Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

PROTEIN KINASE C ALPHA MEDIATES ERLOTINIB RESISTANCE IN LUNG CANCER CELLS

Mahlet B. Abera and Marcelo G. Kazanietz

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

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: PKCα, EMT and erlotinib resistance in lung cancer.

Corresponding author: 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.

Number of pages:

35 text pages

7 figures

60 references

224 words in the Abstract

690 words in the Introduction

852 words in the Discussion

Nonstandard Abbreviation: BSA: bovine serum albumin; DAG: diacylgycerol ; EGFR-epidermal growth factor receptor; EMT: epithelial-to-mesenchymal transition; NSCLC: non-small cell lung cancer; NTC: non-target control; PBS: phosphate-buffered saline; PAGE: polyacrylamide gel electrophoresis; PKC: Protein kinase C; RNAi: RNA interference; TGF-β: transforming growth factor beta; TKI: tyrosine-kinase inhibitor; qPCR: quantitative polymerase chain reaction.

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

Overexpression and mutational activation of the epidermal growth factor receptor (EGFR) plays an important role in the pathogenesis of non-small cell lung cancer (NSCLC). EGFR tyrosine-kinase inhibitors (TKIs) are given as a primary therapy for advanced patients with EGFR activating mutations; however, the majority of these tumors relapse and patients eventually develop resistance to TKIs. To address a potential role of PKC isozymes in the resistance to TKIs, we used the isogenic NSCLC H1650 cell line and its erlotinib-resistant derivative H1650-M3, a cell line that displays a mesenchymal-like morphology driven by TGF-β signaling (PNAS 107:15535-15540, 2010). We found that H1650-M3 cells display remarkable PKCα up-regulation and PKCδ down-regulation. Notably, silencing PKCα from H1650-M3 cells using RNAi caused a significant reduction in the expression of EMT markers vimentin, Zeb2, Snail and Twist. Moreover, pharmacological inhibition or PKCα RNAi depletion and PKCδ restoring sensitized H1650-M3 cells to erlotinib. Whereas ectopic overexpression of PKCα in parental H1650 cells was not sufficient to alter the expression of EMT genes or to confer resistance to erlotinib, it caused down-regulation of PKCδ expression, suggesting a unidirectional cross-talk. Finally, mechanistic studies revealed that PKCα up-regulation in H1650-M3 cells is driven by TGF-β. Our results identified important roles for specific PKC isozymes in erlotinib resistance and EMT in lung cancer cells, and highlight PKCα as a potential target for lung cancer treatment.

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Lung cancer remains one of the major causes of mortality worldwide, accounting for more deaths than any other cancer (Ferlay et al., 2015; Kanne, 2014). Diagnosis of lung cancer normally occurs in late stages of the disease, thus limiting the options for treatment. The most common type of lung cancer (~85 %) is non-small cell lung cancer (NSCLC), which has three main types: squamous cell carcinoma, adenocarcinoma, and large cell carcinoma (Molina et al., 2008; Shames and Wistuba, 2014). Genetic alterations in NSCLC tumors primarily include oncogenic mutations in the epidermal growth factor receptor (*EGFR*) and *KRAS*, as well as inactivation of tumor suppressor genes such as *p53*, *PTEN*, *Rb*, and *p16* (Hollstein et al., 1991; Jin et al., 2010; Reissmann et al., 1993). Mutations in the *EGFR* gene, particularly deletion of exon 19 and L858R mutation in exon 21, occur in 10-50 % of NSCLC patients (Cooper et al., 2013; Gazdar, 2009). Small molecule tyrosine-kinase inhibitors (TKIs) that reversibly inhibit EGFR at the ATP pocket domain, such as erlotinib and gefitinib, currently represent the first line of therapy for EGFR-mutated NSCLC patients (Antonicelli et al., 2013; Steins et al., 2014). Although these therapies are initially efficacious, ultimately most patients develop resistance. Whereas resistance has been attributed in some cases to the acquisition of secondary EGFR mutations or MET amplification (Engelman et al., 2007; Kobayashi et al., 2005), the mechanisms behind the resistance to TKIs are only partially understood. Dissecting the signaling mechanisms driving resistance is crucial for designing combinational therapy regimes to overcome this hurdle and extend life expectancy of NSCLC patients.

Protein kinase C (PKC) represents a group of serine-threonine kinases involved in a variety of cellular functions, including mitogenesis, survival, and motility. The PKC family is

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

composed of 10 members classified into 3 classes: calcium-dependent or conventional PKCs (cPKCα, βI, βII, and γ), calcium-independent or novel PKCs (nPKCδ, ε, η, and θ), and phorbol ester/diacylgycerol (DAG) unresponsive or atypical PKCs (aPKCsζ and ι/λ) (Barry and Kazanietz, 2001; Garg et al., 2013; Griner and Kazanietz, 2007; Newton, 2001; Parker et al., 2014). Decades of research have established key roles for different members of the PKC family in the progression of cancer. It became clear that individual PKC isozymes could act either as tumor promoters or tumor suppressors. For example, PKCβ has been proposed to be involved in lung tumorigenesis, and the PKCβ inhibitor enzastaurin has been examined as a potential therapeutic agent for lung cancer patients (El Osta et al., 2014; Tekle et al., 2008; Vansteenkiste et al., 2012; Willey et al., 2010). Our laboratory recently showed that PKCε, a kinase implicated in cell cycle progression and motility, is required for the tumorigenic and metastatic activities of NSCLC cells (Caino et al., 2012a; Caino et al., 2012b). On the other hand, PKCα and PKCδ negatively modulate NSCLC cell cycle progression (Nakagawa et al., 2005; Oliva et al., 2008; Santiago-Walker et al., 2005; Xiao et al., 2008). Most recently, Hill *et al.* provided direct evidence for a tumor suppressive role for PKCα in KRAS tumorigenesis (Hill et al., 2013). The fact that PKCα promotes NSCLC cell migration (Cheng et al., 2009; O'Neill et al., 2011), suggests divergent roles for this kinase in different stages of lung cancer progression. Likewise, diverse roles for PKCα and other members of the PKC family have been established in survival of NSCLC cells and other cancer cell types (Garg et al., 2013). Additionally, the overexpression of some PKC family members has been also associated with low sensitivity to the irreversible TKI afatinib in lung cell line models (Coco et al., 2014).

Towards the goal of determining a potential involvement of PKC isozymes in TKI resistance in lung cancer, here we took advantage of an isogenic NSCLC cell model of erlotinib-

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

resistance generated by culturing the parental H1650 cell line in the presence of a high concentration of the inhibitor. Erlotinib-resistant H1650 cells display features of epithelial-to-mesenchymal transition (EMT), a phenotype that is maintained by the transforming growth factor beta (TGF-β) pathway (Yao et al., 2010). Our study identified discrete roles for PKC isozymes, specifically PKCα and PKCδ, in erlotinib resistance and EMT in NSCLC cells.

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Reagents

Erlotinib hydrochloride was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The pan-PKC inhibitor GF109203X was purchased from Enzo Life Sciences (Plymouth Meeting, PA). The cPKC inhibitor Gö6976 was obtained from LC laboratories (Woburn, MA).

Cell Culture

The H1650-M3 cell line was derived from parental H1650 cells in the laboratory of Raffaella Sordella, Cold Spring Harbor Laboratory (Yao et al., 2010). Both cell lines were kindly provided by Dr. Sordella, and were cultured in RPMI 1640 medium supplemented with 5 % FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and maintained at 37 °C in a humidified 5 % CO_2 atmosphere.

Real-time PCR

Total RNA was extracted from subconfluent cell cultures using the RNeasy kit from Qiagen (Valencia, CA). Total RNA (1 μg) was reverse transcribed to cDNA using the Taqman reverse transcription reagent kit (Applied Biosystems, Branchburg, NJ). Real-time quantitative PCR (qPCR) was performed essentially as described (Wang et al., 2014) using an ABI PRISM 7700 detection system. The reaction was carried out in triplicate samples containing TaqMan universal PCR MasterMix (Applied Biosystems, Branchburg, NJ), target primers (300 nM), fluorescent probe (200 nM), and 4 μl of transcribed cDNA (6X dilution). Taqman primers 5'end-labeled with 6-carboxyfluorescein (FAM) for PKCα, PKCδ, E-cadherin, Snail, Twist1, vimentin,

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

Zeb2 and 18S rRNA (housekeeping gene) were purchased from Applied Biosystems. PCR product amplification was continuously monitored using the sequence detection system software version 1.7 (Applied Biosystems). Triplicate cycle threshold (Ct) values were averaged and normalized to an average 18S Ct value to calculate the ΔCt. The Δ(ΔCt) was determined by subtracting the control ΔCt value from the experimental ΔCt value. Fold-changes were expressed as $2^{-\Delta(\Delta Ct)}$.

Western blot

Western blot analysis was carried out essentially as previously described (Oliva et al., 2008). Briefly, cells were harvested in lysis buffer (50 mM Tris-HCl, pH 6.8, 10 % glycerol, 2 % SDS, 0.08 % bromophenol blue, and 5 % β-mercaptoethanol). Samples were resolved in 10 % SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MD). After blocking with 5 % milk in 1 % Tween 20/PBS, membranes were incubated with one of the following primary antibodies: anti-PKCα (EMD Millipore Corp., Billerica, MA), anti-PKCε (Santa Cruz Biotechnology), anti-PKCι (Abcam, Cambridge, MA), anti-PKCδ, anti-vimentin, anti-E-cadherin, anti-Snail, anti-phospho-Smad2 (Cell Signaling Technology, Danvers, MA), or anti-vinculin (Sigma-Aldrich, St. Louis, MO). As secondary antibodies, we used either anti-mouse or anti-rabbit antibodies conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA). Bands were visualized by the enhanced chemiluminescence Western blotting detection system, and images were captured using a FujiFILM LAS-3000 system.

RNA interference

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

RNAi duplexes for silencing PKCα were purchased from Dharmacon (Lafayette, CO). The target sequences were as follows: PKCα RNAi #1: CCAUCCGCUCCACACUAAA; PKCα RNAi # 2: GAACAAGGAAUGACUU (Oliva et al., 2008). Control silencer RNAi was purchased from Ambion (Austin, TX). For transfection of RNAi duplexes (25 nM), we used Lipofectamine RNAi/MAX.

Adenoviral infections

Cells were infected with adenoviruses (AdVs) for PKCα, PKCδ, or LacZ (control) using different multiplicities of infection (MOIs), as previously described (Oliva et al., 2008). Adenoviral infections were carried out in RPMI 1640 medium supplemented with 2 % FBS. Four h later, complete medium was added. Experiments were carried at different times after infection, as indicated.

Cell viability assay

Cell viability was determined using the CellTiter 96® AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI), a colorimetric assay that contains MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and phenazine ethosulfate with enhanced chemical stability. Cells seeded into 96-well plates (1×10^4 cells/well) were treated with different concentrations of erlotinib for different times, as indicated. One h after addition of the One Solution Reagent, absorbance was recorded at 490 nm using a 96-well plate reader.

Flow cytometry

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

Subconfluent H1650 cells were detached using 0.02 % EDTA in PBS, washed, pelleted, and resuspended in FACS buffer (PBS pH 7.2, 0.2 % bovine serum albumin). 5×10^6 cells were co-stained with phycoerythrin-conjugated anti-human CD24 and allophycocyanin-conjugated anti-human CD44 (BD Biosciences, San Jose, CA) antibodies. Labeling was performed for 1 h at room temperature in the dark. Labeled cells were washed three times with the FACS buffer and sorted using a BD FACS Aria II cell sorter. Gates were set either at high or low expressions for CD24 and CD44, and subpopulation of cells were collected in FACS buffer for RNA extraction.

Statistical Analysis

All statistical analyses were done using GraphPad Prism v5.03 (GraphPad Software, Inc., San Diego, CA). Data were analyzed using a two-way ANOVA. A p value < 0.05 was considered statistically significant.

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Erlotinib-resistant cells display altered expression of PKC isozymes

Changes in the expression levels of PKC isozymes have been associated with the progression of many types of cancers, including lung cancer, as well as with resistance to chemotherapeutic agents (Bae et al., 2007; Basu et al., 1996; Clark et al., 2003; Felber et al., 2007; Garg et al., 2013). In order to determine if PKC isozymes are implicated in erlotinib resistance, we took advantage of a well-characterized isogenic NSCLC cell model: the parental H1650 cell line and its erlotinib-resistant derivative H1650-M3 (Yao et al., 2010) (Fig. 1A). H1650 cells express conventional PKCα, novel PKCs δ and ε, and atypical PKCs. Western blot analysis revealed a remarkable up-regulation of PKCα in erlotinib-resistant H1650-M3 cells. H1650-M3 cells also have reduced PKCδ levels relative to parental H1650 cells. A slight reduction in PKCε levels was also observed in H1650-M3 cells (Fig. 1B). Densitometric analysis revealed the following levels relative to parental H1650 cells (n=3): 10.90 ± 1.61 (PKCα), 0.40 ± 0.07 (PKCδ), 0.85 ± 0.06 (PKCε), and 1.08 ± 0.13 (PKCι). Determination of mRNA levels for these PKCα and PKCδ led to similar conclusions. Indeed, H1650-M3 cells have 25-fold higher PKCα mRNA levels than parental H1650 cells, whereas PKCδ mRNA levels are reduced by 5-fold in the erlotinib-resistant cell line (Fig. 1C).

PKCα is required but not sufficient to induce erlotinib resistance

To assess a potential association between altered PKCα expression and erlotinib-resistance, we used both pharmacological and RNAi approaches. As PKCα has been implicated in drug resistance in some cancer types (Chen et al., 2010; Lee et al., 2012; Zhao et al., 2012)

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

and its levels are strikingly high in erlotinib-resistant cells, we speculated that this PKC could be involved in acquired resistance to erlotinib in NSCLC cells. Initial experiments showed that treatment of H1650-M3 cells with the pan PKC inhibitor GF109203X increases their sensitivity to erlotinib (10 μM) (Fig. 2A). Gö6976, which preferentially inhibits cPKCs (Martiny-Baron et al., 1993), also enhanced the killing effect of erlotinib in H1650-M3 cells (Fig. 2B). PKCα is the most up-regulated cPKC in this cell line, thus it is likely that this PKC mediates erlotinib resistance.

To unambiguously establish a role for PKCα in erlotinib resistance, we used RNAi. Two different PKCα RNAi duplexes were transfected into H1650-M3 cells, which depleted PKCα by 91 % (PKCα1 RNAi) and 89% (PKCα2 RNAi) relative to a non-target control (NTC) RNAi duplex, as determined by densitometry (Fig. 2C, *left panel*). A dose-response analysis for inhibition of cell viability by erlotinib revealed an IC_{50} ~ 5 μM in NTC H1650 cells (which is similar to parental H1650 cells). On the other hand, IC_{50} in H1650-M3 cells was > 20 μM, as also established in a previous study (Yao et al., 2010). Notably, PKCα depletion sensitizes H1650-M3 cells to erlotinib, as judged by the reduction in IC_{50} (8.7 ±1.4 μM for PKCα1 RNAi; 9.2 ±3.0 μM for PKCα2 RNAi) (Fig. 2C, *right panel*).

To determine if PKCα up-regulation was sufficient to induce erlotinib resistance, PKCα was overexpressed in parental H1650 cells using an adenovirus (AdV). A LacZ AdV was used as control (Fig. 2D, *left panel*). We found that PKCα overexpression failed to alter the response of H1650 cells to the TKI (IC_{50} = 4.7 ±1.3 μM for PKCα AdV; IC_{50} = 5.5 ±2.0 μM for LacZ AdV) (Fig. 2D, *right panel*). Taken together, these data indicate that although PKCα is required

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

for the resistance of NSCLC cells to erlotinib, overexpression of this kinase is not alone sufficient to induce erlotinib resistance.

PKCδ alters the sensitivity of H1650-M3 cells to erlotinib

Our results clearly ascribe a role for PKCα in determining the sensitivity of H1650 cells to erlotinib. The fact that H1650-M3 cells display PKCδ down-regulation relative to parental H1650 cells prompted us to investigate whether changes in PKCδ levels could also dictate the sensitivity to the TKI. PKCδ has been previously shown to mediate the cytotoxic effect of several anti-cancer drugs (Blass et al., 2002; Reyland et al., 1999). To address this issue, we first overexpressed PKCδ in H1650-M3 cells using a PKCδ AdV (Fig. 3A). As shown in Fig. 3B, overexpression of PKCδ in erlotinib-resistant cells caused a reduction in the IC_{50} for erlotinib. This effect was proportional to the expression levels of PKCδ achieved by infecting cells with different MOIs of the PKCδ AdV. Infection of H1650-M3 cells with an MOI=1 pfu/cell did not cause any significant PKCδ overexpression or sensitization to erlotinib (IC_{50} = 24.2 ±0.6 μM for PKCδ AdV; IC_{50} = 24.7 ±2.0 μM for control LacZ AdV). On the other hand, infection with PKCδ AdV at MOI=10 pfu/cell caused significant sensitization (IC_{50} = 8.7 ±1.9 μM for PKCδ AdV; IC_{50} = 26.4 ±0.4 μM for LacZ AdV). At higher MOIs, the sensitivity of H1650-M3 cells was essentially similar to that observed in parental H1650 cells (MOI=30: IC_{50} = 6.3 ±0.5 μM for PKCδ AdV; IC_{50} = 22.2 ±0.4 μM for LacZ AdV; MOI=100: IC_{50} = 4.5 ±0.4 μM for PKCδ AdV; IC_{50} = 19.5 ±1.0 μM for LacZ AdV). Thus, PKCδ down-regulation in H1650-M3 cells contributes to erlotinib resistance.

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

Previous studies have shown that overexpression of one PKC isozyme could alter the expression of other PKC family members. For example, overexpression of PKCα alters the expression of PKCδ and PKCε in various cellular models (Romanova et al., 1998; Tonetti et al., 2000; Ways et al., 1995). As erlotinib-resistant H1650 cells display PKCα overexpression and PKCδ down-regulation relative to the parental cell line, we asked if there is a mutual regulation between these PKCs. To test our hypothesis, we either overexpressed PKCα or depleted PKCδ in parental H1650 cells. Interestingly, PKCα overexpression by adenoviral means reduced PKCδ expression, both at mRNA and protein levels. These effects were proportional to the PKCα overexpression levels achieved by using increased MOIs of the PKCα AdV (Fig. 4A and 4B). Next, to assess if down-regulation of PKCδ alters PKCα expression levels, we silenced PKCδ expression from parental H1650 cells using RNAi. As shown in Fig. 4C, both control and PKCδ-depleted H1650 cells display similar PKCα levels. Furthermore, adenoviral overexpression of PKCδ in erlotinib-resistant H1650-M3 cells failed to induce changes in PKCα expression (Fig. 4D). These results argue for a unidirectional cross-talk whereby overexpression of PKCα in erlotinib-resistant H1650-M3 cells contribute to PKCδ down-regulation, however PKCδ was unable to influence PKCα expression.

PKCα is required for the maintenance of mesenchymal phenotype of H1650-M3 cells

Erlotinib-resistant H1650 cells exhibit mesenchymal properties, driven by the TGF-β pathway (Yao et al., 2010). The mesenchymal phenotype is a hallmark of cancer cells exhibiting aggressive phenotype (Tam et al., 2013). A recent study in breast cancer showed that PKCα is up-regulated in cells that had undergone EMT (Tam et al., 2013). Thus, we speculated that this

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

kinase might contribute to the maintenance of the mesenchymal phenotype of erlotinib-resistant H1650 cells.

First, we investigated if PKCα levels were elevated in a subpopulation of H1650 cells that display stem cell-like properties. Parental H1650 cells were sorted into $CD44^{high}/CD24^{low}$ and $CD44^{low}/CD24^{high}$ enriched populations, and PKCα mRNA levels were determined by qPCR. These experiments revealed PKCα up-regulation in $CD44^{high}/CD24^{low}$ cells (Fig. 5A). As shown in a previous study (Yao et al., 2010), H1650-M3 cells display elevated levels of genes associated with EMT, including vimentin, Snail, Twist, and Zeb2, as well as reduced levels of E-cadherin. To establish a potential link between PKCα up-regulation and the mesenchymal phenotype of H1650-M3 cells, we examined the expression of EMT markers by qPCR after silencing PKCα. Notably, PKCα RNAi depletion caused a significant reduction in vimentin, Snail, Twist, and Zeb expression, suggesting that PKCα mediates the induction of these EMT genes. Expression of the epithelial marker E-cadherin, however, remained unaffected (Fig. 5B). Changes were also validated at the protein level for those markers that could be readily detected by Western blot (64% and 69% reduction for vimentin; 42% and 60% reduction for Snail, using PKCα1 and PKCα2 RNAi, respectively) (Fig. 5C). Despite the PKCα requirement for the expression of EMT markers in H1650-M3 cells, it became apparent that overexpression of this kinase in parental H1650 cells was not sufficient to induce these EMT genes, as determined by qPCR 72 h after infection with increasing MOIs of the PKCα AdV (Fig. 5D). No changes were observed even one week after PKCα AdV infection (data not shown). Altogether, these results indicate that PKCα is required for the expression of genes involved in the maintenance of the mesenchymal phenotype of erlotinib-resistant cells, however its overexpression is not sufficient to induce this phenotypical change.

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

Next, we set to explore whether PKCδ has a role in the expression of genes associated with EMT transition. As PKCδ is down-regulated in H1650-M3 cells, we adenovirally overexpressed PKCδ in these cells and assessed the expression of EMT markers by qPCR. Unlike PKCα silencing, ectopic overexpression of PKCδ in H1650-M3 cells did not change the expression of vimentin, Twist, or Zeb2, although a reduction in Snail levels could be observed. Likewise, PKCδ overexpression did not affect E-cadherin mRNA levels (Fig. 6A). In addition, we also found that PKCδ RNAi depletion from parental H1650 cells failed to change the expression of Snail and E-cadherin (Fig. 6B). Therefore, the involvement of PKCδ is only confined to erlotinib resistance but not to EMT.

PKCα up-regulation in erlotinib-resistant cells is mediated by TGF-β

TGF-β has been widely implicated in EMT in multiple cancer types (Massague, 2012; Moustakas and Heldin, 2012). It has been previously established that activation of the TGF-β signaling pathway mediates EMT and erlotinib resistance in H1650 cells (Yao et al., 2010). Based on this premise, we sought to establish if a causal relationship exists between TGF-β signaling and PKCα expression. H1650-M3 cells were treated with the TGF-β receptor inhibitor LY2109761, and its efficacy to inhibit TGF-β signaling was confirmed by its ability to reduce Smad2 phosphorylation (Fig. 7A). PKC inhibitors GF109203X and Gö6976 did not affect Smad2 phosphorylation, suggesting that PKC does not affect the activation of this pathway. Notably, the TGF-β receptor inhibitor caused a time-dependent reduction in PKCα mRNA level. This effect became noticeable at the protein level 48 h and 72 h after LY2109761 treatment (Fig. 7B). Furthermore, when parental H1650 cells were treated with TGF-β for different times,

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

significant PKCα up-regulation both at mRNA and protein levels could be observed. This effect was quite remarkable after long-term treatment with TGF-β (Fig. 7C and 7D). Therefore, TGF-β signaling is implicated in the overexpression of PKCα observed in erlotinib-resistant cells.

Lastly, we sought to establish an association between PKCα up-regulation and TGF-β signaling in the induction of the mesenchymal phenotype. H1650 cells were infected with PKCα AdV (or LacZ AdV as a control) and then subject to TGF-β treatment. mRNA was extracted one week after treatment and EMT markers were determined by qPCR. As shown in Fig. 7E, overexpression of PKCα potentiated TGF-β induction of vimentin, Snail and Twist, thus establishing the relevance of the TGF-β-PKCα pathway in the induction of the mesenchymal phenotype.

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

Tumor cells harboring activating mutations of EGFR are addicted to this oncogenic stimulus to maintain their proliferative and survival advantages. TKIs such as erlotinib are effective for treatment of advanced NSCLC tumors harboring EGFR activating mutations. However, many patients treated with erlotinib develop resistance to the targeted molecular therapy (Steins et al., 2014; Tang et al., 2013). PKC isozymes have been recognized as key effectors of known oncogenes implicated in drug resistance such as *c-MET, KRAS* and *TGF-β* (Kermorgant et al., 2004; Sakaguchi et al., 2004; Symonds et al., 2011). Moreover, phorbol esters, which are known activators of PKCs, induce multidrug resistance (Fine et al., 1988; Kalalinia et al., 2012).

Here, we present evidence for the involvement of specific PKC isozymes in erlotinib resistance and EMT in NSCLC cells. Using an isogenic cell model, we found considerable changes in the expression of PKC isozymes that are causally associated with resistance to erlotinib. Erlotinib-resistant H1650-M3 cells exhibit elevated PKCα levels, whereas PKCδ expression in these cells is markedly down-regulated. Although this is the first evidence for the involvement of these two PKC isozymes in resistance to this targeted molecular therapy, altered expression of PKCα and PKCδ has been detected in several cancer cell types. For example, elevation of PKCα expression or activity has been reported in pancreatic, colon, prostate, glioma and gastric cancer cells resistant to chemotherapeutic drugs, including cisplatin, doxorubicin and vincristine (Chen et al., 2010; Matsumoto et al., 1995; Wu et al., 2009; Zhao et al., 2012). Interestingly, comparable to what we observed in erlotinib-resistant cells, continuous exposure of MCF-7 breast cancer cells to tamoxifen rendered high levels of PKCα and down-regulation of

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

PKCδ (Li et al., 2012).

Studies have indicated the importance of PKCα overexpression in protecting cancer cells against drug-induced cell death. For example, PKCα overexpression in colon cancer cells attenuates doxorubicin-induced apoptosis by elevating phosphorylation of Bcl-2, Bad, and decreasing PARP cleavage. More importantly, in several cancer models, PKCα overexpression has been associated with increased drug resistance by elevating expression and phosphorylation of the drug efflux pump P-gp encoded by the multidrug resistant gene 1 (*MDR1*) (Lee et al., 2012). The functional importance of PKCα overexpression has been further demonstrated by using pharmacological inhibitors and RNAi. For example, inhibition of PKCα using Gö6976 restores the sensitivity of pancreatic cancer cells to chemotherapeutic drugs (Chen et al., 2010), and silencing PKCα by RNAi reverses drug resistance in ovarian cancer cells (Zhao et al., 2012). In our study, we found that RNAi depletion or inhibition of PKCα using Gö6976 sensitizes erlotinib-resistant NSCLC cells to the TKI.

As previously characterized, H1650-M3 cells have elevated expression of genes associated with EMT and display morphological changes that are reminiscent of the mesenchymal phenotype. Interestingly, parental erlotinib naïve cells possess a small subpopulation of cells that are mesenchymal, erlotinib-resistant and similar to H1650-M3 cells (Yao et al., 2010), indicating that H1650-M3 cells were potentially generated through a selection process that favors the survival of cells that utilize alternate mechanisms to overcome drug-induced death. A recent study by the Weinberg laboratory established that PKCα preferentially supports the maintenance of the mesenchymal cell state through the regulation of the transcription factor FRA1 (Fos-related antigen 1). Additionally, elevated PKCα expression was found in a subpopulation of normal mammary epithelial cells enriched in the mesenchymal

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

surface marker CD44 (Tam et al., 2013). Similarly, our results indicate a correlation between enrichment of mesenchymal phenotype and PKCα expression in NSCLC cells. Inhibition of PKCα in H1650-M3 cells also led to a reduction in the expression of genes associated with the mesenchymal phenotype. Interestingly, although exposure to erlotinib resulted in a differential expression of EMT markers, including up-regulation of vimentin, snail, Twist and Zeb2, as well as down-regulation of E-cadherin, the effect of inhibiting PKCα was limited to the genes associated with mesenchymal phenotype, thus underscoring its role in the maintenance of this phenotype.

In our study, we also identified a functional link between TGF-β and PKCα. TGF-β signaling was shown to be sufficient and required for the induction of erlotinib resistance and EMT in H1650-M3 cells (Yao et al., 2010). We found that inhibition of TGF-β signaling reduced the expression of PKCα in H1650-M3 cells. On the other hand, TGF-β increased the expression of PKCα in parental H1650 cells, indicating that in the process of acquiring an aggressive phenotype, TGF-β up-regulates the expression of PKCα. TGF-β is known to control gene expression by activating the Smad transcription factors (Massague, 2012). The promoter region of PKCα does not display any obvious Smad binding site (data not shown), arguing for the involvement of alternative or indirect mechanisms. It is worth noting that gene profiling analysis in A549 lung adenocarcinoma cells identified PKCα as a TGF-β target gene (Ranganathan et al., 2007).

In summary, our results provide evidence for a role of PKCs in acquired drug resistance to erlotinib and EMT. Elevation of PKCα expression as well as PKCα dependent down-regulation of PKCδ are required for erlotinib resistance, whereas mesenchymal genes are regulated only by PKCα. Our results argue for a potential therapeutic use of PKCα inhibitors to

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

overcome drug resistance and EMT in lung cancer.

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

Authorship Contributions

Participated in research design: Abera, Kazanietz.

Conducted experiments: Abera.

Performed data analysis: Abera, Kazanietz.

Wrote or contributed to the writing of the manuscript: Abera, Kazanietz.

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Antonicelli, A., Cafarotti, S., Indini, A., Galli, A., Russo, A., Cesario, A., Lococo, F.M., Russo, P., Mainini, A.F., Bonifati, L.G., et al. (2013). EGFR-targeted therapy for non-small cell lung cancer: focus on EGFR oncogenic mutation. Int J Med Sci 10, 320-330.

Bae, K.M., Wang, H., Jiang, G., Chen, M.G., Lu, L., and Xiao, L. (2007). 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 Res 67, 6053-6063.

Barry, O.P., and Kazanietz, M.G. (2001). Protein kinase C isozymes, novel phorbol ester receptors and cancer chemotherapy. Curr Pharm Des 7, 1725-1744.

Basu, A., Weixel, K., and Saijo, N. (1996). Characterization of the protein kinase C signal transduction pathway in cisplatin-sensitive and -resistant human small cell lung carcinoma cells. Cell Growth Differ 7, 1507-1512.

Blass, M., Kronfeld, I., Kazimirsky, G., Blumberg, P.M., and Brodie, C. (2002). Tyrosine phosphorylation of protein kinase Cdelta is essential for its apoptotic effect in response to etoposide. Mol Cell Biol 22, 182-195.

Caino, M.C., Lopez-Haber, C., Kim, J., Mochly-Rosen, D., and Kazanietz, M.G. (2012a). Proteins kinase Cvarepsilon is required for non-small cell lung carcinoma growth and regulates the expression of apoptotic genes. Oncogene 31, 2593-2600.

Caino, M.C., Lopez-Haber, C., Kissil, J.L., and Kazanietz, M.G. (2012b). Non-small cell lung carcinoma cell motility, rac activation and metastatic dissemination are mediated by protein kinase C epsilon. PLoS One 7, e31714.

Chen, Y., Yu, G., Yu, D., and Zhu, M. (2010). PKCalpha-induced drug resistance in pancreatic cancer cells is associated with transforming growth factor-beta1. J Exp Clin Cancer Res 29, 104.

Cheng, C.Y., Hsieh, H.L., Sun, C.C., Lin, C.C., Luo, S.F., and Yang, C.M. (2009). IL-1 beta induces urokinase-plasminogen activator expression and cell migration through PKC alpha, JNK1/2, and NF-kappaB in A549 cells. J Cell Physiol 219, 183-193.

Clark, A.S., West, K.A., Blumberg, P.M., and Dennis, P.A. (2003). Altered protein kinase C (PKC) isoforms in non-small cell lung cancer cells: PKCdelta promotes cellular survival and chemotherapeutic resistance. Cancer Res 63, 780-786.

Coco, S., Truini, A., Alama, A., Dal Bello, M.G., Vene, R., Garuti, A., Carminati, E., Rijavec, E., Genova, C., Barletta, G., et al. (2014). Afatinib resistance in non-small cell lung cancer involves the PI3K/AKT and MAPK/ERK signalling pathways and epithelial-to-mesenchymal transition. Targeted oncology. Epub ahead of print.

Cooper, W.A., Lam, D.C., O'Toole, S.A., and Minna, J.D. (2013). Molecular biology of lung cancer. J Thorac Dis 5, S479-S490.

El Osta, M., Liu, M., Adada, M., Senkal, C.E., Idkowiak-Baldys, J., Obeid, L.M., Clarke, C.J., and Hannun, Y.A. (2014). Sustained PKCbetaII activity confers oncogenic properties in a phospholipase D- and mTOR-dependent manner. FASEB J 28, 495-505.

Engelman, J.A., Zejnullahu, K., Mitsudomi, T., Song, Y., Hyland, C., Park, J.O., Lindeman, N., Gale, C.M., Zhao, X., Christensen, J., et al. (2007). MET amplification leads to gefitinib resistance in lung cancer by activating ERBB3 signaling. Science 316, 1039-1043.

Felber, M., Sonnemann, J., and Beck, J.F. (2007). Inhibition of novel protein kinase C-epsilon augments TRAIL-induced cell death in A549 lung cancer cells. Pathol Oncol Res 13, 295-301.

Ferlay, J., Soerjomataram, I., Dikshit, R., Eser, S., Mathers, C., Rebelo, M., Parkin, D.M., Forman, D., and Bray, F. (2015). Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. International journal of cancer Journal international du cancer 136, E359-386.

Fine, R.L., Patel, J., and Chabner, B.A. (1988). Phorbol esters induce multidrug resistance in human breast cancer cells. Proc Natl Acad Sci U S A 85, 582-586.

Garg, R., Benedetti, L.G., Abera, M.B., Wang, H., Abba, M., and Kazanietz, M.G. (2013). Protein kinase C and cancer: what we know and what we do not. Oncogene.

Gazdar, A.F. (2009). Activating and resistance mutations of EGFR in non-small-cell lung cancer: role in clinical response to EGFR tyrosine kinase inhibitors. Oncogene 28 Suppl 1, S24-31.

Griner, E.M., and Kazanietz, M.G. (2007). Protein kinase C and other diacylglycerol effectors in cancer. Nat Rev Cancer 7, 281-294.

Hill, K.S., Erdogan, E., Khoor, A., Walsh, M.P., Leitges, M., Murray, N.R., and Fields, A.P. (2013). Protein kinase Calpha suppresses Kras-mediated lung tumor formation through activation of a p38 MAPK-TGFbeta signaling axis. Oncogene.

Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991). p53 mutations in human cancers. Science 253, 49-53.

Jin, G., Kim, M.J., Jeon, H.S., Choi, J.E., Kim, D.S., Lee, E.B., Cha, S.I., Yoon, G.S., Kim, C.H., Jung, T.H., et al. (2010). PTEN mutations and relationship to EGFR, ERBB2, KRAS, and TP53 mutations in non-small cell lung cancers. Lung Cancer 69, 279-283.

Kalalinia, F., Elahian, F., Hassani, M., Kasaeeian, J., and Behravan, J. (2012). Phorbol ester TPA modulates chemoresistance in the drug sensitive breast cancer cell line MCF-7 by inducing expression of drug efflux transporter ABCG2. Asian Pac J Cancer Prev 13, 2979-2984.

Kanne, J.P. (2014). Screening for lung cancer: what have we learned? AJR American journal of roentgenology 202, 530-535.

Kermorgant, S., Zicha, D., and Parker, P.J. (2004). PKC controls HGF-dependent c-Met traffic, signalling and cell migration. EMBO J 23, 3721-3734.

Kobayashi, S., Boggon, T.J., Dayaram, T., Janne, P.A., Kocher, O., Meyerson, M., Johnson, B.E., Eck, M.J., Tenen, D.G., and Halmos, B. (2005). EGFR mutation and resistance of non-small-cell lung cancer to gefitinib. N Engl J Med 352, 786-792.

Lee, S.K., Shehzad, A., Jung, J.C., Sonn, J.K., Lee, J.T., Park, J.W., and Lee, Y.S. (2012). Protein kinase Calpha protects against multidrug resistance in human colon cancer cells. Mol Cells 34, 61-69.

Li, Z., Wang, N., Fang, J., Huang, J., Tian, F., Li, C., and Xie, F. (2012). Role of PKC-ERK signaling in tamoxifen-induced apoptosis and tamoxifen resistance in human breast cancer cells. Oncol Rep 27, 1879-1886.

Martiny-Baron, G., Kazanietz, M.G., Mischak, H., Blumberg, P.M., Kochs, G., Hug, H., Marme, D., and Schachtele, C. (1993). Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. J Biol Chem 268, 9194-9197.

Massague, J. (2012). TGFbeta signalling in context. Nat Rev Mol Cell Biol 13, 616-630.

Matsumoto, T., Tani, E., Yamaura, I., Miyaji, K., and Kaba, K. (1995). Effects of protein kinase C modulators on multidrug resistance in human glioma cells. Neurosurgery 36, 565-571; discussion 572.

Molina, J.R., Yang, P., Cassivi, S.D., Schild, S.E., and Adjei, A.A. (2008). Non-small cell lung cancer: epidemiology, risk factors, treatment, and survivorship. Mayo Clin Proc 83, 584-594.

Moustakas, A., and Heldin, C.H. (2012). Induction of epithelial-mesenchymal transition by transforming growth factor beta. Semin Cancer Biol 22, 446-454.

Nakagawa, M., Oliva, J.L., Kothapalli, D., Fournier, A., Assoian, R.K., and Kazanietz, M.G. (2005). Phorbol ester-induced G1 phase arrest selectively mediated by protein kinase Cdelta-dependent induction of p21. J Biol Chem 280, 33926-33934.

Newton, A.C. (2001). Protein kinase C: structural and spatial regulation by phosphorylation, cofactors, and macromolecular interactions. Chem Rev 101, 2353-2364.

O'Neill, A.K., Gallegos, L.L., Justilien, V., Garcia, E.L., Leitges, M., Fields, A.P., Hall, R.A., and Newton, A.C. (2011). Protein kinase Calpha promotes cell migration through a PDZ-dependent interaction with its novel substrate discs large homolog 1 (DLG1). J Biol Chem 286, 43559-43568.

Oliva, J.L., Caino, M.C., Senderowicz, A.M., and Kazanietz, M.G. (2008). S-Phase-specific activation of PKC alpha induces senescence in non-small cell lung cancer cells. J Biol Chem 283, 5466-5476.

Parker, P.J., Justilien, V., Riou, P., Linch, M., and Fields, A.P. (2014). Atypical protein kinase Ciota as a human oncogene and therapeutic target. Biochem Pharmacol 88, 1-11.

Ranganathan, P., Agrawal, A., Bhushan, R., Chavalmane, A.K., Kalathur, R.K., Takahashi, T., and Kondaiah, P. (2007). Expression profiling of genes regulated by TGF-beta: differential regulation in normal and tumour cells. BMC Genomics 8, 98.

Reissmann, P.T., Koga, H., Takahashi, R., Figlin, R.A., Holmes, E.C., Piantadosi, S., Cordon-Cardo, C., and Slamon, D.J. (1993). Inactivation of the retinoblastoma susceptibility gene in non-small-cell lung cancer. The Lung Cancer Study Group. Oncogene 8, 1913-1919.

Reyland, M.E., Anderson, S.M., Matassa, A.A., Barzen, K.A., and Quissell, D.O. (1999). Protein kinase C delta is essential for etoposide-induced apoptosis in salivary gland acinar cells. J Biol Chem 274, 19115-19123.

Romanova, L.Y., Alexandrov, I.A., Nordan, R.P., Blagosklonny, M.V., and Mushinski, J.F. (1998). Cross-talk between protein kinase C-alpha (PKC-alpha) and -delta (PKC-delta): PKC-alpha elevates the PKC-delta protein level, altering its mRNA transcription and degradation. Biochemistry 37, 5558-5565.

Sakaguchi, M., Miyazaki, M., Sonegawa, H., Kashiwagi, M., Ohba, M., Kuroki, T., Namba, M., and Huh, N.H. (2004). PKCalpha mediates TGFbeta-induced growth inhibition of human keratinocytes via phosphorylation of S100C/A11. J Cell Biol 164, 979-984.

Santiago-Walker, A.E., Fikaris, A.J., Kao, G.D., Brown, E.J., Kazanietz, M.G., and Meinkoth, J.L. (2005). Protein kinase C delta stimulates apoptosis by initiating G1 phase cell cycle progression and S phase arrest. J Biol Chem 280, 32107-32114.

Shames, D.S., and Wistuba, II (2014). The evolving genomic classification of lung cancer. J Pathol 232, 121-133.

Steins, M., Thomas, M., and Geissler, M. (2014). Erlotinib. Recent Results Cancer Res 201, 109-123.

Symonds, J.M., Ohm, A.M., Carter, C.J., Heasley, L.E., Boyle, T.A., Franklin, W.A., and Reyland, M.E. (2011). Protein kinase C delta is a downstream effector of oncogenic K-ras in lung tumors. Cancer Res 71, 2087-2097.

Tam, W.L., Lu, H., Buikhuisen, J., Soh, B.S., Lim, E., Reinhardt, F., Wu, Z.J., Krall, J.A., Bierie, B., Guo, W., et al. (2013). Protein kinase C alpha is a central signaling node and therapeutic target for breast cancer stem cells. Cancer Cell 24, 347-364.

Tang, J., Salama, R., Gadgeel, S.M., Sarkar, F.H., and Ahmad, A. (2013). Erlotinib resistance in lung cancer: current progress and future perspectives. Front Pharmacol 4, 15.

Tekle, C., Giovannetti, E., Sigmond, J., Graff, J.R., Smid, K., and Peters, G.J. (2008). Molecular pathways involved in the synergistic interaction of the PKC beta inhibitor enzastaurin with the antifolate pemetrexed in non-small cell lung cancer cells. Br J Cancer 99, 750-759.

Tonetti, D.A., Chisamore, M.J., Grdina, W., Schurz, H., and Jordan, V.C. (2000). Stable transfection of protein kinase C alpha cDNA in hormone-dependent breast cancer cell lines. Br J Cancer 83, 782-791.

Vansteenkiste, J., Ramlau, R., von Pawel, J., San Antonio, B., Eschbach, C., Szczesna, A., Kennedy, L., Visseren-Grul, C., Chouaki, N., and Reck, M. (2012). A phase II randomized study of cisplatin-pemetrexed plus either enzastaurin or placebo in chemonaive patients with advanced non-small cell lung cancer. Oncology 82, 25-29.

Wang, H., Gutierrez-Uzquiza, A., Garg, R., Barrio-Real, L., Abera, M.B., Lopez-Haber, C., Rosemblit, C., Lu, H., Abba, M., and Kazanietz, M.G. (2014). Transcriptional regulation of oncogenic protein kinase C (PKC) by STAT1 and Sp1 proteins. J Biol Chem 289, 19823-19838.

Ways, D.K., Kukoly, C.A., deVente, J., Hooker, J.L., Bryant, W.O., Posekany, K.J., Fletcher, D.J., Cook, P.P., and Parker, P.J. (1995). MCF-7 breast cancer cells transfected with protein kinase C-alpha exhibit altered expression of other protein kinase C isoforms and display a more aggressive neoplastic phenotype. J Clin Invest 95, 1906-1915.

Willey, C.D., Xiao, D., Tu, T., Kim, K.W., Moretti, L., Niermann, K.J., Tawtawy, M.N., Quarles, C.C., and Lu, B. (2010). Enzastaurin (LY317615), a protein kinase C beta selective inhibitor, enhances antiangiogenic effect of radiation. Int J Radiat Oncol Biol Phys 77, 1518-1526.

Wu, D.L., Sui, F.Y., Du, C., Zhang, C.W., Hui, B., Xu, S.L., Lu, H.Z., and Song, G.J. (2009). Antisense expression of PKCalpha improved sensitivity of SGC7901/VCR cells to doxorubicin. World J Gastroenterol 15, 1259-1263.

Xiao, L., Caino, M.C., von Burstin, V.A., Oliva, J.L., and Kazanietz, M.G. (2008). Phorbol ester-induced apoptosis and senescence in cancer cell models. Methods Enzymol 446, 123-139.

Yao, Z., Fenoglio, S., Gao, D.C., Camiolo, M., Stiles, B., Lindsted, T., Schlederer, M., Johns, C., Altorki, N., Mittal, V., et al. (2010). TGF-beta IL-6 axis mediates selective and adaptive mechanisms of resistance to molecular targeted therapy in lung cancer. Proc Natl Acad Sci U S A 107, 15535-15540.

Zhao, L.J., Xu, H., Qu, J.W., Zhao, W.Z., Zhao, Y.B., and Wang, J.H. (2012). Modulation of drug resistance in ovarian cancer cells by inhibition of protein kinase C-alpha (PKC-alpha) with small interference RNA (siRNA) agents. Asian Pac J Cancer Prev 13, 3631-3636.

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

FOOTNOTES

This work was supported by grants from the National Institutes of Health [R01 CA139120 and CA089202].

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Figure 1. Altered expression of PKC isozymes in erlotinib-resistant NSCLC cells. (A) Parental (H1650) and erlotinib-resistant (H1650-M3) cells were treated with erlotinib at indicated concentrations and cell viability was determined 24 h later using an MTS assay. (B) Expression of PKC isozymes in parental (H1650) and erlotinib-resistant (H1650-M3) cells was analyzed by Western blot. Similar results were observed in three individual experiments. (C) PKCα and PKCδ mRNA levels in H1650 and H1650-M3 cells were measured by qPCR. Human 18S rRNA was used as an endogenous control for normalization. Results (relative to H1650 cells) were expressed as mean ± S.D. of triplicate samples. Similar results were observed in three additional experiments.

Figure 2. PKCα protects H1650-M3 cells from erlotinib induced cell death. (A) H1650-M3 cells were pre-treated for 1 h with either the pan-PKC inhibitor GF109203X (5 μM) or vehicle. Cells were then treated with erlotinib (10 μM), and cell viability was determined 24 h later using an MTS assay. **, $p<0.01$ *vs.* vehicle. (B) H1650-M3 cells were pre-treated for 1 h with either the cPKC inhibitor Gö6976 (5 μM) or vehicle. Cells were then treated with erlotinib (10 μM), and cell viability was determined 24 h later using an MTS assay. *** $p<0.001$ *vs.* vehicle. (C) H1650-M3 cells were transfected with either PKCα (α1 or α2) or non-target control (*NTC*) RNAi duplexes. After 48 h, cells were treated with erlotinib for 24 h at the indicated concentrations. *Left panel*, PKCα expression by Western blot. *Right panel*, cell viability was determined using an MTS assay. Parental H1650 cells were included for comparison. (D) Parental H1650 cells were infected with either PKCα AdV or LacZ AdV (MOI= 30 pfu/cell).

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

Five days after infection, cells were treated with erlotinib at the indicated concentrations. *Left panel*, PKCα expression by Western blot. *Right panel*, cell viability was determined 24 h later. H1650-M3 cells were included for comparison. Data were expressed as mean ± S.D. of triplicate samples. Similar results were observed in two additional experiments.

Figure 3. PKCδ alters the sensitivity of H1650-M3 cells to erlotinib. (A) H1650-M3 cells were infected with either PKCδ AdV or LacZ AdV at the indicated MOIs. Expression of PKCδ was determined using Western blot. Densitometric analysis is shown as mean ± S.D. (n=3). (B) A viability assay using MTS was carried out 48 h after infection. Data were expressed as mean ± S.D. of triplicate samples. Similar results were observed in two additional experiments.

Figure 4. PKCα modulates the expression of PKCδ in H1650 cells. (A) H1650 cells were infected with either PKCα AdV or LacZ AdV at the indicated MOIs. PKCα and PKCδ mRNA levels were determined by qPCR 72 h after infection. Data are expressed as mean ± S.D. of triplicate samples. Results are expressed as fold-change relative to LacZ AdV. (B) Expression of PKCα and PKCδ was determined by Western blot 72 h after infection with either PKCα AdV or LacZ AdV. (C) Parental H1650 cells were transfected with either PKCδ (δ1 or δ2) or NTC RNAi duplexes. PKCα and PKCδ levels were analyzed 72 h later by Western blot. (D) H1650-M3 cells were infected with either PKCδ AdV or LacZ AdV (MOI=100 pfu/cell). PKCδ and PKCα levels were analyzed 96 h later by Western blot. Similar results were observed in three independent experiments. Densitometric analysis is shown as mean ± S.D. (n=3).

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 5. **PKCα is required for the expression of markers of the mesenchymal phenotype.** (A) Parental H1650 cells were sorted into $CD44^{high}/CD24^{low}$ and $CD44^{low}/CD24^{high}$ sub-populations by flow cytometry. PKCα mRNA levels were determined by qPCR. Data are expressed as mean ± S.D. of triplicate samples. (B) H1650-M3 cells were transfected with either PKCα (α1 or α2) or NTC RNAi duplexes. After 72 h, RNA was extracted for qPCR analysis of selected genes associated with epithelial (E-cadherin) or mesenchymal (vimentin, Snail, Twist, and Zeb2) phenotypes. Results are shown as fold-change relative to parental H1650 cells. Data were expressed as mean ± S.D. of triplicate samples. (C) Expression of epithelial and mesenchymal markers was determined by Western blot. (D) H1650 cells were infected with either PKCα AdV or LacZ AdV at the indicated MOIs. After seven days, expression of E-cadherin, vimentin, Snail, Twist and Zeb2 were determined by qPCR. Similar results were observed in three independent experiments.

Figure 6. Genes involved in the mesenchymal phenotype are not regulated by PKCδ. (A) H1650-M3 cells were infected with either PKCδ AdV or LacZ AdV (MOI=100 pfu/cell). After 96 h, mRNA levels for various mesenchymal (vimentin, Snail, Twist, and Zeb2) or epithelial (E-cadherin) associated genes were measured by qPCR. Results are shown as fold-change relative to control (LacZ AdV-infected) H1650-M3 cells. Data were expressed as mean ± S.D. of triplicate samples. (B) Parental H1650 cells were transfected with either PKCδ (δ1 or δ2) or NTC RNAi duplexes. Expression of PKCδ, E-cadherin and Snail was analyzed by Western blot 72 h later. Similar results were observed in three independent experiments.

Molecular Pharmacology Fast Forward. Published on February 27, 2015 as DOI: 10.1124/mol.115.097725
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 7. TGF-β signaling controls PKCα expression in erlotinib-resistant cells. (A) H1650-M3 cells were pre-treated for 1 h with either the pan-PKC inhibitor GF109203X (5 μM), the cPKC inhibitor Gö6976 (5 μM), the TGF-β receptor inhibitor LY2109761 (5 μM) or vehicle. Cells were then treated with TGF-β (20 ng/ml, 30 min) and phospho-Smad2 levels were determined by Western blot. (B) H1650-M3 cells were treated with the TGF-β receptor inhibitor LY2109761 (5 μM) for the indicated times. PKCα mRNA and protein levels were determined by qPCR and Western blot, respectively. Densitometric analysis is shown as mean ± S.D. (n=3). (C) PKCα mRNA levels in H1650 cells were measured 6 h or 2 weeks after TGF-β treatment. (D) H1650 cells were treated with TGF-β (5 ng/ml) for 24 h, 48 h, 1 week or 2 weeks. PKCα levels were determined by Western blot. Densitometric analysis is shown as mean ± S.D. (n=3). (E) H1650 cells were infected with either PKCα AdV or LacZ AdV (MOI=30 pfu/cell). Twenty-four h after infection, cells were treated with TGF-β (5 ng/ml) for one week. mRNA levels for PKCα, Snail, vimentin, and Twist were measured using qPCR. In all cases, data were expressed as mean ± S.D. of triplicate samples and experiments were reproduced at least three times.

Figure 1

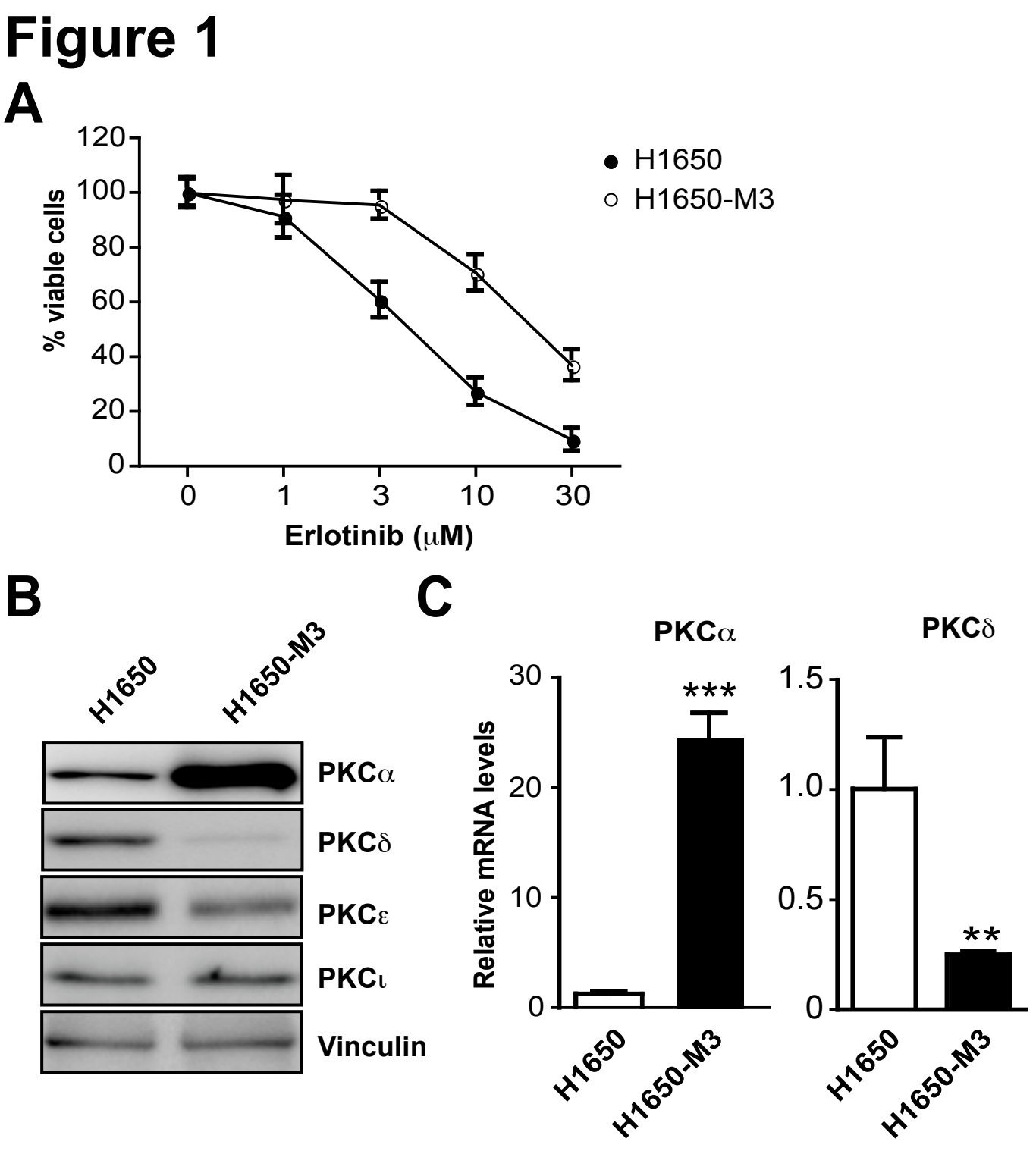

A
120
100
80
60
40
20
0
% viable cells
H1650
H1650-M3
0
1
3
10
30
Erlotinib (μM)
B
H1650
H1650-M3
PKCα
PKCδ
PKCε
PKCι
Vinculin
C
PKCα
PKCδ
30
20
10
0
Relative mRNA levels
***
1.5
1.0
0.5
0
**
H1650
H1650-M3
H1650
H1650-M3

Figure 2

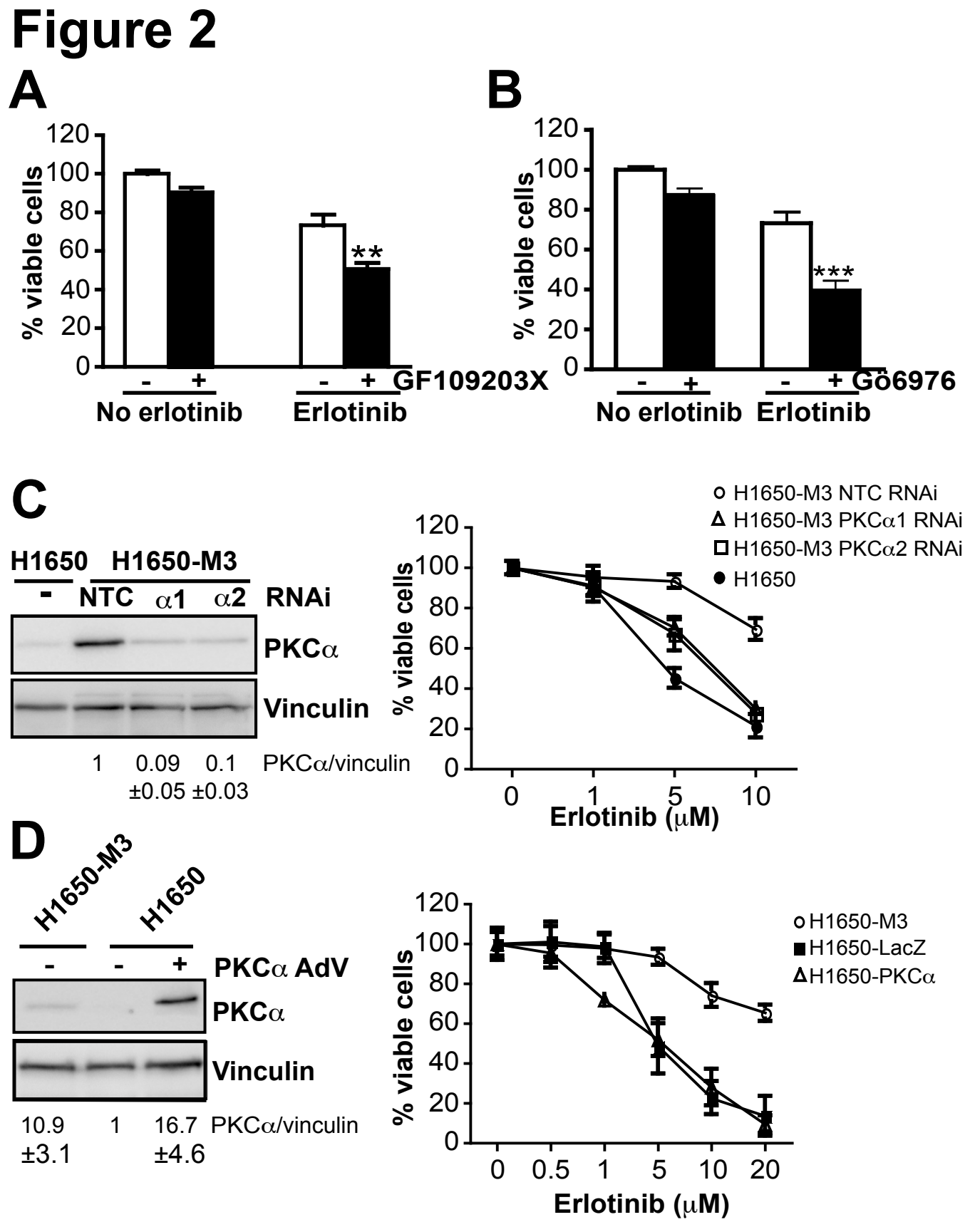
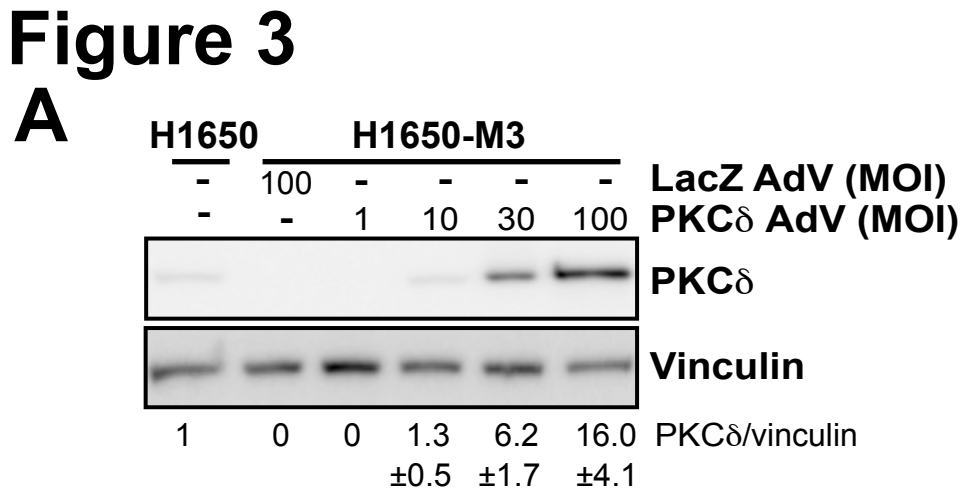

A
% viable cells
120
100
80
60
40
20
0
**
- + GF109203X
- +
No erlotinib
Erlotinib
B
% viable cells
***
- + Gö6976
- +
No erlotinib
Erlotinib
C
H1650
H1650-M3
- NTC α1 α2 RNAi
PKCα
Vinculin
1 0.09 ±0.05 0.1 ±0.03 PKCα/vinculin
H1650-M3 NTC RNAi
H1650-M3 PKCα1 RNAi
H1650-M3 PKCα2 RNAi
H1650
% viable cells
0 1 5 10
Erlotinib (μM)
D
H1650-M3
H1650
- - + PKCα AdV
PKCα
Vinculin
10.9 ±3.1 1 16.7 ±4.6 PKCα/vinculin
H1650-M3
H1650-LacZ
H1650-PKCα
% viable cells
0 0.5 1 5 10 20
Erlotinib (μM)

Figure 3


A
H1650
H1650-M3
- 100 - - - - LacZ AdV (MOI)
- - 1 10 30 100 PKCδ AdV (MOI)
PKCδ
Vinculin
1 0 0 1.3 6.2 16.0 PKCδ/vinculin
±0.5 ±1.7 ±4.1

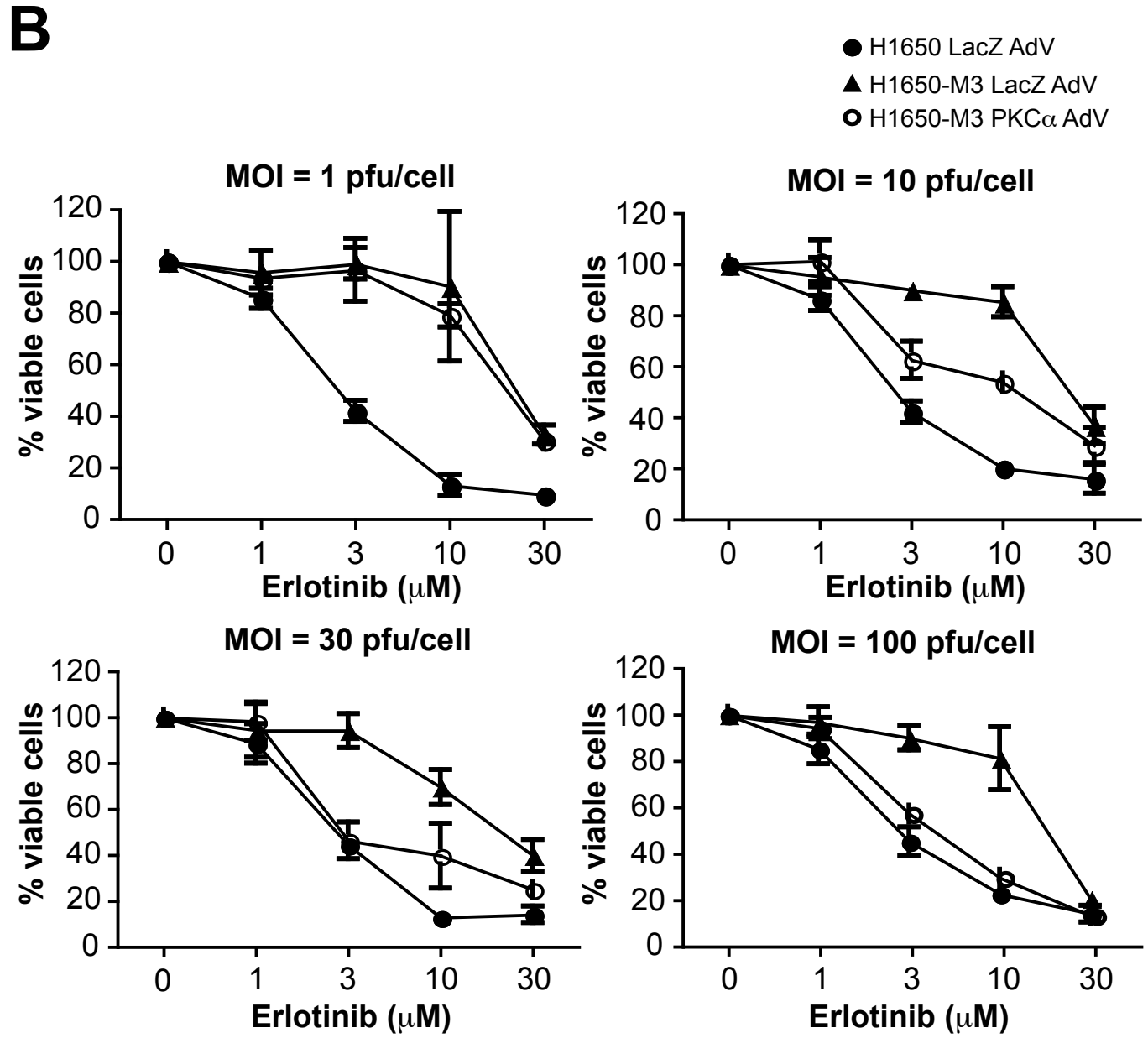

B
H1650 LacZ AdV
H1650-M3 LacZ AdV
H1650-M3 PKCα AdV
MOI = 1 pfu/cell
MOI = 10 pfu/cell
MOI = 30 pfu/cell
MOI = 100 pfu/cell
% viable cells
0 20 40 60 80 100 120
0 1 3 10 30
Erlotinib (μM)

Figure 4

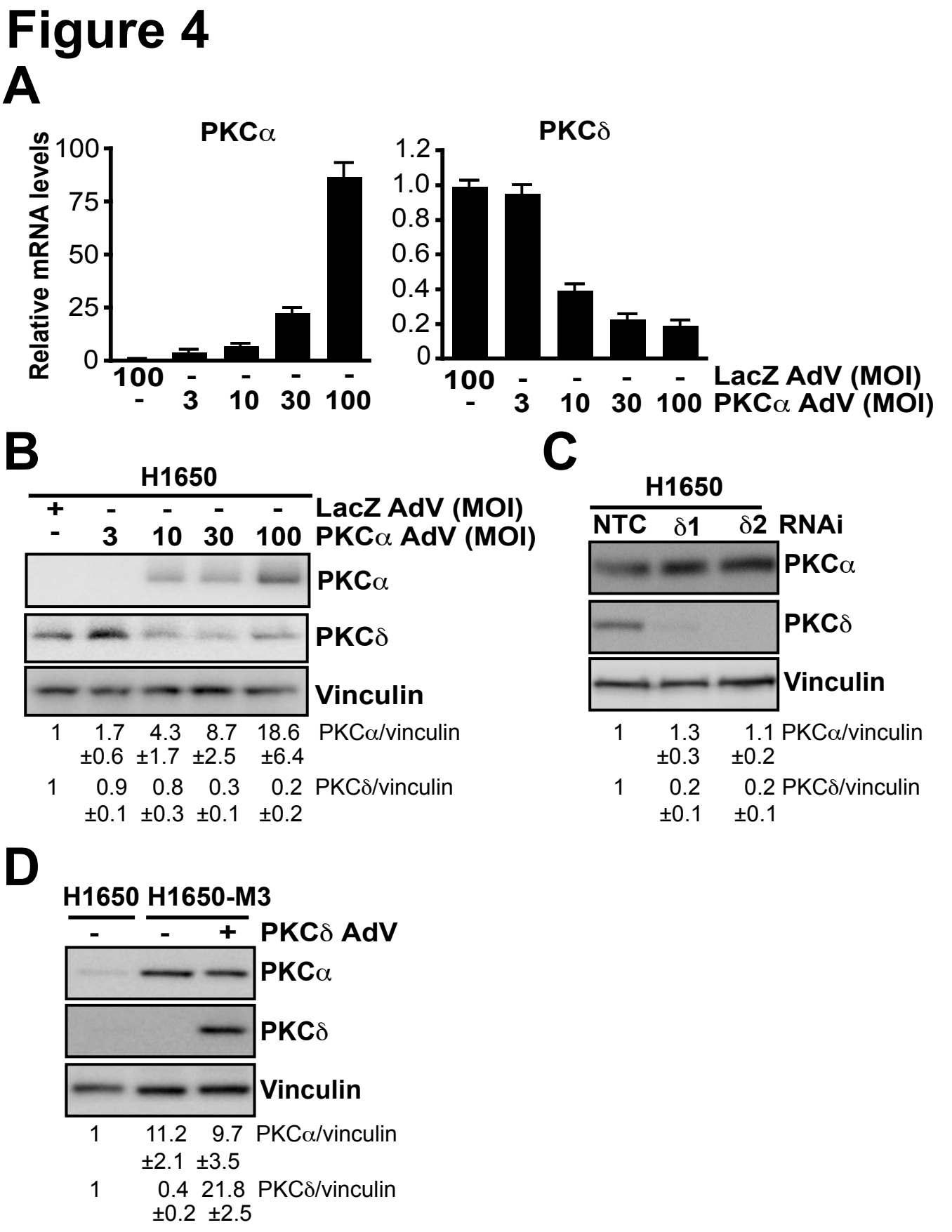

A
PKCα
PKCδ
Relative mRNA levels
100
75
50
25
0
1.2
1.0
0.8
0.6
0.4
0.2
0
100 - - - - LacZ AdV (MOI)
- 3 10 30 100 PKCα AdV (MOI)
B
H1650
+ - - - - LacZ AdV (MOI)
- 3 10 30 100 PKCα AdV (MOI)
PKCα
PKCδ
Vinculin
1 1.7 ±0.6 4.3 ±1.7 8.7 ±2.5 18.6 ±6.4 PKCα/vinculin
1 0.9 ±0.1 0.8 ±0.3 0.3 ±0.1 0.2 ±0.2 PKCδ/vinculin
C
H1650
NTC δ1 δ2 RNAi
PKCα
PKCδ
Vinculin
1 1.3 ±0.3 1.1 ±0.2 PKCα/vinculin
1 0.2 ±0.1 0.2 ±0.1 PKCδ/vinculin
D
H1650 H1650-M3
- - + PKCδ AdV
PKCα
PKCδ
Vinculin
1 11.2 ±2.1 9.7 ±3.5 PKCα/vinculin
1 0.4 ±0.2 21.8 ±2.5 PKCδ/vinculin

Figure 5

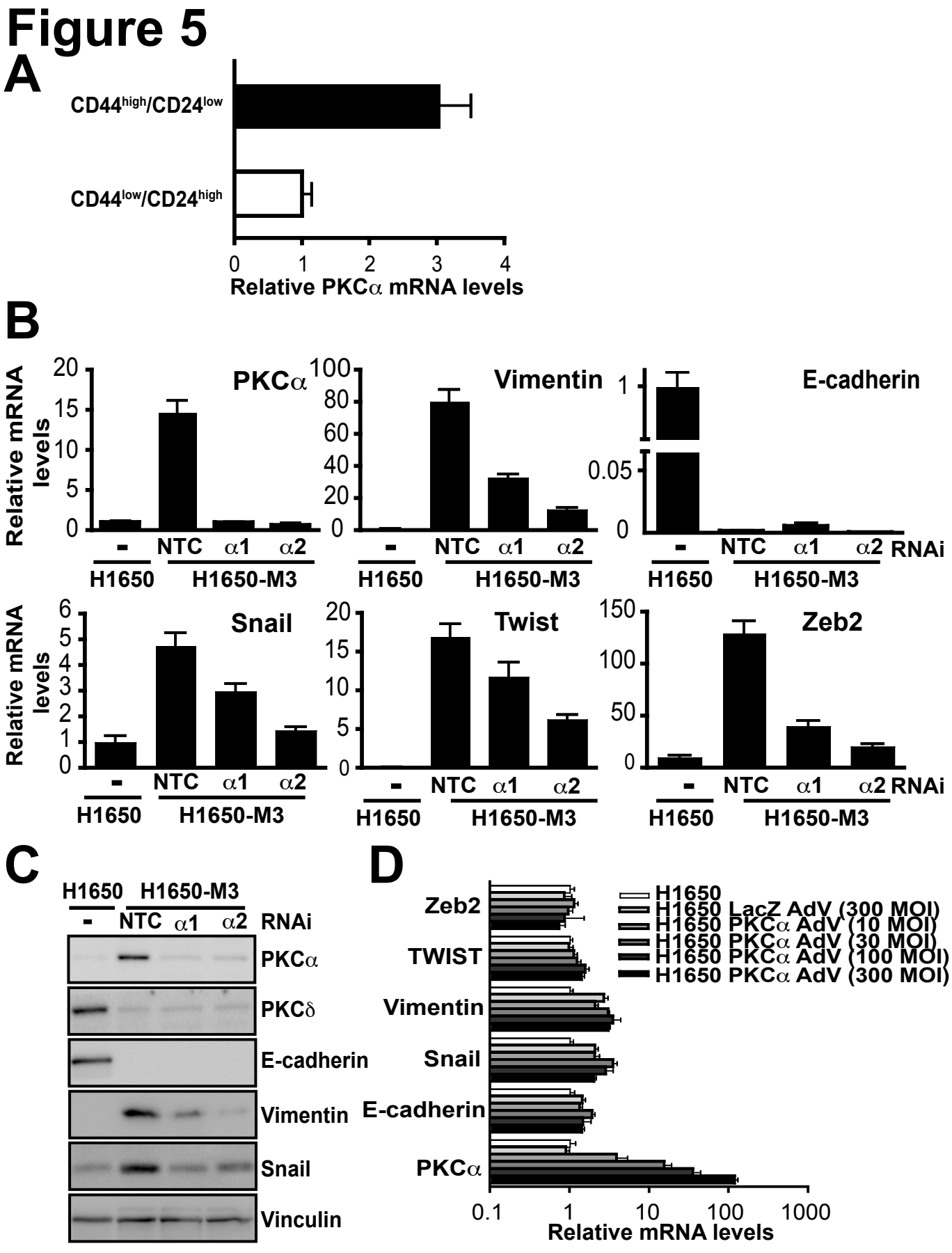

A
CD44high/CD24low
CD44low/CD24high
0
1
2
3
4
Relative PKCα mRNA levels
B
Relative mRNA levels
PKCα
Vimentin
E-cadherin
Snail
Twist
Zeb2
-
NTC
α1
α2
RNAi
H1650
H1650-M3
C
H1650
H1650-M3
PKCα
PKCδ
E-cadherin
Vimentin
Snail
Vinculin
D
Zeb2
TWIST
Vimentin
Snail
E-cadherin
PKCα
H1650
H1650 LacZ AdV (300 MOI)
H1650 PKCα AdV (10 MOI)
H1650 PKCα AdV (30 MOI)
H1650 PKCα AdV (100 MOI)
H1650 PKCα AdV (300 MOI)
0.1
1
10
100
1000
Relative mRNA levels

Figure 6

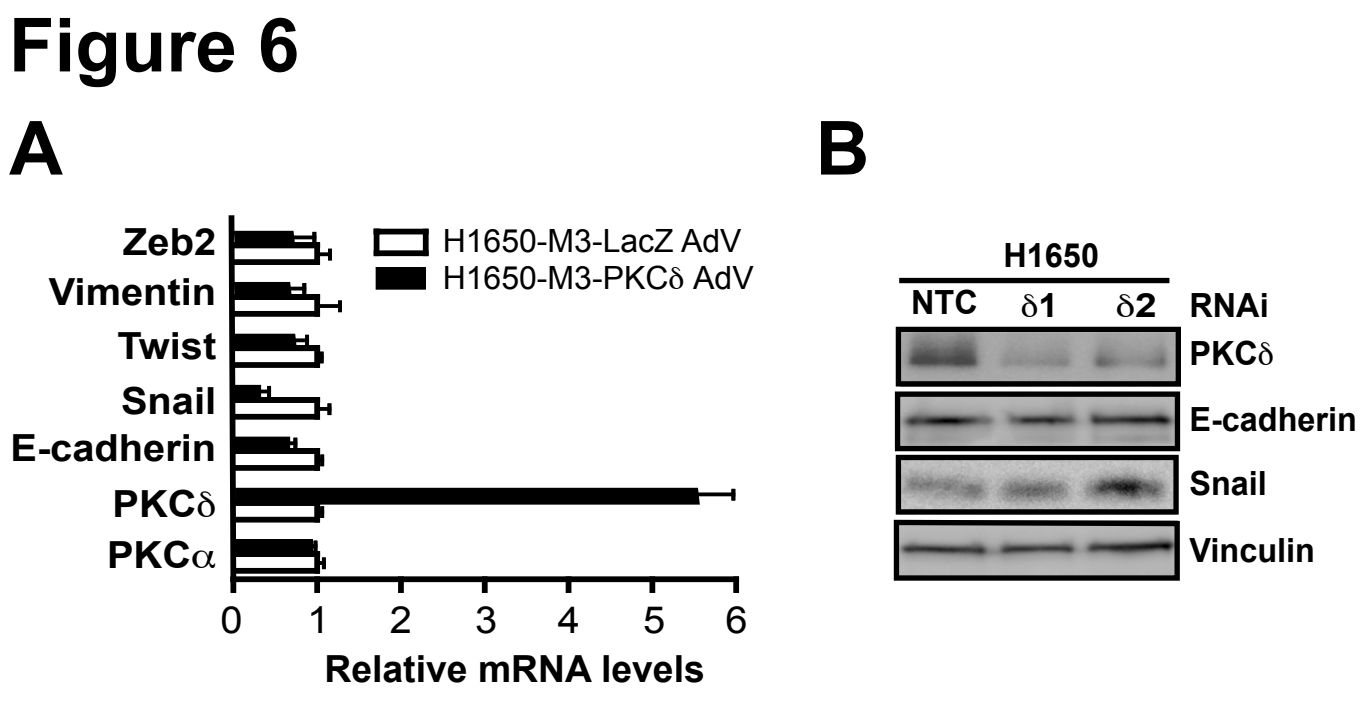
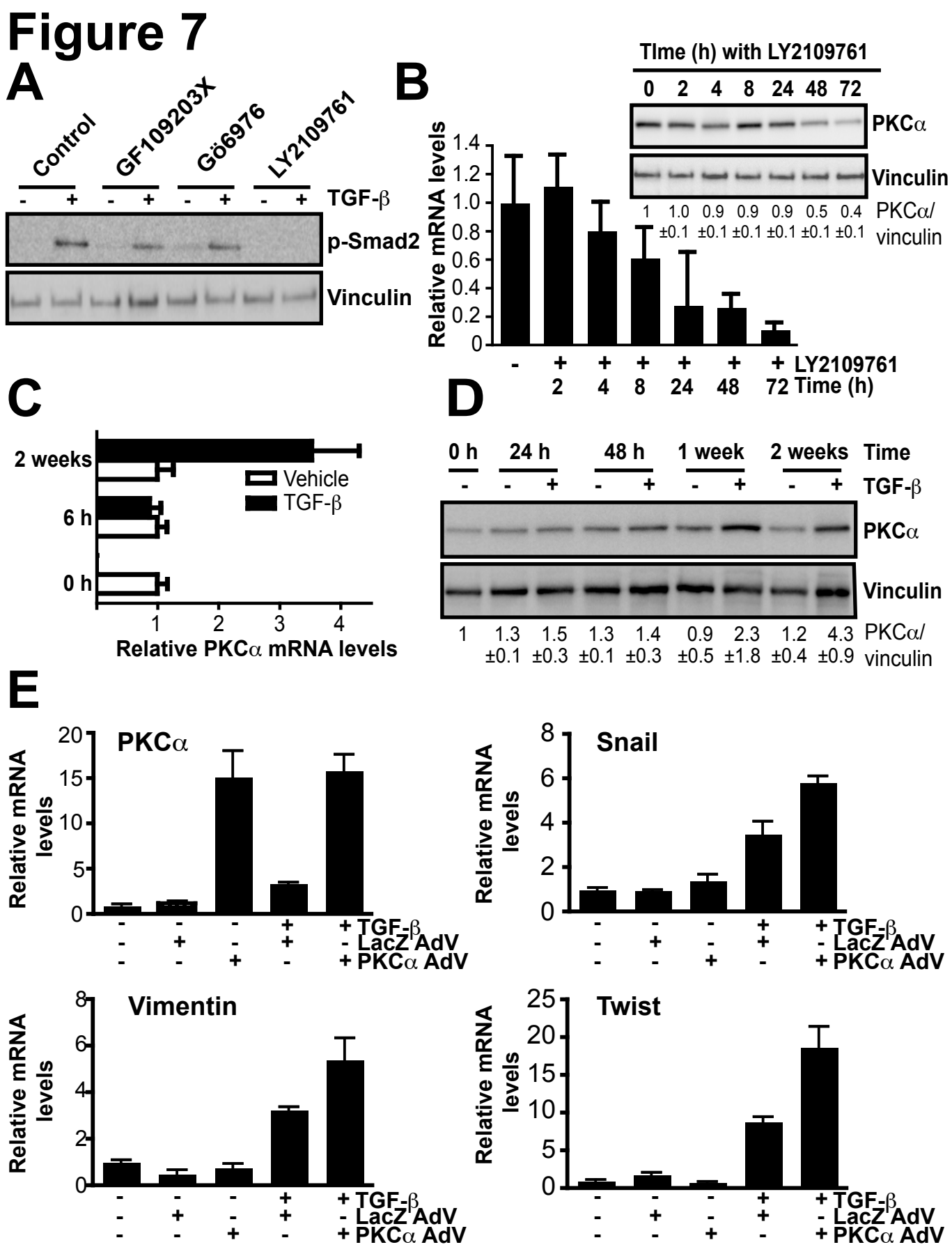

A
Zeb2
Vimentin
Twist
Snail
E-cadherin
PKCδ
PKCα
H1650-M3-LacZ AdV
H1650-M3-PKCδ AdV
0
1
2
3
4
5
6
Relative mRNA levels
B
H1650
NTC
δ1
δ2
RNAi
PKCδ
E-cadherin
Snail
Vinculin

Figure 7


A
Control
GF109203X
Gö6976
LY2109761
TGF-β
p-Smad2
Vinculin
B
TIme (h) with LY2109761
0 2 4 8 24 48 72
PKCα
Vinculin
1 1.0 ±0.1 0.9 ±0.1 0.9 ±0.1 0.9 ±0.1 0.5 ±0.1 0.4 ±0.1
PKCα/ vinculin
Relative mRNA levels
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
+ LY2109761
2 4 8 24 48 72 Time (h)
C
2 weeks
6 h
0 h
Vehicle
TGF-β
1 2 3 4
Relative PKCα mRNA levels
D
0 h 24 h 48 h 1 week 2 weeks Time
TGF-β
PKCα
Vinculin
1 1.3 ±0.1 1.5 ±0.3 1.3 ±0.1 1.4 ±0.3 0.9 ±0.5 2.3 ±1.8 1.2 ±0.4 4.3 ±0.9
PKCα/ vinculin
E
PKCα
Snail
Vimentin
Twist
Relative mRNA levels
+ TGF-β
- LacZ AdV
+ PKCα AdV