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REVIEW ARTICLE

Protein kinase D2: a versatile player in cancer biology

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Abstract

Protein kinase D2 (PKD2) is a serine/threonine kinase that belongs to the PKD family of calcium–calmodulin kinases, which comprises three isoforms: PKD1, PKD2, and PKD3. PKD2 is activated by many stimuli including growth factors, phorbol esters, and G-protein-coupled receptor agonists. PKD2 participation to uncontrolled growth, survival, neovascularization, metastasis, and invasion has been documented in various tumor types including pancreatic, colorectal, gastric, hepatic, lung, prostate, and breast cancer, as well as glioma multiforme and leukemia. This review discusses the versatile functions of PKD2 from the perspective of cancer hallmarks as described by Hanahan and Weinberg. The PKD2 status, signaling pathways affected in different tumor types and the molecular mechanisms that lead to tumorigenesis and tumor progression are presented. The latest developments of small-molecule inhibitors selective for PKD/PKD2, as well as the need for further chemotherapies that prevent, slow down, or eliminate tumors are also discussed in this review.

Introduction

Protein kinases comprise a gene family of 538 members in the human genome [1] and have been involved in a multitude of complex cellular functions and pathways. Despite their diverse functions, protein kinases adopt a strikingly similar active conformation of the catalytic domain [2, 3] catalyzing the transfer of the γ-phosphate of adenosine 5'-triphosphate (ATP) to the substrate's hydroxyl group of either serine, threonine, or tyrosine residues through a process called phosphorylation. Protein phosphorylation regulates most aspects of cell life [4] and deregulation of this process via dysfunctional kinase activity has crystallized as a major mechanism by which tumor cells escape normal physiological constraints on survival and growth [5]. Protein kinase D (PKD) family comprises three highly conserved enzymes in humans: PKD1, PKD2, and PKD3. The evolution of these isoforms appears to be associated with the evolution of vertebrates. Although only two PKD members are expressed in fish, amphibians, and birds, all three PKD isoforms are expressed in mammals. PKD isoform found in amphibians and fish most closely resembles mammalian PKD1, whereas the invertebrate PKD found in *Drosophila* is most closely homologous with PKD3. By contrast, PKD2 seems to have evolved from mammals onwards [6]. PKD1 and PKD2 are the most closely related of the three mammalian PKD isoforms and share similar regulatory molecular mechanisms in various cell types. Members of the PKD family are effectors of diacylglycerol (DAG) signaling and are (often) activated downstream of protein kinase C by multiple stimuli including growth factors and hormones. They are implicated in various functions, both physiological and pathological, such as cell viability and proliferation, growth and differentiation, intracellular trafficking and regulation of the Golgi complex. Several reports described an essential role of PKD isoforms in carcinogenesis and tumor progression. Importantly, PKD members can display tissue-dependent expression and perform isoform-specific functions in different contexts. So far, many studies of PKD function do not specify the PKD isoform studied. Since molecular cloning and first characterization of human PKD2 in our laboratory 16 years ago, a compelling body of evidence on the role of this kinase in tumorigenesis and cancer progression has accumulated. The current review aims to summarize, integrate, and present most of these findings under the aspects of the basic principles in cancer biology.

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Protein structure and regulation

PKD was cloned and characterized in the mid of 1990s [7, 8]. Due to the presence of two phorbol ester and DAG C1-binding domains, PKD was initially regarded by one group as a member of protein kinase C family and termed PKCμ. PKCμ was included in the group of the atypical protein kinase C (aPKC: ξ and ι) that together with the classical protein kinase C (PKC) members (PKCs: α, β1, β2, and γ) and the novel PKCs isoforms (nPKCs: δ, η, ε, and ϕ) form a superfamily of serine/threonine kinases implicated in the regulation of growth, differentiation, transformation, and apoptosis. However, careful examination of the catalytic domain of PKD/PKCμ revealed that the kinase domains show little similarity to the highly conserved regions of the kinase subdomains of the PKC family but are rather related to the Ca^{2+}/calmodulin-dependent protein kinase II-like protein kinases [1]. Furthermore, the substrate specificity of PKD/PKCμ [7, 9] and its sensitivity to inhibitors [8] are unlike those of the PKC family. Therefore, the members of the PKD family were considered as a distinct family of protein kinases belonging to the Ca^{2+}/calmodulin-dependent protein kinase (CAMK) group, later named PKD1. The PKD family also included two other isoforms, PKD2 [10] and PKD3 (PKCν) [11, 12]. All three isoforms of this kinase family share similar structural features such as the highly conserved N-terminal regulatory domain containing two cysteine-rich DAG-binding C1 domains and an auto-inhibitory pleckstrin homology (PH) domain (Fig. 1). Interestingly, PKD1 and PKD2 share ~85% overall identity at the amino-acid level and display an even higher degree of homology in their catalytic domain and C1 domains, which represent the key for controlling the intracellular localization of these kinases. The C1 domains are also thought to be involved in the regulation of PKD activity. Interestingly, however, although deletion of the C1 domains in PKD1 results in elevated kinase activity, similar deletions in PKD2 abrogate its activity. The N-terminal regulatory domain also contains a pleckstrin homology domain involved in auto-inhibition. Partial deletions or a full ablation of the PH domain results in increased kinase activity [13, 14]. The PKD2 mRNA is widely expressed and encodes an 878-amino-acid protein of 105 kDa. C1a/C1b domain plays a complex role in the regulation of nucleocytoplasmic shuttling [15]. PKDs are regulated by phosphorylation. For PKD2, several phosphorylation sites have been identified including Ser706 and Ser710—in the activation loop corresponding to Ser744 and Ser748 in PKD1 [16] and an autophosphorylation site at Ser876, corresponding to Ser916 in PKD1 [10]. The latter phosphorylation site is not present in PKD3 (Fig. 1). PKD2 is also phosphorylated at Ser244 by casein kinase 1, which results in nuclear import [17]. Furthermore, PKD2 is also shown to be regulated via the phosphorylation of several tyrosine residues. For example, PKD2 is phosphorylated at Tyr717 in the P + 1 loop by Abl in oxidative stress conditions in an isoform-specific manner, which results in increased kinase activity [18]. Tyr87 phosphorylation has also been shown in oxidative stress conditions and was thought to be necessary for PKCδ-mediated PKD2 activation in oxidative stress conditions, but recent data suggest that this event might be superfluous to this extent. Canonical activation of PKD is induced by PKC through the phosphorylation of Ser706/710 in the activation loop [19]. As for PKD1, PKD2 becomes activated downstream of G-protein-coupled receptors through the activation of PLCγ, which in turn activates PKCα, PKCε, or PKCη [16]. PKD2 can also be activated by gastrin via the cholecystokinin B receptor [16, 20], hypoxia [21], and by insulin-like growth factor (IGF)-1 [22].

Protein expression and function

In contrast to PKD1, a high steady-state expression level of PKD2 was demonstrated in human pancreas, lung, heart, smooth muscle, and brain [8, 10], whereas kidney and liver display lower levels of PKD2 mRNA. This suggests that various members of PKD family exhibit tissue-specific expression. In fast proliferating tissues, such as testis and colonic crypts, an augmented expression of PKD2 was

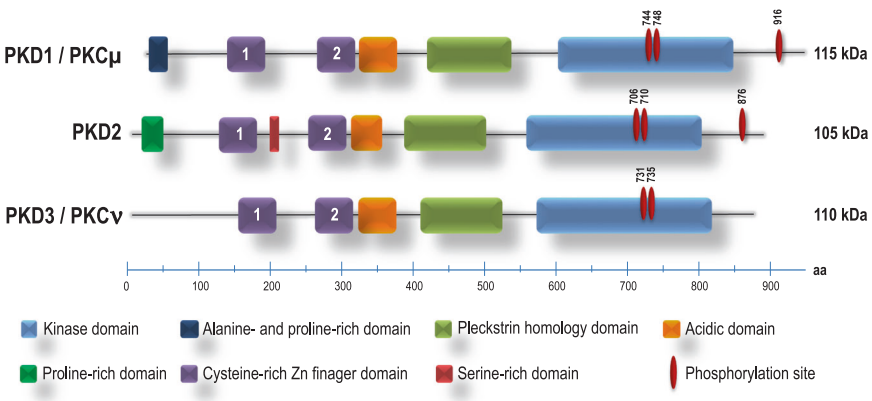

Fig. 1 Molecular architecture of protein kinase D family members: PKD1/PKCμ, PKD2, and PKD3/PKCν. Adapted from Rykx et al. [12]

detected [10]. Additionally, mouse PKD2 but not PKD1, was specifically expressed in T- and B-lymphocytes that comprise the thymus, spleen, and lymph nodes [6]. As PKD1 and PKD2 have a similar (but not identical) molecular weight and are recognized by the same C-terminal antibodies, many older studies of PKD function did not specify which PKD isoform was investigated. Various adult tissues and cell lines show that different PKD isoforms are in some instances functionally redundant. For example, multiple PKD isoforms appear to regulate Golgi organization [23], protein transport [23-26], nuclear factor-kappa B (NF-κB)-dependent cell survival response [13, 27, 28], and release of chemokines by Toll-like receptor-activated epithelial cells [29]. However, there are examples of cells/tissues where expression of a single PKD isoform appears to be predominant suggesting a specific and non-redundant role of a particular member of PKD family. Such an example is the role of PKD1 in regulating insulin secretion in pancreatic β-cells [30]. The observation of distinct PKD pools at different intracellular sites, including Golgi, cytoplasm, nucleus, mitochondria, and plasma membrane [31] further complicates our understanding of the functions of different PKD isoforms. In addition, various PKD isoforms can differently localize within the same cell [31, 32] or shuttle between cytosol and nucleus in response to particular stimuli [33, 34]. Several reports imply PKDs in the organization of the Golgi apparatus, regulating the fission of vesicles from the trans-Golgi network (TGN) [23, 35]. PKD phosphorylates several substrates at the Golgi, including PI4KIIIβ [36], ceramide transferring protein (CERT) [37], and oxysterol-binding protein 1 (OSBP1) [38]. Phosphorylation of PI4KIIIβ results in increased production of phosphatidylinositol-4-phosphate (PI(4)P), which serves as a lipid "platform" to recruit lipid-modifying enzymes and other regulating factors including CERT and OSBP [36-38]. PKD-mediated phosphorylation of CERT and OSBP1 results in decreased affinity for PI(4)P, making PKD an important sensor and mediator of lipid homeostasis at the TGN [37, 38]. Vesicle budding by PKD is thought to occur through its C1a domain mediated binding of DAG at the outer leaflet of the TGN, followed by the recruitment of phospholipase D (PLD) into the ARF1-PKD complex. PLD generates phosphatidic acid (PA) from PtdCho, which can be further converted into Lyso-PA (LPA). LPA can form spontaneous curvatures, promoting vesicle budding [38]. Very recently our lab revealed that PKD2 and not PKD1, is a core factor in the assembly of a functional multiprotein complex at the TGN that regulates matrix metalloproteinase 2 (MMP2) secretion [39]. Importantly, these processes require direct binding of PKD2 to ARF1 GTP-ase.

Many initial studies of PKD function did not specify which PKD isoform was being referred to when role of the kinase in the immune system was investigated. For instance, Sidorenko and colleagues referred to PKD when they first revealed that T-cell receptor (TCR) cross-linking causes activation of kinase [40]. Furthermore, PKD was described to regulate different aspects of T-cell differentiation and development of mature T-lymphocytes [40]. More knowledge about the function of PKD isoforms in immune system comes from Cantrell's group [6, 41]. Using PKD2 and PKD1 transgenic mice and PKD2 gene-trap mutant mice, this study demonstrates that T- and B-lymphocytes specifically express PKD2, but not PKD1 [6, 41]. Furthermore, the catalytic activity of PKD2 is important for optimal T-cell-dependent antibody response in vivo and effective cytokine production after TCR engagement. In this context, PKD2 kinase dead mutant T-lymphocytes fail to produce interleukin-2 (IL-2) and interferon-γ (IFNγ) that are critical for the adaptive immune response, a fact that suggests a role of the kinase in controlling cytokine production [41]. PKD1 and PKD2 display similar subcellular trafficking and activation kinetics in response to antigen receptor in A20 lymphoma B cells and leukemic Jurkat T-cell line [41-43]. Altogether, this study provides evidence that PKD2 has, at least in some settings, a unique non-redundant role in controlling T-cell function during adaptive immune response and urges for further detailed investigations.

PKDs are also involved in vasculogenesis and myogenic differentiation. Formation of vasculature involves the assembly of endothelial cells into tubes that takes place during embryogenesis [44]. In addition, members of PKD family were reported to be involved in cardiac, skeletal, and smooth muscle regulatory processes such as the mediation of the hypertrophic response to angiotensin II or stimulation of myocyte enhancer factor-2 (MEF2) activity in skeletal muscle thus playing a crucial role in heart remodeling [45-49]. Our lab examined the putative participation of PKDs to myogenic differentiation by involving murine C2C12 myoblasts [50]. C2C12 differentiate rapidly and represent a bona fide in vitro model to study the differentiation of myoblasts to myotubes. Although both PKD2 and PKD3 are predominantly expressed in C2C12 cells, only PKD2 was found to be catalytically active during the early phase of differentiation to skeletal muscle cells [50]. Selective depletion of PKD2 was sufficient to prevent differentiation of C2C12 cells [50]. By contrast, ectopic expression of the kinase augments the initiation of myoblast differentiation by activating Mef2D, a transcription activator that specifically binds to the MEF2 element present in regulatory regions of many muscle-specific genes that control cardiac morphogenesis and myogenesis [49].

The role of PKD2 in cancer

Cancer development is a complex process characterized by various epigenetic and genetic modifications that promote resistance to pro-apoptotic stimuli and confer insensitivity

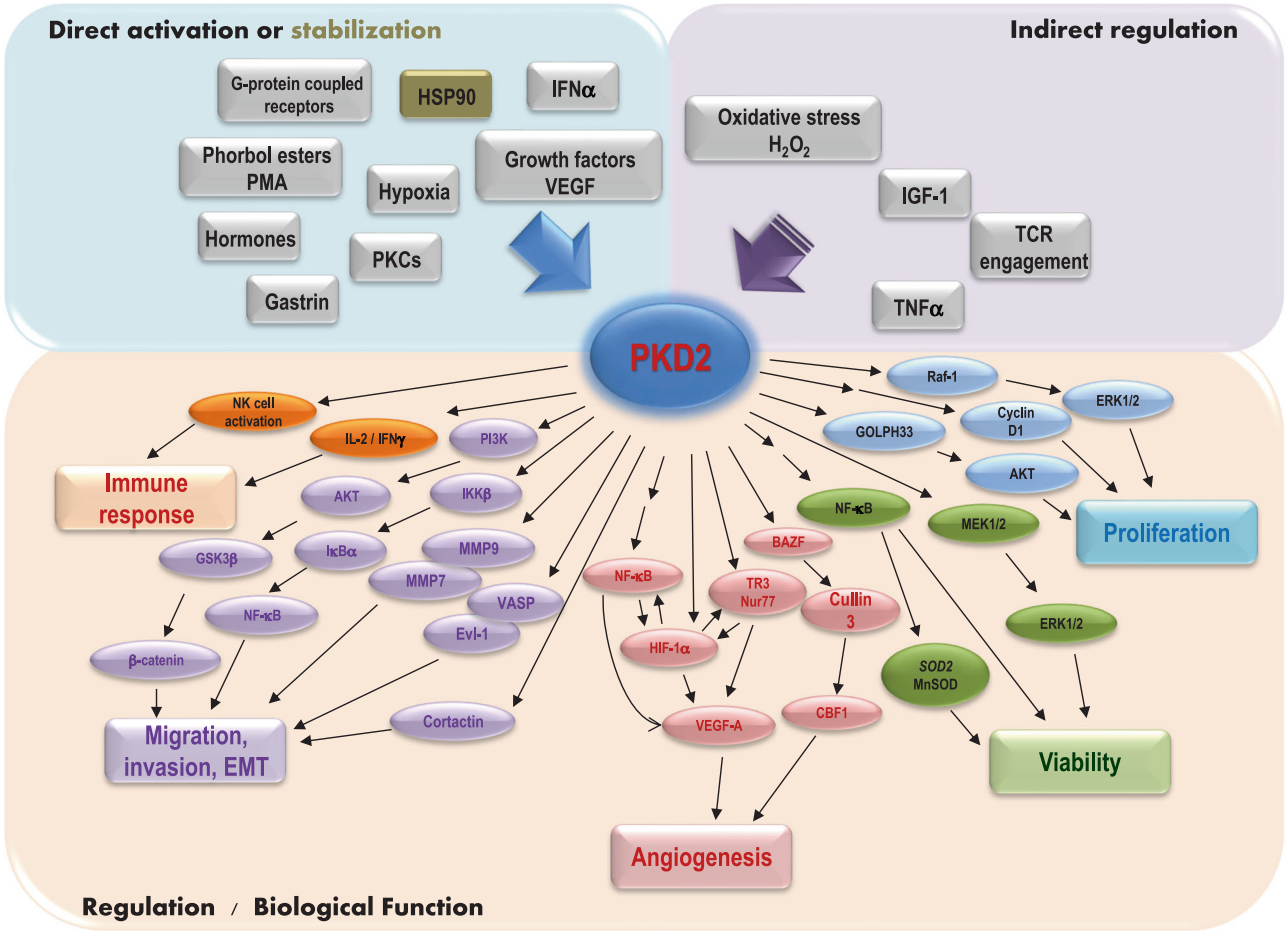

Fig. 2 PKD2 is involved in the regulation of multiple signaling pathways, as well as in the integration of extracellular signals. Separate circuits present the capability of PKD2 to orchestrate cancer cell proliferation (blue), survival (green), angiogenesis (red), motility (violet), and immune response (orange). This depiction is rather simplistic, as there is considerable crosstalk between signaling pathways comprising of PKD2-regulated molecules that participate to more than one cancer hallmark (e.g., NF-κB). Furthermore, due to the fact that each cancer cell is exposed to a variety of signals from its microenvironment, each of these signaling pathways is connected to direct (light blue) or indirect (light violet) signals originating from other cells in the tumor microenvironment

to antigrowth signals while sustaining angiogenesis and immune surveillance. At the beginning of this millennium, Hanahan and Weinberg defined six hallmarks of cancer as distinctive and complementary capabilities that enable tumor growth and metastatic dissemination [51]. Cancer cells are able to sustain proliferative signaling, evade tumor suppressors, capable of invasion and metastasis, resist apoptosis, promote angiogenesis, and enable replicative immortality [51]. The conceptual progress made since the year 2000 enabled not only describing new features crucial to the six hallmark capabilities but also the acquisition of novel hallmarks such as deregulation of the cellular metabolism and avoiding immune destruction [52]. Furthermore, the progress in technology and knowledge in cancer research revealed, solidified, and extended the concept of cancer biology in the sense that various factors such as the tumor microenvironment have to be taken into account and that cancer is not defined by simply enumerating various traits of tumor cells [52]. The heterogeneity in tumor microenvironment, such as for instance the availability of oxygen in different sections of a tumor, often leads to heterogeneity in the expression profiles of cancer cells within that particular tumor. The variation in the expression signatures in different regions of a tumor translates to a perturbation of regulatory signaling pathways, which makes difficult to find a suitable therapy for the whole tumor. The disruption of regulatory pathways in cancer cells is often a source for genomic instability. Prime examples of deregulated molecules are kinases [53], which have become the most intensively pursued anticancer drug targets. Furthermore, kinases are placed at the crossroad/junction of multiple signaling pathways in the intricate intracellular molecular circuitry governing cancer cell biology (Fig. 2). Various kinases, including PKD2, show elevated protein

levels in tumors and might serve as cancer biomarkers. Particularly high PKD2 expression was revealed in multiple epithelial tumors, including pancreatic [21, 54, 55], colorectal [21, 56], prostate [57, 58], gastric [21, 59], breast cancer [60, 61], and hepatocellular carcinoma [62]. High expression of the kinase was also detected in glioblastoma multiforme [63-65] and myeloid leukemia [27]. In the following, we will highlight the most important functions of PKD2/PKD from the perspective of cancer hallmarks as described by Hanahan and Weinberg [51, 52]. As an add-on to these, newly described, as well as potential functions of PKD2 are also presented.

Sustaining proliferative signaling

Normal tissues tightly regulate the production and secretion of growth-promoting signals thereby ensuring the homeostasis and the maintenance of normal tissue architecture and function. By contrast, tumor cells not only deregulate such signals, but also enable signaling mechanisms conveyed largely by growth factors that control the progression through the cell cycle, proliferation, and cell growth. For instance, activation of the extracellular-signal regulated kinase (ERK) pathway culminates with the transactivation of various transcription factors implicated in growth, differentiation, or mitosis. PKD has been shown to prolong ERK signaling via phosphorylation of Ras and Rab interactor 1 at Ser351 [66]. This induces 14-3-3 binding and results in cytoplasmatic sequestration of this interactor, which in turn potentiates the interaction between Ras and the RAF kinase, potentiating ERK signaling [66]. Furthermore, although no direct evidence is present, a putative participation of PKDs to PKC-mediated mitogen-activated protein kinase (MAPK) signaling toward cell proliferation should be also taken into account. Recent evidence suggests that PKDs may play a role in colon cancer. PKD2 is the major isoform in human colon cancer cells, whereas PKD1 is not expressed [67]. Inhibition of PKD with CRT0066101 had anti-proliferative effects on colon cancer cell lines and on tumor growth in a HCT-116 xenograft model. Mechanistically, effects were seen on AKT and ERK signaling after inhibition or knockdown of PKD2 (but not PKD3) [67]. A link between growth factor signaling and cell cycle progression is provided by cyclin D1, which is induced as a secondary response following mitogenic stimulation. Recently, we showed that PKD2 abrogation in glioblastoma cells induced the arrest of tumor cells in the G1 phase of cell cycle, an event that was associated with downregulation of cyclin D1 [63]. Moreover, abrogation (both pharmacologic and genetic) of PKD2 prevented formation of glioblastoma tumors in vivo and corresponded to a significantly reduced number of Ki67-positive tumor cells [63]. Bernhart and colleagues extended these findings to a xenograft mouse model using CRT0066101, a PKD family inhibitor [64]. PKD2 silencing was associated with glioma cell senescence via p53-dependent and -independent pathways [65]. Interestingly, although PKD2 overexpression resulted in enhanced proliferation and migration of gastric cancer cells, ectopic PKD1 had the opposite effects, suggesting that expression level of PKD1/PKD2 may differentially determine the behavior of gastric tumor cells. In a similar manner, silencing of PKD2 but not of PKD1 significantly inhibited the proliferation of primary human endothelial cells [68]. In addition, our lab could demonstrate that PKD2 knockdown in pancreatic cancer cells orthotopically implanted in mouse pancreas was associated with impaired tumor growth [21], further substantiating a pro-proliferative role of PKD2. Ectopic PKD2 expression correlated with augmented expression of Golgi phosphoprotein 3 (GOLPH3) [69], a membrane protein located at the TGN that is involved in vesicle transportation from Golgi to the cell membrane and in the glycosylation of proteins. In this study, high level of GOLPH3 was associated with the activation of phosphoinositide 3-kinase (PI3K)-AKT and enhanced glioma proliferation [69]. Furthermore, GOLPH3 was demonstrated to be a novel oncogene, which is commonly targeted for amplification in human cancers and regulates the response to rapamycin [70]. Growth factors such as gastrin or IGF-1 stimulate PKD activity in addition to their role in tumor cell growth [71-73]. In addition, vascular endothelial growth factor (VEGF) A-mediated PKD activation in endothelial cells was reported to promote both proliferation and migration [74, 75]. Many of these events involve PKC-mediated PKD phosphorylation [17].

Resistance to cell death

Programmed cell death (apoptosis) serves as a natural barrier to cancer development [52, 76, 77]. PKD1 was reported to promote cell survival through the induction of anti-apoptotic proteins survivin and cellular FLICE (FADD-like IL-1β-converting enzyme)-inhibitory protein long form (c-FLIPL) in pancreatic cancer cells [78]. PKD1 and PKD2 protect cells from oxidative stress-induced cell death by activating the transcription factor NF-κB [13, 28, 79, 80]. Interestingly, recent evidence suggests isoform-specific regulation of PKDs in oxidative stress conditions, which is paired by differential requirements for their signaling output to NF-κB. Indeed, PKD2 signaling to NF-κB requires phosphorylation of the kinase at Tyr438 in the human myeloid leukemia cells [27]. However, in contrast to PKD1, activation of NF-κB by PKD2 tyrosine phosphorylation is independent of its catalytic activity. This may suggest that PKD2 acts rather as a chaperone or scaffold and not as a kinase in this process [27]. The production of reactive

oxygen species (ROS) at the mitochondria, an event intimately associated with a variety of human diseases [81-83], has been demonstrated to initiate a mitochondria-to-nucleus signaling cascade in which PKD plays a critical function [80]. In this scenario, elevated mitochondrial ROS (mROS) production results in PKD-dependent activation of NF-κB. Translocation of NF-κB to the nucleus followed by induction of *SOD2* leads to subsequent expression and accumulation of manganese superoxide dismutase (MnSOD), detoxification of ROS, and finally increased cellular survival [13, 80]. Together with other stimuli, ROS also induces apoptosis signal-regulating kinase 1 (ASK1), an upstream activator of c-Jun N-terminal kinase (JNK) and p38 signaling cascades [84]. Using kinase inhibitors and RNA interference, Zhang and colleagues identified PKD1 as s critical upstream mediator in H_2O_2-induced ASK1 activation and showed that 14-3-3 binding to PKD1 plays a critical role in mediating H_2O_2-induced ASK1–JNK activation and endothelial cell apoptosis [85]. PKD1 can also activate JNK downstream of death-associated protein kinase (DAPK) (in a PKC-independent fashion), which results in a pro-death signal and increased necrotic cell death [86]. Furthermore, Chen and colleagues demonstrated a pro-survival and anti-apoptotic role of PKD2 in phorbol myristate acetate (PMA)-induced apoptosis in androgen-sensitive model of human prostatic carcinoma (LNCaP) prostate cancer cells and identified ERK1/2 and NF-κB as downstream effectors [87]. In this study, the authors suggest that PKD2 may signal through non-redundant pathways to modulate PMA-induced apoptosis. Recently, we found that HSP90 chaperone interacts with and stabilizes PKD2 in human cancer cells. Depletion of PKD2 following HSP90 inhibition was corroborated with tumor cell death in vitro, as well as in vivo xenograft models [88]. Implication of the kinase in cell death evoked by HSP90 inhibition together with its ability to mediate stress-induced NF-κB activation and cell survival [13] infer that PKD2 represents an essential molecule involved in HSP90 signaling down to NF-κB and is associated with chaperone-supported cancer cell viability.

Role in EMT

Prior to intravasation, cancer cells often undergo the process of epithelial-to-mesenchymal transition (EMT), an event by which epithelial cells lose their polarization by reorganizing their cytoskeleton and undergoing distinct biochemical changes that allow transition to mesenchymal cells capable to invade, resist apoptosis, and disseminate [89-91]. Although PKD1 was reported to inhibit EMT by controlling the localization of the transcription factor Snail [92, 93] and through E-cadherin phosphorylation [94, 95], the role of PKD2 or PKD3 in the regulation of EMT is poorly understood. Indirect evidence indicates that these two isoforms are potential positive regulators of EMT. Treatment of MDA-MB-231 breast cancer cells with the selective pan-PKD inhibitor CRT0066101 led to reduced expression of EMT markers such as Snail, N-cadherin, MMP9, smooth muscle actin (SMA), and vimentin [96]. Indeed, these data are in line with our own findings showing that ectopic expression of PKD2 in MDA-MB-231 breast or A549 lung cancer cells correlates with acquisition of a mesenchymal phenotype, loss of E-cadherin, and gain of vimentin expression (Azoitei and Seufferlein, unpublished data). Tumor necrosis factor (TNF)α plays an important role in EMT in many cancer cells [97, 98] but also triggers the association of TNFR1 and TRAF2, an event that is required for PKD2 activation and EMT [62]. PKD2 was demonstrated to contribute to TNFα-induced EMT and invasion by increasing the activity of the PI3K/AKT/glycogen synthase kinase 3 beta (GSK-3β)/β-catenin pathway [62]. Phosphorylation of GSK-3β by PKD2 was followed by translocation of β-catenin to the nucleus, regulation of T-cell factor/lymphoid enhancer factor (TCF/LEF)-dependent transcription and enhanced EMT and invasiveness in hepatocellular carcinoma [62].

Invasion and metastasis

The multistep nature of invasion and metastasis has been schematized as a succession of changes in cell biology, beginning with local invasion that leads to intravasation of cancer cells into proximal blood or lymphatic vessels. The journey of cells with the blood/lymph stream to remote distances is followed by the movement of cancer cells from the vessel lumen into the parenchyma of remote tissues (extravasation), establishment of small nodules of cancer cells (micrometastasis), and finally the growth of micrometastatic lesions to macroscopic tumors [51, 52]. PKD1 and PKD2 isoforms show similar structural features and fulfill a variety of overlapping functions [12, 99]. However, their expression in various tumors is not identical. This leads to the fact that tumor-relevant parameters are controlled in an isoform-specific manner, sometimes even in an opposing manner. For instance, PKD1 is downregulated in invasive breast cancer cell lines suggesting an inhibitory role of tumor cell motility [100-102]. The inhibition of tumor cell motility by ectopic PKD1 was also demonstrated in other tumor cells including pancreatic cancer cells. Our lab has investigated the direct cell motility in a pancreatic ductal adenocarcinoma (PDAC) model system and compared the PKD1 and PKD2 invasive features in three-dimensional extracellular matrix (ECM) culture [55, 103]. In this study, PKD1-regulated migration by interacting with cofilin-phosphatase Slingshot-1L (SSH1L) at the cell periphery and in dynamic membrane protrusions [55, 103].

However, although on the one hand PKD1 stable knockdown strongly enhanced invasive outgrowth from tumor clusters, ectopic expression of PKD2 on the other hand significantly enhanced invasion of pancreatic cancer cells in the surrounding the ECM [55, 103]. This is in line with another study showing that abrogation of PKD2 in doxorubicin-resistant Michigan Cancer Foundation-7 (MCF-7) breast cancer cells led to a significant reduction in cell migration [61]. ECM and cancer cells display an intimate interaction. Thus, ECM can influence the behavior of cancer cells, which in turn can cause breakdown of basal membranes by enhanced secretion of MMPs [104]. We identified in pancreatic cancer cells a novel isoform-specific regulatory function for PKD2 upstream of MMP7 and MMP9 in promoting invasion and angiogenesis both in vitro and in vivo [55, 103]. Interestingly, MMP9 is responsible for the release of VEGF [96, 104, 105], a growth factor essential in tumor angiogenesis of whose regulation by PKD2 will be discussed in more detail in the angiogenesis section. The events of invasion and migration promoted by PKD2-regulated MMPs and integrin expression were also reported in glioblastoma [64] and stromal myofibroblasts [106]. Interestingly, in contrast to the effects of PKD2 on MMPs, PKD1 expression in osteosarcoma cells has been shown to reduce invasiveness likely via the decrease of MMP2 expression in these cells. PKD2 and PKD3 also regulate migration and invasion via other mechanisms. For example, in prostate cancer cells, PKD2 interacts with and phosphorylates inhibitor of nuclear factor kappa-B kinase subunit beta (IKKβ) while is responsible for inhibitory kappa B protein alpha (IκBα) degradation [58]. The fact that subsequent nuclear translocation of p65 was primarily dependent on PKD2 rather than PKD3, suggests that PKD2 may play a role in classical NF-κB-mediated cell invasion [58]. In addition, PKD2 also promotes invasion and tumor cell migration by phosphorylating the splice variant of calcium and integrin-binding protein 1 (CIB1a) [107]. Several studies revealed additional substrates for PKDs at the leading edge such as Ena/VASP-like protein (EVL)-1 [108], vasodilator-stimulated phosphoprotein (VASP) [109], and cortactin [110].

Induction of angiogenesis

In order to grow, tumors require oxygen and nutrients, as well as the possibility to evacuate metabolic wastes and CO_2. Angiogenesis, the generation of tumor-associated neovasculature from pre-existing vessels, is the process that ensures all these needs. A large body of evidence demonstrates that the angiogenic switch is orchestrated by countervailing factors that either oppose or induce angiogenesis [111]. A well-known inducer of angiogenesis is VEGF-A, which governs the growth of new blood vessels both in physiological and pathological conditions [112]. VEGF gene expression can be regulated both by hypoxia and by oncogene signaling [113, 114]. Many epithelial tumors are characterized by their ability to grow in hypoxic environment [115-117]. This usually requires upregulation of hypoxia inducible factor 1-alpha (HIF-1α), a transcription factor involved in the cellular response to hypoxia. Hypoxia-mediated VEGF-A expression can be mediated by molecular mechanisms dependent on PKCs [118]. Indeed, our experiments revealed a marked increase in PKD activation under hypoxic conditions [21]. Our lab and others have previously shown that PKD2 modulates the expression of the orphan-nuclear receptor TR3/Nur77, a protein that has been reported to be a stabilizer of HIF-1α and mediator of VEGF expression [21, 119, 120]. Later experiments demonstrated that hypoxia-induced VEGF-A expression and secretion requires expression of TR3 in pancreatic cancer cells, which in turn crucially requires PKD2 [21]. Furthermore, mere abrogation of PKD2 in the tumor cells prevented tumor angiogenesis in two in vivo experimental models, chicken CAM and orthotopic pancreatic tumor xenograft in nude mice [21]. Interestingly, PKD2 is highly expressed not only in tumor cells but also in endothelial cells [21]. Treatment of mice with Vivo-Morpholino splice-blocking oligonucleotides targeting murine PKD2 resulted in impaired blood vessel formation, which resulted in the formation of smaller tumors [21]. Similarly, depletion of PKD2 resulted in decreased basal and VEGF-A-induced endothelial cell sprouting [21]. These data are in line with the fact that PKD2, but not PKD1, is expressed in primary human endothelial cells from various tissues and is required for the expression of VEGFR-2 in endothelial cells [68]. Moreover, in endothelial cells PKD2 is activated by VEGF via tyrosine phosphorylation [74, 75] and contributes to endothelial cell proliferation, migration, and angiogenesis in vitro [68]. A recent study identified PKD2 as a crucial molecule in the stabilization of BCL6-associated zinc-finger (BAZF) protein upon induction by VEGF-A [121]. VEGF-A-induced BAZF forms a complex with cullin 3, an E3 ubiquitin ligase that degrades CBF-1, a transcription factor implicated in Notch signaling [122, 123]. To note, angiogenic sprouting is tightly regulated by the Notch signaling circuit of the VEGFR-Notch ligand, Dll4 [123-125]. Interestingly, HSP90 inhibitors 17-AAG and PU-H71 inhibited PKD2-mediated stabilization of *BAZF-mRNA* [126, 127]. This is in line with our experiments demonstrating that the HSP90 chaperone supports tumor growth and angiogenesis through PKD2 protein stabilization [88]. In this study, we have identified PKD2 as a novel client of the chaperone and demonstrated that ectopic expression of PKD2 restored tumor cell viability and vascularization after HSP90 pharmacologic inhibition in vivo. These results support PKD2 degradation through HSP90 inhibition as a potential

strategy to approach two important cancer features, angiogenesis, and cell viability, with one drug. HSP90 inhibitors have been reported to indirectly regulate HIF-1α [126-128]. The fact that the chaperone interacts both with HIF-1α [128] and PKD2 [88] likely implicates PKD2 in tumor angiogenesis coordinated by HSP90/HIF-1α. Indeed, ectopically expressed PKD2 partially restores HIF-1α protein levels, HIF-1α transcriptional activity, and secreted VEGF-A levels after HSP90 inhibition [88]. Although Choi and colleagues demonstrated that TR3/Nur77 is activated by HIF-1α [129], in the same year Yoo et al. revealed that TR3/Nur77 stabilizes HIF-1α [130]. It still remains to be elucidated whether PKD2 restores HIF-1α through the induction of TR3/Nur77 or whether PKD2 activates TR3/VEGF-A by stabilizing HIF-1α. As the transcription factor NF-κB and its target gene *VEGF-A* are activated by hypoxia, HSP90, and PKD2 [88, 131], it is possible that NF-κB signaling might be connected to the hypoxic response governed by HSP90-PKD2. However, in this study PKD2 only marginally restored hypoxia-induced NF-κB promoter activity during chaperone inhibition, suggesting that additional factors/clients are necessary to transmit HSP90's angiogenic signals via NF-κB pathway [88]. Whether PKD2 activates NF-κB/VEGF-A via upregulation of HIF-1α or whether interaction with other molecules is required (such as IKK [89]) is not fully understood so far. Furthermore, our lab has identified a novel splice variant of CIB1a as a potential substrate of PKD2 [107]. PKD2 interacts with and phosphorylates CIB1a at Ser115, which is associated with tumor cell growth and angiogenesis [107]. Another process through which PKD2 controls tumor angiogenesis is secretion of MMPs. Although PKD2 controls secretion of MMP7 and MMP9, only MMP9 was shown to augment the release of extracellular matrix-bound VEGF-A [107]. The fact that PKD2 controls angiogenesis via regulation of molecules such as CIB1a or MMPs, shown to promote also tumor cell invasion, indicates again the versatility and the implication of this kinase in different aspects of cancer biology.

Regulation of innate and adaptive immune defenses

Human and mouse T- and B-lymphocytes, thymocytes, and spleen cells predominantly express PKD2, but not PKD1 or PKD3 [6]. PKD2 catalytic activity is important for effective cytokine production after TCR stimulation. TCR stimulation results in the translocation of PKD2 to the nucleus, an event associated with the induction of IL-2 and IFNγ production, which play a critical role in adaptive immune responses. TCR engagement induces DAG production and activates PKCs [132-134], which were shown to phosphorylate Ser707 and Ser711 in PKD2. The failure of catalytically inactive PKD2 T-lymphocytes to effectively produce IL-2 and IFNγ suggests that PKD2 has a unique role in controlling T-cell functions during adaptive immune responses [6]. IFNα-stimulated activation of PKD2 requires its phosphorylation at Tyr438, a molecular event also necessary for efficient serine phosphorylation and degradation of IFNAR1 and consequently restriction of magnitude and duration of cellular responses to type 1 interferon including IFNα/β [135]. As IFNα/β are used in treatment of infections, multiple sclerosis, and cancers, deciphering the mechanisms of ligand-inducible activation of PKD2 and its recruitment to the receptor may be of clinical relevance [135]. Notably, IFNα/β antagonizes the process of angiogenesis that is stimulated by VEGF, and both counteracting stimuli activate PKD2. VEGF-mediated recruitment of PKD2 to the IFNAR1 is followed by phosphorylation, ubiquitination, and degradation of the receptor subsequent to inhibition of type 1 IFN signaling. The fact that degradation of the IFN1R is required for efficient VEGF-stimulated angiogenesis [135] adds another level of complexity to the role of PKD2 in cancer progression. Interestingly, PKD2 was recently also implicated in a pathway that drives PDL-1 surface expression downstream of IFN-γ in oral squamous carcinoma, which argues for the combination of (PKD) targeted therapies with the emerging immune therapies [136]. Several other studies revealed that PKD2 does not only play a role in adaptive but also in the innate immune responses. Natural killer (NK) cells were reported to establish the first line of defense against tumor and virus-infected cells [137, 138] and their activation is subdivided in two mechanisms: natural toxicity and CD16-mediated antibody-dependent cellular toxicity. Engagement of CD16 was demonstrated to cause responses in various kinases including PKD2, indicating a role in the signal network orchestrating NK cell effector functions [139, 140]. In this study, application of CID755673, a selective inhibitor of PKD family members, resulted in a significant dose-dependent reduction of NK cell degranulation markers and cytokine release. These data underline PKD2 as a signaling component in NK cell activation and therefore as a molecule with a potential role in cancer immunosurveillance.

Regulation of protein transport and secretion

Altered vesicular sorting or transport of specific proteins has been repeatedly linked to a broad range of cancer types and therefore represents a promising target for therapeutic interventions [141]. PKD isozymes are instrumental in the regulation of protein secretion from the TGN to the plasma membrane [142] by acting as key regulators of membrane fission of transport carriers at the TGN [25, 143-145]. All three isoforms of PKD have been implicated in secretory vesicle biogenesis [17, 26]. In this context, a substantial function of PKD1 in the secretion of several tumor-promoting factors including IL-6, IL-8 and growth-related

oncogene-α in endothelial cells and epithelial cancer cell lines has been characterized [146-148]. PKD2, on the other hand, has been shown to mediate constitutive secretion of MMPs (MMP7 and MMP9) [55], which have both been linked with overall survival in gastric cancer [149] and more generally in tumor metastasis. A vital function of PKD2 was also described in the regulation of peptide hormone release, namely of the neuroendocrine tumor marker chromogranin A, from carcinoid derived pancreatic cells [25]. Furthermore, the isoform PKD3 plays a critical role in the secretion of tumor-promoting factors, for example, in prostate cancer [150]. It is understood that the function of PKDs in vesicle formation is, at least partially, mediated by the phosphorylation and thereby activation of substrate proteins like phosphatidylinositol-4 kinase III β and ceramide transfer protein CERT, followed by changes within the local lipid environment [151, 152]. It is known that PKDs are associated with the TGN by binding to DAG via their C1a zinc-finger domain. PKD2 directly interacts via Pro275 in its C1b domain with ARF1 [153], a GTPase that directly regulates the assembly of budding vesicles by recruiting coat (COPI) and clathrin adaptor proteins to the TGN and by activating lipid-modifying enzymes [154-159]. Mutation of P275 prevents PKD2 localization to the TGN, resulting in a severely reduced secretion of cargo proteins [153]. Interestingly, it was shown that PKD dimerization is essential for effective DAG dependent secretion of membraneous cargo [160]. Furthermore, a recent study has identified a protein complex at the TGN comprising PKD2, ARF1, and the Arf-like GTPase Arl1 [39]. PKD2 acts as a scaffold protein, anchoring the complex at the TGN and recruiting effector proteins like the BAR domain protein arfaptin 2, independent of its kinase function and vital for constitutive secretion processes such as the transport of MMPs [39].

Potential regulation of energy metabolism

The uncontrolled growth of tumors does not only involve deregulated cell proliferation but also adjustments in the energy metabolism. In the early stages of cancer development, tumor cells proliferate beyond the limit of diffusion and face hypoxia, a low oxygen environment reported to prevent energy production by oxidative phosphorylation [161]. The increasing demand for energy by fast proliferating tumor cells in hypoxic microenvironment triggers rapid metabolic reprograming [161-163]. Upon activation of HIF-1α/HIF-2α transcription factors or by Ras oncoproteins, cancer cells undergo a "metabolic switch" by using glycolysis to efficiently maintain cellular bioenergetics in restrictive growth conditions [162-165]. To date, there is no direct evidence on the role of PKD2 in the process of aerobic glycolysis. However, an indirect role of the kinase in energy metabolism could be envisaged upon regarding its involvement in the regulation of HIF-1α. Indeed, we demonstrated that abrogation of PKD2 in pancreatic cancer cells resulted in impaired hypoxia-induced HIF-1α accumulation and transcriptional activity and was associated with decreased tumor growth [21]. Although further and detailed investigations are required, these results might represent the first steps in revealing a novel function for PKD2, namely reprogramming the energy metabolism.

PKD inhibition in cancer therapy

The emergence of PKD2 as a putative therapeutic target for cancer has encouraged the development of potent, selective, and cell-permeable small-molecule inhibitors. Several small-molecule inhibitors such as CID755673 and analogs [166], 2,6-naphthyridine and bipyridyl and analogs [167-169], 3,5-diarylazoles [170], CRT0066101 [56], pteridine [171], and CRT5 [172] were demonstrated to inhibit PKD in vitro and in intact cells. Treatment with CID755673 resulted in a significant reduction of NK cell granulation markers and cytokine release in peripheral blood mononuclear cell population [138]. This study underlines the importance of CaMKII for NK cell signaling and suggests PKD2 as a novel signaling component in activation of NK cells and cancer immunosurveillance. On the other hand, treatment with CRT0066101 was reported to efficiently block the growth of pancreatic and colon tumor xenografts in mice and was associated with G2-M phase arrest and induction of apoptosis [56], whereas CRT5 decreased VEGF-induced endothelial migration, proliferation, and tubulogenesis [172]. Tandon and colleagues described two new pyrazolopyrimidine pan-PKD inhibitors, namely 1-NA-PP1 and IKK-16 that potently inhibited prostate tumor cell migration and invasion [173]. Recently, a more potent PP1 derivative was synthesized showing the potential for this class of molecules as PKD inhibitors [171]. In a targeted screen of 80 chemically diverse compounds, Tandon et al. identified SD-208 that showed moderate inhibitory activity towards PKD with an IC50 of 106 nM in vitro [171]. The (orally available) compound could inhibit prostate cancer cell survival, proliferation, and invasion in vitro and tumor growth in a xenograft model of PC3 cells [171]. PKD2 can be also indirectly targeted by using HSP90 chaperone inhibitors [88] currently under clinical evaluation such as PU-H71, a water-soluble member of the purine class of HSP90 inhibitors [174] or STA-9090, a resorcinol-containing triazole molecule [175]. We have reported that HSP90-dependent PKD2 stabilization results in the activation of NF-κB and its target VEGF-A, which promotes tumor cell proliferation and increased vascularization in hypoxic tumors

[88]. All these initial steps in deciphering the intricate molecular network in which PKD2 is implicated, provide a rational basis for the design and development of new inhibitors that, alone or in combination cocktails with currently available anticancer drugs, may improve the clinical outcome. However, before any PKD-based therapies can be implemented in the clinic, the expression level and pattern of each PKD isoform has to be accurately determined so that the appropriate treatment regimen can be decided upon.

Conclusion and perspectives

There is a compelling body of evidence involving PKD2 in the regulation of multiple signaling pathways, as well as in the integration of extracellular signals that modulate cancer cell morphology, migration, proliferation, and survival (Fig. 2, Table 1, Fig. 3). Various functions of PKD2 in common human tumors were summarized in this review from the perspective of cancer hallmarks described and

Table 1 The table presents the cancer-related functions of each PKD isoform in a particular tumor type

Isoform	Cancer-related function	Tumor type	Activation/regulation	Reference
	Proliferation	Pancreatic		[54]
		Prostate	MMP2/MMP9	[177]
	Invasion	Breast	MMP	[97]
PKD1	Tumor cell viability	Prostate	ERK1/2, NF-κB	[87]
		Pancreatic	Cortactin/SSH1L	[55, 110]
		Breast	MMP	[97]
	EMT	Prostate	Snail	[178]
		Pancreatic	HIF-1α/VEGF	[21]
		Glioma	GOLPH3/AKT	[69]
	Proliferation	Glioblastoma	Cyclin D1/p53	[63-65]
	Invasion	Pancreatic	MMP/Snail	[55, 103]
		Neuroendocrine		[176]
		Prostate	NF-κB/HDAC1/uPA	[58]
	Migration/motility	Pancreatic	CIB1a	[107]
		Breast cancer		[61]
	Tumor cell viability	Myeloid leukemia	NF-κB/Bcr-Abl	[27]
PKD2		Prostate	ERK1/2/NF-κB	[87]
		Colon pancreas lung	HSP90/NF-κB	[88]
	EMT	Hepatocellular	PI3K/GSK-3β/β-catenin	[62]
	Tumor growth	Myeloid leukemia	Bcr-Abl/NF-κB	[27]
		Glioblastoma	p53	[63-65]
		Colorectal		[56]
		Breast		[60]
		Pancreas colon lung	HIF-1α/NF-κB/HSP90	[21, 88]
		Prostate	ERK1/2, NF-κB	[87]
	Secretion	Neuroendocrine	Chromoganin A	[22, 25]
		Pancreatic	MMP7/MMP9	[55, 93]
	Tumor angiogenesis	Pancreatic	HIF-1α/VEGF-A/BAZF	[21, 120, 121]
		Pancreatic	CIB1a/MMP9	[107]
		Colon lung	HSP90/HIF-1α/NF-κB/VEGF-A	[88]
	Proliferation	Prostate	MMP2/MMP9	[177]
		Breast	mTORC-S6K1	[179]
PKD3	Tumor growth	Prostate	Akt/ERK1-2	[57]
		Breast		[60]
	Invasion	Prostate	NF-κB/HDAC1/uPA	[58]

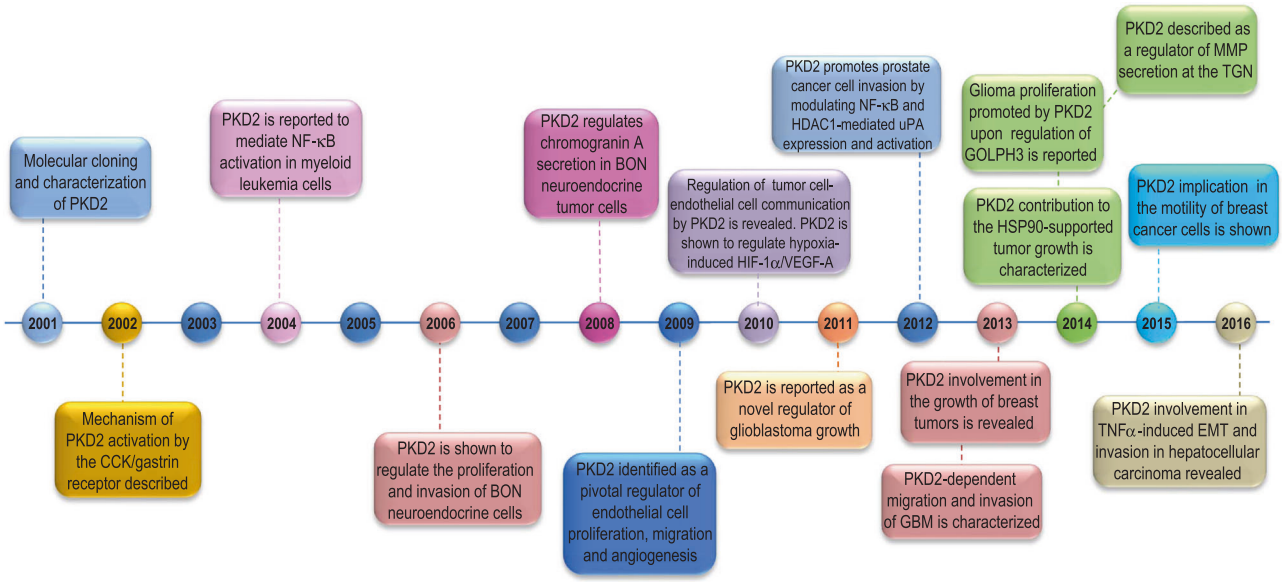

Fig. 3 The timeline presents the most important findings on the role of PKD2 in tumor progression since the molecular cloning of the kinase

revisited by Hanahan and Weinberg in years 2000 and 2011, respectively. Newly described, as well as potential functions of PKD2, not necessarily regarded as a cancer hallmark, are also presented. While multiple studies over the last 16 years identified previously unrecognized mechanisms of action for PKD2, there is still need for a better understanding of differential kinase expression, isoform-specific functions, and activation of different isoforms and molecular cross-signaling. Delineation of potential compensatory actions between various PKD isoforms in a given tumor will help to enhance the therapeutic prospects of PKDs in a successful combinatorial molecular therapy approach.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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