1, neither Akt phosphorylation at Ser\(473\), a main target of mTORC2, nor Erk1/2 phosphorylation were modulated by PKD3 ca overexpression (Fig. 2C). Together, these data provide evidence that the mTORC1-S6K1 signaling pathway is a potential downstream target of PKD3.

**PKD3 in TNBC**
followed by immunoblotting of whole cell lysates with the different antibodies specific for the mTOR signaling pathway. Fig. 3A shows that the S6K1 phospho-specific signal strongly increased upon PDBu stimulation and preincubation of cells with specific pharmacological inhibitors of the PKC/PKD pathway abrogated phospho-S6K1 detection (Fig. 3A, top panel). Similarly, phospho-mTOR (Ser2448) and phospho-S6 signals were reduced by these inhibitors. Gö6976 inhibits PKD family members directly, whereas Gö6983 inhibits PKD indirectly by inhibiting novel PKCs. Preincubation of cells with rapamycin, a potent inhibitor of mTORC1, also blocked the PDBu-induced mTORC1 (Ser2448) and S6K1 phosphorylation, providing evidence that PDBu-induced S6K1 activation is indeed dependent on mTORC1 and excluding the possibility of an mTORC1-independent pathway. Phosphorylation of 4E-BP1 at Thr37/46 was not affected by PKD inhibition, indicating that PKD activation contributes mainly to the mTORC1-S6K1 signaling axis (see also Fig. 2C). Furthermore, mTORC2 phosphorylation at Ser2481 and Akt phosphorylation at Ser473 were not downregulated by the PKD inhibitors, providing further support for the selective activation of mTORC1 by PKD. In fact, phosphorylation of Akt in PKD-inhibited MDA-MB-231 cells was increased (Fig. 3A), which can be explained by the known feedback activation of mTORC2 in response to mTORC1 inhibition (21).

We next determined the effect of PKD3 depletion on the mTOR signaling pathway in MDA-MB-231 and MDA-MB-468 cell lines. We used two independent, specific siRNAs to silence PKD3 (siPKD3-1 and siPKD3-2) and as controls, mock as well as control siRNA (non-targeting, siNon)-transfected cells were used (Fig. 3B). Cells were starved overnight and then stimulated with serum prior to lysis, as serum is known to activate S6K1 in breast cancer cells (20). As seen in Fig. 3B, both siRNAs efficiently knocked down PKD3 in both cell lines (upper panels). Importantly, PKD3 knockdown markedly inhibited the serum-induced phospho-signal at Thr389 of the 85-kDa S6K1 isoform, whereas phosphorylation of Akt at Ser473 was not decreased in PKD3-depleted cells, again indicating that mTORC2 activity was not affected by the loss of PKD3. Other pathways such as the MAPK pathway were not modulated by PKD3 inhibition or depletion based on unchanged Erk1/2 phosphorylation (Fig. 3, A and B). In sum, these data suggest that PKD3 is upstream of mTORC1 and S6K1 in basal-like TNBC cells.

PKD3 Knockdown Impairs Endolysosomal mTOR Activation—To shed light onto the cellular interaction between PKD3 and mTORC1-S6K1, we performed localization studies in MDA-MB-231 cells. The best characterized localization for the PKD isoforms is at the Golgi complex (1, 4), and mTOR has also been described to localize to the Golgi membranes (22, 23). However,
PKD3 was also observed to localize to the nucleus in prostate cancer cells and VAMP2-positive vesicular structures in HEK 293 cells (10, 24). Due to the lack of a commercially available antibody suited for the immunocytochemical detection of endogenous PKD3, we ectopically expressed GFP-tagged PKD3 in MDA-MB-231 cells where it localized mainly to the Golgi compartment, as revealed by costaining with the trans-Golgi marker p230, with smaller amounts found at the plasma membrane and in the cytosol (Fig. 4A). Endogenous mTOR, however, did not localize to the Golgi complex in MDA-MB-231 cells (data not shown) but was instead found to partly colocalize with the endolysosomal marker LAMP1 (Fig. 4B, upper panel). This is in line with findings that mTORC1 is recruited to and activated at endosomal and lysosomal membranes in response to amino acids (25–27). We therefore investigated the colocalization of mTOR with LAMP1 in PKD3-depleted MDA-MB-231 cells. Despite a slight increase in the amount of cytosolic protein, most of the mTOR signal was found to localize to vesicular structures in these cells (Fig. 4B, middle and lower panels). Immunofluorescence staining with the phosphorylation-specific antibody (Ser2448), however, revealed that phospho-mTOR colocalized with LAMP1 in control cells (Fig. 4C, upper panel), whereas in PKD3-depleted cells, a weaker, less defined staining pattern was observed, indicative of a lower amount of active mTORC1 at endolysosomal membranes (Fig. 4C, middle and lower panels). Thus, depletion of PKD3 in MDA-MB-231 cells causes a decrease of active mTORC1 at endolysosomal membranes.

**PKD3 Knockdown Impacts Endolysosomal Trafficking—mTORC1 signaling is involved in the regulation of autophagy and endosomal trafficking, processes that are intimately related (28, 29).** To investigate the consequences of PKD3 knockdown in this regard, we incubated control and PKD3-depleted cells with LysoTracker, which accumulates in acidic organelles that include late endosomes, lysosomes, and autophagolysosomes. We simultaneously analyzed the distribution of microtubule-associated protein LC3, a protein that is lipidated by an ubiquitin-like system involving Atg7 and Atg3 leading to its association with autophagic vesicles (30). The antibody used in the study mainly detects the lipidated LC3-II form, the presence of

![FIGURE 4. PKD3 knockdown impairs endolysosomal mTOR activation. A, MDA-MB-231 cells were plated onto collagen-coated coverslips and transiently transfected with an expression vector encoding GFP-PKD3 WT. The next day, cells were fixed and stained with p230-specific primary and Alexa Fluor 546-conjugated secondary antibodies (red). The images shown are projections of several confocal sections. Scale bar, 20 µm. B and C, MDA-MB-231 cells were plated onto collagen-coated coverslips and transiently transfected with two independent siRNAs specific for PKD3 or control siRNA (siNon). 72 h post transfection, cells were fixed and co-stained with mTOR-specific (B) or phospho-mTOR (Ser2448)-specific (C), and LAMP1-specific primary and Alexa Fluor 488- and 546-conjugated secondary antibodies, respectively (green and red as indicated). Nuclei were visualized by Draq5 staining. The images were acquired using identical settings; shown are projections of several confocal sections. Scale bar, 20 µm.]
which on autophagosomal membranes serves as an indicator of autophagy. Interestingly, cells lacking PKD3 showed a strong accumulation of LysoTracker-containing vesicles, which were increased in size and further positive for LC3 (Fig. 5A).

We next analyzed the distribution of the mannose 6-phosphate receptor, which cycles between the Golgi complex and endosomes where it releases lysosomal enzymes before returning to the Golgi compartment. Knockdown of PKD3 led to the trapping of mannose 6-phosphate receptor in LysoTracker-positive vesicles (Fig. 5B), which was associated with the accumulation of immature forms of the lysosomal protease cathepsin D in these cells, as observed by immunoblotting of respective cell lysates (Fig. 5C). These results are indicative of defective endosome maturation and trafficking associated with the increased formation and/or turnover of autophagolysosomes and the impaired activation of mTORC1 at endolysosomal membranes.

**PKD3 Knockdown Modulates Cell Morphology and Impacts on Cell Proliferation** — The mTORC1-S6K1 signaling pathway is important for the regulation of protein and lipid synthesis, processes that are important for cell proliferation and organi- mal growth (14, 15). We therefore investigated related biological effects of PKD3 depletion on MDA-MB-231 and MDA-MB-468 cells. Microscopic analysis of cell morphology revealed that PKD3-depleted cells were less spread than the controls (Fig. 6A), but they were not smaller in overall cell size as assessed by FACS analysis (data not shown). Next, control and PKD3-depleted MDA-MB-231 and MDA-MB-468 cells were subjected to proliferation assays. Relative viable cell numbers were determined by crystal violet staining on days 3 and 5 days post plating. In both cell lines, PKD3 knockdown moderately decreased the relative viable cell number after 3 days and markedly decreased the relative viable cell number by almost 50% after 5 days (Fig. 6B). This is in line with results using a pharmacological PKD inhibitor (NB-142-70). Here, a dose-dependent inhibition of cell proliferation with an IC50 of ~5 µM was observed for MDA-MB-231 cells (data not shown). At 5 µM, cell proliferation of both, MDA-MB-231 and MDA-MB-468 cells, was reduced by almost 40% after 3 days and 65% after 5 days (Fig. 6C). In summary, we have shown that the elevated expression of...
PKD3 in TNBC cells provides a molecular connection between the Golgi and endolysosomal compartments to enhance proliferative mTORC1-S6K1 signaling.

DISCUSSION

In the majority of human tumors, the mTORC1 pathway is aberrantly activated, sustaining the increased proliferation of tumor cells even under nutrient-limiting conditions (15, 31). In early stage TNBC in particular, positive phospho-mTOR staining was correlated with significantly worse overall as well as recurrence-free survival (32). Here, we show that TNBC cells and basal-like breast cancer tissues exhibit increased PKD3 protein and transcript levels, respectively. Overexpression of catalytically active PKD3 resulted in the hyperactivation of S6K1, whereas PKD3 knockdown reduced S6K1 phosphorylation. Interestingly, we did not observe any changes in the phosphorylation of Erk1/2 and Akt, kinases whose activation has previously been implicated in the context of PKD3-positive prostate cancer (10), indicating a selective effect of PKD3 on S6K1 in breast cancer cells. The two main S6K1 isoforms, p70 and p85, derive from the same gene and are produced by alternative translational initiation (16). In both MDA-MB-231 and MDA-MB-468 cells, the p85 isoform showed a stronger activation in response to serum or phorbol ester compared with that of the more abundant p70 isoform. The N-terminal extension of the 85-kDa isoform comprises a nuclear localization signal (16); however, in breast cancer cells, both isoforms were mainly observed in the cytosol (20), and their functional differences are poorly understood. Our data suggest that p85 S6K1 is the main serum-responsive isoform in TNBC cells that is regulated by PKD3 via mTORC1 signaling. In fact, this is also the S6K1 isoform that appears to be predominantly phosphorylated in all breast cancer cell lines apart from MCF7 (see Fig. 1B).

In this study, we show for the first time that PKD3, which is predominantly associated with Golgi membranes, is required for the integrity of the endolysosomal system and mTORC1 activation at endomembranes in TNBC cells. Based on our data, we propose that PKD3 provides a molecular link between protein synthesis and sorting at the level of the Golgi complex and the catabolic processes occurring at the endolysosomal compartment via a mechanism that remains to be defined. Although PKD kinases are best studied in the context of the constitutive trafficking of cargo proteins to the plasma membrane, there is recent evidence that PKD also affects the delivery of specific proteins to the lysosomes. Kinase-inactive PKD1 and PKD2 inhibited the exit of the lysosomal transmembrane protein mucolipin 1 from the Golgi (33), suggesting that PKD

FIGURE 6. PKD3 knockdown affects cell spreading and reduces cell proliferation. A, MDA-MB-231 and MDA-MB-468 cells were plated onto collagen-coated coverslips and transiently transfected with two independent siRNAs specific for PKD3 or control siRNA (siControl). 72 h after transfection, cells were fixed and stained with Alexa Fluor 546 conjugated phalloidin (red). The images shown are projections of several confocal sections. Scale bar, 20 μm. B, MDA-MB-231 and MDA-MB-468 cells were transiently transfected with two siRNAs specific for PKD3. Control siRNA (siNon) as well as mock-transfected cells were used as controls. 24 h after transfection, 2500 cells were seeded in sextuple into 96-well plates, and cell density was measured via crystal violet staining after 3 and 5 days. Data show the mean of five (MDA-MB-231) and three (MDA-MB-468) independent experiments, respectively. All values were normalized to the initial cell density at 6 h after plating. Error bars represent S.E. *, p ≤ 0.05; **, p ≤ 0.01; ***, p ≤ 0.001. PKD3 knockdown in both experiments was verified by parallel Western blotting (blots not shown). In C, MDA-MB-231 and MDA-MB-468 cells were seeded in triplicate into 96-well plates (2500 cells per well). The next day, cells were treated with 5 μM NB-142–70 (PKD inhibitor) and grown for 24, 48, 72, and 120 h. Cell density was measured via crystal violet staining and is presented as the percentage of control cells. Data show the mean of two independent experiments; error bars represent S.E.
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directly impacts lysosome protein composition. Because the cycling of the mannose 6-phosphate receptor, which is responsible for transporting most lysosomal enzymes, was perturbed in PKD3-depleted cells, PKD3 appears to have a more global role in endosomal maturation and trafficking. By regulating lipid homeostasis at the TGN membranes, PKD may indirectly affect the function of the endolysosomal system, by altering its lipid composition as a consequence of constitutive vesicular transport processes. For example, phosphatidic acid is a lipid second messenger that is necessary for mTOR activation (34). Conversely, cholesterol accumulation in the endolysosomal compartment negatively affected the fusion of lysosomes with endocytic and autophagic vesicles (35) and inhibited mTOR activation in endothelial cells (36). Moreover, depletion of the lipid transfer protein CERT, which shuttles ceramide from the endoplasmic reticulum to Golgi membranes, increased autophagic flux and synergized with paclitaxel in mediating LAMP2-mediated cell death (37), most likely via a mechanism involving ceramide accumulation within the endoplasmic reticulum. These studies exemplify the various crosstalk mechanisms between the Golgi and endolysosomal system, all of which may potentially impact mTORC1 activation. Of note, in some cells, GFP-tagged PKD3 was also observed on vesicle-like structures; however, these did not stain positively for mTOR (data not shown). Nevertheless, we cannot completely rule out that endogenous PKD3 also localizes to endolysosomal membranes where it acts as an upstream regulator of mTORC1.

Silencing of PKD3 in MDA-MB-231 cells led to the accumulation of LC3 on LysoTracker-positive vesicles that are likely to represent defective late endosomes and/or autophagolysosomes. Compared with control cells, the size of these acidic vesicles was drastically increased, indicative of a disturbed endolysosomal flux presumably affecting both endosomal maturation and autophagy. Reminiscent of our observations, when early to late endosomal conversion was blocked by overexpression of constitutively active Rah5, mTORC1 remained localized to hybrid early/late endosomes but failed to be activated in response to nutrients (26). Autophagy is known to have a tumor-suppressive function and constitutive mTORC1 activation may contribute to tumor progression by inhibiting autophagy (31, 38). However, in established cancers, autophagy can promote cell survival under a variety of stresses and may even be required for tumor maintenance (38). PKD was recently reported to induce autophagy in response to reactive oxygen species by direct phosphorylation and activation of Vps34, a PI3K required for autophagosome formation (39). These results seem to be in contradiction with our findings; however, Eisenberg-Lerner and Kimchi (39) investigated the induction of autophagy by the PKD1 isoform and under conditions of oxidative stress, which obviously differ from our setting in PKD1-negative TNBC cells where PKD3 exerts a protective effect on basal autophagy. Of note, Vps34 has also been described to activate mTOR and induce S6K1 phosphorylation on Thr389 to inhibit autophagy (40, 41), making it intriguing to speculate that the different PKD isoforms engage the same substrate to trigger opposing cellular outcomes.

Indeed, emerging evidence suggests that the different PKD isoforms do not simply have redundant but in some instances may even have antagonizing functions. For example, PKD1 expression appears to be required for the maintenance of epithelial characteristics, and its loss is associated with increased cell migration and invasion (5). By contrast, in lung cancer cells, PKD3 was identified as part of a bone metastatic signature (42), and silencing of PKD3 in prostate cancer cells was shown to reduce in vitro proliferation, migration, invasion, and xenograft growth in nude mice (43). Mechanistically, PKD3 promoted the expression and secretion of cytokines and extracellular proteases such as matrix metalloproteinase 9 and urokinase-type plasminogen activator via activation of NFκB and histone deacetylase signaling pathways, thereby positively contributing to cell migration and invasion (43, 44). Furthermore, silencing of PKD2 in MCF7 cells promoted cell spreading (7), whereas PKD3 knockdown in the same cell line (data not shown) and in MDA-MB-231 cells as shown in this study impaired cell spreading.

In MDA-MB-231 and MDA-MB-468 cells, the magnitude of pharmacological PKD inhibition on cell proliferation was similar to that upon PKD3 ablation. Both cell lines express PKD2 but no or relatively little PKD1 (9), indicating that PKD3 is the main PKD isoform in TNBC cells and PKD2 plays a minor contribution in these cells. For the treatment of TNBC, several targeted agents, including mTOR inhibitors such as RAD001, are currently being evaluated in clinical trials in combination with chemotherapy, however, thus far with limited success (45, 46). Both, PKD3 knockdown and pharmacological PKD inhibition were more effective with respect to growth inhibition of MDA-MB-231 and MDA-MB-468 cells than rapamycin (data not shown) to which these cells were relatively insensitive. This is most likely due to the fact that PKD3 is required for general Golgi sorting and secretory function in TNBC cells and thus signals through pathways additional to mTORC1. We previously reported that PKD3 stimulates cell spreading and migration through GIT1 phosphorylation, which is involved in the trafficking of the focal adhesion protein paxillin (11). The rounded cell morphology seen upon PKD3 silencing may thus be explained, at least in part, by impaired trafficking of GIT1-paxillin complexes. Due to the important function of PKD in the cellular homeostasis of different tissues, the therapeutic inhibition of PKD is a delicate issue. Nevertheless, based on our data, the development of an isoform-specific pharmacological PKD3 inhibitor may hold promise for the targeted treatment of TNBC.

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