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.

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

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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 isoforms comprise three classes: conventional (cPKC: α, β, γ), novel (nPKC: δ, ε, η, θ), and atypical (aPKC: ξ, ζ). cPKC and nPKC isoforms 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 isoforms are activated by binding to diacylglycerol (DAG) and Ca²⁺, whereas nPKC isoforms are activated solely by DAG, events that engage PKC at membranes. Prolonged activation of cPKC and nPKC isoforms 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 isoforms bind neither Ca²⁺ nor DAG.

PKC has proved an intractable target in cancer therapeutics (Kang, 2014). PKCα 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 isoforms, there is conflicting evidence as to whether they act as oncogenes or as tumor suppressors. For example, PKCα 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 PKCε 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, PKCζ 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 APCMin/+ model (Oster and Leitges, 2006). Based on the dogma that PKC isoforms contribute positively to cancer progression, many PKC inhibitors have entered clinical trials; however, they have been ineffective (Mackay and Twelves, 2007).

SUMMARY

Protein kinase C (PKC) isoforms 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 isoforms generally function as tumor suppressors, indicating that therapies should focus on restoring, not inhibiting, PKC activity.
Correction of one patient-identified, heterozygous, loss-of-function (LOF) PKC genes and tumor suppressors known to be regulated by PKC. That they may cooperate with co-occurring mutations in oncogenes and family membership and assessed their functional impact. Specifically, we asked how these cancer-associated mutations affect the processing phosphorylations of these agonists to activate the mutants. Lastly, we asked how these mutations affected the processing phosphorylations of PKC. PKC\(\gamma\) 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 PKC\(\xi\) (G61W, PKC\(\beta\) (G61W), and PKC\(\gamma\) (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 PKC. PKC\(\gamma\) (D193N) was present in colorectal and ovarian cancers.

**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) isoforms (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 isoforms (Table 1 and Table S1).

**PKC Mutations in the Regulatory C1 and C2 Domains Are LOF**

The C1 domains of cPKC and nPKC isoforms 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 (PKC\(\xi\) 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 (PKC\(\xi\) 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 isoforms, 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. PKC\(\gamma\) 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 PKC\(\xi\) (G61W), PKC\(\beta\) (G61W), and PKC\(\gamma\) (G62H) 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 isoforms is also critical for activation, as it mediates Ca\(^{2+}\)-dependent pre-targeting to plasma membrane, where these isoforms bind DAG and become activated (Newton, 2003). One mutation identified within the C2 domain of PKC\(\gamma\) (D193N) was present in colorectal and ovarian cancers.
<table>
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<tr>
<th>Mutation</th>
<th>Activity</th>
<th>Domain</th>
<th>Cancer(s)</th>
<th>Residue Importance</th>
<th>Allele Frequency</th>
<th>Other Mutations</th>
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<td>G23E</td>
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(Continued on next page)
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.

Mutations examined in this study.

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

No response to physiological stimuli.

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 PKCβi A509V mutations ablated activity by preventing processing phosphorylations, and both exhibited dominant-negative roles (Figures 3G–3J). PKCβi 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, PKCδ A444V, PKCβII Y417H, PKCβIII 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 PKCδ R162H pseudosubstrate mutation showed reduced agonist-stimulated and basal activity (Figures S1K and S1L). The PKCβi P619Q C-terminal tail mutation, residing within a conserved PXBP 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,
Analysis of cancer types most frequently harboring PKC mutations revealed that, although PKC isoforms are mutated across many cancers, PKC mutations are enriched in certain cancers (Figure 4D). Namely, PKC isoforms 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 isoforms 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 branch 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 PKCα 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 PKCα by overexpressing WT PKCαIII. 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

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 uM 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 PKCα (PDB 2UZP) highlighting Asp193 and Asp254 residues involved in Ca2+ binding. (G) Normalized FRET ratio changes (mean ±SEM) showing PKC activity as read out by CKA1 upon elevation of intracellular Ca2+ stimulated by thapsigargin (5 μM), followed by PDBu (200 nM). (H) Normalized FRET ratio changes (mean ±SEM) showing translocation of YFP-tagged PKCα constructs toward membrane-localized CFP upon stimulation of COS7 cells with thapsigargin (5 μM) followed by PDBu (200 nM). Data are representative traces from individual cells of three independent experiments. See also Figure S1.

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

(A) Solution structure of the C1A domain of PKCα (PDB 2E73) showing the corresponding PKCα His75 residue that coordinates Zn2+ 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 CKA1 in COS7 cells co-expressing CKA1 and either mCherry-tagged WT, mutant PKCα, or no exogenous PKC (endogenous). (C) (Left) Representative YFP images of the indicated PKC isoforms under basal and PDBu-treated conditions (200 nM; 15 min) showing relocalization of 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).
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 PKC mutation (Figure 5I) 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 capable of forming the compact multicellular aggregates formed by the DLD1 parental cells (Figure 5D). Moreover,
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 PKCβII 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 5Fa 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
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 PKCγ (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, PKCζ 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 PKCζ such as would occur from nonsense mutations or gene deletions also conferred a growth advantage (Figure 5E), indicating that PKCζ is haploinsufficient for tumor suppression.

A tumor-suppressive role of PKC is supported by PKC gene knockout mouse models and cellular studies. PKCζ-deficient (Prkca−/−) mice developed spontaneous intestinal tumors (Oster and Leitges, 2006). In an APCMin−/− background, loss of PKCζ induced more aggressive tumors and decreased survival (Oster and Leitges, 2006), and in the context of oncogenic Kras, PKCζ 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 APCMin−/− background (Ma et al., 2013). Knockdown of PKCζ 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 PKCζ in colon cancer cells (Choi et al., 1990) or of PKCζ 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 PKCζ and PKCα (Craven and DeRubertis, 1994) or PKCζ and PKCα 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 PKCζ 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 PKCζ levels correlated with increased grade in endometrial cancer and glioma (Reno et al., 2008; Mandil et al., 2001). PKCζ was downregulated in colon and hepatocellular carcinomas, and lower PKCζ expression was associated with poorer long-term survival (Davidson et al., 1994; Lu et al., 2009). However, increased PKCζ protein and DNA copy number levels have been observed in certain cancers (Perry et al., 2014; Regala et al., 2005). PKCζ 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 PKCζ Mutation Reduces Growth in Soft Agar, Suspension, and a Xenograft Model

(A) Immunoblot (left) and quantification (right; mean ±sEM) of PKCζIII, 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) 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 (mm3) ±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 PKC\(\alpha\), copy number levels inversely correlate with protein levels in breast cancer (Myhre et al., 2013), the cancer in which PKC\(\alpha\) 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 PKC\(\alpha\).

The recent discovery that germline LOF mutations in PKC\(\alpha\) 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 PKC\(\alpha\) mutation have reduced levels of PKC\(\alpha\) (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 PKC\(\alpha\) stabilizes p53, thus promoting apoptosis (Abbas et al., 2004; Yoshida et al., 2006), but the role of other PKC isozymes is less clear. K\(R\)\(A\)\(S\) 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., 2008). 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 PKC\(\zeta\) 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 PKC\(\zeta\)-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 PKC\(\zeta\)-a or PKC\(\zeta\)-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

<table>
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<th>Gene</th>
<th>PKCα (50)</th>
<th>PKCβ (90)</th>
<th>PKCγ (102)</th>
<th>PKCa (47)</th>
<th>PKCe (57)</th>
<th>PKCε (51)</th>
<th>PKCζ (81)</th>
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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 nicksense Cas9 enzymes (Fam et al., 2013). CRISPR-targeted clones were expanded and qDNA was extracted using a Quick-qDNA MiniPrep Kit (Zymo Research Corporation) and were screened for the presence of two wild-type alleles by PCR using primers spanning the AS09 locus, followed by restriction digest with BtgZI. This restriction site was only present in the WT allele, and correction of the AS09T 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
term mice (Harlan) were housed in compliance with the University of California San Diego Institutional Animal Care and Use Committee. 3 x 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 x length x 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 and Else Schilling American Cancer Society Professor and holds the Renato C.J.M., and J.B. were supported by Cancer Research UK. T.H. is a Frank L. Fuld Scholar in Cancer Research. T.H. is supported by National Research Service Award NS080939 and the James S. McDonnell Foundation to F.B.F., and NIH CA82683 to T.H. C.E.A. was supported by the UCSD Graduate Training Program in Cellular and Molecular Pharmacology (T32 GM007752) and the NSF grant in Cellular and Molecular Pharmacology (T32 GM007752) and the NSF Core for the TUNEL staining, Meghdad Rahdar for cell sorting, and Jack Dixon for helpful comments, the Moores Cancer Center Histology Core for the tetracycline-inducible PKCδ2. J. Clin. Endocrinol. Metab. (2004). Inhibition of human p53 basal transcription by down-regulation of pro-apoptotic Bcl-XL on mitochondria and induces apoptosis. Mol. Cell 21, 481–493.


