PATZ1 induces PP4R2 to form a negative feedback loop on IKK/NF-κB signaling in lung cancer

SUPPLEMENTARY FIGURES

Supplementary Figure S1: Resurgence of phospho-AktS473 at the late phase of PIP3 and growth factor stimulation is not followed up by activation of IKK/NF-κB signaling, COX-2 and Snail in A549 lung cancer cells. Cells were treated for various durations with 5 μM of PIP3 and 100 ng/ml of EGF, HGF or IGF-1 as indicated. Cells were examined by immunoblotting for protein expression. β–actin was used as a loading control. Blots are representative of three independent experiments.
Supplementary Figure S2: In the late phase, PGE2 increases PP4R2 as well as the association of PP4C with PP4R2 and IKKα/β to dephosphorylate phospho-IKKα/β. A549 cells were treated with PGE2 (20 μg/ml) for various durations as indicated. A. The cell extracts were immunoprecipitated with anti-PP4C or control IgGs antibodies, and the pull-down proteins were detected by immunoblotting. B. Myc:PP4R2 (55 kDa), Myc:PP4C (40 kDa) and Flag:phospho-IKKβ (95 kDa) were isolated from Myc:PP4R2-, Myc:PP4C- or Flag:DA-IKKβ-transfected CL1-0 cells respectively. These isolated proteins were co-incubated as indicated in vitro. IKK phosphorylation was assessed by immunoblotting. Blots are representative of three independent experiments.
Supplementary Figure S3: PP4C knockdown results in the upregulation of IKK/NF-κB signaling, COX-2 and Snail without affecting the association of PP4R2 with phospho-IKK at the late phase of PGE2 stimulation. Lung cancer cells (A549) were transfected with PP4C siRNA (siPP4C) or control scrambled siRNA (siCont) for 48 h and then treated with PGE2 (20 μg/mL) for the various durations as indicated. A and B. Cells were examined by immunoblotting for protein expression. C. The cell extracts were immunoprecipitated with anti-phospho-IKKα/βS176/180 or control IgGs antibodies, and the pull-down proteins were detected by immunoblotting (top panel). PP4C activity was determined by using phosphopeptide K-R-pT-I-R-R as substrate (bottom panel). Blots are representative of three independent experiments. Data represent means ± s.d. of three independent experiments; *, P < 0.05 by t-test.
Supplementary Figure S4: PATZ1 directly activates PP4R2 gene transcription. A. Overexpression of PATZ1 increased PP4R2 reporter activity in a dose-dependent manner. Parental cells (A549 and CL1-5; C) were cotransfected with reporter plasmids pGL3-PP4R2, pRL-TK and plasmids of empty vector (EV; 1 μg) or PATZ1 (pPATZ1; 0.25-1 μg) as indicated for 24 h. The transfected cells were examined by luciferase reporter assay. B. Knockdown of PATZ1 reversed PIP3-induced PP4R2 reporter activity. Lung cancer cells (A549 and CL1-5) were transfected with control scrambled siRNA (-siPATZ1) or PATZ1 siRNA (+siPATZ1) for 48 h and then treated with PIP3 (5 μM). The treated cells were examined by luciferase reporter assay. C. Analysis of the interaction between PATZ1 and the PP4R2 promoter by ChIP assay. A549 cells treated with PIP3 (5 μM) were cross-linked with formaldehyde and chromatin immunoprecipitation was performed with antibodies against PATZ1. Recruitment of PATZ1 to the PP4R2 promoter was analyzed by SYBR green real time PCR with primers for the PP4R2. Data represent means ± s.d. of three independent experiments. *, P < 0.05 and **, P < 0.01 by t-test.
Supplementary Figure S5: Knockdown of PP4R2 or PP4C but not PP4R1 increases phospho-NF-κB, COX-2, Snail and MMP-2 activity as well as migration/invasion of the lung cancer cells. Lung cancer cells (A549, H1299, CL1-0, CL1-5) were transfected either without (C) or with control scrambled siRNA (siCont), PP4R1 siRNA (siPP4R1), PP4R2 siRNA (siPP4R2) or PP4C siRNA (siPP4C) for 48 h as indicated. 

A. The cell lysates were examined by immunoblotting for protein expression.

B and C. The activities of MMP-2 and MMP-9 in cell-conditioned media were analyzed by gelatin zymography after culturing cells in serum-free medium for 24 h. The migration and invasion abilities of cells were examined by transwell assay after 20 h incubation. Blots are representative of three independent experiments. Data represent means ± s.d. of three independent experiments; *, P < 0.05 and **, P < 0.01 by t-test.
Supplementary Figure S6: Knockdown of PATZ1 attenuates PP4R2 and increases phospho-NF-κB, COX-2, Snail and MMP-2 activity as well as migration/invasion of the lung cancer cells. Lung cancer cells (A549, H1299, CL1-0, CL1-5) were transfected either without (C) or with control scrambled siRNA (siCont) or PATZ1 siRNA (siPATZ1) for 48 h. A. The cell lysates were examined by immunoblotting for protein expression. B and C. The activities of MMP-2 and MMP-9 in cell-conditioned media were analyzed by gelatin zymography after culturing cells in serum-free medium for 24 h. The migration and invasion abilities of cells were examined by transwell assay after 20 h incubation. Blots are representative of three independent experiments. Data represent means ± s.d. of three independent experiments; *, P < 0.05 and **, P < 0.01 by t-test.
Supplementary Figure S7: Overexpression of PP4R2 or PP4C but not PP4R1 attenuates phospho-NF-κB, COX-2, Snail and MMP-2 activity as well as migration/invasion of the lung cancer cells. Lung cancer cells (A549, H1299, CL1-0, CL1-5) were transfected either without (C) or with control empty vector (EV), pCMV6-PP4R1 (pPP4R1), pCMV6-PP4R2 (pPP4R2) or pCMV6-PP4C (pPP4C) for 48 h as indicated. A. The cell lysates were examined by immunoblotting for protein expression. B and C. The activities of MMP-2 and MMP-9 in cell-conditioned media were analyzed by gelatin zymography after culturing cells in serum-free medium for 24 h. The migration and invasion abilities of cells were examined by transwell assay after incubation for 20 h. Blots are representative of three independent experiments. Data represent means ± s.d. of three independent experiments; *P < 0.05 by t-test.
Supplementary Figure S8: Knockdown of PP4R2 and PP4C reverses PATZ1-inhibited IKK/NF-κB signaling, MMP-2 activity and migration/invasion of lung cancer cells. Lung cancer cells (A549, H1299 and CL1-5) were transfected either without (C) or with empty vector (EV), PATZ1 plasmid (pPATZ1), control scrambled siRNA (siCont), PP4R1 siRNA (siPP4R1), PP4R2 siRNA (siPP4R2) or PP4C siRNA (siPP4C) for 48 h as indicated. A. The cell lysates were examined by immunoblotting for protein expression.
B and C. The activities of MMP-2 and MMP-9 in cell-conditioned media were analyzed by gelatin zymography after culturing cells in serum-free medium for 24 h. The migration and invasion abilities of cells were examined by transwell assay after incubation for 20 h. Blots are representative of three independent experiments. Data represent means ± s.d. of three independent experiments; *P < 0.05 by t-test.