SUPPLEMENTAL MATERIALS

MATERIALS AND METHODS

Reagents and cell lines— LPA (18:1) was purchased from Avantis Lipid (Alabaster, AL). FTI-277, GGTI-298, PKC inhibitor Calphostin C, bisindolylmaleimide I, Gö6976 and U0126 were purchased from Calbiochem (San Diego, CA). The antibodies used in the study were as follows: Anti-uPA mAb from America Diagnostica (Greenwich, CO); anti-PKCα, β, γ, θ and ζ polyclonal antibodies from BD Transduction Laboratory (Lexington, KY); anti-phospho-PKCα(Ser657) polyclonal antibody, anti-PKCα, anti-Ras and anti-Bcl10 mAbs from Upstate Biotechnology (Lake Placid, NY); Anti-phospho-PKCδ(Thr505) and PKCδ polyclonal antibodies from Cell Signaling (Boston, MA); Anti-Ras polyclonal antibody from Santa Cruz Biotechnology (Santa Cruz, CA); Anti-CARMA1, 3 and anti-MALT1 polyclonal antibodies from GeneTex (San Antonio, TX). Recombinant adenovirus (Ad) containing dominant negative CARMA3 (CARMA3ΔCARD) and dominant negative PKCα, θ, and ζ (KR mutants) were obtained from Cell Biolabs (San Diego, CA). Control Ad (empty vector) and Ad containing dominant negative H-Ras have been described elsewhere (Bian et al., 2004). OVCAR3 and SK-OV3 cell lines were maintained in DMEM containing 10% fetal calf serum at 37^0C in a humidified 5% CO_2 incubator. SK-OV3 cells stably expressing constitutively active H-Ras(V12) or K-Ras(Q61) were generated as previously described (Bian et al., 2004).

siRNA Design— All siRNAs were designed using web-based BLOCK-iT™ RNAi Designer (www.invitrogen.com). The top 3 ranked siRNA sequences were chosen for each target gene. The siRNA sequences for CARMA3 are

CCTAGAAGTTCAGGAGAAA, GCTCATCCGTAACCTGCTA and TCAGCAGCATGTCAGACAT. The siRNA sequences for Bcl10 are GGACCTCACTGAAGTGAAG, GTGAAGAAGGACGCCTTAG and CCCTCACCGAGGAGGACCT. The siRNA sequences for MALT1 are GCCTGTGTCTGCTGAAGTT, GGAACACACTGAAGTTCTT and GCATTATCACTCATTGCAA. The siRNAs were purchased from Invitrogen (Carlsbad, CA). Control siRNA was obtained from Applied Biosystems (Foster City, CA).

Matrigel invasion assay— Cell invasion was analyzed using matrigel invasion chambers (Cell Biolabs, San Diego, CA) according to the manufacturer's protocol. Briefly, SK-OV-3 cells were infected with control Ad or Ad containing dominant negative PKCα or CARMA3 for 24 hr and then starved for another 24 hrs. Cells (50,000cells/well) were added to each chamber and allowed invade matrigel for 48 hrs at 37^0C, 5% CO_2 atmosphere. To induce SK-OV-3 cell invasion, 10μM LPA or 50ng/ml TNFα was added into the medium in the underwells. The matrigel and non-invading cells in the chamber were removed using cotton-swabs and the invading cells on the bottom of invasion chamber stained with crystal violet. The number of invasive cells was counted under the microscope.

RESULTS

All three classes of PKCs can induce NF-κB activation and uPA upregulation. To determine the ability of PKC isoforms to activate NF-κB, NF-κB promoter luciferase reporter gene plasmid was cotransfected into SK-OV3 cells with expression vector encoding constitutively active PKCα, θ or ζ mutant (AE mutant) for 24 hrs. Expression of constitutively active PKCα, θ or ζ mutants all significantly increased NF-κB promoter

activity over the vector control (Fig.S2B). In a parallel experiment, we introduced these constitutively active PKC expression constructs into SK-OV3 cells for 48 hrs followed by immunoblotting to detect uPA protein. All these constitutively active mutants were able to elevate endogenous uPA level (Fig.S2C). Similar results were also obtained with OVCAR3 cells (data not shown). These results suggest that PKC isoforms in all three classes possess ability to activate NF-κB and upregulate uPA expression in ovarian cancer cells.

The activity of PKCα is required for elevated NF-κB activity and uPA expression in cells expressing constitutively active Ras mutant— We previously showed that ovarian cancer cells expressing constitutively active Ras mutant (H-RasV12 or K-RasQ61) displayed elevated NF-κB activity and uPA expression (Li et al., 2005). To determine whether PKCα was involved in elevated NF-κB activity and uPA expression in these cells, we treated H-RasV12 or K-RasQ61-expressing SK-OV3 cells with calphostin C or Gö6976 for 24 hrs and subsequently analyzed NF-κB promoter activity and uPA expression. Treatment of these inhibitors inhibited NF-κB activity and uPA expression over 70% in these cells (Fig.S5A), suggesting that PKCα is involved in Ras-mediated NF-κB activation and uPA upregulation. In a parallel experiment, we also examined PKCα activity in these constitutively active Ras-expressing SK-OV3 cells. Immunoblotting with anti-phospho-PKCα(Ser657) polyclonal antibody showed that PKCα activity was elevated in cells expressing these constitutively active Ras mutants in comparison with the vector control cells (Fig.S5B). However, treatment of these cells with FTI-277 but not GGTI-298 or MEK1/2 inhibitor U0126 decreased PKCα activity (Fig.S5B). These results suggest that Ras alone is sufficient to activate PKCα.

PKCδ is not involved in LPA-induced uPA upregulation— In a recent study, PKCδ was shown to mediate LPA-induced NF-κB activation in NIH-3T3 cells (Cummings et al., 2004). To investigate the potential involvement of PKCδ in LPA-induced NF-κB activation and uPA upregulation in ovarian cancer cells, we first determined whether LPA could activate PKCδ in ovarian cancer cells. Immunoblotting with anti-phospho-PKCδ(Thr505) antibody showed that the levels of phospho-PKCδ(Thr505) was similar in the entire 1-hr period of TNFα stimulation in both SK-OV-3 and OVCAR3 lines (Fig.S6A), suggesting that TNFα was unable to activate PKCδ in these two ovarian cancer cell lines. In further experiments, we treated SK-OV-3 and OVCAR3 cells with PKCδ inhibitor rhottlerin or expressed dominant negative PKCδ in these cells followed by determining LPA-induced NF-κB activation and uPA upregulation. Inhibiting PKCδ by either rhottlerin or dominant negative mutant exhibited no significant effect in both LPA-induced events (Fig.S6B). These results thus rule out the involvement of PKCδ in LPA-induced NF-κB activation and uPA upregulation in ovarian cancer cells.

REFERENCES

Bian D, Su S, Mahanivong C, Cheng RK, Han Q, Pan ZK, Sun P and Huang S. (2004). *Cancer Res*, **64,** 4209-17.

Cummings R, Zhao Y, Jacoby D, Spannhake EW, Ohba M, Garcia JG, Watkins T, He D, Saatian B and Natarajan V. (2004). *J Biol Chem*, **279,** 41085-94.

Li H, Ye X, Mahanivong C, Bian D, Chun J and Huang S. (2005). *J Biol Chem*, **280,** 10564-71.

FIGURE LEGEND FOR SUPPLEMENTARY FIGURES

Fig.S1. PKC inhibitors do not inhibit TNFα-induced NF-κB activation and uPA upregulation. A. SK-OV-3 and OVCAR3 cells were treated with 0.5μM calphostin C (Cal C), 10μM bisindolylmaleimide I (Bis I) or 5μM Gö6976 for 2 hrs, and then 50ng/ml TNFα added to cells for 4 or 16 hrs. ***A.*** Following 4-hr TNFα stimulation, cells were harvested and subsequently analyzed for NF-κB promoter activity. ***B.*** Following 16-hr TNFα stimulation, cells were lysed and cell lysates subjected to immunoblotting to detect uPA and actin with the respective antibodies.

Fig.S2. All three classes of PKC isoforms possess ability to activate NF-κB and upregulate uPA expression. ***A.*** Lysates of SK-OV3 and OVCAR3 cells were subjected to immunoblotting to detect PKC isoforms with the respective antibodies. + denotes the positive control provided by manufacturer. ***B.*** To determine the effect of constitutively active PKCα, θ and ζ on NF-κB promoter activity, cells were transfected with pNF-κB-Luc, pAc-RLuc and mammalian expression vector encoding constitutively active PKCα, θ or ζ at 10:0.3:30 ratio for 24 hrs and then starved for another 24 hrs. Cells were lysed and cell lysates analyzed for NF-κB promoter activity. SK-OV-3 cells cotransfected with pNF-κB-luc and expression vector encoding constitutively active PKCα, PKCθ or PKCζ for 24 hrs and then analyzed for NF-κB promoter activity. Control is the cells transfected with pNF-κB-luc and empty expression vector. ***C.*** SK-OV3 cells were transfected with empty vector (control) or vector encoding constitutively active PKCα, PKCθ or PKCζ for 24 hrs. Cells were switched to serum-free medium for another 24 hr and then analyzed for uPA and actin with the respective antibodies.

Fig.S3. PKCα activity is not required for TNFα-induced NF-κB activation and uPA upregulation. A. SK-OV-3 and OVCAR3 were cotransfected with pNF-κB-luc for 24 hrs and then infected with control Ad or Ad containing dominant negative PKCα for another 24 hrs. After 24-hr starvation, cells were stimulated with 50ng/ml TNFα for 4 hrs followed by assays to determine NF-κB promoter activity. ***B.*** SK-OV-3 or OVCAR3 cells were infected with control Ad or Ad containing dominant negative PKCα for 24 hrs, and then starved for 24 hrs. Cells were stimulated with 50ng/ml TNFα for 16 hrs followed by immunoblotting to detect uPA and actin with the respective antibodies.

Fig.S4. CARMA3, Bcl10 and MALT1 are not involved in TNFα-induced NF-κB activation and uPA upregulation. *A.* Untransfected or pNF-κB-luc-transfected SK-OV-3 or OVCAR3 cells were infected with control Ad or Ad containing CARD-deleted CARMA3 [Ad.CARMA3(-)] for 24 hrs and then starved for another 24 hrs. Transfected cells were stimulated with 50ng/ml TNFα for 4 hrs followed by assaying NF-κB promoter activity. Untransfected cells were also stimulated with 50ng/ml TNFα for 16 hrs and then subjected to immunoblotting to detect uPA. ***B.*** SK-OV3 and OVCAR3 cells were transfected with 100nM control or CARMA3 siRNAs for 2 days. Part of siRNA-treated cells were transfected with pNF-κB-luc for 24 hrs, then starved for 24 hrs and stimulated 50ng/ml TNFα for 4 hrs followed by analysis of NF-κB promoter activity. Remaining siRNA-treated cells were starved for 24 hrs and then stimulated with 50ng/ml TNFα for 16 hrs. Cells were lysed and cell lysates subjected to immunoblotting to detect uPA and actin with the respective antibodies. ***C.*** SK-OV3 cells were transfected with 100nM control or Bcl10 siRNAs for 2 days. Part of the cells was again transfected with pNF-κB-luc for 24 hrs and then starved for 24 hrs followed by 50ng/ml TNFα stimulation

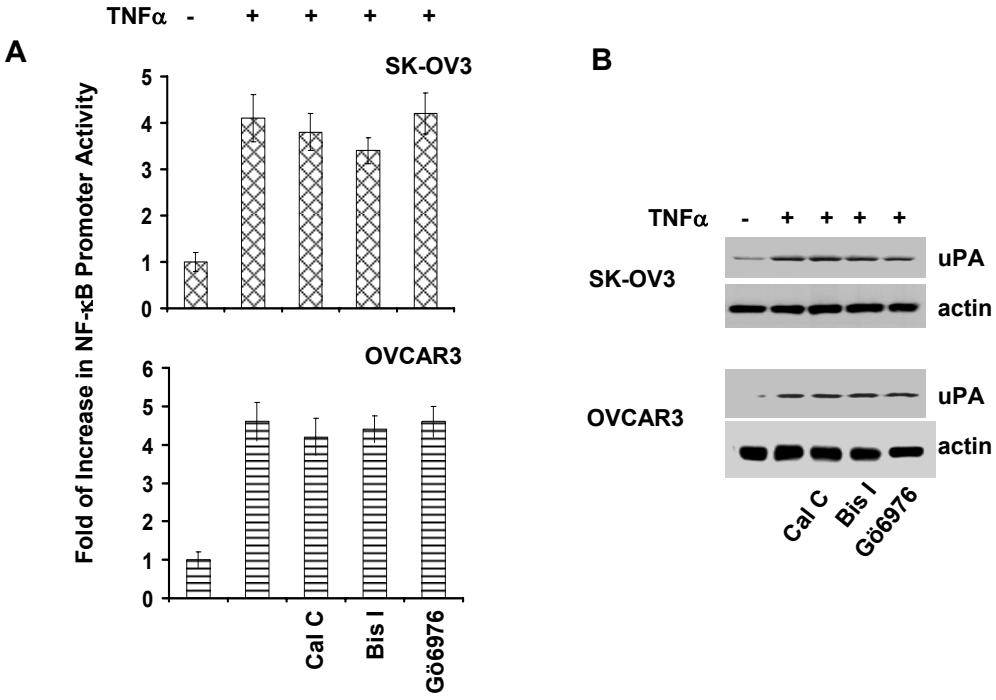
for 4 hrs. Cells were harvested and assayed for NF-κB promoter activity. The remaining cells were starved for 24 hrs and then stimulated with 50ng/ml TNFα for 16 hrs followed by immunoblotting to detect uPA and actin with the respective antibodies. ***D.*** SK-OV3 cells were transfected with 100nM control or MALT1 siRNAs for 2 days. Part of the cells was again transfected with pNF-κB-luc for 24 hrs and then starved for 24 hrs followed by 50ng/ml TNFα stimulation for 4 hrs. Cells were harvested and assayed for NF-κB promoter activity. The remaining cells were starved for 24 hrs and then stimulated with 50ng/ml TNFα for 16 hrs followed by immunoblotting to detect uPA and actin with the respective antibodies.

Fig.S5. Elevated NF-κB activity and uPA expression in constitutively active Ras-expressing cells are dependent on PKC activity. ***A.*** SK-OV-3 cells expressing constitutively active Ras mutants (SK-OV3/H-RasV12 or SK-OV3/K-RasQ61) were treated with 0.5μM calphostin C or 5μM Gö6976 for 24 hrs, then harvested and lysates either analyzed for NF-κB promoter activity or uPA protein level. ***B.*** SK-OV3/H-RasV12 or SK-OV3/K-RasQ61 cells were either treated with 1μM FTI-277, 1μM GGTI-298, 2μM U0126 or left untreated for 24 hrs. Cells were then lysed and cell lysates subjected to immunoblotting to detect phosphor-PKCα(Ser657) and PKCα with the respective antibodies.

Fig.S6. PKCδ is not involved in LPA-induced uPA upregulation. ***A.*** SK-OV-3 or OVCAR3 cells were starved for 24 hrs and then stimulated with 10μM LPA for various times. Cells were lysed and cell lysates subjected to immunoblotting to detect phospho-PKCδ(Thr505) and PKCδ with the respective antibodies. ***B.*** In one set, SK-OV-3 or OVCAR3 cells were infected with control Ad or Ad containing dominant negative PKCδ

for 24 hrs and then starved for another 24 hrs. In second set, 24-hr-starved cells were treated with 5μM rhottlerin for 2 hrs. Cells were then stimulated with 10μM LPA for 16 hrs followed by immunoblotting to detect uPA protein with uPA mAb.

Fig.S1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3
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Bis I
Gö6976
B
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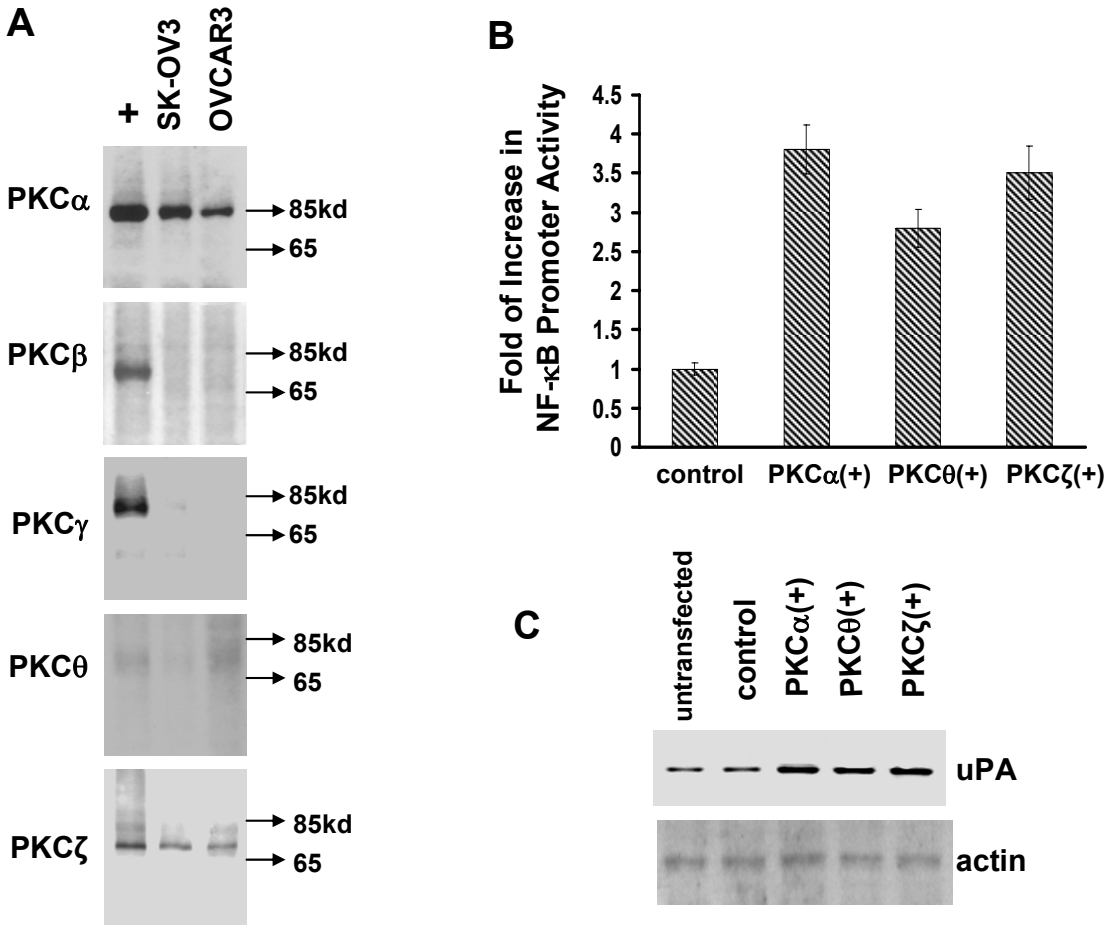
Fig.S2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2
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1
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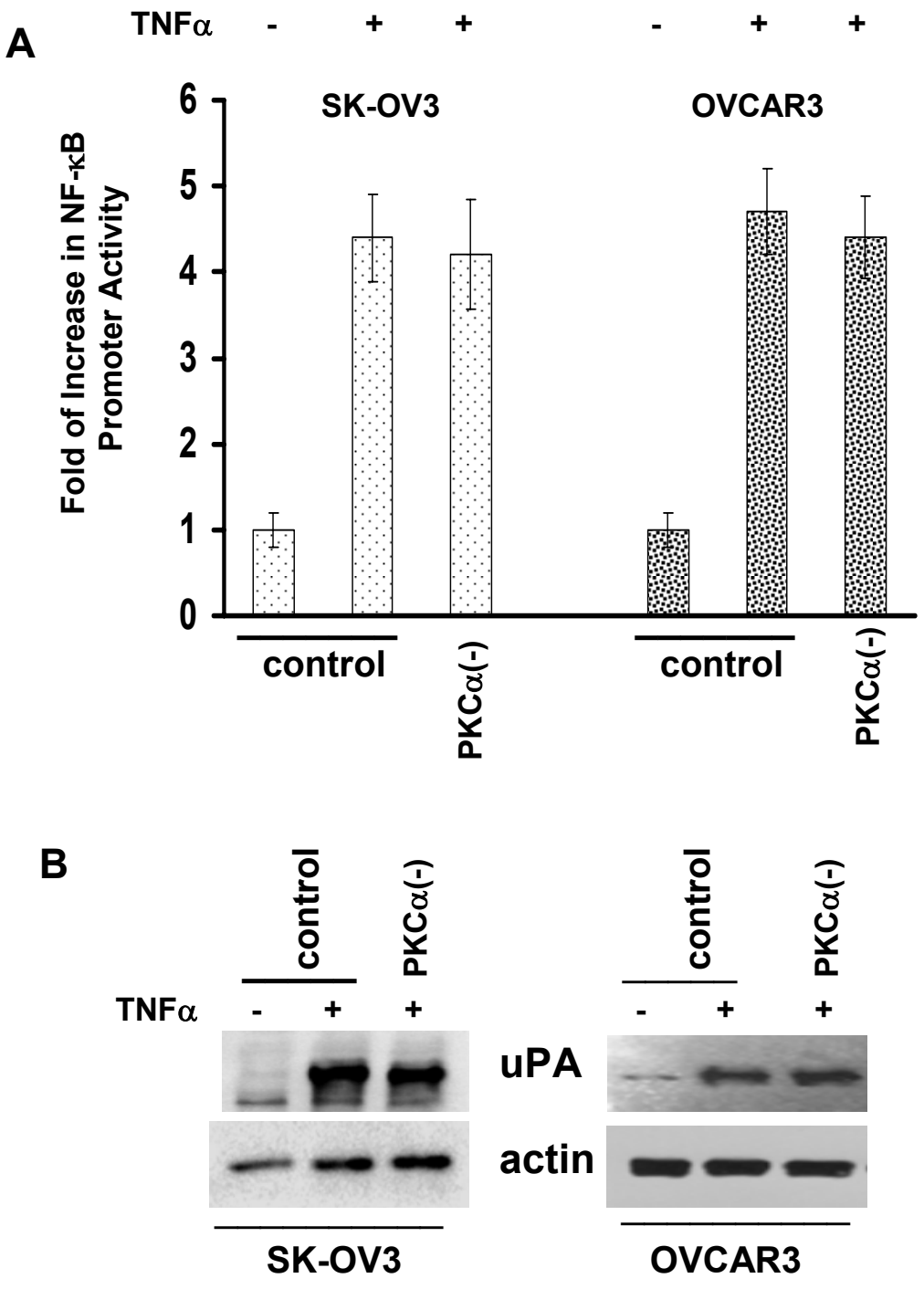
Fig.S3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control
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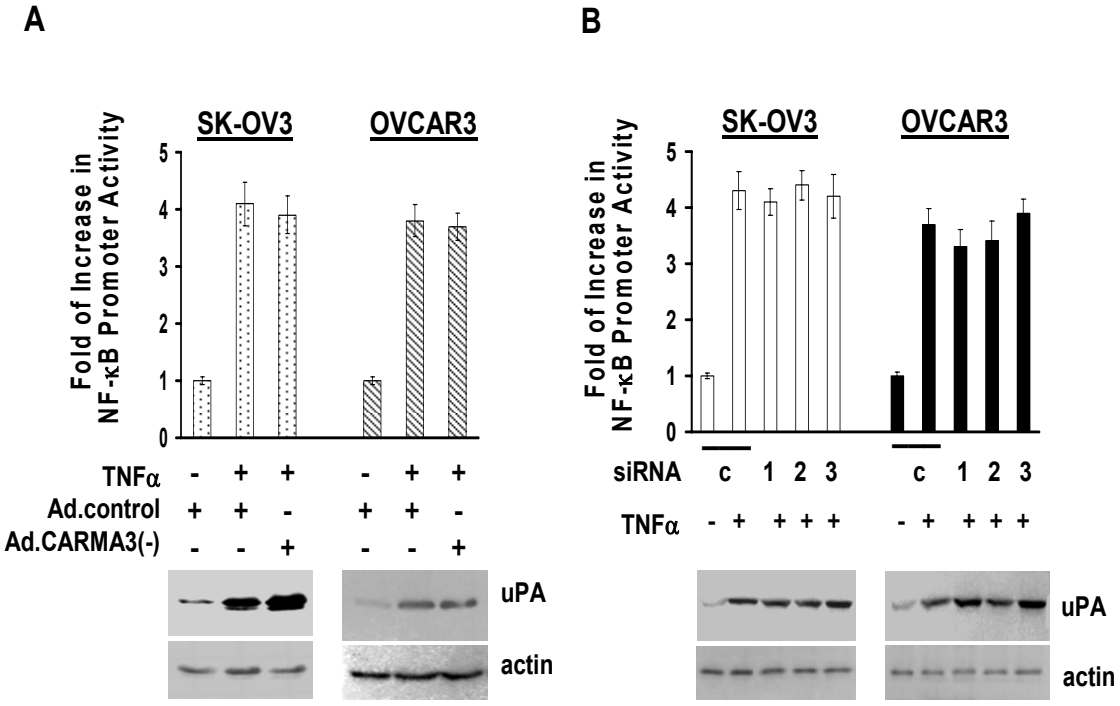
Fig.S4A-B


A
B
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Fold of Increase in NF-κB Promoter Activity
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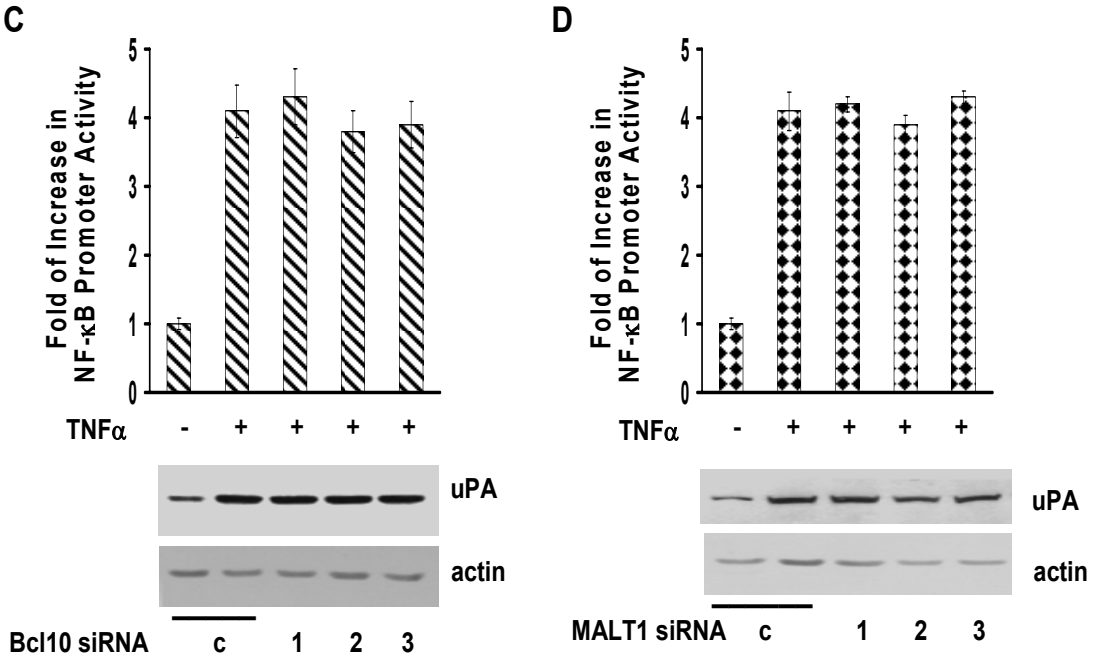
Fig.S4C-D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actin
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Fold of Increase in NF-κB Promoter Activity
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Fig.S5

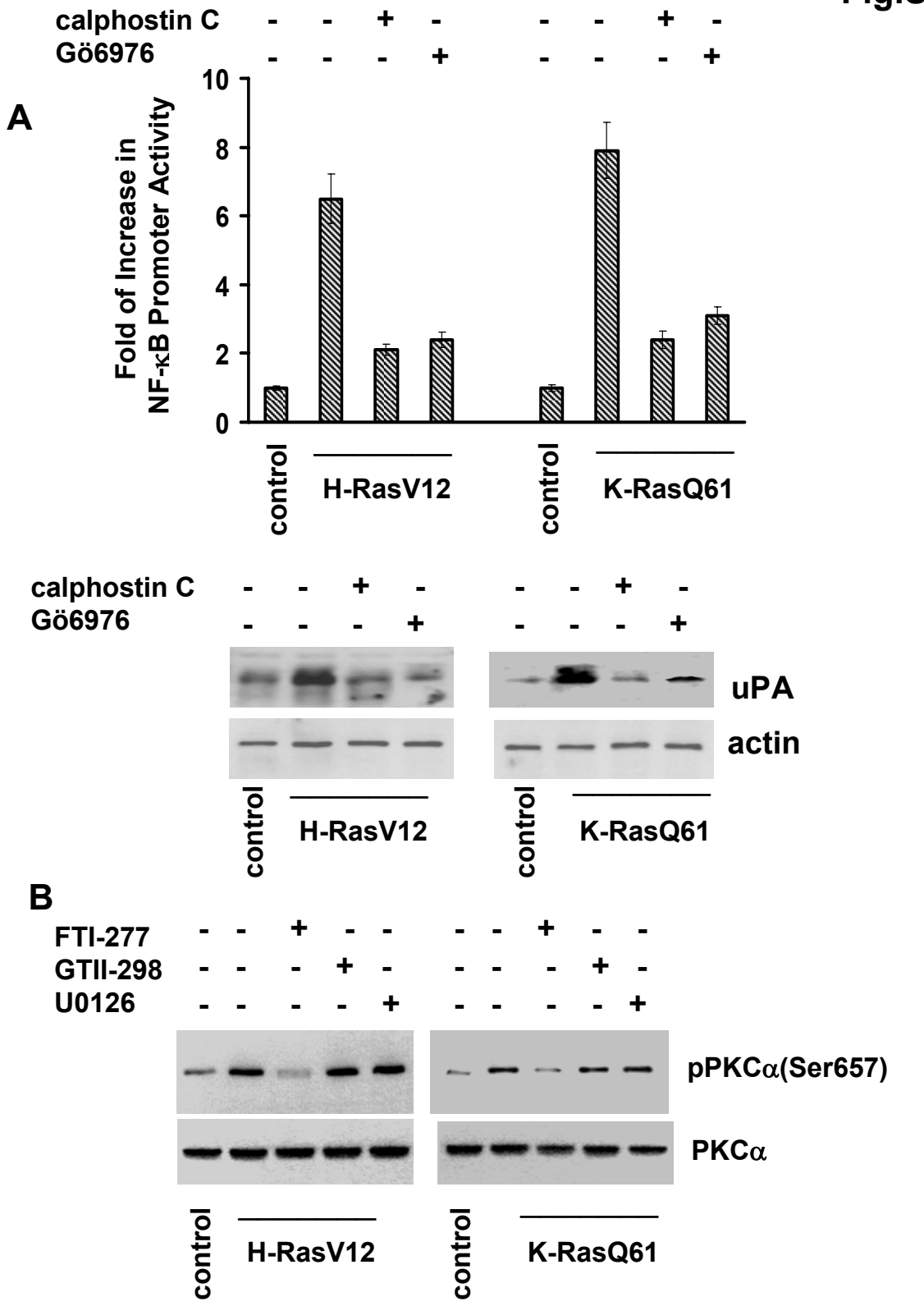
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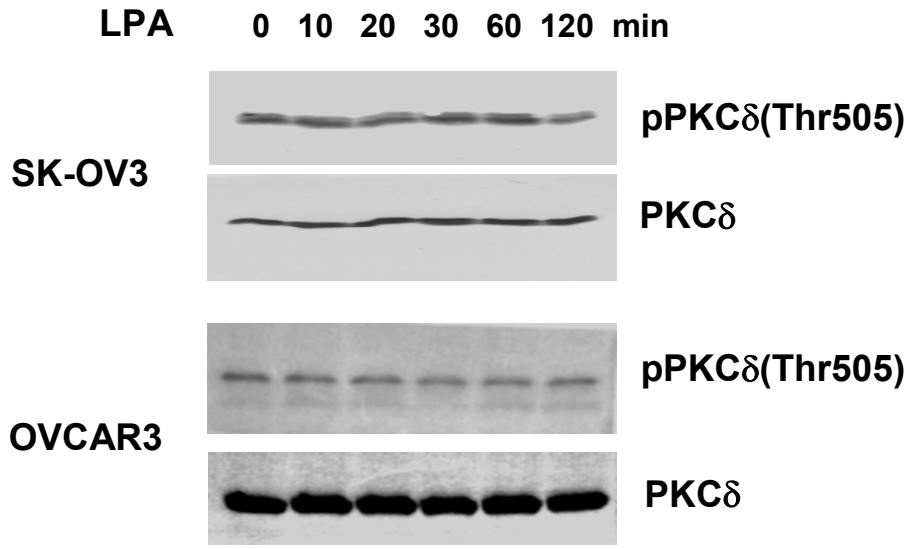
Fig.S6

A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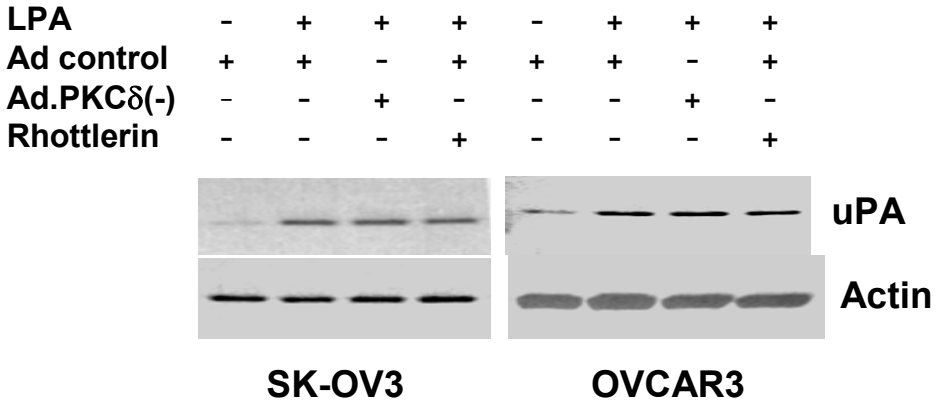
B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SK-OV3
OVCAR3