HHS Public Access

Author manuscript

*Oncogene*. Author manuscript; available in PMC 2019 September 28.

Published in final edited form as:

*Oncogene*. 2019 July ; 38(27): 5396–5412. doi:10.1038/s41388-019-0796-4.

# DISTINCTIVE REQUIREMENT OF PKCε IN THE CONTROL OF Rho GTPases IN EPITHELIAL AND MESENCHYMALLY-TRANSFORMED LUNG CANCER CELLS

**Victoria Casado-Medrano**, **Laura Barrio-Real**, **Anita Wang**, **Mariana Cooke**, **Cynthia Lopez-Haber**, and **Marcelo G. Kazanietz**[*]

Department of Systems Pharmacology and Translational Therapeutics, Perelman School of Medicine, University of Pennsylvania, Philadelphia, PA 19104-6160, USA

## Abstract

Diacylglycerol (DAG)/phorbol ester-regulated protein kinase C (PKC) isozymes have been widely linked to tumor promotion and the development of a metastatic phenotype. PKCε, an oncogenic member of the PKC family, is abnormally overexpressed in lung cancer and other cancer types. This kinase plays significant roles in proliferation, survival and migration; however its role in epithelial-to-mesenchymal transition (EMT) has been scarcely studied. Silencing experiments in non-small lung cancer (NSCLC) cells revealed that PKCε or other DAG-regulated PKCs (PKCα and PKCδ) were dispensable for the acquisition of a mesenchymal phenotype induced by transforming growth factor beta (TGF-β). Unexpectedly, we found a nearly complete down-regulation of PKCε expression in TGF-β-mesenchymally transformed NSCLC cells. PMA and AJH-836 (a DAG-mimetic that preferentially activates PKCε) promote ruffle formation in NSCLC cells via Rac1, however they fail to induce these morphological changes in TGF-β-mesenchymally transformed cells despite their elevated Rac1 activity. Several Rac Guanine nucleotide Exchange-Factors (Rac-GEFs) were also up-regulated in TGF-β-treated NSCLC cells, including Trio and Tiam2, which were required for cell motility. Lastly, we found that silencing or inhibiting PKCε enhances RhoA activity and stress fiber formation, a phenotype also observed in TGF-β-transformed cells. Our studies established a distinctive involvement of PKCε in epithelial and mesenchymal NSCLC cells, and identified a complex interplay between PKCε and small GTPases that contributes to regulation of NSCLC cell morphology and motile activity.

### Keywords

PKCε; TGF-β; EMT; Rho; Rac; migration; lung cancer



[*]Correspondence and reprints requests should be addressed to Marcelo G. Kazanietz (marcelog@upenn.edu) at the Department of Systems Pharmacology and Translational Therapeutics, Perelman School of Medicine, University of Pennsylvania, 1256 Biomedical Research Building II/III, 421 Curie Blvd., Philadelphia, PA 19104-6160. Phone: (215)-898-0253; fax: (215)-746-8941.

CONFLICT OF INTEREST

The authors have nothing to disclose.

## INTRODUCTION

Protein kinase C (PKC) serine-threonine kinases represent the most prominent cellular targets for the lipid second messenger diacylgycerol (DAG) and the phorbol esters, natural products with tumor promoter activity. Based on their distinctive biochemical and structural features, DAG/phorbol ester-responsive PKCs are classified into calcium-dependent "classical/conventional" cPKCs (α, βI, βII, and γ) and calcium-independent "novel" nPKCs (δ, ε, η, and θ). Decades of research have proven that individual members of the PKC family control signaling pathways crucial for cell proliferation, survival, differentiation, and motility, with an exquisite degree of isozyme- and cell type-specificity (1-3).

PKC isozymes often display altered expression and/or activity in cancer, and in some cases this is causally linked to disease progression (1, 4). In this regard, recent studies highlighted a key association between PKCε up-regulation and the progression of lung, prostate, and breast cancer (5-7). PKCε has been recognized as a cancer biomarker that participates in central steps of the metastatic cascade, namely cancer motility, invasiveness, and the secretion of metalloproteases (6, 8-10). Genetically engineered PKCε transgenic and knock-out mouse models further helped delineating fundamental roles for this kinase in cancer initiation and progression as well as revealed cooperative effects with defined oncogenic and tumor suppressor stimuli, thus highlighting the attractiveness of this kinase as a potential target for cancer therapy (11-13). As an example, transgenic overexpression of PKCε in the mouse prostate confers a preneoplastic phenotype that progresses to invasive malignant carcinoma upon loss of a single allele of the tumor suppressor Pten (6). In human non-small cell lung cancer (NSCLC), PKCε is abnormally up-regulated compared to normal lung epithelium, and silencing PKCε expression markedly reduces the ability of NSCLC cells to form tumors in athymic nude mice. RNAi depletion of PKCε in NSCLC cells alters the expression profile of pro-apoptotic and pro-survival genes, thus underscoring its prominent role in NSCLC cell survival and growth maintenance (7, 10, 14).

As might be anticipated from its central role in tumor progression and metastasis, PKCε signals through multiple cascades, including the Erk, PI3K/Akt, NF-κB and Stat3 pathways (6, 15-18). In NSCLC cells, phorbol esters and growth factors induce remarkable morphological changes, including the formation of ruffles, via activation of PKCε and Rac1, a member of the Rho small GTPase family. Notably, RNAi depletion or pharmacological inhibition of PKCε in NSCLC cells impairs ruffle formation, consequently reducing their migratory and invasive capacities (10, 19). Recent evidence suggested that PKC isozymes, including PKCε, are involved in epithelial-to-mesenchymal transition (EMT), a sequential process of biochemical and cellular events that constitute the onset of metastatic dissemination (18, 20-23). EMT involves the expression of specific transcription factors, reorganization of cytoskeletal structures, inhibition of cell-cell contacts, and secretion of extracellular matrix (ECM)-degrading enzymes, ultimately leading to the acquisition of enhanced migratory and invasive capacities (24, 25).

In this study we investigated whether a potential relationship between PKCε and EMT exists in NSCLC cells. Although we found that PKCε is dispensable for EMT, our results revealed a distinctive involvement of this kinase in the control of actin-rich structures depending on

whether NSCLC cells are in epithelial or mesenchymal states. In addition, we identified novel relationships between PKCε and Rho GTPases that greatly impact on the architecture and motile activity of NSCLC cells.

## RESULTS

### PKCε and other DAG-regulated PKCs are dispensable for TGF-β induced EMT in NSCLC cells

In order to investigate a potential involvement of PKCε in EMT, we used an established TGF-β treatment protocol (26-28). A549 and H358 NSCLC cells were treated with TGF-β (10 ng/ml) for 6 days to generate A549-T6 and H358-T6 cell lines, respectively. TGF-β-treated cells lost the cuboidal epithelial morphology to acquire a characteristic elongated shape (Fig. 1a), and display elevated motile activity characteristic of the mesenchymal phenotype, as determined using a Boyden chamber (Fig. 1b). Morphological changes in A549-T6 and H358-T6 cells were accompanied by loss of E-cadherin expression and vimentin up-regulation, as determined by Western blot (Fig. 1c) and Q-PCR (Fig. 1d). Similar morphological changes and vimentin up-regulation in response to TGF-β treatment were observed in “normal” immortalized, non-transformed human bronchial epithelial HBEC cells, although E-cadherin changes in these cells were marginal (Fig. S1).

Based on a previous study in mammary models suggesting the involvement of PKCε in EMT (17), we asked if this kinase plays any role in the acquisition of a mesenchymal phenotype in NSCLC cells. To address this question, we silenced PKCε expression from A549 and H358 cells using two different RNAi duplexes (ε1, ε2), which cause nearly complete PKCε depletion (Fig. 1f) that lasted for at least 4 days (data not shown). We found that PKCε-depleted NSCLC cells still developed an elongated mesenchymal shape in response to TGF-β treatment (Fig. 1e). Consistent with this result, E-cadherin down-regulation and vimentin up-regulation by TGF-β were essentially the same in NSCLC cells subject to PKCε or non-target control (NTC) RNAi (Fig. 1f). PKCε depletion did not cause any significant changes in TGF-β-induced phosphorylation of Smad2/3, a proximal event in the TGF-β cascade (Fig. 1g), further supporting the concept that PKCε is not involved in TGF-β signaling to drive EMT. For all these experiments, similar results were observed with two additional PKCε RNAi duplexes (ε3 and ε4) (Fig. S2).

In addition to PKCε, NSCLC cells express DAG/phorbol ester-responsive PKCα and PKCδ isozymes, which play roles in cancer progression, including in lung cancer (29, 30). To assess if these PKCs have any roles in EMT, we performed similar experiments in which PKCα or PKCδ were knocked down using two specific RNAi duplexes (α1/α2 and δ1/δ2, respectively). We observed that, similarly to PKCε, neither PKCα nor PKCδ were required for TGF-β-induced mesenchymal transformation in A549 or H358 NSCLC cells, as judged by analysis of cell morphology (Fig. 2a), expression of EMT markers (Fig. 2b), and Smad2/3 phosphorylation (Fig. 2c). Likewise, PKCα and PKCδ RNAi did not affect TGF-β-induced vimentin up-regulation or Smad2/3 phosphorylation in HBEC cells (Fig. S3A and S3B). Furthermore, the pan-PKC inhibitor GF109203X or the cPKC inhibitor Gö6976 (31) had no effect on Smad2 phosphorylation by TGF-β in NSCLC cells (Fig. 2d), further reinforcing the concept that DAG/phorbol ester responsive PKCs expressed in NSCLC cells

are dispensable for TGF-β pathway activation. Similar result was observed in HBEC cells (Fig. S3C).

### TGF-β promotes PKCε down-regulation

As PKCε up-regulation/activation has been previously linked to motile and invasive phenotypes in cancer cells, including NSCLC cells (4, 10), we next examined whether changes in PKCε levels could be associated with mesenchymal transformation induced by TGF-β. To our surprise, PKCε expression in A549-T6 and H358-T6 cells, which display elevated motile activity (Fig. 1b), was prominently down-regulated (~70% and ~80% reduction, respectively) relative to their corresponding epithelial counterpart cell lines (Fig. 3a). PKCδ expression levels were slightly reduced (~20-30%), and PKCα expression levels were essentially unchanged under the same experimental conditions. Similar PKCε down-regulation by TGF-β treatment was observed in H322 and H1299 NSCLC cell lines as well as in HBEC cells (Fig. S4). Moreover, similar effects were observed in prostate, ovarian and pancreatic cancer cell lines (Fig. S5). A subsequent time-course analysis revealed a progressive reduction in PKCε levels that became noticeable 2 days after initiation of TGF-β treatment (Fig. 3b, *upper panels*). Densitometric analysis showed that PKCε down-regulation in A549 and H358 cells follows a similar time-dependency to E-cadherin down-regulation and vimentin up-regulation (Fig. 3b, *lower panels*).

### Epithelial and mesenchymal NSCLC cells exhibit differential PKCε requirement for actin cytoskeleton reorganization and motility

The unexpected PKCε down-regulation observed by TGF-β treatment led us to speculate a differential involvement of this kinase in the motile activity of epithelial and mesenchymally transformed NSCLC cells. Knocking down PKCε in “epithelial” A549 and H358 cells using ε1 and ε2 RNAi duplexes reduced their ability to migrate by ~60%, as determined with a Boyden chamber assay. Of course, as endogenous PKCε levels are essentially null in TGF-β-transformed NSCLC cells, one would predict a limited effect of PKCε RNAi on the motile activity of cells in mesenchymal state. Indeed, despite the higher motility of A549-T6 and H358-T6 cells relative to their epithelial counterparts, delivery of ε1 and ε2 RNAi duplexes into these cells did not significantly affected migration (Figs. 3c and 3d). Thus, PKCε is only required for the motility of NSCLC cells in an epithelial state, whereas it is largely dispensable for the migratory activity of mesenchymally-transformed NSCLC cells.

We have previously reported that phorbol esters and DAG analogues promote the formation of ruffles in NSCLC cells, an effect largely mediated by PKCε (10, 19). The reduced PKCε expression in TGF-β-transformed cells led us to speculate that important differences in the formation of these actin-rich structures might exist between epithelial and mesenchymal NSCLC cells. To address this issue, parental A549 and A549-T6 cells were treated with the phorbol ester PMA (100 nM, 30 min) and the formation of ruffles was analyzed by phalloidin-rhodamine staining. As seen before (10), PMA induced a prominent formation of peripheral ruffles in A549 cells, an effect that was substantially reduced upon PKCε RNAi depletion (Fig. 4a). A549-T6 cells maintained their characteristic elongated shape in response to the phorbol ester, however the formation of actin-rich structures was barely detected, other than some small structures that could be occasionally observed. As expected

from the low PKCε levels present in mesenchymally-transformed A549 cells, subjecting these cells to PKCε RNAi had no effect.

To further confirm the differential requirement of PKCε in ruffle formation in epithelial *vs.* mesenchymal state, we took advantage of AJH-836, a DAG-mimetic analogue characterized in our laboratory that preferentially activates PKCε relative to other PKCs (19). Like PMA, AJH-836 also caused a prominent formation of ruffles in parental A549 cells that was sensitive to PKCε RNAi (see also Ref. (19)). In A549-T6 cells, however, AJH-836 did not cause any obvious formation of peripheral ruffles in A549-T6 cells (Fig. 4a), as described above for PMA.

As a second approach to determine ruffle formation, we stained cells for cortactin (Fig. 4b). In agreement with phalloidin staining experiments, we found significant cortactin staining in A549 “epithelial” cells in response to either PMA or AJH-836. This effect is not observed in cells subject to PKCε RNAi depletion. Moreover, no obvious cortactin staining in response to either PKC activator can be observed in A549 cells subject to TGF-β treatment. A quantitative analysis of staining is shown in Fig. 4c. Altogether, these experiments indicate that PKCε is relevant for the formation of peripheral ruffles only in epithelial NSCLC cells.

## TGF-β-transformed NSCLC cells display enhanced activation of Rho GTPases

We have previously reported that in NSCLC cells, PKCε controls the activation of Rac1, a small G-protein involved in actin cytoskeleton reorganization and the formation of dynamic membrane structures required for cell locomotion (10, 32-34). In the next set of experiments, we examined if mesenchymal transformation of NSCLC cells is associated with changes in the activity of Rac1. Fig. 5a shows that TGF-β-transformed A549 cells display a significant elevation in active Rac1 levels relative to their corresponding epithelial counterpart, as determined with a PBD “pull-down” assay (35). Mesenchymally transformed cells also display a significant increase in the activity of Cdc42, another member of the Rho GTPase family implicated in the regulation of cellular architecture (36).

We next investigated whether changes in RhoA activity also occur in cells transformed to a mesenchymal state. The small GTPase RhoA plays a central role in regulating actin-binding proteins required for the assembly of stress fibers (37). As shown in Fig. 5a, there was a significant elevation in Rho-GTP (active) levels in A549-T6 cells relative to parental A549 cells, as determined with a rhotekin RBD “pull-down” assay (38). Higher levels of active Rac1, Cdc42, and RhoA were also observed in H358 cells transformed with TGF-β (data not shown).

To determine a potential involvement of PKCε in the regulation of Rho GTPases, we re-expressed this kinase in the mesenchymal–like cells using a FLAG-tagged PKCε vector, to obtain A549-T6-PKCε cells. Ectopic expression of PKCε in mesenchymally-transformed A549 lung cells reduced the levels of activated Rac1, Cdc42, and RhoA in A549-T6 cells (Fig. 5b). Thus, while PKCε is important for the motility of NSCLC cells in the epithelial state, down-regulation of this kinase in mesenchymally-transformed cells is a permissive input for the activation of Rac1 activity, as well as the activity of other GTPases of the Rho family.

### Changes in the expression of Rac-GEFs in mesenchymal NSCLC cells

Activation of Rho small G-proteins is mediated by Guanine nucleotide Exchange Factors (GEFs), a large family of proteins with discrete specificities towards different Rho GTPases (39-41). Overexpression of Rac-GEFs that contribute to the motile phenotype has been described in various cancer types (42, 43). We wished to determine if the expression of Rac-GEFs changes in mesenchymally-transformed cells. We generated a Q-PCR array for the simultaneous detection of 26 Rac-GEFs. Three sets of A549 cells were used for this analysis: non-treated, TGF-β-treated (6 days), and TGF-β-treated cells (6 days) followed by 6 days without TGF-β, a condition in which the mesenchymal phenotype is reversed (A549-T6-REV). The morphology of A549-T6-REV cells is similar to that of parental A549 cells (Fig. S6a). In addition, A549-T6-REV cells have high E-cadherin and low vimentin levels, as determined by Western blot (Fig. S6b) and immunofluorescence (Fig. S6c), and display high PKCε levels (Fig. S6b). The “cut-off” established as the limit for detection for the Q-PCR was Ct=35. Experimental Ct values are shown in Supplementary Table S1, and revealed detectable expression for 16 Rac-GEFs (Fig. 6a). Up-regulation in A549-T6 relative to parental A549 was found for 7 Rac-GEFs, which could be reversed upon TGF-β removal (DOCK2, DOCK4, NGEF, RasGRF2, TIAM2, TRIO, VAV2) (Table S1, Fig. 6a and Fig. 6b). Rac-GEF up-regulation in mesenchymally transformed cells was validated in independent Q-PCR experiments performed both in A549/A549-T6 and H358/H358-T6 cell lines (Fig. 6c). To determine a potential role of these Rac-GEFs in the motility of TGF-β-transformed NSCLC cells, we used RNAi, which caused a nearly complete depletion in the corresponding mRNA levels relative to NTC RNAi (Fig. 6d). Silencing Tiam2 and TRIO caused the largest reduction in the motility of A549-T6 cells, whereas depletion of other Rac-GEFs caused no effects or marginal reductions in migratory capacity (Fig. 6e and 6f).

### PKCε depletion/inhibition is permissive for the formation of Rho-mediated stress fibers in NSCLC cells

As shown in Fig. 5, TGF-β-transformed cells display elevated RhoA-GTP levels. RhoA plays a central role in regulating actin-binding proteins required for the assembly of stress fibers (37). Consistent with the elevated RhoA activity in mesenchymally-transformed cells, confocal microscopy analysis revealed an evident increase in actin stress fibers in A549-T6 cells compared to parental cells. A similar effect was observed in other NSCLC cell lines (H358 and H1299) subject to TGF-β treatment (Fig. 7a).

The negative regulation of RhoA activity by PKCε (see Fig. 5) prompted us to analyze its potential functional consequences. Notably, there was a noticeable increase in stress fibers in epithelial A549 cells subjected to PKCε RNAi depletion. This effect was observed with 4 different RNAi duplexes (ε1-ε4) (Fig. 7b) and resembles the phenotype seen in TGF-β-transformed cells. The formation of stress fibers in PKCε-depleted cells was abolished by treatment with the Rho kinase (ROCK) inhibitor Y27632 (Fig. 7c), hence supporting the involvement of the Rho pathway. The efficacy of Y27632 was confirmed by its ability to impair the phosphorylation of the ROCK substrate MYPT1 (Fig. S7a).

To further substantiate the inhibitory role that PKCε has on stress fiber formation, we took advantage of εV1-2, a TAT-fused permeable peptide that selectively inhibits PKCε (44). We

previously reported that this inhibitor blocks the formation of ruffles in response to PKCε activation by PMA and AJH-836 (10, 19). Treatment of A549 cells with εV1-2 (10 μM, 60 min) caused a marked increase in the formation of stress fibers. On the other hand, a TAT peptide used as a control had no effect. The effect of εV1-2 was sensitive to the ROCK inhibitor Y27632 (Fig. 7d), which reduces phospho-MYPT1 levels (Fig. S7b).

Finally, we asked if pharmacological PKCε inhibition could have an impact on RhoA activity. A time-dependent increase in RhoA-GTP levels by εV1-2 was observed, with maximum effect at 15-30 min, whereas the TAT control peptide had no effect (Fig. S7c). Fig. 7e shows a representative Rho-GTP “pull-down” assay of εV1-2-treated cells at 30 min with the corresponding densitometric analysis. Altogether, these results suggest that PKCε negatively regulates RhoA activation and RhoA-mediated morphological changes in NSCLC cells.

## DISCUSSION

A large body of evidence links PKC isozymes to multiple steps of cancer progression, including cancer cell migration, invasiveness and metastatic dissemination. However, the specific roles of individual members of the PKC family in the different stages of progression remain partially understood and have been a subject of controversy (4, 45). Most notably, PKCε emerged as an oncogenic member of the PKC family, with significant involvement in tumorigenesis and metastasis (4, 6, 8, 15, 21). PKCε is overexpressed in many epithelial cancers, and has been widely implicated in pathways regulating cell division, motility, and survival, making it an attractive target for cancer therapy (4, 17). Several links have also been established between PKCε, actin cytoskeleton reorganization, invasive capacity, and metalloprotease secretion required for ECM degradation (8, 10, 19, 46). Based on recent suggestions implicating PKCε in EMT (18, 20, 22, 23), we decided to explore this issue in more detail in NSCLC cellular models that display elevated PKCε expression and depend on PKCε for tumorigenic and metastatic activity (10, 14). Our results showed that despite the involvement of PKCε in NSCLC cell motility and ruffle formation, this kinase is not required for the transition to a mesenchymal state. A similar conclusion was attained for other DAG/phorbol ester-regulated PKCs, namely PKCα and PKCδ, which also have recognized roles in lung cancer progression (29, 30). Silencing PKCε or other relevant PKCs in NSCLC cells did not affect Smad2/3 activation by TGF-β, arguing that acquisition of the mesenchymal phenotype in this setting is independent of the activation of the DAG/PKC pathway. However, ectopic overexpression of PKCε induces a mesenchymal phenotype in MCF-10A mammary epithelial cells, and these morphological features could be partially reversed in TGF-β-transformed cells upon PKCε knockdown (23). Contrasting results with regard to the PKCε requirement for Smad phosphorylation have been reported in fibroblasts and renal epithelial cells (22, 33), thus suggesting a distinctive cell context-specific involvement of PKCε in the transition to the mesenchymal state.

The unanticipated down-regulation of PKCε by TGF-β, but no other PKCs, raises interesting functional and mechanistic issues. The first relates to the relevance of the dynamic regulation of PKCε expression in physiological and pathophysiological settings. For example, expression levels of PKCε greatly influence apoptotic/survival responses

(47-50). Muscle differentiation and erythropoiesis involve PKCε up-regulation (51-54), whereas megakaryocytopoiesis, colonic mucosa and vascular endothelial differentiation are accompanied by PKCε down-regulation (55-57). Notably, hypoxic signaling, which promotes EMT and is linked to metastasis, induces down-regulation of PKCε in tumor cells (47). In our study, we found that PKCε down-regulation in EMT is a permissive signal for the activation of Rho GTPases that control cell architecture and motile capacity. A second important question is: what mechanisms drive PKCε down-regulation by TGF-β? It is well known that PKCs can be down-regulated by persistent treatment with phorbol esters and related DAG-mimetics (58). However, one would predict distinctive mechanisms taking place upon TGF-β receptor activation, which does not primarily couple to DAG generation and PKC activation. A likely possibility is that PKCε is transcriptionally repressed during the acquisition of the mesenchymal phenotype via genetic and/or epigenetic mechanisms. As yet, the mechanisms controlling the transcription of the PKCε gene (*PRKCE*) are only partially understood (59, 60). *PRKCE* is also regulated by specific miRNAs (61-63), including miR-222, which inhibits PKCε expression and controls EMT in NSCLC cells (64). As PKCε is subject to ubiquitination, and its degradation involves lysosomal mechanisms distinctive to those mediating PKCα and PKCδ degradation (Casado Medrano *et al.*, manuscript in preparation), another attractive hypothesis is that TGF-β-regulated ubiquitylating enzymes such as SMURF1, USP15, or Nedd4L (65-67) contribute to PKCε down-regulation in EMT. This area of research is currently under investigation in our laboratory.

PKCε has been linked to a migratory phenotype in cancer cells (5, 46). In prostate cancer cells, described a pro-migratory pathway driven by the chemokine CXCL13 that involves PKCε (6). In lung cancer cells, DAG-mimetics acting through PKCε promote the formation of actin-rich structures required for cell motility, and targeted disruption of PKCε reduces motility though Rac1 inactivation (10, 19), supporting previously reported hierarchical PKCε-Rac relationships (32-34, 68). Mechanistically, one likely hypothesis is that PKCε phosphorylates and thereby controls the activity of Rac regulatory proteins, such as Rac-GEFs, exchange factors that can be regulated via phosphorylation by PKCs and other kinases (39, 69, 70). PKCs can also inhibit the activity of Rac-GAPs responsible for Rac1 inactivation (71, 72). Direct binding of PKCε to actin (73, 74) may also contribute to the regulation of motile structures. Based on our results, the assumption is that different mechanisms independent of PKCε must take place in the control of cell motility in a mesenchymal state. Indeed, the PKCε-mediated permissive signal for Rac1 activation operating in NSCLC cells in the epithelial state is annulled in TGF-β-transformed cells due to PKCε down-regulation. The high motility state and elevated Rac1 activity in mesenchymally-transformed cells may therefore rely on alternative pathways independent of PKCε. Not surprisingly, we found significant changes in the pattern of expression of Rac-GEFs between epithelial and TGF-β-transformed NSCLC cells, suggestive of distinctive Rac activation mechanisms in either state. Interestingly, a dependency of lung adenocarcinoma metastasis on TGF-ε-mediated activation of the Rac-GEF DOCK4 has been described (75). Identifying direct substrates and effectors of PKCε implicated in the regulation of Rac signaling will provide important insights into the pathways controlling cell motility and metastatic dissemination in cancer cells.

Our results showing a negative regulatory role of PKCε in Rho signaling also enlightened unexpected links between this PKC isozyme and small GTPases that control the actin cytoskeleton. Available data indicate an extensive and complex signaling cross-talks between TGF-β and Rho GTPases in EMT, both in normal and neoplastic epithelial cells (76). Early studies by Moses and co-workers reported that TGF-β-induced epithelial to mesenchymal transdifferentiation involves the activation of RhoA, and that EMT could be inhibited by expression of dominant-negative Rho/ROCK mutants (77). The requirement of the Rho/ROCK pathway in TGF-β signaling, including in cytoskeleton rearrangement and EMT, was subsequently described in other models (78). Similar to our results, other studies also reported elevated Rho activity in mesenchymally-transformed cells (78, 79). Given that PKCε inhibition leads to RhoA activation and the induction of stress fiber formation, we propose that the loss of expression of this kinase in TGF-β-transformed cells acts as a permissive mechanism for the activation of this GTPase in a mesenchymal state. This conclusion is further supported by the evident formation of stress fibers in NSCLC cells upon PKCε RNAi depletion or treatment with the PKCε inhibitor εV1-2, an effect that is sensitive to the ROCK inhibitor Y27632. Our results also recapitulate the negative relationship between PKCε and RhoA activation status found during megakaryocyte differentiation (53). A negative regulation of RhoA-dependent pathways by PKCε has also been reported in astrocytes (80).

In summary, our studies underscore a differential requirement for PKCε in NSCLC cells depending on whether they are in epithelial or mesenchymal states. Despite the fact that PKCε is dispensable for TGF-β-induced EMT, this kinase has important roles in the formation of actin rich structures and migration of NSCLC cells in an epithelial state. PKCε down-regulation in TGF-β-mesenchymally transformed cells releases a “brake” that facilitates RhoA activation and enables the formation of stress fibers. This conclusion is summarized in the model depicted in Fig. 8. Our results highlight the high levels of complexity in the regulation of small GTPase function by upstream kinases in EMT transdifferentiation and motile/invasive processes involved in the metastatic dissemination of cancer cells.

## MATERIAL AND METHODS

### Cell lines and materials

All cell lines were obtained from ATCC (Manassas, VA) and are fully authenticated (see ATCC homepage). Human NSCLC (A549, H358, H1299 and H322), prostate cancer (PC3 and DU145), and ovarian cancer (SKVO3) cell lines were cultured in RPMI medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Human pancreatic cancer cell lines (PANC1 and HPAFII) were cultured in DMEM medium supplemented with 10% FBS, 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. Immortalized human bronchial epithelial HBEC cells were cultured in KSFM medium supplemented with 50 μg/ml bovine pituitary extract and 5 ng/ml EGF. Human TGF-β was purchased from Peprotech (Rocky Hill, NJ). PKC inhibitors GF109203X and Gö6976 were purchased from TOCRIS (Bristol, UK). The ROCK inhibitor Y27632 was obtained from Calbiochem (Burlington, MA).

### RNA interference (RNAi)

ON-TARGET Plus small interfering RNAs (siRNAs) were purchased from Dharmacon (Lafayette, CO). For PKCs we used the following siRNAs: CCAUCCGCUCCACACUAAA (α1) and GAACAAGGAAUGACUU (β2) for PKCα; CCAUGAGUUUAUCGCCACC (δ1) and CAGCACAGAGCGUGGGAAA (δ2) for PKCδ; and J-004653-06-0002 (ε1), J-004653-07-0002 (ε2), J-004653-07-0002 (ε3) and J-004653-09-0002 (ε4) for PFKCε. For DOCK2, DOCK4, NGEF, RasGRF2, Tiam2, TRIO and Vav2 depletion we used ON-TARGET plus SMARTpool (Catalog# L-019915, L-017968, L-009354, L-024516, L-008434, L-00547 and L-005199, respectively). ON-TARGET Plus non-targeting pool (Catalog # D-001810) was used as a control. siRNAs were transfected with Lipofectamine RNAi/Max (Invitrogen-Life Technologies, Grand Island, NY), as previously described (81).

### TGF-β treatment

For EMT experiments, cells were treated with TGF-β (10 ng/ml). For most experiments, a 6-day treatment was used, and TGF-β was replaced every 2 days. In some cases, cells were subject to RNAi 3 days before the 6-day TGF-β treatment was completed. For Smad2/3 phosphorylation experiments, cells were treated with TGF-β (10 ng/ml) for 2 h.

### Transfections

pCDNA3-Flag or pCDNA3-PKCε-Flag (Addgene, Cambridge, MA) plasmids were transfected using Lipofectamine 2000, as recommended by the manufacturer (Thermo Fisher Scientific, Walthan, MA).

### Western blot analysis

Western blots were carried out as previously described (35), using the following antibodies: anti-Rac1 clone 23A8 (catalog # 05-389, Upstate Biotechnology, Lake Placid, NY), anti-phospho-Erk1/2 (catalog # 9101S), anti-PKCδ (catalog # 2058S), anti-PKCε (catalog # 2683S), anti-phosphoSmad2/3 (catalog # 8828S), anti-phospho-MYPT1 (catalog # 5163S), anti-vimentin (catalog # 3390S), anti-Cdc42 (catalog # 2466S), anti-RhoA (catalog # 2117S, Cell Signaling Technology, Beverly, MA), anti-PKCε (catalog # sc-208, Santa Cruz Biotechnology, Dallas, TX), anti-vinculin (catalog # V9131, Sigma-Aldrich, St. Louis, MO), anti-β-actin (catalog # A5441, BD Biosciences, Franklin Lakes, NJ), and anti E-cadherin (catalog # MAB1838, RD Systems, Minneapolis, MN).

### Quantitative real-time PCR (Q-PCR)

Total RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA). Reverse transcription of RNA was done using the Taqman reverse transcription reagent kit (Applied Biosystems, Branchburg, NJ). For the GEFs array, TaqMan Assays (primers and probes) 5′ end-labeled with 6-carboxyfluorescein (6-FAM) for 26 GEFs and 5 housekeeping genes (ACTB, B2M, HRPT1, TBP and UBC) for normalization were purchased from Applied Biosystems. Q-PCR amplifications were performed using an ABI PRISM 7300 Detection System in a total volume of 20 μl containing Taqman Universal PCR Master Mix (Applied Biosystems), commercial target primers (300 nM), fluorescent probe (200 nM), and 1 μl of cDNA. PCR product formation was continuously monitored using the Sequence Detection

System software version 1.7 (Applied Biosystems). The FAM signal was normalized to endogenous UBC (housekeeping gene).

### Pull-down assays

Rac1-GTP and Cdc42-GTP levels were determined with a pull-down assay using the p21-binding domain (PBD) of Pak1, as described previously (35). To determine RhoA-GTP levels, rhotekin beads bound to agarose (Millipore, Burlington, MA) were used in the pull-down assay, and detection of RhoA-GTP was carried out by Western blot using an anti-RhoA antibody (1:1000 dilution).

### Cytoskeleton morphology assays and migration

Assessment of ruffles was done after phalloidin staining, as previously described (42). Briefly, cells were serum starved for 24 h and stimulated for 30 min with either 100 nM PMA or 1 μM AJH-836, as described before (10, 19). Following fixation with 4% formaldehyde, cells were was stained with phalloidin-rhodamine, and cell nuclei were counterstained with DAPI. Slides were visualized by fluorescence microscopy.

For immunofluorescence, cells growing on glass coverslides at low confluence were fixed with 4% formaldehyde, stained with cortactin, E-cadherin or Vimentin antibodies, and nuclei counterstained with DAPI. Slides were visualized by confocal microscopy, using a Zeiss LSM 710 microscope. Immunofluorescence experiments were carried out using the following antibodies: rhodamine-phalloidine (catalog # R415, Thermo Fisher Scientific, Waltham, MA), cortactin (catalog # 3503S, Cell Signaling Technology), E-cadherin (catalog # MAB1838, RD Systems MN) and vimentin (catalog # NB300-223, Novus biological, Centennial, CO). As secondary antibodies we used Alexa 488 (catalog # A11001), Alexa 555 (catalog # A21428), and Alexa 549 (catalog # A-11042) (Thermo Fisher Scientific). For quantification of ruffles, five random fields were selected. Ruffle area was measured by thresholding for signal intensity using ImageJ.

For migration analysis, we used a Boyden chamber assay with 12 μM pore diameter polycarbonate membranes. The lower chamber contained RPMI medium with 10% FBS. Migration was assessed for 16 h at 37°C, and migratory cells in each well were counted by contrast microscopy in 5 independent fields.

### Statistical analysis

Sample sizes for cellular studies for each experimental condition were three to five in most cases, based on established reproducibility of the assays, and this is indicated in figure legends. Experiments were replicated at least three times. Statistical analysis was done with *t*-test or ANOVA using GraphPad Prism 3.0. In all cases, a p-value $<0.05$ was considered statistically significant.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

This work was supported by grant R01-ES026023 and R01-ES026023-S1 from the National Institutes of Health to M.G.K.

## REFERENCES

1. Griner EM, Kazanietz MG. Protein kinase C and other diacylglycerol effectors in cancer. Nature reviews Cancer. 2007;7(4):281–94. [PubMed: 17384583]
2. Isakov N Protein kinase C (PKC) isoforms in cancer, tumor promotion and tumor suppression. Seminars in cancer biology. 2018;48:36–52. [PubMed: 28571764]
3. Wu-Zhang AX, Newton AC. Protein kinase C pharmacology: refining the toolbox. The Biochemical journal. 2013 ;452(2): 195–209. [PubMed: 23662807]
4. Garg R, Benedetti LG, Abera MB, Wang H, Abba M, Kazanietz MG. Protein kinase C and cancer: what we know and what we do not. Oncogene. 2014;33(45):5225–37. [PubMed: 24336328]
5. Pan Q, Bao LW, Kleer CG, Sabel MS, Griffith KA, Teknos TN, et al. Protein kinase C epsilon is a predictive biomarker of aggressive breast cancer and a validated target for RNA interference anticancer therapy. Cancer research. 2005;65(18):8366–71. [PubMed: 16166314]
6. Garg R, Blando JM, Perez CJ, Abba MC, Benavides F, Kazanietz MG. Protein Kinase C Epsilon Cooperates with PTEN Loss for Prostate Tumorigenesis through the CXCL13-CXCR5 Pathway. Cell reports. 2017;19(2):375–88. [PubMed: 28402859]
7. Bae KM, Wang H, Jiang G, Chen MG, Lu L, Xiao L. Protein kinase C epsilon is overexpressed in primary human non-small cell lung cancers and functionally required for proliferation of non-small cell lung cancer cells in a p21/Cip1-dependent manner. Cancer research. 2007;67(13):6053–63. [PubMed: 17616661]
8. Gutierrez-Uzquiza A, Lopez-Haber C, Jernigan DL, Fatatis A, Kazanietz MG. PKCepsilon Is an Essential Mediator of Prostate Cancer Bone Metastasis. Molecular cancer research : MCR. 2015;13(9):1336–46. [PubMed: 26023164]
9. Gorin MA, Pan Q. Protein kinase C epsilon: an oncogene and emerging tumor biomarker. Molecular cancer. 2009;8:9. [PubMed: 19228372]
10. Caino MC, Lopez-Haber C, Kissil JL, Kazanietz MG. Non-small cell lung carcinoma cell motility, rac activation and metastatic dissemination are mediated by protein kinase C epsilon. PloS one. 2012;7(2):e31714. [PubMed: 22384062]
11. Hafeez BB, Zhong W, Weichert J, Dreckschmidt NE, Jamal MS, Verma AK. Genetic ablation of PKC epsilon inhibits prostate cancer development and metastasis in transgenic mouse model of prostate adenocarcinoma. Cancer research. 2011;71(6):2318–27. [PubMed: 21406403]
12. Benavides F, Blando J, Perez CJ, Garg R, Conti CJ, DiGiovanni J, et al. Transgenic overexpression of PKCepsilon in the mouse prostate induces preneoplastic lesions. Cell cycle (Georgetown, Tex). 2011;10(2):268–77.
13. Garg R, Blando JM, Perez CJ, Lal P, Feldman MD, Smyth EM, et al. COX-2 mediates pro-tumorigenic effects of PKCepsilon in prostate cancer. Oncogene. 2018.
14. Caino MC, Lopez-Haber C, Kim J, Mochly-Rosen D, Kazanietz MG. Proteins kinase Cvarepsilon is required for non-small cell lung carcinoma growth and regulates the expression of apoptotic genes. Oncogene. 2012;31(20):2593–600. [PubMed: 21996750]
15. Aziz MH, Manoharan HT, Church DR, Dreckschmidt NE, Zhong W, Oberley TD, et al. Protein kinase Cepsilon interacts with signal transducers and activators of transcription 3 (Stat3), phosphorylates Stat3Ser727, and regulates its constitutive activation in prostate cancer. Cancer research. 2007;67(18):8828–38. [PubMed: 17875724]
16. Garg R, Blando J, Perez CJ, Wang H, Benavides FJ, Kazanietz MG. Activation of nuclear factor kappaB (NF-kappaB) in prostate cancer is mediated by protein kinase C epsilon (PKCepsilon). The Journal of biological chemistry. 2012;287(44):37570–82. [PubMed: 22955280]
17. Basu A, Sivaprasad U. Protein kinase Cepsilon makes the life and death decision. Cellular signalling. 2007;19(8): 1633–42. [PubMed: 17537614]

18. Hafeez BB, Fischer JW, Singh A, Zhong W, Mustafa A, Meske L, et al. Plumbagin Inhibits Prostate Carcinogenesis in Intact and Castrated PTEN Knockout Mice via Targeting PKCepsilon, Stat3, and Epithelial-to-Mesenchymal Transition Markers. Cancer prevention research (Philadelphia, Pa). 2015;8(5):375–86.
19. Cooke M, Zhou X, Casado-Medrano V, Lopez-Haber C, Baker MJ, Garg R, et al. Characterization of AJH-836, a DAG-lactone with selectivity for novel PKC isozymes. The Journal of biological chemistry. 2018.
20. Gandellini P, Folini M, Longoni N, Pennati M, Binda M, Colecchia M, et al. miR-205 Exerts tumor-suppressive functions in human prostate through down-regulation of protein kinase Cepsilon. Cancer research. 2009;69(6):2287–95. [PubMed: 19244118]
21. Jain K, Basu A. The Multifunctional Protein Kinase C-epsilon in Cancer Development and Progression. Cancers. 2014;6(2):860–78. [PubMed: 24727247]
22. Wang LY, Diao ZL, Zheng JF, Wu YR, Zhang QD, Liu WH. Apelin attenuates TGF-beta1-induced epithelial to mesenchymal transition via activation of PKC-epsilon in human renal tubular epithelial cells. Peptides. 2017;96:44–52. [PubMed: 28847490]
23. Jain K, Basu A. Protein Kinase C-epsilon Promotes EMT in Breast Cancer. Breast cancer : basic and clinical research. 2014;8:61–7. [PubMed: 24701121]
24. Kang Y, Massague J. Epithelial-mesenchymal transitions: twist in development and metastasis. Cell. 2004;118(3):277–9. [PubMed: 15294153]
25. Gonzalez DM, Medici D. Signaling mechanisms of the epithelial-mesenchymal transition. Science signaling. 2014;7(344):re8. [PubMed: 25249658]
26. Pang MF, Georgoudaki AM, Lambut L, Johansson J, Tabor V, Hagikura K, et al. TGF-beta1-induced EMT promotes targeted migration of breast cancer cells through the lymphatic system by the activation of CCR7/CCL21-mediated chemotaxis. Oncogene. 2016;35(6):748–60. [PubMed: 25961925]
27. Brown KA, Aakre ME, Gorska AE, Price JO, Eltom SE, Pietenpol JA, et al. Induction by transforming growth factor-beta1 of epithelial to mesenchymal transition is a rare event in vitro. Breast cancer research : BCR. 2004;6(3):R215–31. [PubMed: 15084245]
28. David CJ, Huang YH, Chen M, Su J, Zou Y, Bardeesy N, et al. TGF-beta Tumor Suppression through a Lethal EMT. Cell. 2016;164(5):1015–30. [PubMed: 26898331]
29. Symonds JM, Ohm AM, Carter CJ, Heasley LE, Boyle TA, Franklin WA, et al. Protein kinase C delta is a downstream effector of oncogenic K-ras in lung tumors. Cancer research. 2011;71(6): 2087–97. [PubMed: 21335545]
30. Hill KS, Erdogan E, Khoor A, Walsh MP, Leitges M, Murray NR, et al. Protein kinase Calpha suppresses Kras-mediated lung tumor formation through activation of a p38 MAPK-TGFbeta signaling axis. Oncogene. 2014;33(16):2134–44. [PubMed: 23604119]
31. Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, et al. Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. The Journal of biological chemistry. 1993;268(13):9194–7. [PubMed: 8486620]
32. Ong ST, Freeley M, Skubis-Zegadlo J, Fazil MH, Kelleher D, Fresser F, et al. Phosphorylation of Rab5a protein by protein kinase C is crucial for T-cell migration. The Journal of biological chemistry. 2014;289(28): 19420–34. [PubMed: 24872409]
33. Leask A, Shi-Wen X, Khan K, Chen Y, Holmes A, Eastwood M, et al. Loss of protein kinase Cepsilon results in impaired cutaneous wound closure and myofibroblast function. Journal of cell science. 2008;121(Pt 20):3459–67. [PubMed: 18827016]
34. Gorshkova I, He D, Berdyshev E, Usatuyk P, Burns M, Kalari S, et al. Protein kinase C-epsilon regulates sphingosine 1-phosphate-mediated migration of human lung endothelial cells through activation of phospholipase D2, protein kinase C-zeta, and Rac1. The Journal of biological chemistry. 2008;283(17): 11794–806. [PubMed: 18296444]
35. Lopez-Haber C, Barrio-Real L, Casado-Medrano V, Kazanietz MG. Heregulin/ErbB3 Signaling Enhances CXCR4-Driven Rac1 Activation and Breast Cancer Cell Motility via Hypoxia-Inducible Factor 1alpha. Molecular and cellular biology. 2016;36(15):2011–26. [PubMed: 27185877]
36. Hall A Rho GTPases and the control of cell behaviour. Biochemical Society transactions. 2005;33(Pt 5):891–5. [PubMed: 16246005]

37. Ridley AJ, Hall A. The small GTP-binding protein rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell. 1992;70(3):389–99. [PubMed: 1643657]
38. Guilluy C, Dubash AD, Garcia-Mata R. Analysis of RhoA and Rho GEF activity in whole cells and the cell nucleus. Nature protocols. 2011;6(12):2050–60. [PubMed: 22134128]
39. Rossman KL, Der CJ, Sondek J. GEF means go: turning on RHO GTPases with guanine nucleotide-exchange factors. Nature reviews Molecular cell biology. 2005;6(2): 167–80. [PubMed: 15688002]
40. Garcia-Mata R, Burridge K. Catching a GEF by its tail. Trends in cell biology. 2007;17(1):36–43. [PubMed: 17126549]
41. Bustelo XR. RHO GTPases in cancer: known facts, open questions, and therapeutic challenges. Biochemical Society transactions. 2018;46(3):741–60. [PubMed: 29871878]
42. Sosa MS, Lopez-Haber C, Yang C, Wang H, Lemmon MA, Busillo JM, et al. Identification of the Rac-GEF P-Rex1 as an essential mediator of ErbB signaling in breast cancer. Molecular cell. 2010;40(6):877–92. [PubMed: 21172654]
43. Cook DR, Solski PA, Bultman SJ, Kauselmann G, Schoor M, Kuehn R, et al. The ect2 rho Guanine nucleotide exchange factor is essential for early mouse development and normal cell cytokinesis and migration. Genes & cancer. 2011;2(10):932–42. [PubMed: 22701760]
44. Mochly-Rosen D, Das K, Grimes KV. Protein kinase C, an elusive therapeutic target? Nature reviews Drug discovery. 2012; 11(12):937–57. [PubMed: 23197040]
45. Newton AC, Brognard J. Reversing the Paradigm: Protein Kinase C as a Tumor Suppressor. Trends in pharmacological sciences. 2017;38(5):438–47. [PubMed: 28283201]
46. Pan Q, Bao LW, Teknos TN, Merajver SD. Targeted disruption of protein kinase C epsilon reduces cell invasion and motility through inactivation of RhoA and RhoC GTPases in head and neck squamous cell carcinoma. Cancer research. 2006;66(19):9379–84. [PubMed: 17018591]
47. Gobbi G, Masselli E, Micheloni C, Nouvenne A, Russo D, Santi P, et al. Hypoxia-induced down-modulation of PKCepsilon promotes trail-mediated apoptosis of tumor cells. International journal of oncology. 2010;37(3):719–29. [PubMed: 20664941]
48. Meshki J, Caino MC, von Burstin VA, Griner E, Kazanietz MG. Regulation of prostate cancer cell survival by protein kinase Cepsilon involves bad phosphorylation and modulation of the TNFalpha/JNK pathway. The Journal of biological chemistry. 2010;285(34):26033–40. [PubMed: 20566643]
49. Pardo OE, Wellbrock C, Khanzada UK, Aubert M, Arozarena I, Davidson S, et al. FGF-2 protects small cell lung cancer cells from apoptosis through a complex involving PKCepsilon, B-Raf and S6K2. The EMBO journal. 2006;25(13):3078–88. [PubMed: 16810323]
50. Shankar E, Sivaprasad U, Basu A. Protein kinase C epsilon confers resistance of MCF-7 cells to TRAIL by Akt-dependent activation of Hdm2 and downregulation of p53. Oncogene. 2008;27(28): 3957–66. [PubMed: 18317451]
51. Bassini A, Zauli G, Migliaccio G, Migliaccio AR, Pascuccio M, Pierpaoli S, et al. Lineage-restricted expression of protein kinase C isoforms in hematopoiesis. Blood. 1999;93(4):1178–88. [PubMed: 9949160]
52. Gaboardi GC, Ramazzotti G, Bavelloni A, Piazzi M, Fiume R, Billi AM, et al. A role for PKCepsilon during C2C12 myogenic differentiation. Cellular signalling. 2010;22(4):629–35. [PubMed: 19954762]
53. Gobbi G, Mirandola P, Carubbi C, Masselli E, Sykes SM, Ferraro F, et al. Proplatelet generation in the mouse requires PKCepsilon-dependent RhoA inhibition. Blood. 2013; 122(7): 1305–11. [PubMed: 23838351]
54. Di Marcantonio D, Galli D, Carubbi C, Gobbi G, Queirolo V, Martini S, et al. PKCepsilon as a novel promoter of skeletal muscle differentiation and regeneration. Experimental cell research. 2015;339(1): 10–9. [PubMed: 26431586]
55. Gobbi G, Mirandola P, Sponzilli I, Micheloni C, Malinverno C, Cocco L, et al. Timing and expression level of protein kinase C epsilon regulate the megakaryocytic differentiation of human CD34 cells. Stem cells (Dayton, Ohio). 2007;25(9):2322–9.
56. Gobbi G, Di Marcantonio D, Micheloni C, Carubbi C, Galli D, Vaccarezza M, et al. TRAIL up-regulation must be accompanied by a reciprocal PKCepsilon down-regulation during

differentiation of colonic epithelial cell: implications for colorectal cancer cell differentiation. Journal of cellular physiology. 2012;227(2):630–8. [PubMed: 21465464]

57. Galli D, Carubbi C, Masselli E, Corradi D, Dei Cas A, Nouvenne A, et al. PKCepsilon is a negative regulator of PVAT-derived vessel formation. Experimental cell research. 2015;330(2): 277–86. [PubMed: 25433270]
58. Leontieva OV, Black JD. Identification of two distinct pathways of protein kinase Calpha down-regulation in intestinal epithelial cells. The Journal of biological chemistry. 2004;279(7): 5788–801. [PubMed: 14638691]
59. Wang H, Gutierrez-Uzquiza A, Garg R, Barrio-Real L, Abera MB, Lopez-Haber C, et al. Transcriptional regulation of oncogenic protein kinase C (PKC) by STAT1 and Sp1 proteins. The Journal of biological chemistry. 2014;289(28): 19823–38. [PubMed: 24825907]
60. Zhang H, Meyer KD, Zhang L. Fetal exposure to cocaine causes programming of Prkce gene repression in the left ventricle of adult rat offspring. Biology of reproduction. 2009;80(3):440–8. [PubMed: 18945988]
61. Zhang X, Li D, Li M, Ye M, Ding L, Cai H, et al. MicroRNA-146a targets PRKCE to modulate papillary thyroid tumor development. International journal of cancer. 2014;134(2):257–67. [PubMed: 23457043]
62. Wang Y, Men M, Yang W, Zheng H, Xue S. MiR-31 Downregulation Protects Against Cardiac Ischemia/Reperfusion Injury by Targeting Protein Kinase C Epsilon (PKCepsilon) Directly. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology. 2015;36(1): 179–90.
63. Zhao W, Wang P, Ma J, Liu YH, Li Z, Li ZQ, et al. MiR-34a regulates blood-tumor barrier function by targeting protein kinase Cepsilon. Molecular biology of the cell. 2015;26(10): 1786–96. [PubMed: 25788289]
64. Garofalo M, Croce CM. microRNAs: Master regulators as potential therapeutics in cancer. Annual review of pharmacology and toxicology. 2011;51:25–43.
65. Gao S, Alarcon C, Sapkota G, Rahman S, Chen PY, Goerner N, et al. Ubiquitin ligase Nedd4L targets activated Smad2/3 to limit TGF-beta signaling. Molecular cell. 2009;36(3):457–68. [PubMed: 19917253]
66. Iyengar PV. Regulation of Ubiquitin Enzymes in the TGF-beta Pathway. International journal of molecular sciences. 2017; 18(4).
67. Sun X, Xie Z, Ma Y, Pan X, Wang J, Chen Z, et al. TGF-beta inhibits osteogenesis by upregulating the expression of ubiquitin ligase SMURF1 via MAPK-ERK signaling. Journal of cellular physiology. 2018;233(1):596–606. [PubMed: 28322449]
68. Slupsky JR, Kamiguti AS, Harris RJ, Cawley JC, Zuzel M. Central role of protein kinase Cepsilon in constitutive activation of ERK1/2 and Rac1 in the malignant cells of hairy cell leukemia. The American journal of pathology. 2007;170(2):745–54. [PubMed: 17255340]
69. Fleming IN, Elliott CM, Collard JG, Exton JH. Lysophosphatidic acid induces threonine phosphorylation of Tiam1 in Swiss 3T3 fibroblasts via activation of protein kinase C. The Journal of biological chemistry. 1997;272(52):33105–10. [PubMed: 9407095]
70. Montero JC, Seoane S, Garcia-Alonso S, Pandiella A. Multisite phosphorylation of P-Rex1 by protein kinase C. Oncotarget. 2016;7(47):77937–49. [PubMed: 27788493]
71. Levay M, Settleman J, Ligeti E. Regulation of the substrate preference of p190RhoGAP by protein kinase C-mediated phosphorylation of a phospholipid binding site. Biochemistry. 2009;48(36): 8615–23. [PubMed: 19673492]
72. Griner EM, Caino MC, Sosa MS, Colon-Gonzalez F, Chalmers MJ, Mischak H, et al. A novel cross-talk in diacylglycerol signaling: the Rac-GAP beta2-chimaerin is negatively regulated by protein kinase Cdelta-mediated phosphorylation. The Journal of biological chemistry. 2010;285(22): 16931–41. [PubMed: 20335173]
73. Zeidman R, Troller U, Raghunath A, Pahlman S, Larsson C. Protein kinase Cepsilon actin-binding site is important for neurite outgrowth during neuronal differentiation. Molecular biology of the cell. 2002; 13(1): 12–24. [PubMed: 11809819]
74. Prekeris R, Mayhew MW, Cooper JB, Terrian DM. Identification and localization of an actin-binding motif that is unique to the epsilon isoform of protein kinase C and participates in the

regulation of synaptic function. The Journal of cell biology. 1996;132(1-2):77–90. [PubMed: 8567732]

75. Yu JR, Tai Y, Jin Y, Hammell MC, Wilkinson JE, Roe JS, et al. TGF-beta/Smad signaling through DOCK4 facilitates lung adenocarcinoma metastasis. Genes & development. 2015;29(3):250–61. [PubMed: 25644601]
76. Ungefroren H, Witte D, Lehnert H. The role of small GTPases of the Rho/Rac family in TGF-beta-induced EMT and cell motility in cancer. Developmental dynamics : an official publication of the American Association of Anatomists. 2018;247(3):451–61. [PubMed: 28390160]
77. Bhowmick NA, Ghiassi M, Bakin A, Aakre M, Lundquist CA, Engel ME, et al. Transforming growth factor-beta1 mediates epithelial to mesenchymal transdifferentiation through a RhoA-dependent mechanism. Molecular biology of the cell. 2001; 12(1):27–36. [PubMed: 11160820]
78. Ungefroren H, Gieseler F, Kaufmann R, Settmacher U, Lehnert H, Rauch BH. Signaling Crosstalk of TGF-beta/ALK5 and PAR2/PAR1: A Complex Regulatory Network Controlling Fibrosis and Cancer. International journal of molecular sciences. 2018; 19(6).
79. Chang MT, Asthana S, Gao SP, Lee BH, Chapman JS, Kandoth C, et al. Identifying recurrent mutations in cancer reveals widespread lineage diversity and mutational specificity. Nature biotechnology. 2016;34(2): 155–63.
80. Burgos M, Pastor MD, Gonzalez JC, Martinez-Galan JR, Vaquero CF, Fradejas N, et al. PKCepsilon upregulates voltage-dependent calcium channels in cultured astrocytes. Glia. 2007;55(14): 1437–48. [PubMed: 17676593]
81. Barrio-Real L, Lopez-Haber C, Casado-Medrano V, Goglia AG, Toettcher JE, Caloca MJ, et al. P-Rex1 is dispensable for Erk activation and mitogenesis in breast cancer. Oncotarget. 2018;9(47): 28612–24. [PubMed: 29983884]

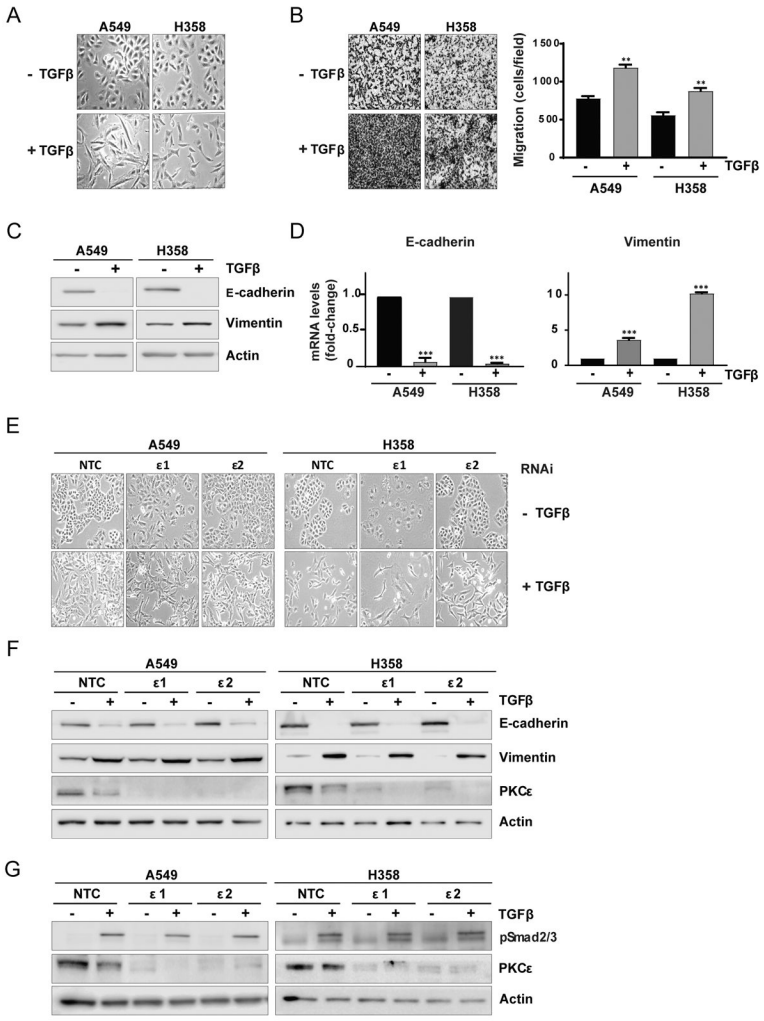


**Figure 1. PKCε is not required for EMT in A549 and H358 NSCLC cells.**

(A) Morphological changes in NSCLC cells treated with TGF-β (10 ng/ml, 6 days) (T6). Representative micrographs are shown. Magnification: 20x. (B) Enhanced motility of TGF-β-treated NSCLC cells, as determined using a Boyden chamber. Results are expressed as mean ± S.D. of 5 random fields. Similar results were observed in 2 additional experiments. **, p<0.01. (C) Expression of E-cadherin and vimentin in T6 *vs.* parental NSCLC cells, as determined by Western blot. (D) Expression of E-cadherin and vimentin in T6 *vs.* parental NSCLC cells, as determined by Q-PCR. Data represent the mean ± S.E.M. of 3 independent experiments. Results are expressed as fold-change relative to parental cells. ***, p<0.001 *vs.* non-treated cells. (E) Morphological changes in A549 and H358 cells subject to either PKCε (ε1 and ε2 duplexes) or non-target control (*NTC*) RNAi, after 6 days of treatment with TGF-β. Magnification: 20x. (F) Expression of E-cadherin and vimentin by Western blot in A549 and H358 cells (parental and T6) subject to either PKCε or NTC RNAi. (G) Changes in Smad2/3 phosphorylation upon TGF-β stimulation (10 ng/ml, 2 h) in NSCLC cells subject to either PKCε or NTC RNAi.

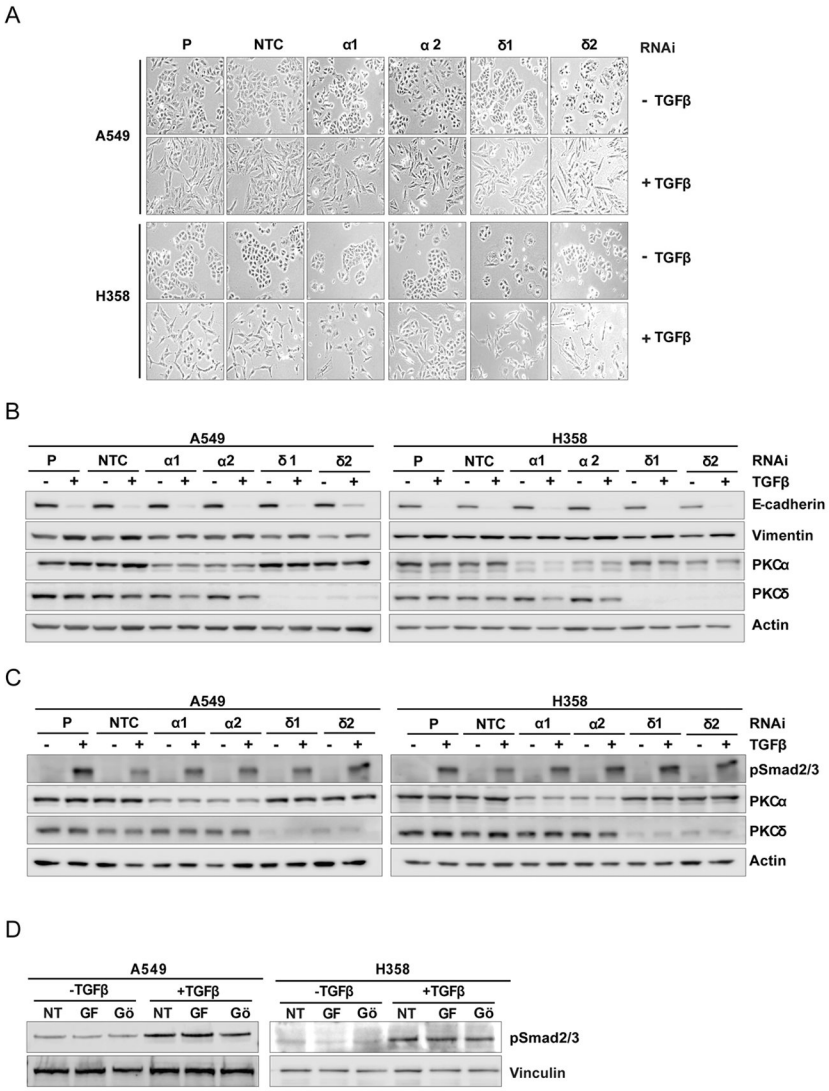


**Figure 2. PKCα and PKCδ are dispensable for TGF-β-induced EMT in NSCLC cells.**

(A) A549 and H358 cells were subject to either PKCα or PKCδ RNAi, using two different duplexes (α1 or α2, and δ1 or δ2, respectively), or NTC RNAi. Representative micrographs showing morphological changes in response to TGF-β (10 ng/ml, 6 days) are shown. Magnification: 20x. (B) Expression of E-cadherin and vimentin by Western blot in A549 and H358 cells (parental and T6) subject to PKCα, PKCδ, or NTC RNAi, after 6 days of treatment with TGF-β. (C) Changes in Smad2/3 phosphorylation upon TGF-β stimulation (10 ng/ml, 2 h) in A549 and H358 cells subject to PKCα, PKCδ, or NTC RNAi. (D) Effect of PKC inhibitors GF109203X (*GF*) and Gö6976 (*Gö*) (5 μM) on Smad2/3 phosphorylation by TGF-β (10 ng/ml, 2 h). Similar results were observed in two additional experiments.

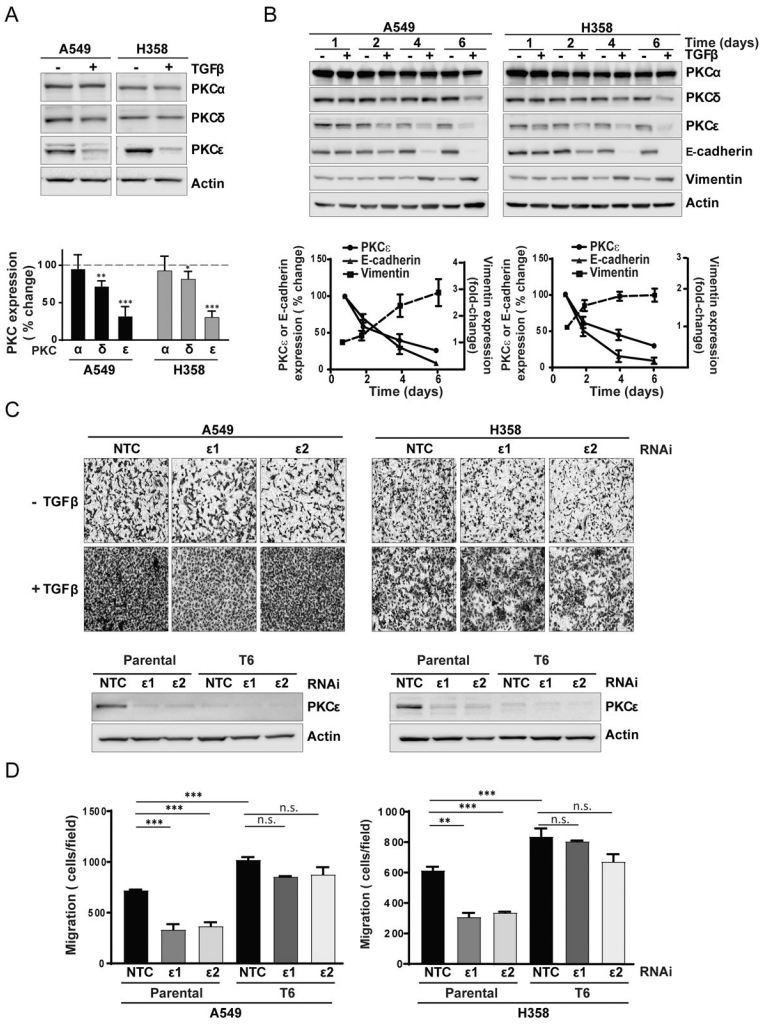


**Figure 3. PKCε is down-regulated during EMT in NSCLC cells.**

(A) Expression of PKC isozymes in A549 and H358 cells after TGF-β treatment (10 ng/ml, 6 days). *Upper panel*, representative Western blot. *Lower panel*, PKCε expression, as determined by Q-PCR. Results are expressed as mean ± S.E.M. of 3 independent experiments. (B) Time-course analysis of PKCα, PKCδ, and PKCε expression in response to TGF-β (10 ng/ml, 1-6 days). *Upper panel*, representative Western blots. *Lower panel*, densitometric analysis of PKCε down-regulation. The graph also shows the changes in expression of E-cadherin and vimentin. Results are expressed as mean ± S.E.M. of 3 independent experiments. (C) *Upper panel*, migration of parental and TGF-β-treated (T6) NSCLC cells subject to either PKCε (ε1 and ε2 duplexes) or NTC RNAi, as determined with a Boyden chamber. Representative images are shown. *Lower panel*, Western blot showing PKCε silencing in parental and TGF-β-treated cells. (D) Quantification of migration (cells/field) in each well. Data are expressed as mean ± S.D. of 5 random fields. Similar results were observed in an additional experiment. **, $p<0.01$; ***, $p<0.001$. *n.s.*, not significant.

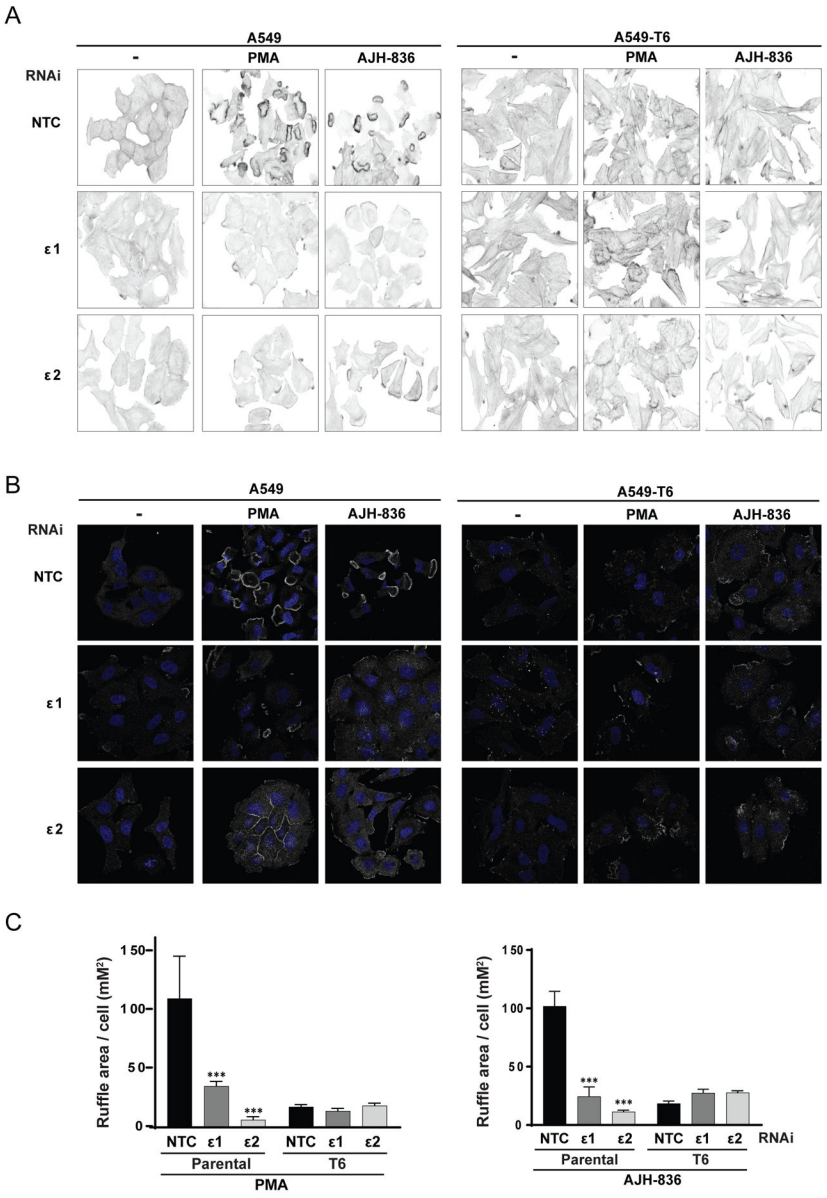


**Figure 4. PKCε is required for ruffle formation in epithelial A549 cells.**

(A) Parental A549 and T6-A549 cells were transfected with two different PKCε RNAi (ε1 or ε2) or NTC RNAi duplexes, serum starved, and treated for 30 min with PMA (100 nM) or the DAG-lactone AJH-836 (1 μM), fixed, and stained with phalloidin-rhodamine. Representative micrographs are shown. Magnification: 40x. (B) Similar experiments were carried out using cortactin staining. Magnification: 63x. (C) Quantitative analysis of cortactin staining. Magnification: 20x. Data are expressed as mean ± S.D. of 5 random fields. Similar results were observed in an additional experiment. ***, $p<0.001$.

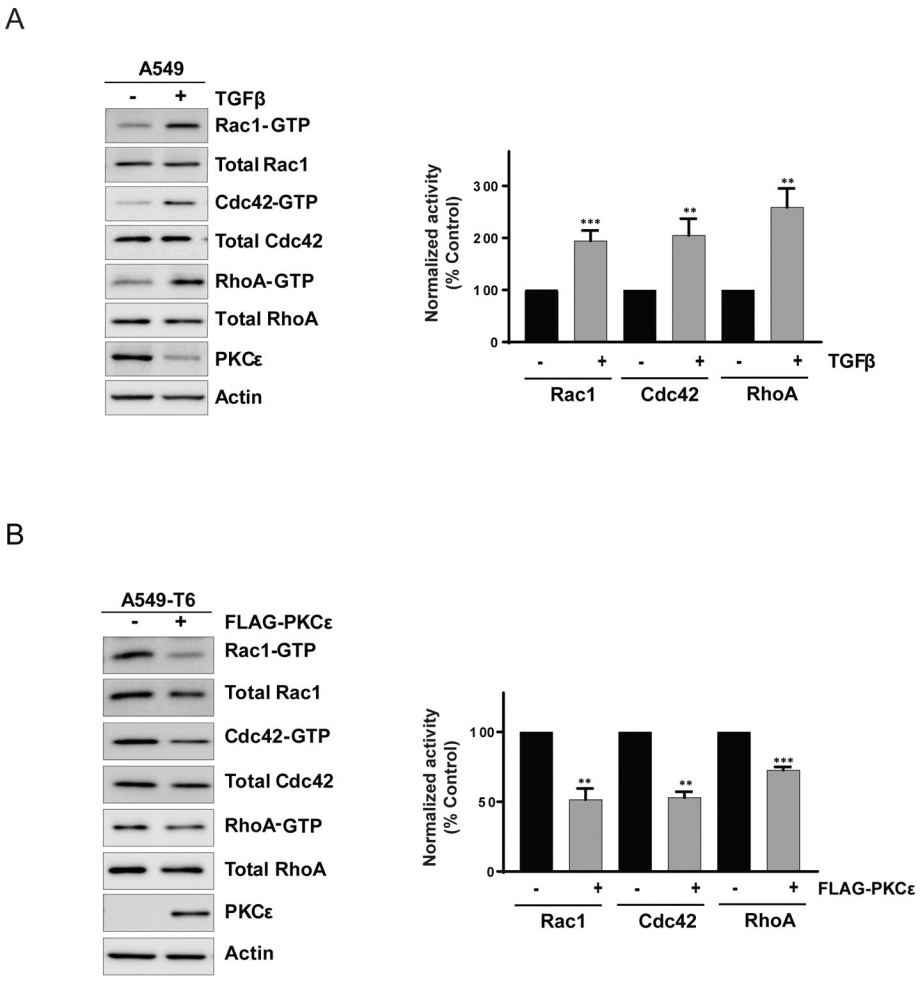


**Figure 5. TGF-β-treated cells display elevated Rac1, Cdc42, and RhoA activities.**

A549 cells were treated with TGF-β (10 ng/ml, 6 days) and the levels of active Rac1, Cdc42, and RhoA were then determined in cell extracts. For Rac1-GTP and Cdc42-GTP, a PBD "pull-down" assay was used. For RhoA-GTP, we used a rhotekin RBD "pull-down" assay. *Left*, representative experiments. *Right*, quantification of Rac1-GTP, Cdc42-GTP, and RhoA-GTP levels, normalized to the corresponding total levels of the GTPases, and expressed as % relative to parental cells not treated with TGF-β. Data expressed as mean ± S.E.M. (n=3). **, p<0.01; ***, p<0.001 *vs.* parental cells. (C) TGF-β-treated (T6) A549 cells were transfected with a FLAG-tagged PKCε expression vector, and 30 h later active levels of Rac1, Cdc42 and RhoA were determined using a "pull-down" assay. *Left*, representative experiments. *Right*, quantification of Rac1-GTP, Cdc42-GTP, and RhoA-GTP levels, normalized to the corresponding total levels of the GTPases, and expressed as % relative to cells transfected with empty vector. Data are expressed as mean ± S.E.M. (n=3). **, p<0.01; ***, p<0.001 *vs.* cells transfected with empty vector.

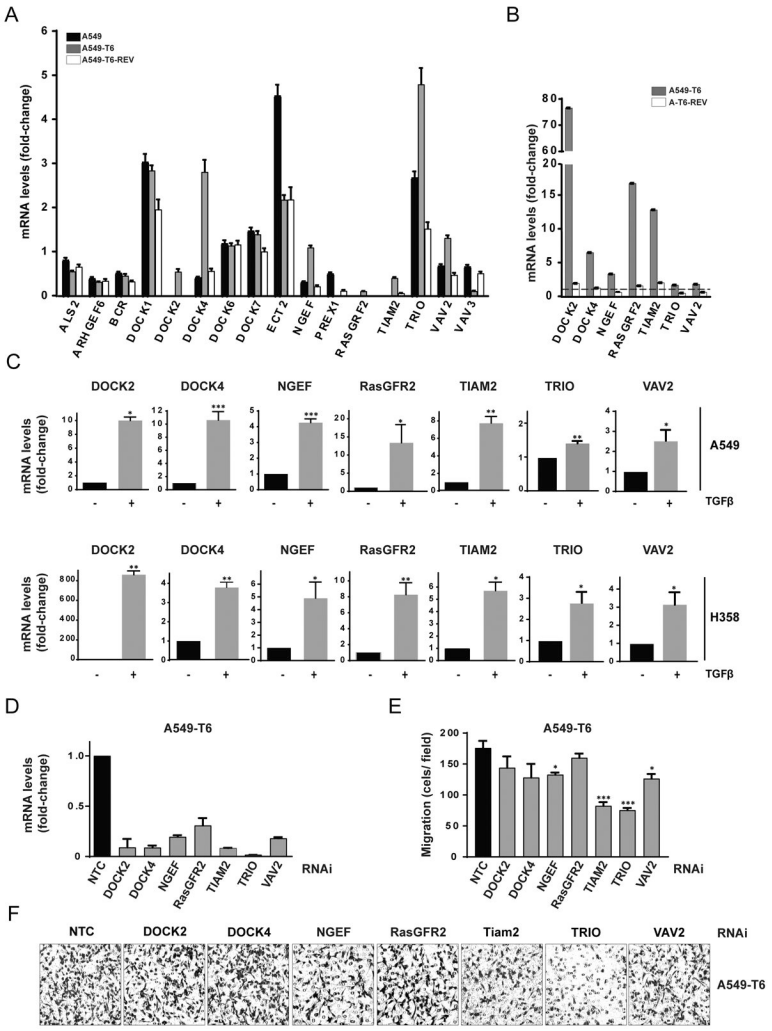


**Figure 6. Differential expression of Rac-GEFs in epithelial *vs*. mesenchymal NSCLC cells.**

(A) mRNA was obtained from parental A549 cells, cells treated with TGF-β (10 ng/ml) for 6 days (A549-T6 cells), and T6 cells subsequently cultured in the absence of TGF-β ("reverted") A549-T6-REV cells. cDNA was prepared by reverse transcription and used to assess the expression of 26 Rac-GEFs and 5 housekeeping genes (ACTB, B2M, HRPT1, TBP and UBC) using Q-PCR. Results represent the fold-change ($2^{-\Delta\Delta Ct}$) in mRNA expression levels for each Rac-GEF according to its respective treatment. Ct values were normalized to the expression of the 5 housekeeping genes (−ΔCt), and then compared with the average expression of the 26 GEFs in the 3 treatments (ΔΔCt). For those Rac-GEFs with Ct>35 in parental A549 cells, expression was considered as undetectable. (B) Representation of Rac-GEFs regulated by TGF-β treatment. Results are expressed as fold-change ($2^{-\Delta\Delta Ct}$) relative to parental A549 cells. (C) Validation Q-PCR assays for selected Rac-GEFs in A549 (*upper panels*) and H358 cells (*lower panels*) subject to TGF-β treatment. Results are expressed as fold-change relative to parental cells. Data expressed as mean ± S.E.M. (n=3). *, p<0.05, **, p<0.01; ***, p<0.001 *vs*. parental cells. (D) A549-T6 cells were transfected with RNAi (after 6 days of TGF-β treatment) for selected Rac-GEFs or NTC RNAi, as indicated. Depletion of Rac-GEFs, expressed as relative to NTC, is shown, and expressed as

mean ± S.E.M. (n=3). (E) Migration of A549-T6 cells subject to RNAi for selected Rac-GEFs or NTC, as determined with a Boyden chamber. Results are expressed as mean ± S.D. of 5 random fields. Similar results were observed in 2 additional experiments. (F) Representative images of migration experiments. *, $p<0.05$; ***, $p<0.001$ *vs.* NTC.

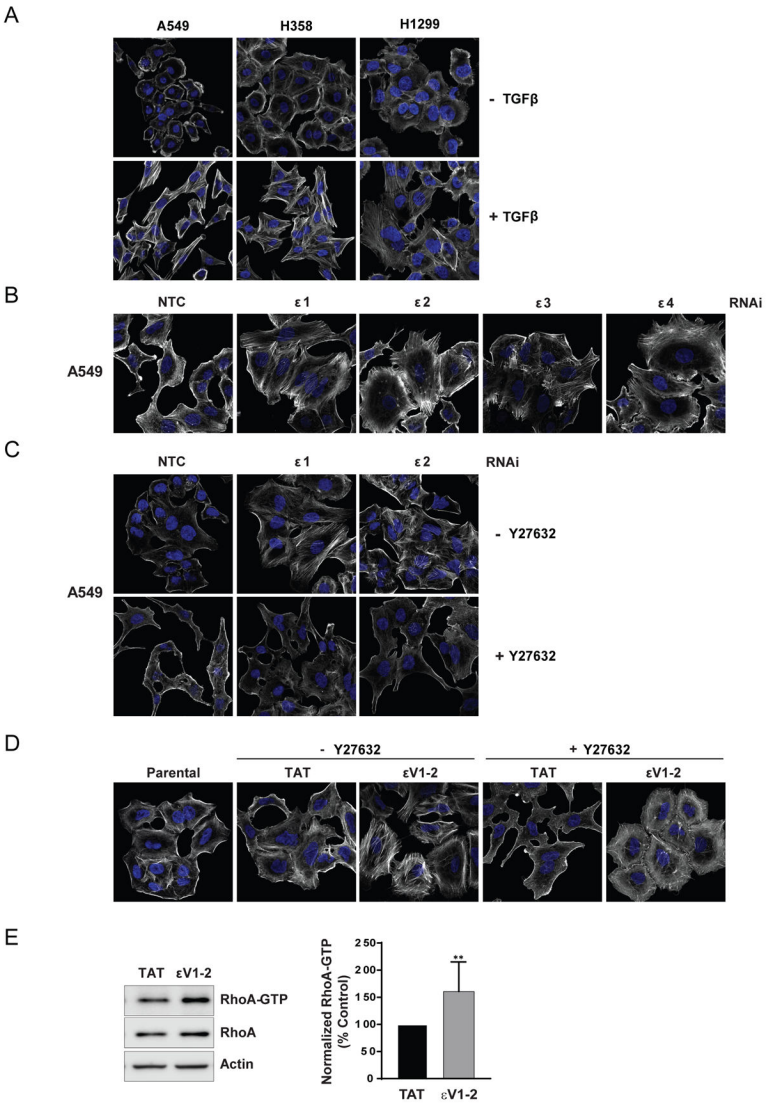


**Figure 7. PKCε negatively regulates RhoA-dependent stress fiber formation.**

(A) NSCLC cells (A549, H358 and H1299) cells were treated with TGF-β (10 ng/ml, 6 days), fixed, stained with phalloidin-rhodamine, and visualized by confocal microscopy. Magnification: 63x. (B) Stress fiber formation was analyzed by confocal microscopy in A549 cells 48 h after transfection of different PKCε RNAi duplexes (ε1, ε2, ε3 or ε4) or a non-target-control (*NTC*) RNAi duplex. (C) Confocal microscopy analysis of A549 cells subject to either PKCε RNAi (ε1 or ε2) or NTC RNAi, serum starved for 24 h, and then treated with the ROCK inhibitor Y27632 (10 μM, 24 h). (D) Confocal microscopy analysis of serum-starved (24 h) A549 cells treated with Y27632 (10 μM) for 30 min, followed by treatment with either εV1-2 (10 μM) or the TAT control peptide for 1 h. (E) RhoA-GTP levels were determined after 30min treatment with either εV1-2 or TAT. *Left*, representative experiment. *Right*, quantification of RhoA-GTP levels, normalized to total RhoA, and expressed as % relative to parental cells. Data expressed as mean ± S.E.M. (n=4). **, $p<0.01$ *vs.* parental cells.

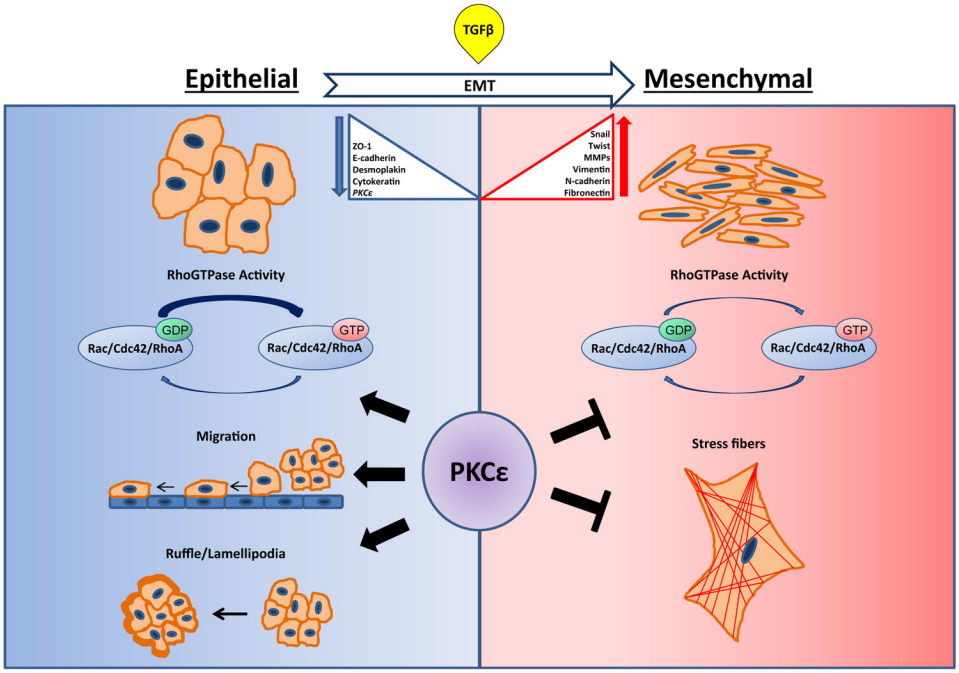


**Figure 8. Model depicting the proposed mechanism of PKCε-mediated regulation of EMT in NSCLC.**

Distinctive involvement of PKCε in the control of the actin cytoskeleton in epithelial or mesenchymal NSCLC cells.