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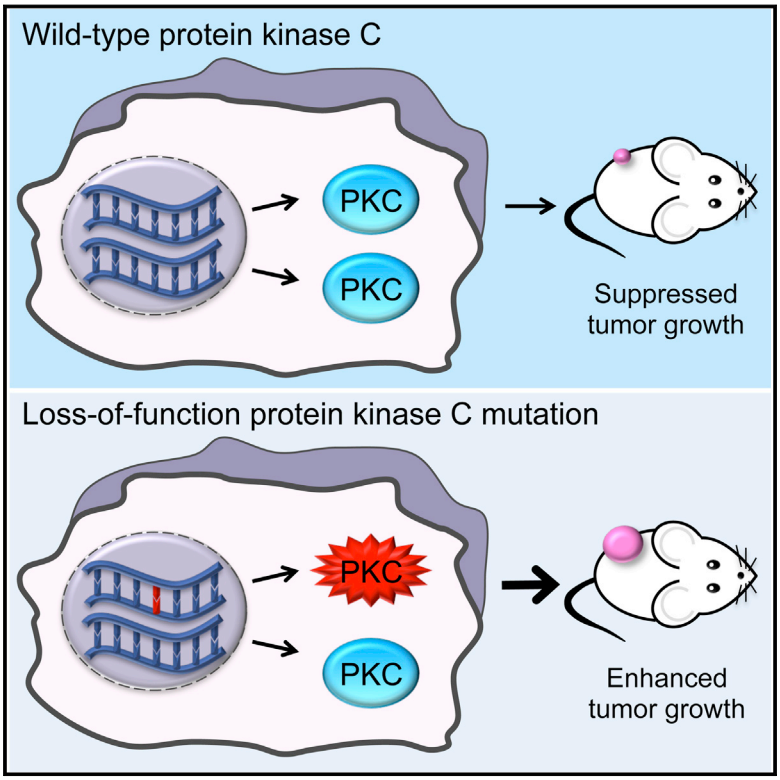
Cancer-Associated Protein Kinase C Mutations Reveal Kinase's Role as Tumor Suppressor

Graphical Abstract



Highlights

- Cancer-associated PKC mutations are LOF and can act in a dominant-negative manner
- Correcting a heterozygous PKCβ LOF mutation reduces tumor volume
- Hemizygous deletion shows PKC is haploinsufficient for tumor suppression
- Therapeutic strategies should aim to restore PKC activity instead of inhibiting it

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In Brief

Cancer-associated kinase mutations have generally been characterized as oncogenic, but an analysis of PKC mutations reveals that the majority are loss of function, indicating a tumor-suppressive role for this kinase and a shift in therapeutic strategies targeting PKC.

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Cancer-Associated Protein Kinase C Mutations Reveal Kinase's Role as Tumor Suppressor

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SUMMARY

Protein kinase C (PKC) isozymes have remained elusive cancer targets despite the unambiguous tumor promoting function of their potent ligands, phorbol esters, and the prevalence of their mutations. We analyzed 8% of PKC mutations identified in human cancers and found that, surprisingly, most were loss of function and none were activating. Loss-of-function mutations occurred in all PKC subgroups and impeded second-messenger binding, phosphorylation, or catalysis. Correction of a loss-of-function PKCβ mutation by CRISPR-mediated genome editing in a patient-derived colon cancer cell line suppressed anchorage-independent growth and reduced tumor growth in a xenograft model. Hemizygous deletion promoted anchorage-independent growth, revealing that PKCβ is haploinsufficient for tumor suppression. Several mutations were dominant negative, suppressing global PKC signaling output, and bioinformatic analysis suggested that PKC mutations cooperate with co-occurring mutations in cancer drivers. These data establish that PKC isozymes generally function as tumor suppressors, indicating that therapies should focus on restoring, not inhibiting, PKC activity.

INTRODUCTION

The protein kinase C (PKC) family has been intensely investigated in the context of cancer since the discovery that it is a receptor for the tumor-promoting phorbol esters (Castagna et al., 1982). This led to the dogma that activation of PKC by phorbol esters promotes carcinogen-induced tumorigenesis (Griner and Kazanietz, 2007), yet targeting PKC in cancer has been unsuccessful.

The PKC family contains nine genes that have many targets and thus diverse cellular functions, including cell survival, proliferation, apoptosis, and migration (Dempsey et al., 2000). PKC isozymes comprise three classes: conventional (cPKC: α, β, γ), novel (nPKC: δ, ε, η, θ), and atypical (aPKC: ζ, ι). cPKC and nPKC isozymes are constitutively phosphorylated at three priming sites (activation loop, turn motif, and hydrophobic motif) to structure PKC for catalysis (Newton, 2003). A pseudosubstrate segment maintains PKC in an autoinhibited conformation that is relieved by second-messenger binding. cPKC isozymes are activated by binding to diacylglycerol (DAG) and Ca^{2+}, whereas nPKC isozymes are activated solely by DAG, events that engage PKC at membranes. Thus, these PKC isozymes have two prerequisites for activation: constitutive processing phosphorylations and second-messenger-dependent relocalization to membranes. Prolonged activation of cPKC and nPKC isozymes with phorbol esters leads to their dephosphorylation and subsequent degradation, a process referred to as downregulation (Hansra et al., 1996; Young et al., 1987). aPKC isozymes bind neither Ca^{2+} nor DAG.

PKC has proved an intractable target in cancer therapeutics (Kang, 2014). PKCι was proposed to be an oncogene in lung and ovarian cancers (Justilien et al., 2014; Regala et al., 2005; Zhang et al., 2006), and PKCε was categorized as an oncogene because of its ability to transform cells (Cacace et al., 1993). However, for most PKC isozymes, there is conflicting evidence as to whether they act as oncogenes or as tumor suppressors. For example, PKCδ is considered a tumor suppressor because of its pro-apoptotic effects (Reyland, 2007). However, it promotes tumor progression of lung and pancreatic cancers in certain contexts (Mauro et al., 2010; Symonds et al., 2011). Similarly, both overexpression and loss of PKCζ in colon cancer cells have been reported to decrease tumorigenicity in nude mice or cell lines, respectively (Luna-Ulloa et al., 2011; Ma et al., 2013). Likewise, PKCα was reported to both induce (Walsh et al., 2004; Wu et al., 2013) and suppress colon cancer cell proliferation (Gwak et al., 2009) and to suppress colon tumor formation in the $APC^{Min/+}$ model (Oster and Leitges, 2006). Based on the dogma that PKC isozymes contribute positively to cancer progression, many PKC inhibitors have entered clinical trials; however, they have been ineffective (Mackay and Twelves, 2007).

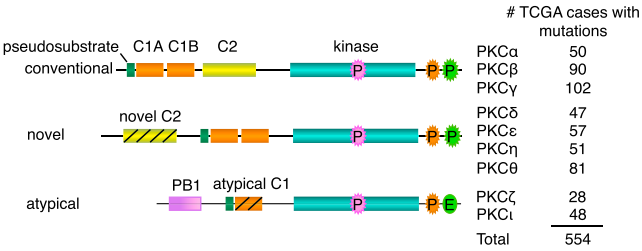


Figure 1. A Multitude of Cancer-Associated Mutations Have Been Identified within the Nine PKC Genes

(Left) Domain structure of conventional (α, β, γ), novel (δ, ε, η, θ), and atypical (ζ, ι) PKC members showing priming phosphorylation sites: activation loop (pink), turn motif (orange), and hydrophobic motif (green). (Right) Number of TCGA cases with cancer-associated mutations (missense, nonsense, insertions, deletions, splice site, or translation start site) identified within each of the PKC genes.

In fact, a recent meta-analysis of controlled trials of PKC inhibitors combined with chemotherapy versus chemotherapy alone revealed that PKC inhibitors significantly decreased response rates and disease control rates in non-small cell lung cancer (Zhang et al., 2014). Why has inhibiting PKC failed in the clinic? It has been well established that prolonged or repetitive treatment with phorbol esters depletes cPKC and nPKC isozymes from cells (Blumberg, 1980; Nelson and Alkon, 2009), bringing into question whether loss of PKC, rather than its activation, promotes tumorigenesis.

PKC is frequently mutated in human cancers. To uncover whether loss or gain of PKC function contributes to cancer progression, we selected mutations throughout the primary sequence and family membership and assessed their functional impact. Specifically, we asked how these cancer-associated mutations alter the signaling output of PKC using our genetically encoded reporter, C kinase activity reporter (CKAR) (Violin et al., 2003). Characterization of 46 of these mutations revealed that most reduced or abolished PKC activity and none were activating. Bioinformatic analysis of all PKC mutations revealed that they may cooperate with co-occurring mutations in oncogenes and tumor suppressors known to be regulated by PKC. Correction of one patient-identified, heterozygous, loss-of-function (LOF) PKCβ mutation in a colon cancer cell line significantly decreased tumor size in mouse xenografts, indicating that loss of PKC function enhances tumor growth. Our data are consistent with PKC isozymes functioning generally as tumor suppressors, reversing the paradigm that their hyperactivation promotes tumor growth.

RESULTS

A Multitude of Cancer-Associated Mutations Have Been Identified within the Nine PKC Genes

554 mutations (as of October 2014), of which most are heterozygous, have been identified in diverse cancers (Cerami et al., 2012; Gao et al., 2013) within cPKC (242), nPKC (236), and aPKC (76) isozymes (Figure 1). These mutations reside throughout the entire coding region, with no apparent mutational hotspots. Therefore, we conducted a comprehensive study of mutations within PKC domains and within interdomain regions to determine how they affect PKC signaling to contribute to cancer pathogenesis. 46 mutations of both conserved and non-conserved residues were selected from all three classes of PKC isozymes (Table 1 and Table S1).

PKC Mutations in the Regulatory C1 and C2 Domains Are LOF

The C1 domains of cPKC and nPKC isozymes are critical for their activation because they mediate PKC translocation to membranes via binding to DAG. Thus, we investigated how C1 domain mutations alter PKC translocation and activation. To measure agonist-dependent PKC activity, COS7 cells co-expressing the FRET-based PKC reporter (CKAR) and equal levels of either wild-type (WT) or mutant mCherry-tagged PKC were stimulated with the cell-permeable DAG, DiC8, or the phorbol ester, phorbol 12,13-dibutyrate (PDBu), and phosphorylation-dependent FRET ratio changes were recorded. Phorbol esters serve as an effective although non-physiological tool to maximally activate PKC because they bind with 100-fold higher affinity to C1 domains compared to DAG (Mosior and Newton, 1998). A mutation identified in a colorectal cancer tumor altered a residue (PKCα H75Q) required for coordination of Zn^{2+} and thus for folding of the C1 domain (Figure 2A). This mutation ablated agonist-stimulated activity, as evidenced by a lower FRET ratio trace compared with that of cells containing only endogenous PKC (Figure 2B). This lower activity suggests that the mutant is dominant negative toward global PKC output. Within a head and neck cancer patient, a mutation altered a critical residue (PKCα W58L) required for controlling the affinity for DAG, but not phorbol ester (Dries et al., 2007) (Figure 2A). This mutation also abolished DiC8-induced and basal activity but retained some PDBu-induced activity, consistent with this residue selectively regulating DAG affinity (Figures 2B and S1A). Because membrane translocation is a prerequisite for activation of cPKC isozymes, we compared the translocation of YFP-tagged WT and mutant PKC to membrane-targeted CFP using FRET (Antal et al., 2014). Mutation of either residue impaired translocation upon stimulation with DiC8, phorbol ester (Figure 2C), or the natural agonist UTP (Figure 2D), accounting for the inability of these agonists to activate the mutants. Lastly, we asked how these mutations affected the processing phosphorylations of PKC. PKCα H75Q, but not W58L, was unphosphorylated, likely because the misfolded C1A domain of the H75Q mutant prevented its processing (Figure 2E). Three additional mutations within the C1A domains of PKCα (G61W), PKCβ (G61W), and PKCγ (Q62H) also exhibited reduced agonist-induced PKC activity (Figures S1B–S1D). Our analysis of nine C1 domain mutations revealed that five reduced or abolished activity while none were hyperactivating (Tables 1 and S1). Inactivation occurred by altering two key inputs required for PKC function: disruption of binding to DAG or processing by phosphorylations.

The C2 domain of cPKC isozymes is also critical for activation, as it mediates Ca^{2+}-dependent pre-targeting to plasma membrane, where these isozymes bind DAG and become activated (Newton, 2003). One mutation identified within the C2 domain of PKCγ (D193N) was present in colorectal and ovarian cancers

Table 1. Loss-of-Function PKC Mutations in Cancer

Mutation[a]	Activity	Domain	Cancer(s)	Residue Importance	Allele Frequency	Other Mutations[b]
γ G23E	none[c]	PS	colorectal	adding negative charge to pseudosubstrate	N/A	γ G23W δ G146R ι G128C
ε R162H	low		head and neck	non-conserved	0.15	
α W58L	none[c]	C1A	head and neck	DAG binding; conserved in all C1a domains	0.22	γ W57splice θ W171*
α G61W	low		lung	conserved in cPKC C1a domains	0.05	β G61W
β G61W	low		lung	conserved in cPKC C1a domains	0.06	α G61W
γ Q62H	none[c]		lung	conserved in all PKC isozymes	0.45	α Q63H ε Q197P
α H75Q	none[d]		colorectal	coordinates Zn^{2+}; conserved in all C1 domains	N/A	η H284Y ι H179Y
γ D193N	none[c]	C2	colorectal/melanoma/ovarian	Ca^{2+} binding site	0.28	
γ T218M	none[c]		stomach	non-conserved	0.42	γ T218R
γ D254N	low		endometrial/ovarian	Ca^{2+} binding site	0.43	
α G257V	none[c]		lung	conserved in cPKC isozymes	0.12	
γ F362L	none[c]	Kinase	endometrial	conserved in cPKC and nPKC isozymes	0.21	γ F362fs β F353L
β Y417H	none[c]		liver	conserved in cPKC isozymes	0.67	γ Y431F
ζ E421K	none[d]		breast	APE motif; conserved in most protein kinases	N/A	α E508K ι E423D
α F435C	none[c]		endometrial	conserved in cPKC and nPKC isozymes	0.31	
α A444V	low		endometrial/breast	conserved in cPKC and nPKC isozymes	0.27	β A447T γ A461T γ A461V δ A454V θ A485T ι S359C
γ G450C	none[c]		endometrial/lung/liver	conserved in cPKC isozymes	0.41	ε R502*
α D481E	low		colorectal	DFG motif; conserved in most protein kinases	N/A	β D484N γ D498N ι D396E
β A509V	none[d]		breast	APE motif; conserved in most protein kinases	N/A	α A506V α A506T β A509T
β A509T	none[c]		colorectal	APE motif; conserved in most protein kinases	0.53	α A506V α A506T β A509V
γ P524R	none[d]		pancreatic	APE motif; conserved in most protein kinases	N/A	γ P524L δ P517S ε P576S θ P548S
δ D530G	none[d]		colorectal	anchors the conserved regulatory spine; conserved in all eukaryotic kinases	N/A	β D523N γ D537G γ D537Y
δ P568A	none[c]		head and neck	conserved in all PKC isozymes	0.16	δ P568S β P561H γ P575H
β G585S	low		lung	conserved in all PKC isozymes	N/A	η G598V
η K591E	low		breast	reversal of conserved charge	N/A	η K591N θ R616Q
η R596H	none[d]		colorectal	conserved in all PKC isozymes	0.50	
η G598V	none[d]		lung	conserved in all PKC isozymes	N/A	β G585S

(*Continued on next page*)

Table 1. *Continued*

Mutation[a]	Activity	Domain	Cancer(s)	Residue Importance	Allele Frequency	Other Mutations[b]
β P619Q	none[d]	C-tail	endometrial	PXXP motif; conserved in AGC kinases	0.48	

PKC mutations showing no activity with any agonist, no activity with physiological stimuli, or reduced activity in response to physiological stimuli. Allele frequencies were obtained from cBioPortal.

[a]Mutations examined in this study.

[b]Other mutations present at the same/corresponding residue in the same/other PKC isozymes.

[c]Kinase-dead.

[d]No response to physiological stimuli.

and in melanoma. Another (D254N) was found in endometrial and ovarian cancers. Because both of these Asp residues (Figure 2F) coordinate Ca^{2+} (Medkova and Cho, 1998), we monitored their activation upon elevation of intracellular Ca^{2+} with thapsigargin, a sarco/endoplasmic reticulum Ca^{2+}-ATPase inhibitor (Rogers et al., 1995). In contrast to WT PKCγ, neither mutant was activated (Figure 2G) nor translocated to the plasma membrane (Figure 2H) following thapsigargin addition, consistent with impaired Ca^{2+} binding. However, both mutants retained full responses to phorbol esters, consistent with unimpaired C1 domains. To further substantiate the inability of the mutants to bind Ca^{2+}, we monitored PKC oscillatory translocation stimulated by histamine-induced oscillatory Ca^{2+} release in HeLa cells (Violin et al., 2003). Whereas WT PKCγ exhibited oscillatory translocation in some cells, the C2 domain mutants were unresponsive to histamine (Figure 2I). Thus, these C2 domain mutations dampen PKCγ activity because they impede Ca^{2+} binding. Mutation of two other C2 domain residues that are not directly involved in Ca^{2+} binding (PKCγ T218M and PKCα G257V) also caused LOF (Figure S1D and S1E); PKCα G257V was LOF because it was not processed by phosphorylation (Figure S1F), whereas the remaining C2 domain mutants were (data not shown). Our analysis of six C2 domain mutations revealed four LOF mutations and no hyperactivating ones (Tables 1 and S1).

PKC Mutations in the Kinase Domain Are LOF

We next evaluated 21 kinase domain mutations, two of which were within PKCδ: D530G in colorectal cancer and P568A in head and neck cancer (Figure 3A). Asp530 functions as an anchor for the kinase regulatory spine, a highly conserved structural element of eukaryotic kinases (Kornev et al., 2006; Kornev et al., 2008); not surprisingly, the D530G mutant was kinase dead and not primed by phosphorylation (Figures 3B and 3C). Mutation of the conserved Pro568 to Ala also prevented a response to natural agonist stimulation but maintained some PDBu-stimulated activity, likely because a small pool of this mutant was phosphorylated (Figures 3B and 3C).

Strikingly, all three PKCη mutations examined (K591E, R596H, and G598V) altered its subcellular localization by pre-localizing it at the plasma membrane prior to stimulation (Figure 3D). However, despite constitutive membrane association, these mutants had reduced basal and stimulated activity as read out by a phospho-(Ser) PKC substrate antibody (Figure 3E) because they were not processed by phosphorylation (Figure 3F). We have previously shown that unprocessed nPKC isozymes have exposed C1 domains that induce constitutive membrane association (Antal et al., 2014).

A number of mutations were present within the highly conserved APE motif that is involved in substrate binding and allosteric activation of kinases (Kornev et al., 2008). PKCγ P524R and PKCβ A509V mutations ablated activity by preventing processing phosphorylations, and both exhibited dominant-negative roles (Figures 3G–3J). PKCβ A509T (colorectal cancer) also showed loss of function in response to UTP but was modestly activated by the potent ligand PDBu (Figure 3I), likely because a small pool of it was phosphorylated (Figure 3J). A LOF mutation that prevented processing of the atypical PKCζ was also found within the APE motif (E421K; Figure S1G).

Further analysis revealed that 16 out of 21 kinase domain mutations that we analyzed (Tables 1 and S1) resulted in full or partial LOF, with the majority preventing processing by phosphorylation. For example, PKCα F435C, PKCα A444V, PKCβII Y417H, PKCβII G585S, and PKCγ G450C had impaired phosphorylation and reduced activity (Figures S1C–S1F and S1H–S1J). However, partial LOF mutations were also observed in cases in which phosphorylation was maintained—PKCα D481E (Figures S1B and S1F) and PKCγ F362L (Figures S1D and S1J), suggesting that these mutations likely decrease PKC's intrinsic catalytic activity.

The Majority of Cancer-Associated PKC Mutations Are LOF

Our analysis of 46 mutations present within eight of the PKC genes revealed that ~61% (28) of them were LOF and none were activating (Figure 4A). A lack of identification of activating mutations is not an artifact of our assays, as activating PKC mutations that increase PKC affinity for DAG or decrease autoinhibition are readily detectable (data not shown). LOF mutations were identified within cPKC (α, β, γ), nPKC (δ, ε, η), and aPKC (ζ) isozymes and occurred within the C1, C2, and kinase domains as well as the pseudosubstrate and C-terminal tail (Figure 4B). For example, the PKCγ G23E pseudosubstrate mutation was not processed by phosphorylation (Figure S1J) and thus lacked any UTP-stimulated activity (Figure S1D), and the PKCε R162H pseudosubstrate mutation showed reduced agonist-stimulated and basal activity (Figures S1K and S1L). The PKCβ P619Q C-terminal tail mutation, residing within a conserved PXXP motif required for processing (Gould et al., 2009), was also LOF as it prevented PKC phosphorylation (Figure S1H). Overall, PKC LOF occurred by diverse mechanisms, most commonly by preventing processing phosphorylations or ligand binding, and as such, there were no mutational hotspots for loss of function. However, we identified seven LOF mutation "warmspots" (Sun et al., 2007) that fell within highly conserved regions of PKC—one within the pseudosubstrate and six within the kinase domain (Figure 4C). Thus,

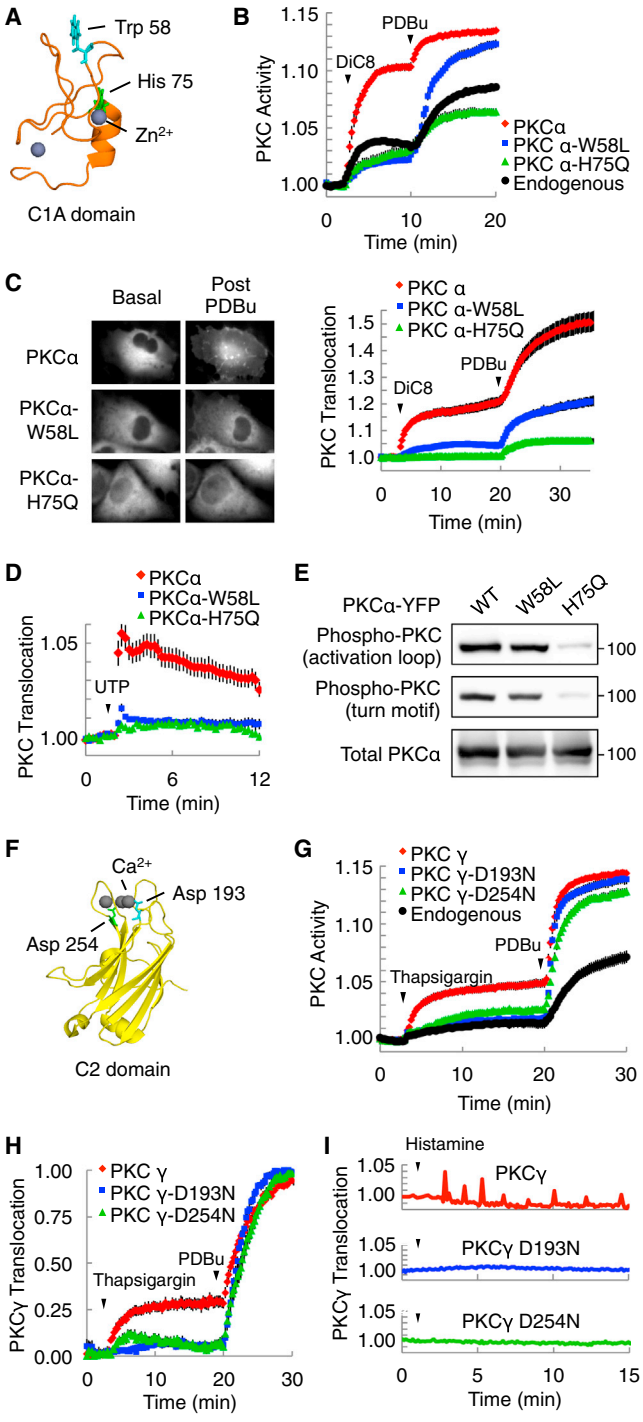


Figure 2. PKC Mutations in the Regulatory C1 and C2 Domains Are LOF

(A) Solution structure of the C1A domain of PKCγ (PDB 2E73) showing the corresponding PKCα His75 residue that coordinates Zn^{2+} and PKCα Trp58.

(B) Normalized FRET ratio changes (mean ±SEM) representing DiC8- (10 μM) followed by PDBu- (200 nM) induced PKC activity as read out by CKAR in COS7 cells co-expressing CKAR and either mCherry-tagged WT, mutant PKCα, or no exogenous PKC (endogenous).

(C) (Left) Representative YFP images of the indicated PKC isozymes under basal and PDBu-treated conditions (200 nM; 15 min) showing relocalization of WT, but not mutant PKCα, to membranes. (Right) Normalized FRET ratio changes (mean ±SEM) quantifying translocation of YFP-tagged PKCα proteins toward a membrane-targeted CFP upon stimulation with 10 μM DiC8, followed by 200 nM PDBu.

(D) Normalized FRET ratio changes (mean ±SEM) showing PKC translocation following UTP (100 μM) stimulation.

(E) Immunoblot showing the phosphorylation state of the indicated YFP-tagged PKCα proteins.

(F) Crystal structure of the C2 domain of PKCγ (PDB 2UZP) highlighting Asp193 and Asp254 residues involved in Ca^{2+} binding.

(G) Normalized FRET ratio changes (mean ±SEM) showing PKC activity as read out by CKAR upon elevation of intracellular Ca^{2+} stimulated by thapsigargin (5 μM), followed by PDBu (200 nM).

(H) Normalized FRET ratio changes (mean ±SEM) showing translocation of YFP-tagged PKCγ constructs toward membrane-localized CFP upon stimulation of COS7 cells with thapsigargin (5 μM) followed by PDBu (200 nM). Data were normalized to the maximal amplitude of translocation for each cell and then scaled from 0 to 1 using the equation: X = (Y − Ymin)/(Ymax − Ymin), where Y = normalized FRET ratio, Ymin = minimum value of Y, and Ymax is maximum value of Y.

(I) Normalized FRET ratio changes displaying oscillatory translocation of YFP-tagged WT PKCγ, but not PKCγ mutants D193N and D254N, in HeLa cells co-expressing membrane-targeted CFP and stimulated with 10 μM histamine. Data are representative traces from individual cells of three independent experiments.

See also Figure S1.

inactivating mutations targeted conserved regulatory elements and frequently hit the same residue, whereas mutations that exhibited no difference from WT occurred more randomly (Table S1).

Analysis of cancer types most frequently harboring PKC mutations revealed that, although PKC isozymes are mutated across many cancers, PKC mutations are enriched in certain cancers (Figure 4D). Namely, PKC isozymes are mutated in 20%–25% of melanomas, colorectal cancers, or lung squamous cell carcinomas but are mutated in <5% of ovarian cancers, glioblastoma, or breast cancers (Cerami et al., 2012; Gao et al., 2013). Additionally, nPKC isozymes are most commonly mutated in gastrointestinal cancers (pancreatic, stomach, and colorectal), which have a lower mutation burden than melanomas and lung cancers, highlighting their importance in this type of cancer (Figure 4D). The majority of PKC mutations are heterozygous, with an allele frequency varying from 0.05 to 0.67 for the mutations characterized (Tables 1 and S1). This indicates that PKC mutations can be truncal events in regards to tumor heterogeneity and exist in a majority of the cells within a tumor or can be branchal events acquired later in tumorigenesis as the tumor progresses to a more aggressive stage. This is consistent with PKC mutations being co-driver events that enhance tumorigenesis mediated by primary drivers.

Dominant-Negative PKCβ Mutation Confers a Tumor Growth Advantage

Because the majority of PKC mutations examined were LOF, we tested whether we could rescue HCT116 colon cancer cells that have a heterozygous LOF frameshift mutation in the C2 domain of PKCβ by overexpressing WT PKCβII. This resulted in a dramatic reduction in anchorage-independent growth (Figure S2A), a hallmark of cellular transformation. Thus, we next used CRISPR/Cas9-mediated genome editing to ask whether

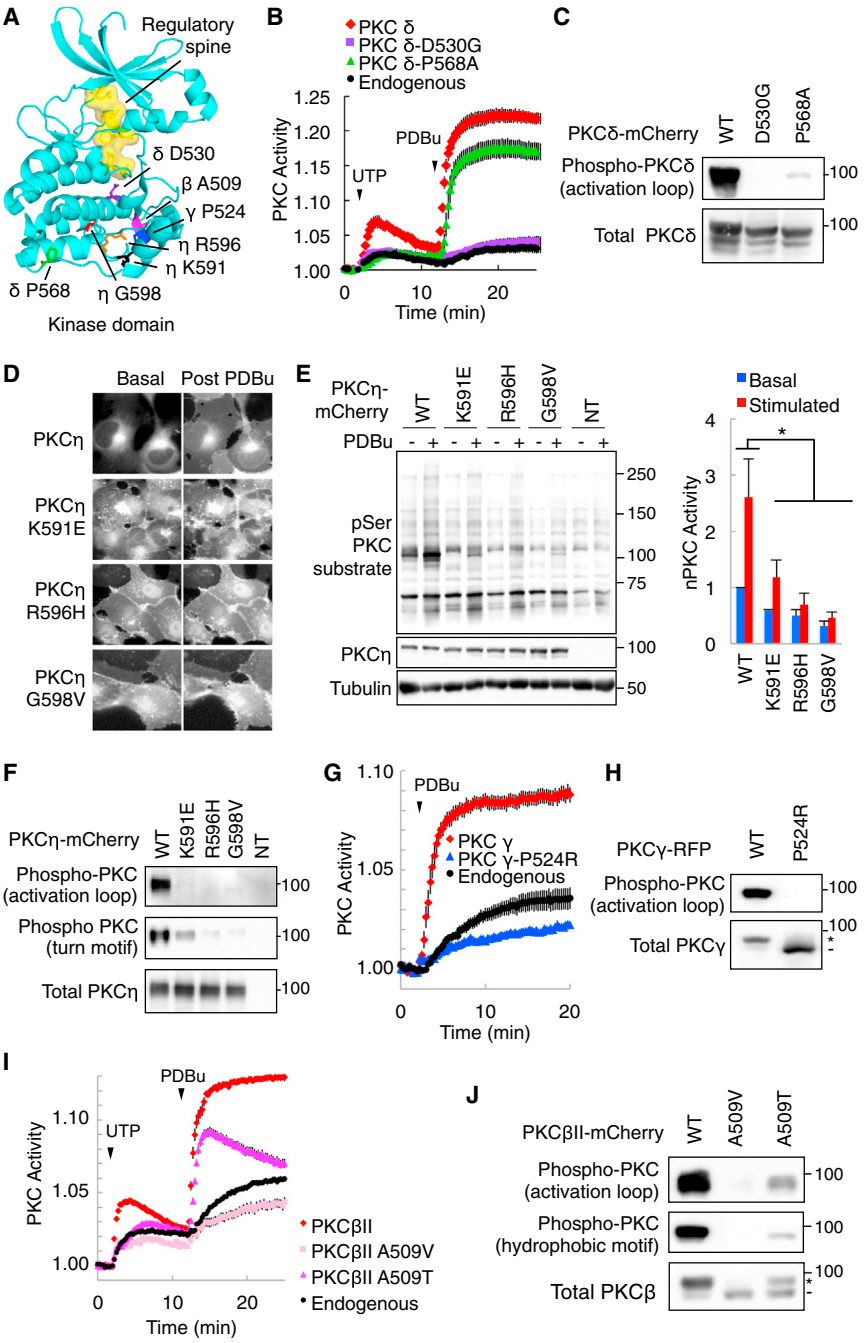


Figure 3. PKC Mutations in the Kinase Domain Are LOF

(A) Crystal structure of the kinase domain of PKCβII (PDB 2I0E) highlighting cancer-associated residues and the regulatory spine (yellow space filling).

(B) Normalized FRET ratio changes (mean ±SEM) showing PKC activity of PKCδ constructs in COS7 cells co-expressing the plasma membrane-targeted, PKCδ-specific reporter PM-δCKAR. Cells were stimulated with UTP (100 μM) followed by PDBu (200 nM).

(C) Immunoblot analysis of the phosphorylation state of PKCδ WT and mutants.

(D) Representative mCherry images of mCherry-tagged PKCη WT or mutants showing localization under basal conditions and 15 min post 200 nM PDBu addition to COS7 cells.

(E) (Left) Immunoblot showing PKC substrate phosphorylation. COS7 cells overexpressing the indicated constructs were pre-treated with 4 μM Gö6976 for 10 min to inhibit cPKC isozymes and were then stimulated or not with 200 nM PDBu to activate nPKC isozymes. (Right) Immunoblots were quantified and normalized to total PKCη levels and tubulin. Data represent averages of three independent experiments ±SEM. Comparisons for basal and stimulated activity were made using a repeated-measures one-way ANOVA followed by post hoc Dunnett's multiple comparison test. *$p < 0.05$ as compared with the WT group.

(F) Immunoblot analysis of the phosphorylation state of mCherry-tagged PKCη WT and mutants.

(G) Normalized FRET ratio changes (mean ± SEM) showing PKC activity from COS7 cells co-expressing CKAR and RFP-tagged PKCγ mutants stimulated with 200 nM PDBu.

(H) Immunoblot depicting PKCγ WT and P524R phosphorylation. The asterisk denotes phosphorylated and the dash unphosphorylated PKCγ.

(I) Normalized FRET ratio changes (mean ±SEM) showing PKC activity of PKCβII constructs in COS7 cells co-expressing CKAR. Cells were stimulated with UTP (100 μM) followed by PDBu (200 nM).

(J) Immunoblot depicting mCherry-tagged PKCβII WT and mutant phosphorylation. The asterisk denotes phosphorylated and the dash unphosphorylated PKCβII.

See also Figure S1.

reverting an endogenous LOF allele to WT would also rescue cell growth. We used DLD1 colon cancer cells because they harbor a PKCβ A509T LOF mutation (Figure 3I) to assess whether a heterozygous LOF PKC mutation could confer a survival advantage, as most cancer-associated PKC mutations are heterozygous. We reverted the mutation to WT in three isogenic clones (Figures S2B and S2C) and confirmed that no sequence alterations existed within the top two most likely predicted off-targets (data not shown). Correction of the A509T mutation in the endogenous PKCβ (*PRKCB*) allele caused a slight but reproducible increase in the PKCβ levels and a >2-fold increase in PKCα levels, although neither reached statistical significance (Figure 5A). Immunoblot analysis with a phospho-(Ser) PKC substrate antibody revealed significantly higher basal PKC activity in the corrected cells (Figure 5B). This is consistent with the DLD1 parental cells having reduced PKC activity because of the LOF PKCβ mutation and the lower PKCα levels. We next tested the ability of these cells to grow in suspension. Consistent with having higher PKC activity and a more tumor-suppressive phenotype, the corrected cells were less viable in suspension (Figure 5C) because they were less capable of forming the compact multicellular aggregates formed by the DLD1 parental cells (Figure 5D). Moreover,

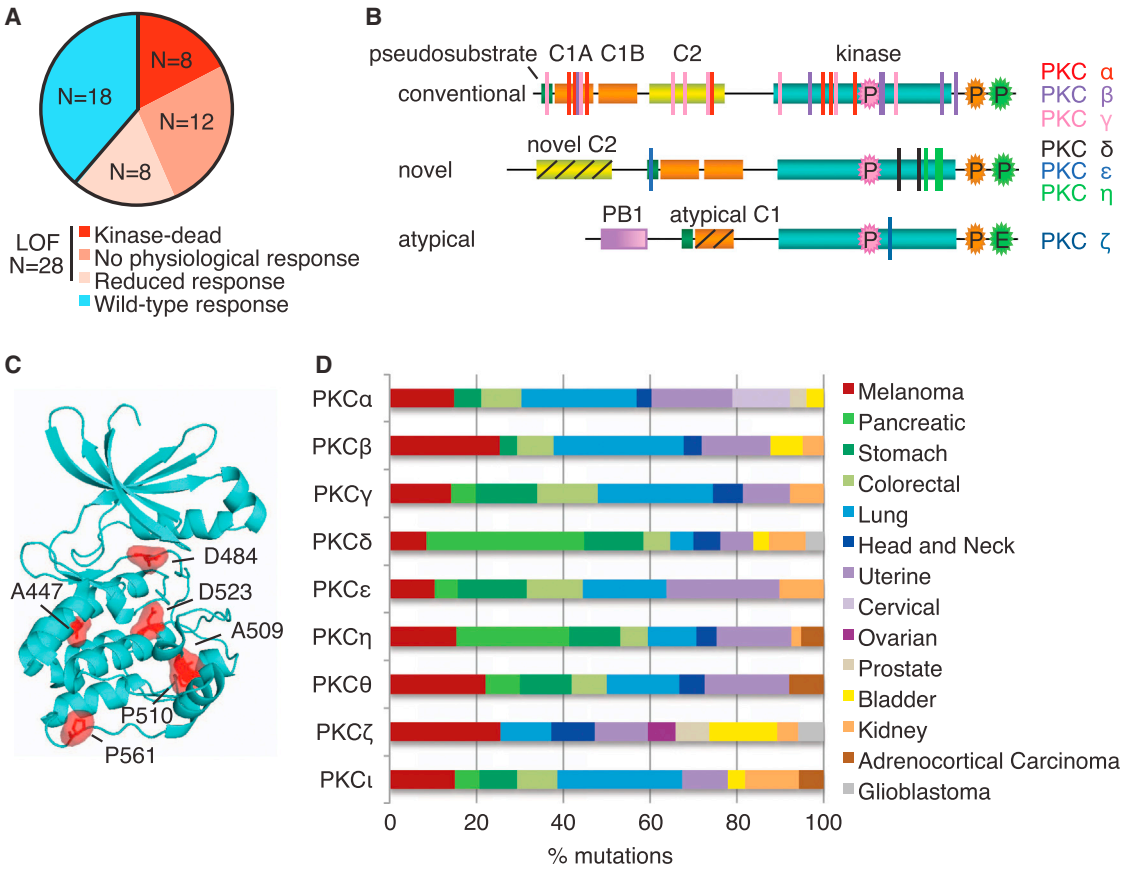


Figure 4. The Majority of PKC Mutations Are LOF

(A) Pie chart of the functional impact of the investigated PKC mutations, with bright red representing mutations that lack any activity, medium red representing mutations that show no response to physiological stimuli (DAG or Ca^{2+} elevation) but some response to non-physiological phorbol esters, light red representing mutations that display reduced activity to physiological stimuli compared to the corresponding WT isozyme, and blue representing no difference from the corresponding WT PKC isozyme.

(B) Domain structure of cPKC, nPKC, and aPKC isozymes, overlaid with the LOF mutations color coded by isozyme.

(C) Crystal structure of the kinase domain of PKCβII (PDB 2I0E) highlighting "warmspot" residues mutated in at least four tumor samples within the various PKC isozymes.

(D) Bar graph depicting the percentage of mutations distributed in the indicated cancers for each PKC isozyme.

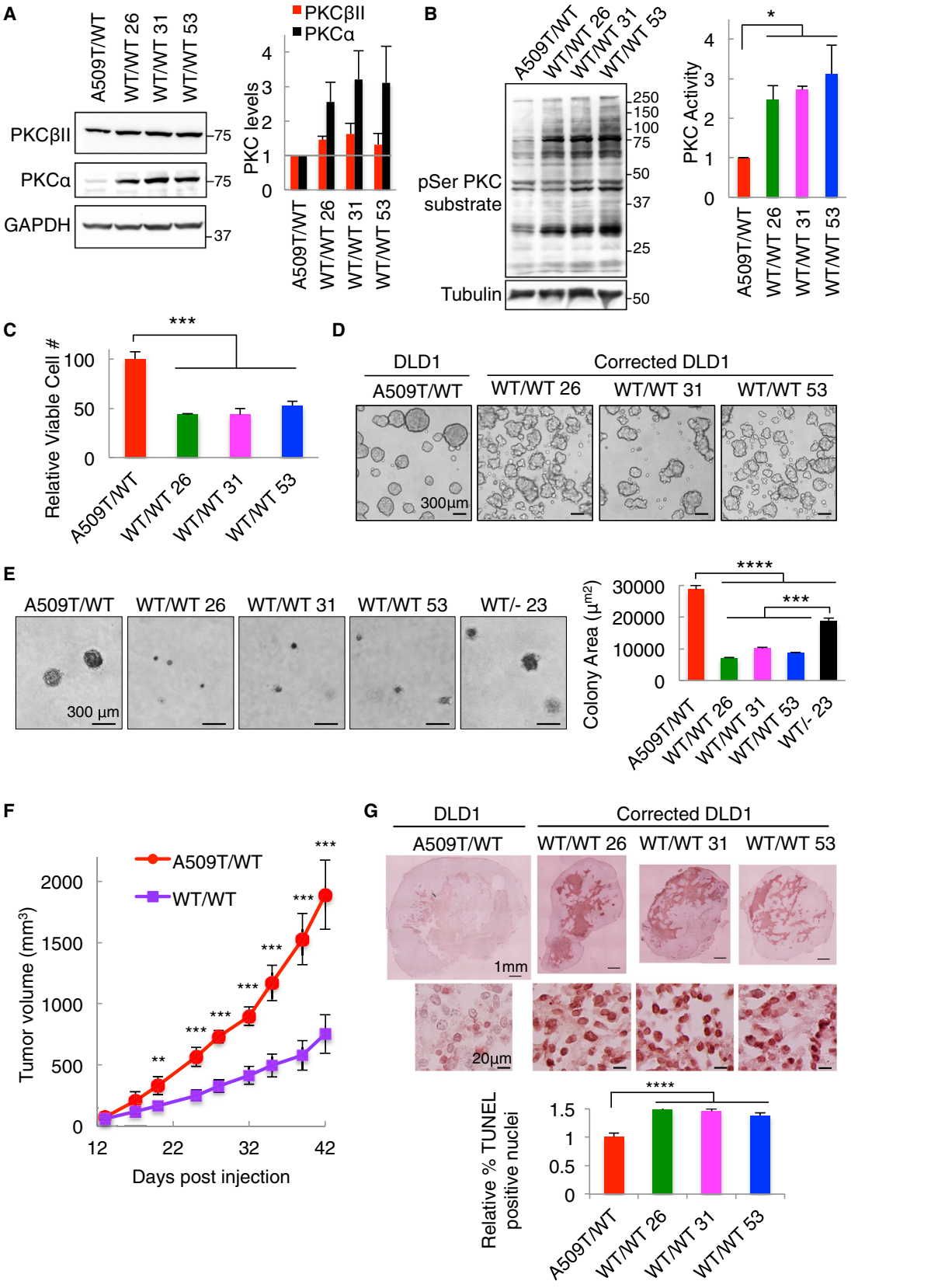
the corrected clones had decreased anchorage-independent growth potential (Figure 5E). These results corroborate those obtained from the HCT116 cells overexpressing PKCβII, demonstrating that partial loss of PKCβ activity is necessary for growth in soft agar. However, in a 2D proliferation assay, the DLD1-corrected cells proliferated at similar rates to the DLD1 parental cells (Figure S2D), indicating that it is not the proliferation rates that differ between these cells but, rather, their ability to grow in the absence of anchorage.

To determine whether PKC displays haploinsufficiency, we knocked out the mutant PKCβ allele in DLD1 cells by creating a frameshift deletion using genome engineering (Figure S2E). This hemizygous clone (WT/- 23), containing only one WT allele and thus expressing lower PKCβII levels (Figure S2F), exhibited significantly increased anchorage-independent growth potential compared to cells containing two WT alleles, indicating that PKCβII is haploinsufficient for tumor suppression (Figure 5E). Additionally, the PKCβ hemizygous cells did not grow as well as the PKCβ A509T mutated cells in soft agar, indicating that this mutation had a dominant-negative effect.

To definitively establish whether a heterozygous LOF PKCβ mutation facilitates tumor growth in vivo, the DLD1 parental or corrected cells were subcutaneously injected into the flanks of nude mice and tumor growth was monitored. Consistent with our cellular data, the tumors derived from the corrected cells were significantly smaller than those from the DLD1 parental cells (Figures 5F and S2G). This reduced growth correlated with increased apoptosis as assessed by TUNEL staining of tumor sections (Figure 5G). These data demonstrate that a heterozygous, dominant-negative PKCβ mutation can significantly increase tumor growth, thus establishing PKCβ as a tumor suppressor.

DISCUSSION

Here we establish that clinical trials targeting PKC have been based on the wrong assumption; it is not inactivation of PKC



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but, rather, activation that suppresses tumor growth. Thus, we propose that therapies should target mechanisms to restore the PKC signaling output rather than reduce it. Our comprehensive analysis revealed that 61% of the PKC mutations characterized were LOF and none were activating. We did not account for nonsense mutations or deletions, so an even higher proportion of PKC mutations are LOF. Corroborating our data, three other LOF PKC mutations have been previously described. A LOF PKCα mutation (D294G in C2 domain) was identified in three types of cancer (Alvaro et al., 1993; Prévostel et al., 1997; Zhu et al., 2005) and a LOF PKCζ mutation (S514F in the kinase domain) was identified in colorectal cancer (Galvez et al., 2009). A partial LOF mutation in PKCι (R471C), present in three distinct cancers, disrupted substrate binding and induced abnormal epithelial polarity (Linch et al., 2013). To our knowledge, no gain-of-function PKC mutations have been observed in cancer. The identification of LOF mutations throughout the PKC family and in diverse cancers supports a general role for PKC isozymes as tumor suppressors.

Strikingly, several LOF PKC mutations (e.g., PKCβ A509V, PKCγ P524R, and PKCα W58L, H75Q, and G257V) acted in a dominant-negative manner by decreasing global endogenous PKC activity. Moreover, the presence of mutant PKCβ A509T protein in DLD1 cells reduced PKCα levels. One mechanism for this cross-PKC dominant-negative effect is that the LOF PKC impairs the priming phosphorylations of other PKCs, thus reducing their steady-state levels. This is supported by a prior study demonstrating that unprocessed kinase-dead PKC isozymes prevent the phosphorylation of other PKC isozymes, likely because their phosphorylation requires common titratable components (Garcia-Paramio et al., 1998). This dominant-negative role of LOF mutations is corroborated by studies showing that kinase-dead PKC isozymes function in a dominant-negative manner to exhibit tumorigenic effects on cells (Galvez et al., 2009; Hirai et al., 1994; Kim et al., 2013; Lu et al., 1997). Importantly, although some PKC mutations were dominant negative, loss of PKC such as would occur from nonsense mutations or gene deletions also conferred a growth advantage (Figure 5E), indicating that PKC is haploinsufficient for tumor suppression.

A tumor-suppressive role of PKC is supported by PKC gene knockout mouse models and cellular studies. PKCα-deficient (*Prkca*$^{-/-}$) mice developed spontaneous intestinal tumors (Oster and Leitges, 2006). In an APC$^{Min/+}$ background, loss of PKCα induced more aggressive tumors and decreased survival (Oster and Leitges, 2006), and in the context of oncogenic Kras, PKCα deletion increased lung tumor formation (Hill et al., 2014). Deletion of PKCζ in mice that are PTEN haploinsufficient resulted in larger, more invasive prostate tumors and enhanced intestinal tumorigenesis in an APC$^{Min/+}$ background (Ma et al., 2013). Knockdown of PKCδ in colon cancer cells increased tumor growth in nude mice (Hernández-Maqueda et al., 2013). Conversely, overexpression of PKC revealed a protective role. Re-expression of PKCβI in colon cancer cells (Choi et al., 1990) or of PKCδ in keratinocytes (D'Costa et al., 2006) or overexpression of PKCζ in colon cancer cells (Ma et al., 2013) or in Ras-transformed fibroblasts (Galvez et al., 2009) decreased tumorigenicity in nude mice.

Clinical data reveal lower PKC protein levels and activity in tumor tissue compared with cognate normal tissue, also supporting a tumor-suppressive role for PKC. Total PKC activity was significantly lower in human colorectal cancers versus normal mucosa because of decreased PKCβ and PKCδ (Craven and DeRubertis, 1994) or PKCβ and PKCε protein levels (Pongracz et al., 1995). PKCα protein was downregulated in 60% of human colorectal cancers (Suga et al., 1998), and PKCζ was downregulated in renal cell carcinoma (Pu et al., 2012) and non-small cell lung cancer (Galvez et al., 2009). Decreased PKCβ and PKCδ levels correlated with increased tumor grade in bladder cancer (Koren et al., 2000; Langzam et al., 2001; Varga et al., 2004), and decreased PKCδ levels correlated with increased grade in endometrial cancer and glioma (Reno et al., 2008; Mandil et al., 2001). PKCη was downregulated in colon and hepatocellular carcinomas, and lower PKCη expression was associated with poorer long-term survival (Davidson et al., 1994; Lu et al., 2009). However, increased PKCι protein and DNA copy number levels have been observed in certain cancers (Perry et al., 2014; Regala et al., 2005). PKCι is part of the 3q26 amplicon, and its increased DNA copy number levels correlate with increased mRNA expression (Figure S3). However, DNA copy number

Figure 5. Correction of a Heterozygous LOF PKCβ Mutation Reduces Growth in Soft Agar, Suspension, and a Xenograft Model

(A) Immunoblot (left) and quantification (right; mean ±SEM) of PKCβII, PKCα, and GAPDH levels in the DLD1 cells.

(B) Immunoblot (left) and quantification (right; mean ±SEM) of phospho-(Ser) PKC substrates. Comparisons were made using a repeated-measures one-way ANOVA followed by post hoc Dunnett's multiple comparison test. *$p < 0.05$ as compared with the DLD1 parental cells. Data represent the mean of three independent experiments ± SEM.

(C) Relative viable cell number (mean ±SEM) as assessed by a trypan blue exclusion assay after 72 hr in suspension from three independent experiments. Comparisons were made by using a one-way ANOVA followed by post hoc Dunnett's Multiple Comparison test. ***$p < 0.001$ as compared with the DLD1 parental cell group.

(D) Representative phase contrast images of DLD1 cells grown in suspension for 24 hr.

(E) (Left) Colony formation assay in soft agar. (Right) Quantification of colony area (mean ±SEM) for colonies with a diameter ≥50 μm from three to six independent experiments. Comparisons were made using a one-way ANOVA followed by post hoc Tukey's multiple comparison test. ****$p < 0.0001$ and ***$p < 0.001$ as compared with the DLD1 parental cell group.

(F) Tumor growth is presented as the mean tumor volume (mm^3) ±SEM, with the red representing data from mice injected with the DLD1 parental cells (A509T/WT; five mice) and purple representing data of the three corrected clones (17 mice total). Comparisons were made using a two-tailed, unpaired Student's t test for each time point. **$p < 0.005$ and ***$p < 0.0005$.

(G) (Top) Representative fields from TUNEL-stained slides of tumors derived from the DLD1 cells. (Bottom) Quantification of TUNEL-positive nuclei (mean ±SEM). Comparisons were made using a one-way ANOVA followed by post hoc Dunnett's Multiple Comparison test. ****$p < 0.0001$ as compared with the DLD1 parental cell group.

See also Figure S2.

and mRNA levels do not correlate for cPKC genes (Figure S3). In fact, for PKCα, copy number levels inversely correlate with protein levels in breast cancer (Myhre et al., 2013), the cancer in which PKCα is most amplified (Cerami et al., 2012; Gao et al., 2013). A number of studies reported increased mRNA expression of other PKC genes in cancer; however, mRNA expression and protein levels often poorly correlate (Myhre et al., 2013). Thus, clinical data of this sort are consistent with a tumor-suppressive function of PKC isozymes, although there might be context specific exceptions for PKCι.

The recent discovery that germline LOF mutations in PKCδ are causal drivers of autoimmune lymphoproliferative syndrome and systemic lupus erythematosus, disorders associated with the acquisition of cancer-associated phenotypes, supports a bona fide tumor-suppressive role of PKC in humans (Belot et al., 2013; Kuehn et al., 2013; Salzer et al., 2013). Both diseases are characterized by increased proliferation and decreased apoptosis of B cells (Belot et al., 2013; Kuehn et al., 2013), and patients frequently develop lymphomas (Bernatsky et al., 2005; Mellemkjaer et al., 1997). Moreover, we found that siblings homozygous for a LOF PKCδ mutation have reduced levels of PKCζ (data not shown), supporting a dominant-negative role of LOF mutations.

How could decreased PKC activity enhance tumorigenesis? One possibility is that PKC isozymes suppress oncogenic signaling by repressing signaling from oncogenes or stabilizing tumor suppressors. Supporting this, unbiased bioinformatic analysis of tumor samples harboring PKC LOF mutations revealed that *TP53* (p53) is one of most frequently mutated genes in tumors harboring LOF mutations for each PKC isozyme (Table 2). PKC might promote the tumor-suppressive function of p53 by stabilizing the WT protein. Considerable evidence suggests that phosphorylation by PKCδ stabilizes p53, thus promoting apoptosis (Abbas et al., 2004; Yoshida et al., 2006), but the role of other PKC isozymes is less clear. *KRAS* was also among the top ten genes mutated in cancers harboring PKC mutations for seven of the PKC isozymes (Table 2), specifically with mutation at Gly12 (Table S3). This argues that PKC might suppress Kras signaling, such that loss of PKC would be required for Kras to exert its full oncogenic potential. Consistent with this, PKC modulates both the activity and localization of Kras through phosphorylation of Ser181 (Bivona et al., 2006). Although the role of this phosphorylation site in tumors remains controversial (Barceló et al., 2014), our analysis is consistent with loss of PKC enhancing its oncogenic potential. In fact, the DLD1 and HCT116 cells used in our assays contained an oncogenic Kras mutation (G13D) that is necessary for the ability of these cells to grow in soft agar (data not shown). This suggests that LOF PKC mutations are not major cancer drivers but, rather, co-drivers that contribute to cancer progression.

We also analyzed which kinase or cancer census genes (genes implicated in cancer) are significantly more commonly mutated (>15-fold) in tumors harboring PKC mutations versus tumors lacking PKC mutations (Table S4). This allowed us to identify proteins that might be important co-drivers or represent novel genetic dependencies for PKC. The tumor suppressor LATS2, which inhibits the Hippo pathway, and the kinases ROCK1 and ROCK2, which are required for the anchorage independent growth and invasion of non-small cell lung cancer cells, were among the top 20 mutated proteins that were significantly enriched in tumors harboring PKC mutations (Table S4). Our analysis suggests that mutations in these genes provide a greater proliferative advantage upon loss of PKC signaling. We also performed an analysis of cancer-specific genes frequently co-mutated with PKC in lung cancer, colorectal cancer, or melanoma. This revealed very little overlap in co-mutated genes between the three cancers and also between the three classes of PKC isozymes (Table S5), suggesting that the individual PKC isozymes regulate distinct pathways in different cancers. Interestingly, cancers with a high PKC mutation burden, such as melanoma and colorectal cancers, show little PKC amplification. Conversely, cancers that have higher PKC amplification rates, such as breast and ovarian cancers, have few PKC mutations (Cerami et al., 2012; Gao et al., 2013), consistent with PKC mutations having a smaller or different role in breast and ovarian cancers.

The foregoing data provide a mechanism for why inhibiting PKC has proved unsuccessful and, in fact, detrimental in cancer clinical trials: it is not gain of function but, rather, LOF that confers a survival advantage. Therefore, therapeutic strategies should target ways to restore PKC activity. Bryostatin-1, a PKC agonist, also failed as a therapeutic and, in fact, exhibited counter-therapeutic effects in cervical cancer (Nezhat et al., 2004), likely because it downregulates PKC (Szallasi et al., 1994). Therefore, strategies to activate PKC without downregulating it hold significant clinical potential. An important ramification of this study is that drugs that inhibit proteins involved in the processing of PKC cause loss of PKC. Notably, both mTOR and HSP90 inhibitors, currently in use in the clinic (Don and Zheng, 2011; Neckers and Workman, 2012), prevent processing of PKC (Gould et al., 2009; Guertin et al., 2006) and would thus have the detrimental effect of removing its tumor suppressive function. Restoring PKC activity would have to accompany other chemotherapeutics, given that PKC isozymes act as the brakes, not the primary drivers, to oncogenic signaling. Our finding that decreased PKC activity enhances tumor growth challenges the concept of inhibiting PKC isozymes in cancer and underscores the need for therapies that restore or stabilize PKC activity in cells.

EXPERIMENTAL PROCEDURES

FRET Imaging and Analysis

Cells were imaged as described previously (Gallegos et al., 2006). For activity measurements, cells were co-transfected with the indicated mCherry-tagged PKC and CKAR or plasma membrane-targeted CKAR, as indicated. For translocation experiments, cells were co-transfected with the indicated YFP-tagged PKC and membrane-targeted CFP.

Generation of CRISPR Cell Lines

The CRISPR/Cas9 genome-editing system was employed to generate DLD1 cell lines in which the PKCβ A509T mutation was reverted to WT or knocked out. For the nuclease method, DLD1 cells were transiently transfected with the hSpCas9 vector containing the gRNA PKCβ-a, the PAGE-purified 70-mer ssODN (Figure S2B), and pMAX-GFP. For the double nickase method, DLD1 cells were transfected with two hSpCas9n vectors containing either gRNA PKCβ-a or PKCβ-b, the ssODN, and pMAX-GFP. GFP$^+$ cells were sorted 72 hr later. To reduce off-target mutagenesis, one of the clones (WT/WT 53) was made using a double-nicking approach that requires the

Table 2. Top 20 Genes with Mutations that Co-Occur with PKC Mutations

PKCα (50)	PKCβ (90)	PKCγ (102)	PKCδ (47)	PKCε (57)	PKCη (51)	PKCθ (81)	PKCι (48)	PKCζ (28)
BLID (7)	TP53 (42)	TP53 (52)	**KRAS** (13)	GNG4 (5)	SPINK7 (5)	TP53 (42)	SPRR2G (6)	TNP1 (3)
TP53 (23)	KRTAP6-2 (6)	CDKN2A (17)	TP53 (22)	**KRAS** (11)	RPL39 (3)	CDKN2A (13)	TP53 (26)	TP53 (15)
KRTAP19-5 (4)	PCP4 (4)	**KRAS** (16)	CDKN2A (9)	DEFB114 (4)	**KRAS** (11)	**KRAS** (14)	CDKN2A (10)	CNPY1 (3)
SPRR2E (4)	**KRAS** (12)	HTN1 (4)	CD52 (3)	CNPY1 (5)	DEFB114 (4)	SPANXN5 (5)	BANF1 (5)	SPATA8 (3)
REG3A (8)	OR4A15 (21)	SPRR2G (5)	CNPY1 (4)	SVIP (4)	PLN (3)	DEFB110 (4)	LACRT (7)	SPANXN3 (4)
H3F3C (6)	POM121L12 (18)	DEFB115 (6)	SPINK13 (4)	CXCL10 (5)	DEFB115 (5)	KRTAP15-1 (8)	CXCL9 (6)	KRTAP19-5 (2)
MLLT11 (4)	REG1A (10)	DNAJC5B (12)	ATP5E (2)	KRTAP19-3 (4)	LELP1 (5)	DEFB119 (5)	**KRAS** (9)	VPREB1 (4)
PI3 (5)	NRAS (11)	REG3G (10)	RPL39 (2)	COX7C (3)	DEFB116 (5)	PPIAL4G (9)	RETNLB (5)	GNG4 (2)
SNURF (3)	PLN (3)	SPATA8 (6)	COX7B2 (3)	KRTAP19-8 (3)	KRTAP19-8 (3)	DPPA5 (6)	WFDC10B (4)	ATP6V1G3 (3)
CDKN2A (7)	GNG4 (4)	REG1A (9)	OR4K1 (11)	SPINK7 (4)	IAPP (4)	CRYGB (9)	DEFB110 (3)	CDKN2A (4)
GNG3 (3)	CDKN2A (9)	POM121L12 (16)	FDCSP (3)	TP53 (18)	NPS (4)	SPANXN2 (9)	TMSB15B (2)	DEFB119 (2)
DAOA (6)	DEFA4 (5)	TRAT1 (10)	CARTPT (4)	BANF1 (4)	WFDC10B (4)	KRTAP19-3 (4)	GNG7 (3)	LGALS1 (3)
RPL39 (2)	OR2L13 (16)	HIST1H2AA (7)	DUSP22 (7)	TMSB15B (2)	S100A7L2 (5)	DYNLRB2 (6)	CNPY1 (4)	SCGB1D1 (2)
SVIP (3)	LCE1B (6)	SPINK13 (5)	BANF1 (3)	DEFA4 (4)	CNPY1 (4)	SPATA8 (5)	LSM8 (4)	NANOS2 (3)
PLN (2)	SPANXN3 (7)	CCK (6)	DYNLL2 (3)	POM121L12 (12)	TP53 (17)	KRTAP19-8 (3)	KRTAP19-5 (3)	CCL17 (2)
FAM19A2 (5)	KRTAP19-3 (4)	OR4K1 (16)	LYRM5 (3)	GYPA (6)	DPPA5 (5)	RIPPLY3 (9)	SPANXN5 (3)	NRAS (4)
CPLX4 (6)	TRAT1 (9)	OR4A5 (16)	ATP6V1G3 (4)	DYNLRB2 (5)	DEFB131 (3)	POM121L12 (14)	CSTL1 (6)	CCL1 (2)
SEC22B (8)	IFNB1 (9)	CCL7 (5)	DEFB128 (3)	HIST1H2BB (5)	SPINK13 (4)	OR4N2 (14)	DEFA4 (4)	PATE4 (2)
CTXN3 (3)	KRTAP19-8 (3)	B2M (6)	MAP1LC3B2 (4)	HIST1H2BI (5)	RPL10L (9)	DEFB115 (4)	SPANXD (4)	POM121L12 (6)
KRTAP19-3 (3)	KRTAP8-1 (3)	PCP4 (3)	GPX5 (7)	FGFR1OP2 (10)	SPRR2A (3)	OTOS (4)	EDDM3A (6)	CRIPT (2)

Data were normalized based on gene length, and the number of co-occurring cases is listed in parentheses. Two genes are highlighted: *TP53* is underlined, and *KRAS* is in bold.

cooperation between two nickase Cas9 enzymes (Ran et al., 2013). CRISPR-targeted clones were expanded and gDNA was extracted using a Quick-gDNA MiniPrep Kit (Zymo Research Corporation) and were screened for the presence of two wild-type alleles by PCR using primers spanning the A509 locus, followed by restriction digest with BtgZI. This restriction site was only present in the WT allele, and correction of the A509T mutation introduced this site into the other allele. The presence a WT allele at both loci was confirmed by Sanger sequencing (Eton Bioscience).

Xenograft Model

Athymic Nude-*Foxn1*^{nu} mice (Harlan) were housed in compliance with the University of California San Diego Institutional Animal Core and Use Committee. 3×10^6 DLD1 cells in 100 μl PBS were injected subcutaneously into the right flank of each 4-week-old female mouse. Tumor dimensions were recorded twice weekly and tumor volume was calculated as 1/2 × length × width^2. Mice were euthanized 43 days after injection, and tumors were excised. One tumor was excluded, as it did not engraft well (DLD1p), and another was excluded, as it was not subcutaneous (WT/WT 31).

SUPPLEMENTAL INFORMATION

Supplemental Information includes Extended Experimental Procedures, three figures, and five tables and can be found with this article online at http://dx.doi.org/10.1016/j.cell.2015.01.001.

AUTHOR CONTRIBUTIONS

L.L.G., J.B., T.H., and A.C.N. initiated the study. C.E.A. and A.C.N. conceived the experiments and wrote the manuscript. C.E.A. performed the experiments with assistance from E.K. for imaging and immunoblots and from C.Z. for the xenograft model. F.B.F. advised on the use of the xenograft model. A.M.H., C.W., C.J.M., and J.B. performed the bioinformatic analysis. N.L.S. and E.W.T. made the tetracycline-inducible PKCβII HCT116 cells.

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