Equivocal, explicit and emergent actions of PKC isoforms in cancer

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Abstract | The maturing mutational landscape of cancer genomes, the development and application of clinical interventions and evolving insights into tumour-associated functions reveal unexpected features of the protein kinase C (PKC) family of serine/threonine protein kinases. These advances include recent work showing gain or loss-of-function mutations relating to driver or bystander roles, how conformational constraints and plasticity impact this class of proteins and how emergent cancer-associated properties may offer opportunities for intervention. The profound impact of the tumour microenvironment, reflected in the efficacy of immune checkpoint interventions, further prompts to incorporate PKC family actions and interventions in this ecosystem, informed by insights into the control of stromal and immune cell functions. Drugging PKC isoforms has offered much promise, but when and how is not obvious.

1,2-diacylglycerol (DAG). A neutral lipid component of membranes, serving in the biosynthesis of more complex lipids and as a signalling lipid.

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https://doi.org/10.1038/s41568-020-00310-4

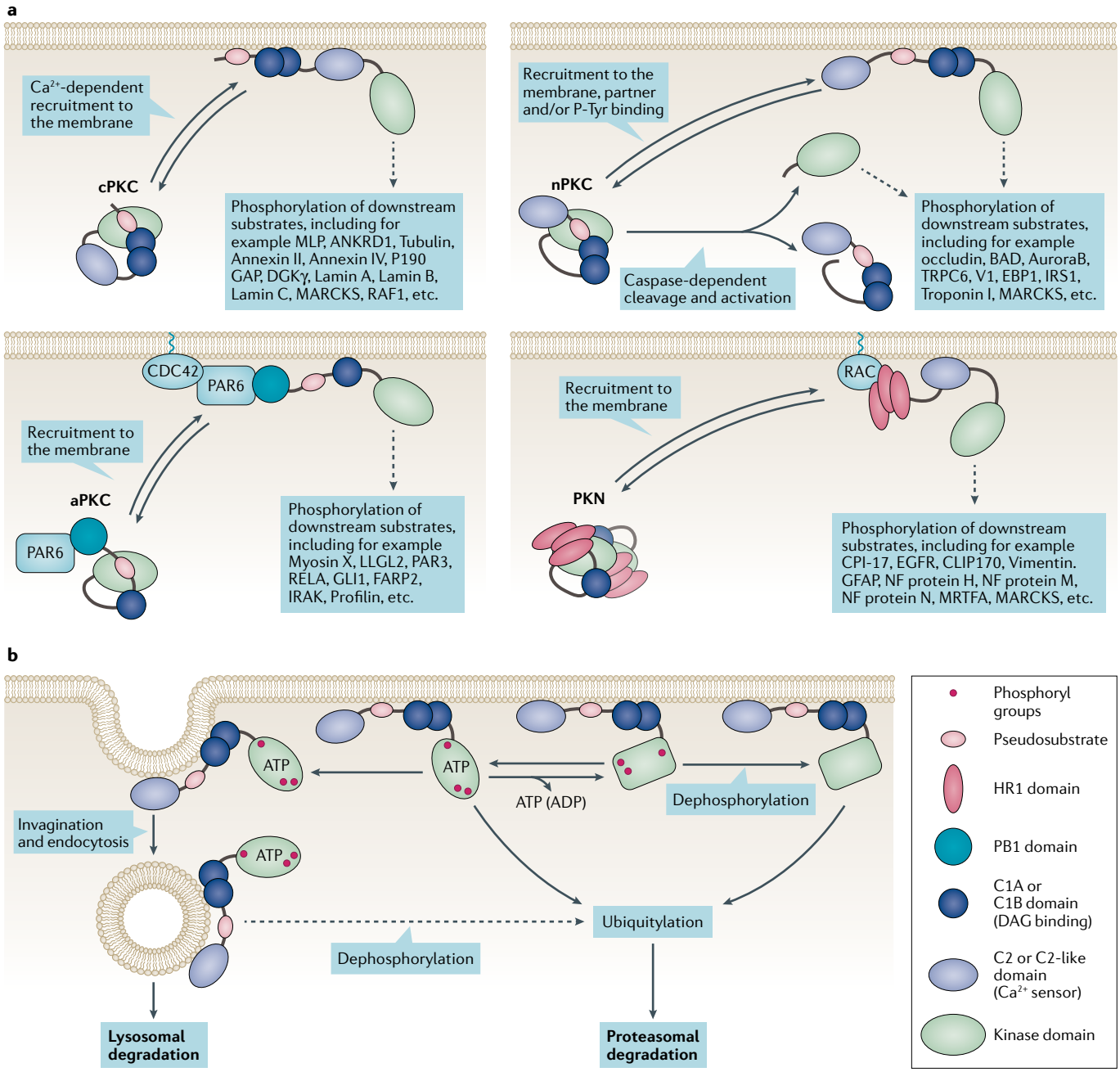
The protein kinase C (PKC) family of serine/threonine protein kinases, comprising the 'classical' PKC (cPKC), 'novel' PKC (nPKC), 'atypical' PKC (aPKC) and PKN subfamilies, is one of the defining families of the AGC kinase class[1]. They retain a modular structure, consisting of domain permutations in their N-terminal regulatory regions, linked via variable sequences to highly conserved C-terminal kinase domains[2]. In *Saccharomyces cerevisiae*, the singular *PKC1* gene encodes a protein retaining domains characteristic of the greatly expanded mammalian family[3]. A subset of PKCs (cPKCs and nPKCs) are responsive to the second messenger 1,2-diacylglycerol (DAG) and feature in many signalling cascades downstream of the broad class of phosphoinositide-specific phospholipases (reviewed elsewhere[4]), which are themselves linked to a spectrum of G protein and tyrosine kinase-associated receptors (see REF.[5]). Other family members respond directly (PKNs) or through partner proteins (aPKCs) to membrane-active, small G proteins, downstream of the exchange factors that control them (recently reviewed elsewhere[6]).

The potential impact of PKCs on cancer has been the subject of extensive investigation, greatly influenced by the pioneering work from Nishizuka's laboratory that identified 'PKC' as a target for certain tumour promoters[7]. What has emerged in the intervening decades, informed by cancer genomics, ex vivo studies and in vivo models, is a complex picture that presents practical and conceptual challenges to the field. Here, we provide an overview of PKC functional attributes, elaborating on properties that influence target validation in cancer. The Review will then focus on the cPKC and nPKC families as DAG and/or tumour promoter-responsive kinases, discussing promoter and suppressor activities in experimental studies and associated with cancer genomics. Finally, we comment on PKC pharmacology and clinical trials. To note, there are over 12,000 publications in the PKC–cancer area and not all will be referenced; rather, exemplars of critical findings and commentaries will be featured, so we beg indulgence of those in the field who have contributed greatly but are conspicuous by their absence.

Regulation and function of PKCs

Turning PKCs on

Canonical activation of cPKCs, which include PKCα, PKCβ and PKCγ, and of nPKCs, which include PKCδ, PKCε, PKCη and PKCθ, involves the binding of membrane-resident DAG, inducing conformational changes and the release of the autoinhibitory pseudosubstrate site, triggering catalytic activity-dependent downstream events[8] (FIG. 1a). A similar conformational principle operates for aPKCs, which include PKCζ and PKCι, albeit effected physiologically through the protein binding of CDC42 and PAR6 or p62 (REF.[9]) to their regulatory domains in a spatially constrained manner (see recent review[10]). A related scenario pertains to the activation of proteins of the PKN subfamily, which include PKN1–3, responding to RHO or RAC[11]; however, this is likely complicated by autoinhibitory dimerization in the basal state as reported for PKN2 (REF.[12]). In all cases, the membrane-recruited PKCs take on a de-inhibited,



open conformation, competent to phosphorylate substrates and associate with conformation-dependent partners[13,14]. There is the potential for dissociation from the membrane of scaffold-bound, active PKC, but evidence for this is scarce[15]. Experimentally, cPKC, aPKC or nPKC isoforms can be expressed as open-conformer, gain-of-function mutants through mutation of the gene regions encoding their autoinhibitory pseudosubstrate sites (see REF.[16]).

The catalytic potential of PKCs is dictated by 'priming' phosphorylations in their catalytic domains that are largely conserved in AGC family members and executed by common PDK1 and mTORC2 pathways[17–20] (see animated model for PKCε; Supplementary video). Autophosphorylation of the hydrophobic priming site has also been proposed (reviewed elsewhere[21]), but this does not appear to dominate behaviour in cells[22]. Integrity of the kinase domain for priming is nevertheless a necessity, requiring competence to bind nucleotide that acts to protect the phosphorylated kinase domain from dephosphorylation[22–24].

Open PKC conformers are required for the upstream kinases to act upon them, for example, the action of PDK1 on PKN1 requires RAC and/or RHO in cells[25] and, similarly, PKC recruitment to membranes appears critical for PDK1 input (reviewed elsewhere[26]). PKC kinase domain priming phosphorylations are typically retained under autoinhibited conditions, such that PDK1 and/or mTORC2 activity is not required to impact short-term actions. Acute inhibition of these upstream kinases has limited effect on PKC isoform phosphorylation, but knockout of the gene encoding PDK1 has a

Conformer
Used in a generic manner to indicate a particular protein conformation.

◀ Fig. 1 | **Domain organization, activation and downregulation pathways for the PKC family. a** | Domain organization and activation. For the 'classical' protein kinase C (cPKC) subfamily, calcium increases membrane association through C2 domains, promoting C1A/B sensing and engagement of 1,2-diacylglycerol (DAG) at the membrane. This leads to dissociation of the pseudosubstrate site from the catalytic domain, permitting substrate engagement. For the 'novel' PKC (nPKC) subfamily, C2 domain interactions with partner proteins recruit isoforms to the membrane. Membrane occupancy enables efficient C1A/B-mediated DAG monitoring and binding, and pseudosubstrate release, enabling catalysis. Some nPKCs are subject to caspase-dependent V3 domain cleavage, leading to kinase activation. For 'atypical' PKC (aPKC) isoforms, partitioning defective protein 6 (Par6) interacts with the N-terminal PB1 domain, enabling membrane recruitment through Par6–CDC42 binding. The single C1 domains of aPKCs do not bind DAG but have non-specific membrane binding activity, possibly enabling release of the pseudosubstrate site and activation of kinase function. aPKCs are held in membrane compartments by other proteins in addition to these core functions. For the PKNs, extrapolating from the PKN2 behaviour, the cytosolic autoinhibited dimer is activated by recruitment to the membrane through its HR1a/b domains at the N terminus. These make a bivalent contact with isoprenylated, GTP-bound (active), RAC or RHO family proteins at the membrane, leading to dissociation of the dimer and activation. The additional input from the C2 domain is likely to be through supplementary membrane-partner interactions. **b** | Activation-induced degradation pathways for PKC. In some cell types, degradation proceeds through the loss of nucleotide pocket occupation through ATP or ADP, altered conformation of the kinase domain and efficient dephosphorylation. This is followed by ubiquitylation and proteasomal degradation. Alternatively, activation-induced endocytosis leads to degradation in lysosomes (can facilitate ubiquitylation-dependent degradation, possibly involving dephosphorylation). CDC42, cell division control protein 42; CLIP170, cytoplasmic linker protein 170; DGKγ, diacylglycerol kinase-γ; EBP1, ErbB3-binding protein 1; EGFR, epidermal growth factor receptor; FARP2, FERM, ARH/RhoGEF and pleckstrin domain protein 2; GAP, GTPase accelerating/activating protein; GFAP, glial fibrillary acidic protein; IRAK, interleukin-1 receptor-associated kinase; IRS1, insulin receptor substrate 1; LLGL2, lethal (2) giant larvae protein; MARCKS, myristoylated alanine-rich C-kinase substrate; MLP, muscle LIM protein; MRTFA, myocardin-related transcription factor A; NF, neurofilament; P-Tyr, phospho-tyrosine; TRPC6, transient receptor potential cation channel subfamily C member 6.

more profound effect[27], as does prolonged inhibition of mTORC2 function[20]. This relative stability of priming phosphorylation contrasts starkly with the related AGC kinases of the AKT-PKB family (see REF.[28]) and makes these priming modifications poor read-outs of PKC activity (see Challenges for target validation).

PKCs are basophilic kinases[29] with overlapping substrate recognition as demonstrated in *Drosophila*[30] and in mammalian cells[31]. This overlapping specificity has profound functional consequences, as is evident in a double *Prkce* (encoding PKCε) and *Prkcd* (encoding PKCδ) knockout mouse that is embryonic lethal whereas neither individual knockout displays a developmental phenotype[32]. Beyond their intrinsic specificities, many isoforms have extensive interactomes, associating with scaffolds and partners that impact localization (reviewed elsewhere[13,14]) as well as substrate docking, as documented for aPKC[33].

Turning PKCs off

The inactivation of PKCs is, in part, dictated by the loss of the typically transient triggers that switch them on. Metabolism of DAG will lead to membrane dissociation of cPKC or nPKC, and then the regulatory domain will reassociate with the catalytic domain through interaction between the pseudosubstrate and substrate binding pocket[34], and likely other inter-domain interactions[35], leading to the accumulation of the primed, latent protein in the cytosol. Beyond this simple reversal of activation, activation-associated downregulation of PKC protein levels has been characterized for the DAG-responsive cPKC and nPKC isoforms. However, how acute or chronic activation impacts regulation of protein levels of aPKC and PKN isoforms is not clear. Downregulation of PKC isoform protein levels is associated with cell type-specific patterns of endomembrane trafficking, dephosphorylation, ubiquitylation and degradation of the respective PKC isoform (FIG. 1b). The extent to which one or other degradative pathway dominates, the activity of specific protein phosphatases, E3 ligases and endocytic requirements, reflect the cell model and the PKC isoform that is affected.

Activation-induced downregulation of PKCα protein levels was originally linked to degradation of PKC protein[36]. Subsequently, evidence indicated that PKC downregulation (PKCα, PKCδ, PKCε) was associated with ubiquitylation[37–39] and also with dephosphorylation and caveolin-dependent endocytosis[40,41]. Two distinct pathways acting in parallel were later reported for PKCα: one involving the ubiquitylation of plasma membrane active, primed protein and its degradation through the proteasome; the second engaging caveolin-dependent traffic and non-proteasomal degradation[42]. Two separate endocytic pathways were reported by Lum et al.[43], and the sequential operation of cholesterol-dependent endocytosis of ubiquitylated PKCα with delivery to the proteasome provides yet another route to downregulation[44].

Various E3 ligases have been proposed to drive PKC ubiquitylation and proteasomal degradation in different contexts, including RINCK, LUBAC and MDM2 (REFS[45–47]). Interestingly, the LUBAC complex preferentially bound activated cPKC, consistent with the observed activation-induced ubiquitylation[47]. Contrasting with these emerging players, molecular details of membrane traffic-dependent, non-proteasomal degradation are limited.

Priming site dephosphorylation of PKCs is a prelude to degradation in many contexts. In the inactive state, PKC priming site dephosphorylation is limited by the interaction between the regulatory and catalytic domains, as indicated by the finding that the phosphatase PHLPP1 suppresses the accumulation of primed PKCβ when there are mutations in the inhibitory pseudosubstrate site[48]. In the membrane-associated active state, dephosphorylation is governed by nucleotide pocket occupation[22]. cPKCs may require peptidyl-prolyl isomerization of the turn motif priming site (phospho-Thr-Pro) by PIN1 to enable dephosphorylation and ubiquitylation[49]. The often transient nature of DAG production physiologically means that, under many circumstances, activation-induced dephosphorylation may have a limited impact on cPKCs and nPKCs. However, there are contexts in which dephosphorylated PKCs accumulate, reflecting either reduced action of upstream kinases or the increased dephosphorylation of primed PKCs under conditions of protection from degradation (see, for example, REFS[40,50,51]).

Challenges for target validation

Consideration of PKC isoforms as drug targets sits squarely with the generic demands of any intervention programme — what is the clinical evidence for action

or inaction playing a critical role in a given disease setting, and what is the expectation of a suitable therapeutic index? For patients with cancer, target validation draws, in part, on the evidence of observed somatic changes impacting function (see PKC gene mutations in cancer), transcriptional and protein-level changes that also may reflect gain or loss of function and evidence of downstream pathway dysregulation. For PKC genes, like any other, these patient-derived data need interpretation in the context of our understanding of the intrinsic isoform properties, their physiological roles and experimental tumour models (see cPKC and nPKC in tumour models).

A substantial gap in addressing these validation issues is the lack of biomarker evidence that speaks to PKC activation or inactivation in tumour settings (see FIG. 2a). In an experimental context, isoform activation has been monitored through rapid fractionation protocols (for example, REF.[52]), fluorescently tagged isoforms as initially reported by Saito and colleagues[53] and direct compartment-directed activity monitors[54]. However, these approaches do not lend themselves

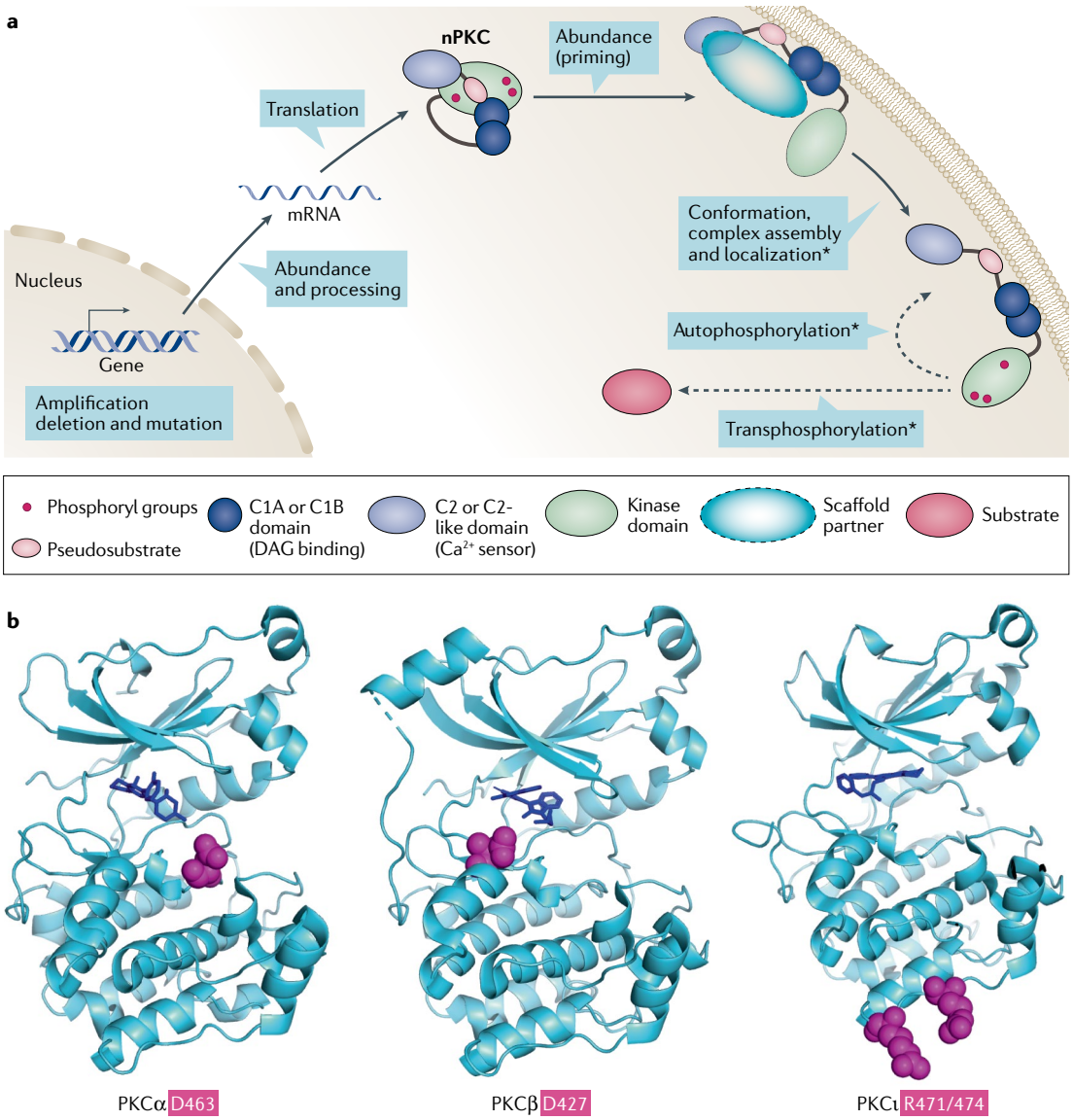


Fig. 2 | **Biomarkers of PKC action and inaction. a** | Protein kinase C (PKC) isoform attributes that have been considered biomarkers to inform on roles in pathological settings: genomic alterations, transcriptional and translational changes, the extent of priming phosphorylation, subcellular localization, complex formation, conformation, self-phosphorylation (autophosphorylation) and substrate phosphorylation (transphosphorylation). There is richness in the data showing levels of expression or concentration of molecules (mRNA in particular) but a paucity of functional data (for example, substrate phosphorylation; the processes for which there is lack of functional data are denoted by an asterisk in the figure), and development of this latter, functional information would be of significant value. The value of priming phosphorylation data as a PKC functional read-out is doubtful. **b** | Sites of penetrant mutations in the kinase domains of PKCα and PKCβ are indicated in the context of their solved kinase domain structures, alongside the hotspot but infrequent kinase domain mutation in the PKCι substrate docking motif. DAG, 1,2-diacylglycerol; nPKC, 'novel' PKC.

Box 1 | **The 1,2-diacylglycerol non-responsive, 'atypical' PKC isoforms in cancer**

'Atypical' protein kinase C (aPKC) isoforms, which include PKCζ and PKCι, are involved in a wide range of cellular functions including the maintenance of polarity, proliferation, cytoskeletal functions, apoptosis and growth factor signalling[175–178]. Unsurprisingly, there are numerous reports associating aPKC deregulation to cancer.

Patient tumour profiling, although of uncertain interpretation for PKC (see text), has generally implicated PKCι as pro-oncogenic. Chromosome 3q26, where the *PRKCI* gene is located, is commonly amplified in human cancer, and both the transcript and protein have been inversely correlated with patient outcomes[179–186]. Infrequent, hotspot mutation of the gene region encoding the polarity-required substrate docking site in PKCι has also been observed[33] (FIG. 2b). By contrast, PKCζ has been implicated as a tumour suppressor in colon cancer, correlating with reduced expression[187].

In several cancer models, a body of literature has accumulated from the Moscat laboratory indicating that PKCι has a suppressive role in tumorigenesis (recently reviewed elsewhere[188]). In prostate cancer cell lines, PKCι knockout induced a neuroendocrine phenotype, increased proliferation and tumour growth, an effect mediated by increased serine biosynthesis[189]. Combined knockout of *Prkcz* (encoding PKCζ) and *Prkci* in the mouse intestine led to the formation of serrated colon tumours with impaired IFNγ expression and decreased $CD8^+$ T cell infiltration suggestive of deficient immune surveillance[190]. In studies of human peripheral blood mononuclear cells, PKCζ was shown to modulate the activation of NF-κB in monocytes and macrophages[191] with an anticipated impact on the behaviour of the tumour niche. Juxtaposed to these experimental observations is the requirement for PKCι in mutant-RAS induced lung, colon and pancreatic tumours[185,192,193] and the ex vivo reversal of RAS-transformed phenotype with PKCι-selective inhibition[61].

For aPKC isoforms, the contrasting literature prescribes the need for direct insight into the roles of aPKC in tumour growth in patients through application of biomarkers informing on aPKC action or inaction.

to pathology. As detailed above, the levels of priming phosphorylations required for function do not typically correlate with levels of activation, and chronic activation can actually induce dephosphorylation and degradation. Thus, measurements that are related to PKC protein levels do not, of themselves, provide insight into pathway function.

Intramolecular events have been investigated as activation markers, specifically autophosphorylation[55–58]. This has been exploited in pathological samples for PKCα using imaging methodologies not easily adapted to routine use[56]. It also transpires that, in cells, 'autophosphorylation' for PKCε, although potentially dependent upon membrane recruitment and conformational activation, is executed in *trans*, limiting biomarker utility[57].

There is a wealth of data on higher or lower levels of expression of PKC isoforms in cancers (Supplementary Figure 1), but this is not coupled to defined downstream events that provide insight into action or inaction. For these highly regulated signalling proteins that do not themselves appear to signal via concentration-dependent oligomerization (aPKC might be an exception in some circumstances[59]), variations in expression alone may not impact signal output without other contributing factors that influence signal input or downstream signal termination. Is the increased expression of PKCι and PKCζ, and the reduction of PKCβ and PKCθ, meaningful in pancreatic ductal adenocarcinoma, and is a reverse functional interpretation valid for the inverse pattern of expression reported for renal clear cell carcinoma (Supplementary Figure 1), or are these, in fact, bystander transcriptomic changes that reflect programming within the tumour, which might be prognostic signatures, but do not assert gain or loss of function?

Ultimately, understanding the context-dependent molecular mechanisms of PKC isoform action will provide the much-needed biomarkers that give insight into pathway operation in tumours and pharmacodynamic biomarkers for trials; specifically, pathophysiological mechanisms, which do not always reflect amplified or muted physiology. This is well exemplified by PKCι that is an established regulator of cell polarity, a property considered tumour-suppressive and characteristically lost in transformed cells[60]. PKCι operates in a sweet spot to control polarity, and too little or too much activity prevents polarization; this is not a concentration-dependent titration of interacting partners but a property that can be reversed by catalytic inhibitors[61]. Such behaviour likely underlies the aPKC suppressor–promoter question (see BOX 1).

cPKC and nPKC in tumour models

Against a backdrop of the roles of cPKC and nPKC as mediators of downstream signalling for tumour growth-promoting signals, or tumour promoters, numerous cell transformation and in vivo mouse models have been assessed for the tumour promoters' dependence upon PKC family members. This has created a varied and sometimes conflicting profile of promoter and suppressor actions.

Phorbol ester-mediated tumour promotion

In mouse skin pretreated with a sub-threshold dose of a carcinogen such as DMBA, phorbol esters will promote the formation of papillomas followed by conversion to overt carcinomas on continued exposure (reviewed elsewhere[62]). The initiation event is stable, requiring DMBA metabolism to a genotoxic form (reviewed elsewhere[63]), and it has been established that this genotoxic form frequently induces RAS mutations[64]. The tumour promotion process elicited by phorbol esters itself has multiple stages, with an irreversible first step, a chronic phase that is, at least initially, reversible and a progression phase that is irreversible[63]. Phorbol esters represent only one class of tumour promoters and impact both the presumptive tumour as well as the tumour microenvironment (TME) where a clear inflammatory driver is involved[65].

The DAG-responsive cPKC and nPKC isoforms are the founding members of the class of targets for the phorbol esters[7,66]. Molecularly, phorbol esters act in a membrane context by mimicking DAG to engage the C1 domains of cPKCs and nPKCs causing activation[67]. Underlining their importance as targets, structurally unrelated tumour promoters also act on PKCs, including mezerin, teleocidin and aplysiatoxin[68–70]. However, not all tumour promoters in this particular mouse skin model target PKC; additional targets of mouse skin tumour promoters include the ER calcium-ATPase (the target of thapsigargin)[71] and protein phosphatase 1/2A (inhibited by okadaic acid[72]).

Phorbol ester-mediated downregulation of PKC protein levels in the mouse skin promotion model has been documented[73,74]. That phorbol esters induced acute

activation of PKCs, followed by chronic downregulation of PKC protein levels, begs the question of whether cPKC and nPKCs in this context function as oncogenic drivers and/or as tumour suppressors. This complexity in interpreting causation is heightened by two further considerations. Firstly, PKC isoforms are not the only C1 domain-containing proteins in the human genome (discussed elsewhere[75]) and, although not all C1 domains bind phorbol esters with high affinity, the tumour promotion response to these C1-binding promoting agents is likely a complex pattern of action on multiple targets. Secondly, the behaviour of these initiation–promotion models reflects an interplay of both the somatically altered target cell (for example, HRAS mutant target cell[64]) and the inflammatory cellular environment elicited by these promoters (discussed elsewhere[65]). Notably, PKC isoforms and other tumour promoter targets are expressed both in the emerging tumour and in the infiltrating inflammatory cells, stroma and vasculature, questioning the combinatorial nature of C1 domain protein engagement in these individual cell types and making resolution of essential promoter or suppressor actions more difficult to dissect.

Suppressors or promoters

Constitutive knockout of genes encoding PKC isoforms in mice (all are viable except *Prkci* or *Pkn2* knockout mice[76,77]) do not predispose to cancer in the manner of a classic tumour suppressor (for example, p53 or APC), although an increase in spontaneous colorectal lesions has been reported in *Prkca*$^{-/-}$ mice[78]. The impact of changes in PKC activity or expression has been assessed more widely in mouse models of cancer in the context of other treatments and/or driver mutations and, here, gain-of-function and loss-of-function alterations indicate a mixed pattern of behaviours, as exemplified below.

For *Prkca*, transgenic expression in the basal layer of the epidermis sensitizes to phorbol ester-driven inflammatory responses and to papilloma–carcinoma conversion in mice[79,80]. However, in the *Prkca* knockout mouse, whereas the absence of PKCα reduces the inflammatory response to phorbol ester promotion, the knockout also leads to enhanced tumour formation[81]. These somewhat contradictory observations likely reflect the complex interplay of diverse cellular responses and that the altered PKCα expression impinges on different cell types in these models. In the *Apc*$^{+/Min}$ mouse model of CRC, *Prkca* knockout increases the tumour growth rate and aggressiveness but not the incidence[78]. It would be of interest to determine whether this effect of PKCα deficiency is dependent on its specific loss in the follicle-derived tumour cells or impacts through the microenvironment.

A tumour-suppressive role for PKCδ has been reported[82]. In the mouse skin promotion context, transgenic expression of *Prkcd* has a selective effect in suppressing phorbol ester-induced tumour formation but not that promoted by ultraviolet[83], suggesting that there are distinct PKCδ-dependent and independent signalling pathways operating in this model. It would be informative to determine whether the phorbol ester effect (that is, PKCδ activation) is dominant over ultraviolet action when co-administered in this model. Knockout of *Prkcd* in mice leads to a lymphoproliferative response with altered B cell self-tolerance[84,85]. Interestingly, in a patient with an autoimmune lymphoproliferative syndrome-like disease, a mutation in *PRKCD* was identified associated with a substantial loss of protein expression[86]. The lymphoproliferation phenotype of this germline alteration indicates specificity in the wiring of B cell controls, with PKCδ acting in a tolerogenic, physiological feedback to promote B cell anergy and, in this cellular context, to be proliferation-suppressive. However, suppressive actions cannot be attributed exclusively to PKCδ, as shown in an MMTV-ERBB2 transformation model[87] and also in urethane-induced lung tumours in mice[88] where PKCδ plays promoting roles.

Tumour or microenvironment action

The extent to which PKC activation or absence impacts the stroma, the innate or the adaptive immune system, is germane to defining promotion and/or suppressor functions. These latter terms typically refer to the tumour autonomous behaviour and not to the TME dependencies; however, experimentally we do not often distinguish the site of action.

Many isoforms control aspects of immune cell function. PKCβ is known to influence B cell responses in mice[89] and was recently shown to regulate mTORC1 signalling in mouse B cells, influencing gene expression and metabolic reprogramming[90]. PKCα regulates T cell-dependent interferon production and B cell IgG2a/b class-switching[91]; and PKCε influences T cell differentiation[92] and macrophage function[93]. PKCθ regulates T cell receptor (TCR)-induced NFAT and NF-κB activation[94,95] and prevents stabilization of $CD4^+CD25^+$ regulatory T cells[96,97], supporting tumour immune recognition. Conversely, PKCη associates with cytotoxic T lymphocyte-associated protein 4 (CTLA4) at the regulatory T cell immune synapse, enabling immune suppression[98]. This likely relates to the reported tumour-suppressive effects of PKCη[99] and its broader regulation of adaptive and innate immune cell functions[100,101].

The influence of PKCs on immune cells and, more generally, the TME questions where experimental organismal inactivation impacts tumorigenesis, and there are few examples in which this issue has been addressed directly. In the MMTV-PyMT model of breast cancer, PKCβ has been found to promote tumour formation[102]. Allograft of an MMTV-PyMT tumour (PKCβ replete) into a *Prkcb*$^{-/-}$ recipient mouse has shown that the requirement for PKCβ for tumour growth in this model operates through its expression in tumour-associated cells[102]. A similar tumour-conducive effect of PKCβ in stroma has recently been described in a model of B cell malignancy[103].

Allograft experiments have shown that the seeding of melanoma-derived lung tumours is compromised in *Pkn3* knockout mice[104], consistent with the small interfering RNA-mediated knockdown of *Pkn3* inhibiting metastasis in vivo[105] although contrasting with the tumour-directed effects observed for *Pkn3* knockdown in an orthotopic prostate cancer mouse model[106].

MMTV-ERBB2 transformation model
A transgenic mouse model with expression of the receptor tyrosine kinase ERBB2 under the control of the mammary gland selective MMTV promoter.

Evidently, the vasculature and tumour niche can be impacted by PKC isoform action or inaction, and this may also contribute to the distinctive responses observed with C1 domain-targeting PKC activators employed clinically (see below), the bryostatins and epoxytiglianes. The bryostatins are PKC activators[107] with a context-dependent, variable ability to invoke PKC downregulation in cell culture[108,109]. Remarkably, bryostatin 1 can protect from phorbol ester-induced tumour promotion[110]. The target cell types that mediate this tumour-suppressive behaviour is not known. Using intratumoural injection, the PKC activators belonging to epoxytiglianes have been shown to have efficacy in treating mouse cancer models[111] and also in treating canine mast cell tumours[112]. As such, tigilanol tiglate has been approved for the treatment of canine mast cell tumours by the European Medicines Agency (EMA). Intratumoural injections produce high local concentrations, and the extent to which the responses to epoxytiglianes are PKC-dependent rather than acting through other C1 domain targets and physicochemical effects remains to be seen. It is also noted that there is evidence of vasculature targeted effects for the haemorrhagic necrosis observed in response to tiglianes[111].

PKC gene mutations in cancer

The mutational landscape of human cancer has provided some profound insights into drivers of disease, exemplified by the penetrant mutation of *BRAF* in melanoma[113]. For PKC genes, there is a spectrum of patient-specific, private mutations across cancer genomes and some rare penetrant mutations.

Private mutations in PKCs

Recent studies have addressed the breadth of mutations found in PKC genes in human cancers and concluded that these proteins play a suppressive role (reviewed elsewhere[21]). Direct analysis of the numerous private *PRKCB* mutations indicated that they are loss-of-function mutations, and one studied in detail (A509T) was shown to be dominant, rationalizing the heterozygous nature of these mutations[114]. The reversion of this *PRKCB*^{A509T} mutation in a naturally occurring cancer cell setting (DLD1 colon cancer cells) and the associated tumour growth rate reduction supports a tumour-suppressive role of PKCβ and reinforces the idea that specific genetic context is critical in these functional assessments.

Although consistent with a tumour suppressor role, the penetration and pattern of these diverse PKC mutations begs the question of whether, in patients, these are bystander events or contributors to disease and/or disease progression. The penetrance of cancer-associated mutations for *PRKCA* (encoding PKCα) is similar to non-synonymous mutations seen in correspondingly sized genes from the clotting cascade (for example, genes encoding protein S and protein C, based on data from cBioPortal). Aggregating data from 15 tumour groups, there is no significantly greater frequency of non-synonymous mutations in *PRKCA* that would reflect a selective advantage, and neither is there any pattern of mutational change that indicates a tissue-specific behaviour; rather, a higher incidence for one gene in a particular tumour type reflects a higher incidence for all genes. So, is there mutation selection or are these bystander events? This remains to be resolved and will require further analysis alongside a wider assessment of the dominance or recessive behaviour of these heterozygous mutations that are predicted to confer a loss of function.

cPKC mutations in rare cancers

High-penetrance somatic variants provide robust evidence for their role in diseases. For PKC, this is a small collection of smoking guns with just two relatively rare tumour types where cPKC gene mutations are highly penetrant, adult T cell leukaemia lymphoma (ATLL) and chordoid gliomas. The issue here is how we interpret the functionality of these somatic variants.

PRKCB mutation in ATLL. ATLL is associated with HTLV-1 infection, a retrovirus endemic in certain areas of the world. The virus establishes lifelong latency in T cells leading to an ATLL lifetime risk of 4–7% (REF.[115]). In a comprehensive survey of the ATLL mutational landscape, somatic changes were documented along the TCR–NF-κB pathway, including frequent mutations in genes encoding phospholipase Cγ (PLCγ; 36%) and PKCβ (33%)[116]. Mutations along this pathway have been predicted as gain-of-function mutations, including those found in *PRKCB*; in the case of inhibitory inputs to this pathway, somatic changes have been assigned as loss of function providing a consistent view of pathway activation[116].

The most penetrant ATLL mutation in *PRKCB* results in an amino acid substitution at D427 in the kinase domain (FIG. 2b), typically D427N. Both the pattern of mutations in genes of the TCR pathway and the limited functional data available suggest that this D427 mutation is an activating mutation. Based upon homology modelling informed by a substrate peptide-bound kinase domain structure of PKCι[117], it is inferred that the D427 residue lies proximal to the substrate binding pocket of PKCβ, such that substitution may compromise binding of the autoinhibitory pseudosubstrate. Although these interactions are not the totality of the regulatory domain–catalytic domain interface[35], it is the case that point mutations and deletions in the inhibitory pseudosubstrate sequence lead to a more active and open conformer in cells[34]. The implication is that, as an open conformer, the mutated PKCβ is activated and/or downregulated (see above). This has yet to be resolved directly, although it has been reported for B cells that PKCβ is required to support the NF-κB pathway through CARD11 and IKK[118], consistent with the gain-of-function analysis predicted in T cells[116]. If PKCβ activation is causative in driving tumour growth, might current PKCβ-directed drugs work? Not necessarily for this D427 mutation, as manipulation of the homologous region of PKCι has been shown to influence substrate interactions[119] and pharmacology[120].

The specific nature of these effects in PKCβ will require further analysis. It will also be of interest to understand whether this hotspot mutation is associated

Bryostatins
Trace bioactive cyclic polyketides first identified in marine bryozoan *Bugula neritina*; they likely originate from the symbiont *B. neritina*.

Epoxytiglianes
Bioactive compounds originally identified in the kernels of *Fontainea picrosperma* fruits and related to phorbol esters (tigliane family of diterpenes).

Private mutations
Those rare mutations that appear only once in cancer genomes, that is, are private to that patient.

with a particular clinical course, segregating with one of the four ATLL subtypes originally defined[121]. Might D427 mutations generate unique actions distinct from that consequent to PLCγ gene mutation, or other ATLL-associated *PRKCB* mutations?

***PRKCA* mutation in chordoid glioma.** Chordoid gliomas are rare, slow-growing, low-grade tumours originating in the third ventricle of the brain[122]. Although well circumscribed, access and the precise location mean surgical intervention can be associated with a high risk of morbidity[123]. Notably, in two recent publications, it was found that there was an essentially fully penetrant, heterozygous mutation in *PRKCA* associated with these tumours[124,125]. This consistent D463H mutation is at the highly conserved aspartate residue that is responsible for positioning the incoming substrate side chain hydroxyl and is a residue essential for catalytic activity as originally defined for the analogous aspartate 166 residue in PKA[126] (FIG. 2b).

At face value, the chordoid glioma-associated mutation in *PRKCA* is a simple, dominant loss-of-function mutant. This is supported by the predicted loss of catalytic potential, reduced half-life and altered subcellular distribution of the D463H mutant[124]. There are four considerations that suggest this is an over-simplistic interpretation. First, there are many routes to a loss of function in these proteins, and the singular mutation identified in these chordoid tumours (always histidine to date) clearly does not reflect an entirely random process. Second, it is known that mutations at this aspartate residue of PKCα and the equivalent in other family members, although blocking catalytic activity, serve to maintain kinase domain conformation, as judged by priming phosphorylations; this contrasts with the experimentally more commonly used kinase-inactivating mutation at the conserved lysine 368 residue[22]. The implication is that the D463H mutation will specifically (but possibly not uniquely) permit a retention of conformation and priming site phosphorylations in the absence of activity. Third, although acknowledging the limitations of mouse models for slow-growing tumours, tumour formation in the central nervous system of *Prkca* knockout mice has not been reported[127]. Evidently, simple loss of function is not a tumour driver or mice are poor surrogates of humans in this context. Last, there is an interesting precedent set for distinctive scaffolding behaviour of PKCα in another central nervous system tumour. In glioblastoma cell models, PKCα expression is associated with protection from apoptosis, with survival compromised on inhibiting expression below a threshold level[128]. This behaviour is not phenocopied by catalytic site inhibitors, but is blocked by the C1 domain-directed inhibitor calphostin C. These observations suggest that PKCα plays some scaffold role in a survival pathway independent of catalytic activity[128].

It appears in chordoid glioma that one allele of *PRKCA* encodes a catalytically incompetent enzyme, but one which may retain partner interaction capabilities. This may be a dominant effect on the wild-type protein encoded by the second allele or related to pathway operation through scaffolding functions. A definitive view on gain or loss of function and their effect on tumour growth will be derived from unravelling mechanisms, which in turn should inform on interventions in this difficult to treat disease — either way, the potential drug candidate is unlikely to be a catalytic inhibitor of PKCα.

Emergent dependencies and their origins

PKC and cell cycle controls

Echoing what is described above regarding the landscape of PKC action in transformation, there is a related complexity to the reported actions of PKC isoforms across the breadth of cell cycle controls (reviewed elsewhere[129,130]). This complexity is particularly well illustrated in the review by Black and Black, where the positive and negative proliferative impacts of PKC family members and their cell type-specific behaviours are clearly illustrated[129].

With respect to cell cycle entry (G0 > G1 transition) of arrested cells in culture, a great deal of evidence exists for the engagement of PKC isoforms in response to growth factor and hormone action (FIG. 3a). However, excepting some haematopoietic cell types[129], there is little clarity over whether these specific responses play out as critical to cell cycle progression in vivo, reflected in the generally normal development of individual PKC gene knockout mice (see above). Belying this developmental normality of murine knockouts, there is published evidence for the involvement of specific PKC isoforms in aspects of cell cycle progression including both positive effects on CDKs via the inhibitor $p27^{kip1}$ (REF.[131]) and negative effects on CDKs as observed for PKCη association with the cyclin E–Cdk2–p21 complex acting via p21 (REF.[132]) (FIG. 3b). There are also observations relating to the organizational requirements associated with cell cycle progression as reported for the DAG-dependent disassembly of nuclear lamin B1 during the cell cycle[133], consistent with the observation that lamins are targets for PKC[134,135], although it is noted that DAG also modifies intrinsic membrane behaviour associated with nuclear envelope formation[136]. The extent to which these influences of PKC on cell cycle progression reflect the nutrient-rich, overindulged, stressed and/or transformed state of the cell culture models remains to be determined. However, it would provide a rationalization of observations if, for example, the controls exerted on CDKs reflected responses to covert stress inherent in cell culture models. This brings us to the third class of controls where stress is definitively involved.

PKCε dependency in transformed cells

There is an emergent property associated with PKCε that is linked to a distinct subset of transformed cells[137]. This manifests as a requirement for PKCε to alleviate the threat of sister chromatid non-disjunction in these particular cells (FIG. 3c). The subset of transformed cells where PKCε is engaged has been defined experimentally as those cell types that do not arrest in G2 in response to the Topoisomerase 2α (Topo2α) catalytic inhibitor, ICRF193 (REF.[138]). This arrest pathway has long been known, but until recently there was a somewhat limited description of its requirements[139,140].

Topoisomerase 2α (Topo2α). One of two genes in mammals that catalyse the resolution of intertwined, catenated DNA, through double-strand cutting, strand passage and religation reactions.

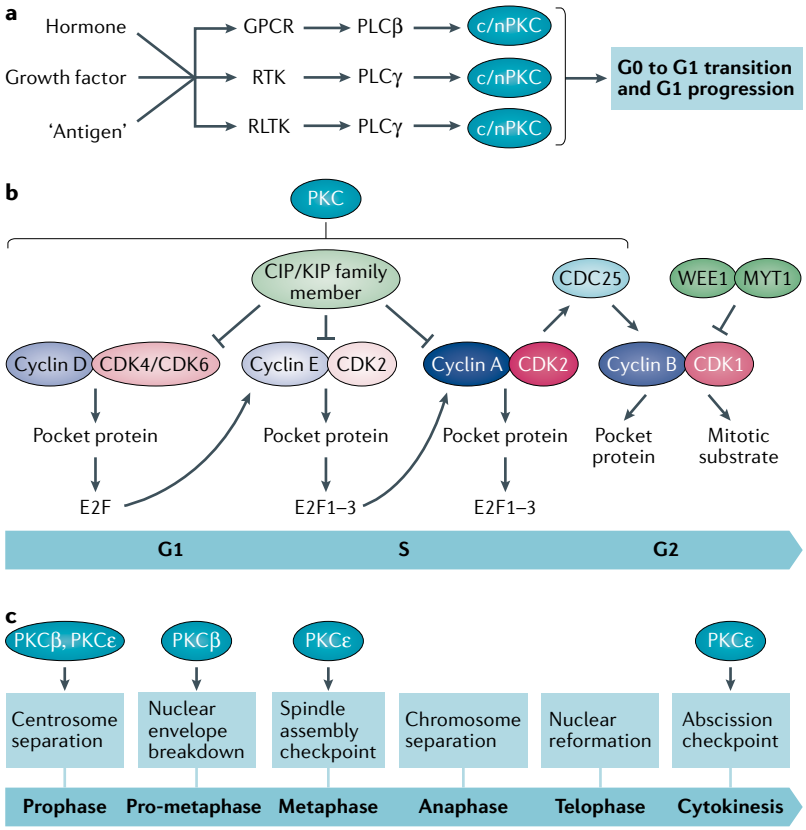


Fig. 3 | **Cell cycle controls and PKC. a** | Various growth-promoting stimuli acting through their cell surface receptors (G protein-coupled receptors (GPCRs) and tyrosine kinase-associated/linked receptors) can act on different members of the phospholipase C (PLC) gene family to trigger signalling cascades through protein kinase C (PKC) family members. These events are circumstantially linked to entry into cell cycle, that is, a G0 to G1 transition and early G1 progression. Ligands engaging GPCRs (7-transmembrane receptors) act through activated heterotrimeric G protein subunits ($G\alpha_q$.GTP, $G\alpha_{11}$.GTP and βγ) to activate members of the PLCβ class of phosphodiesterases, responsible for the hydrolysis of $PI4,5P_2$ and the generation of IP_3 and 1,2-diacylglycerol (DAG), the latter activating PKC isoforms. For ligands acting on receptor tyrosine kinases (RTK) or receptor-linked tyrosine kinases (RLTK), SH2 domain-dependent recruitment and phosphorylation of PLCγ proteins will also lead to IP_3 and DAG production, and consequent PKC activation. **b** | During G1 progression, and entry into and progression through the S phase, there are a series of interconnected events that sequentially cause activation of cyclin/cyclin-dependent kinase (CDK) complexes. These events have been reported to be influenced by PKC isoforms in various cellular settings, including: cyclin D expression, PKCα, PKCβ, PKCδ, PKCε, PKCη and PKCζ; CDK4,6 activity and PKCα; cyclin E expression, PKCδ, PKCε, PKCη and PKCι; CDK2 activity, PKCα, PKCδ and PKCη; CDK inhibitor (CIP/KIP) expression, PKCα, PKCβ, PKCδ, PKCε, PKCη, PKCθ and PKCζ; cyclin A expression and PKCδ; and CDC25 activity and PKCβ. **c** | Progression through M phase is impacted by PKCβ and PKCε as indicated. CDC25, cell division control protein 25; CIP/KIP, CDK interacting protein/kinase inhibitory protein; cPKC, 'classical' PKC; nPKC, 'novel' PKC.

When prompted, the failure of this G2 arrest leads to engagement of PKCε where it influences prometaphase–metaphase transition[141], the metaphase–anaphase transition[137,142] and, finally, the abscission checkpoint[143]. During transit through the M phase, PKCε exerts control on centrosome separation[141] as also reported for PKCβII[144]. At the metaphase–anaphase transition and at cytokinesis, PKCε has been shown to act via phosphorylation of Aurora B[142,143]. In both contexts, the PKCε phosphorylation of Aurora B at S227 switches its specificity towards critical sites on Topo2α[142] and Borealin[143]. The engagement of PKCε in these cancer genome-protective processes suggests that PKCε offers an interventional opportunity in the context of a subset of tumours — defining which tumours should be tractable through mechanism-specified biomarkers.

Borealin
One of the components of the chromosome passenger complex (CPC), alongside INCENP and survivin, regulating the localization and activity of the co-associated Aurora B, which completes the CPC.

The PKC pharmacopoeia and cancer trials

There is a long history of small-molecule inhibitors of PKC dating back to the mid-1980s and the work by Hidaka and colleagues that recognized the druggability of kinases[145]. Many chemotypes followed over the years, including the notoriously non-specific indolocarbazole, staurosporine[146] and the somewhat more selective bisindolylmaleimides[147], alongside many other inhibitors as reviewed elsewhere[148,149]. There are also multiple pharmacological activators of cPKCs and nPKCs as noted above, including the tiglianes[150] and bryostatin 1 (REF.[151]); these agents as well as numerous catalytic site inhibitors have been used clinically with broadly, but not exclusively, disappointing outcomes.

Drugs, trials and tribulations

The extent to which there is a need for exquisite drug specificity is moot, but for targeted therapeutics the line of sight into the clinic is inevitably focused through the lens of the target. For PKC there have been some significant specificity challenges, clouding interpretation of many preclinical and clinical studies exploiting the PKC inhibitor inventory. This is reflected in the wealth of literature around the effects of 'PKC inhibitors' such as rottlerin and chelerythrine, which actually target other cellular functions (see recent examples[152,153]). For the staurosporine derivative midostaurin (PKC412), originally developed as a more selective PKC inhibitor[154], the evolving clinical history has led to US Food and Drug Administration (FDA) approval for its use in acute myeloid leukaemia, albeit through its action on FLT3 (reviewed elsewhere[155]). There are various trials investigating PKC412 in acute myeloid leukaemia and PKC412 in myelodysplastic syndrome. A second staurosporine derivative, potent against PKC isoforms, UCN-01 (7-hydroxystauropsorine) was subsequently identified as a potent CHK1 inhibitor[156] but, unlike midostaurin, has not fared well in clinical trials.

Enzastaurin is a PKCβ preferential inhibitor and has been employed in many clinical trials (reviewed elsewhere[157]). Its ineffectiveness to date is hard to interpret with a lack of molecular data from these clinical studies. Even the original dose-escalation phase I trial failed to report any pharmacodynamic data, did not reach dose-limiting toxicity and settled on pharmacokinetic behaviour to define the 525 mg daily dose for the expansion cohort[158]. There are no data to indicate whether PKCβ or other targeted PKCs in the tumour (or stroma) are blocked at this dose.

In respect of cPKC and nPKC activators there is limited specificity for cPKC or nPKC isoforms and other binding-competent C1 domain proteins[75]. With no pharmacodynamic data it is hard to assess actions in terms of targeting PKC clinically. Nevertheless, the protection from phorbol ester-induced tumour promotion[110] led to early-phase oncology trials of bryostatins (reviewed elsewhere[159]). FDA orphan status was designated to bryostatin

in combination with paclitaxel for oesophageal cancer in 2001, but subsequent trials did not support further development[160]. A phase I trial has been completed for tigilanol tiglate, a second class of activator[161]. As noted above, defining the targets of action for these agents introduced intratumourally is complex; nevertheless, following the recent approval of this PKC activator in a veterinary setting, evidence from efficacy studies in patients is eagerly awaited.

Uveal melanoma

Intraocular melanoma (uveal melanoma) is associated with penetrant driver mutations in the *GNAQ*, *GNA11*, *BAP1*, *EIF1AX* and *SF3B1* genes[162]. *GNAQ* and *GNA11* encode the heterotrimeric G protein α-subunits that trigger activation of the β-class of phosphoinositide-specific phospholipase C proteins[163], elevating DAG levels and, hence, recruiting and activating cPKC and nPKC (and other DAG-responsive targets). In this context and with the, to date, intractability of phosphoinositide-specific phospholipase C inhibitors, there has been interest in targeting PKC isoforms. Currently, among the 33 active trials in uveal melanoma, three are targeting PKC. The first trial employs the orally available drug sotrastaurin (AEB071)[164], a maleimide derivative with potent PKC inhibitory activity[165]. This agent was well tolerated in phase I studies and showed modest activity, principally stable disease[166]. The other active uveal melanoma trials (phase I/II) involve another orally available drug, IDE196 also known as LXS196 (REFS[167,168]). IDE196 was well tolerated and showed modest activity in a reported phase I trial[169]. The outcomes of further efficacy trials and combination studies are awaited.

PKC inhibitors in other cancers

There have been numerous trials for other PKC inhibitors in various cancers. PKCβ upregulation in diffuse large B cell lymphoma has prompted a series of trials. The PKCβ discriminating drug enzastaurin has shown some limited single-agent efficacy in diffuse large B cell lymphoma[170] and is currently in a trial in combination with the standard of care treatment R-CHOP[171]. A second PKCβ selective drug, MS-533, is in trial in chronic lymphocytic leukaemia and small lymphocytic leukaemia[172]. There is no published information on the specificity of this agent. There is an active trial for auranofin in combination with sirolimus[173]; a related cysteine-alkylating gold compound has been reported to specifically target aPKCι[174].

Concluding remarks

Preclinical investigation has yielded a complex landscape of PKC family actions in experimental cancers, the translation of which into the clinical setting is generally hampered by a lack of mechanistic insights that afford robust biomarkers for pathological settings. Reciprocally, the unbiased 'omics data derived from patient tumour biopsies have yielded limited insights to distinguish driver from bystander events and, where providing clear direction, leave open significant issues in relation to interpretation of gain, change or loss of function.

The lack of straightforward correlations reflects the ambiguity of isoform steady-state concentration as a marker for anything other than, perhaps, a complete absence. Similarly, the priming phosphorylation state of isoforms is not simply reflective of their action; at most, this reveals latent potential. There is a need for insight into the non-redundant pathological mechanisms at play and for this to be understood both in a tumour cell context as well as in the TME. Mechanisms will afford the biomarkers required to address the action or inaction of isoforms clinically and, importantly, resolve where gain, change or loss of function operates in guiding the nature of any intervention.

Decades on from the linking of PKC to the action of tumour promoters[7], the drugging of these kinases still offers much promise, but when and how remains moot and there is much to be done to resolve this.

Published online 11 November 2020

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Acknowledgements
The authors thank A. Fields and M. Reyland for commenting on the manuscript. They also acknowledge support from the Francis Crick Institute, which receives its core funding from Cancer Research UK (FC001130), the UK Medical Research Council (FC001130) and the Wellcome Trust (FC001130). M.L. is supported by the National Institute for Health Research, the University College London Hospitals Biomedical Research Centre (no grant numbers apply).

Author contributions
P.J.P. contributed to all aspects of the article. V.C., M.L. and P.C. contributed to researching data for the article and reviewing and/or editing the manuscript before submission. S.J.B., M.C., J.J.T.M., S.M., N.Q.M., T.S. and L.W. contributed to writing the article and reviewing and/or editing it before submission.

Competing interests
The authors declare no competing interests.

Peer review information
Nature Reviews Cancer thanks M.G. Kazanietz, M. Leitges and the other, anonymous, reviewer(s) for their contribution to the peer review of this work.

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Supplementary information
Supplementary information is available for this paper at https://doi.org/10.1038/s41568-020-00310-4.

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