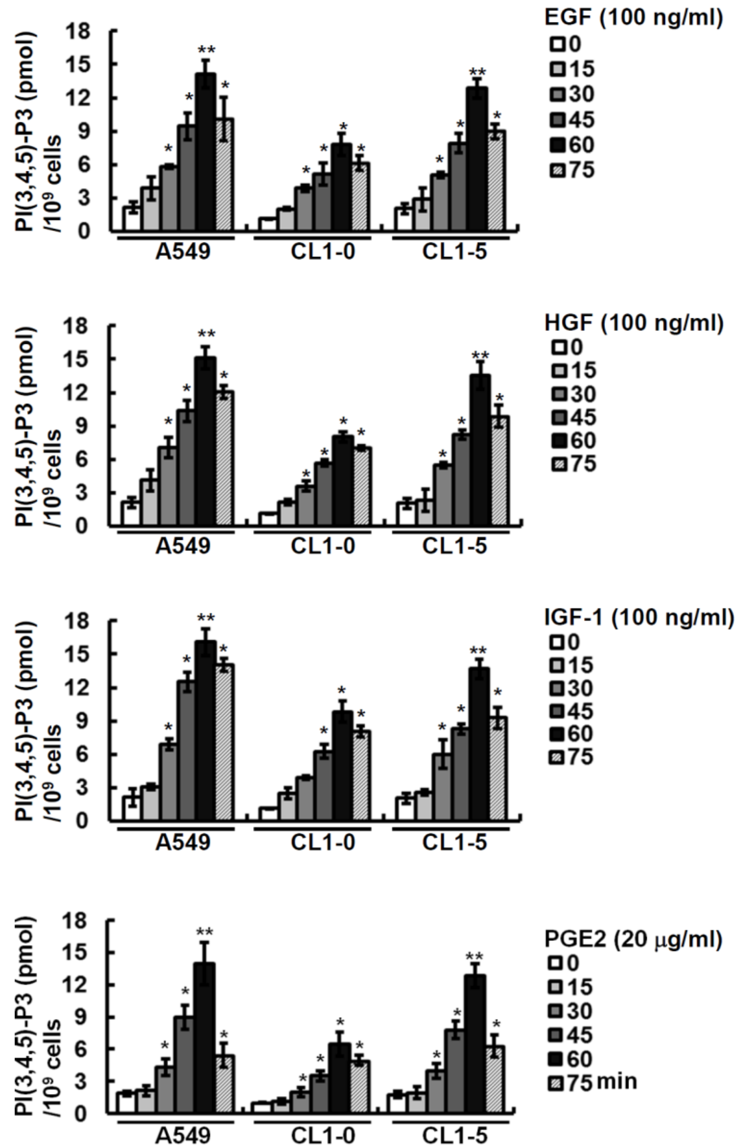
MIG-7 and phosphorylated prohibitin coordinately regulate lung cancer invasion/metastasis

Supplementary Materials

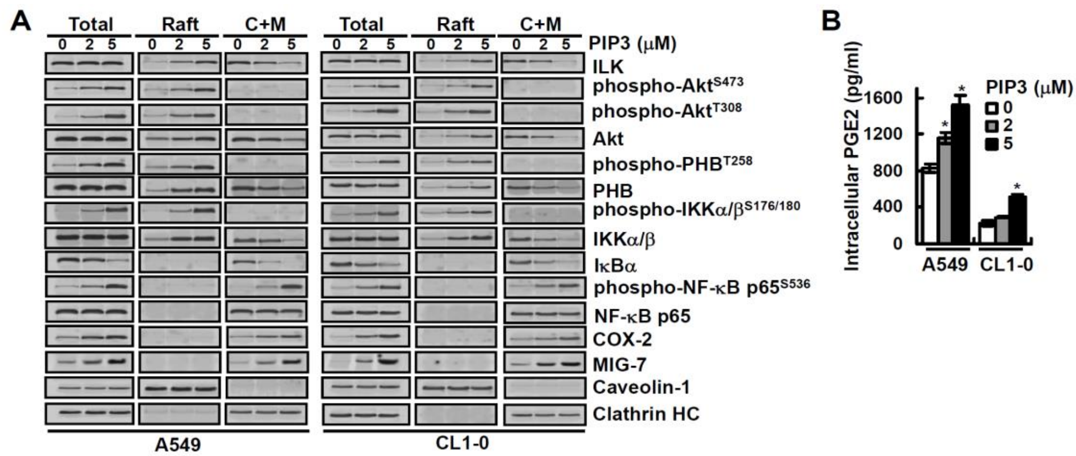
Materials

Recombinant human EGF, HGF and IGF-1 were obtained from R&D Systems (Abingdon, UK). pUSEamp, pUSEamp-myr-*Akt*1 (dominant active) and pUSEamp-*Akt*1 K179M (dominant negative) were purchased from Upstate Biotechnology (Lake Placid, NY). pCMV6-*MEKK1* (wild type) and pCMV-*MEKK1* K1108M (dominant negative) were purchased from ORIGENE (Rockville, MD). Control siRNAs, EP4-siRNA, EP2-siRNA, EP1-siRNA, IKKα/β-siRNA and Raf-1-siRNA were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The MIG7-1 and MIG7-2 shRNA-encoding sequences cloned in pGPU6/Neo siRNA expression vectors were obtained from GenDiscovery Biotechnology (Taipei, Taiwan).[8] Three siRNAs for the 3′UTR of PHB (siRNA ID 143790, 11963 and 11867) were synthesized and a siRNA control was supplied by Invitrogen [9]. Mouse anti-Vimentin (V9) and recombinant PGE2 were purchased from Sigma-Aldrich (St Louis, MO). Phosphatidylinositol (3,4,5)-trisphosphate diC16 (PI(3,4,5)P3 diC16) was purchased from Echelon Biosciences (Salt Lake City, UT). Rabbit anti-EP4 antibodies were obtained from Cayman Chemicals (Ann Arbor, MI). Rabbit anti-MIG-7 (ab83494), mouse anti-MEKK1 (ab55653), rabbit anti-MEKK3 and rabbit

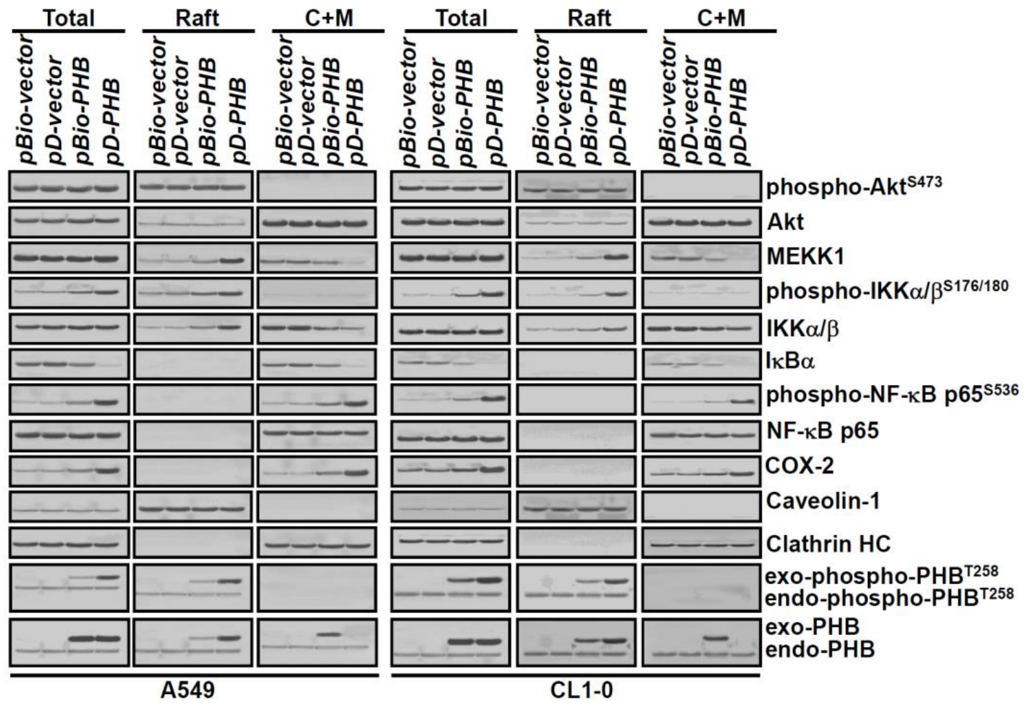
anti-NIK antibodies were purchased from Abcam (Cambridge, UK). Rabbit-anti-PHB (H-80), rabbit-anti-IKKα/β (H-470), mouse anti-β-actin (C-4), goat anti-COX-2 (M-19), rabbit anti-E-cadherin (H-108), rabbit anti-ZEB1 (H-102), rabbit-anti-Snail (H-130) and rabbit anti-Twist (H-81) antibodies as well as horseradish peroxidase-conjugated anti-mouse IgG, horseradish peroxidase-conjugated anti-goat IgG and horseradish peroxidase-conjugated anti-rabbit IgG antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit-anti-ILK, rabbit anti-PI3Kp85, rabbit anti-PI3Kp85^{Y458}, rabbit anti-Akt, rabbit anti-phospho-Akt^{Ser473}, rabbit anti-GSK-3β, rabbit-anti-phospho-GSK-3β^{Ser9}, rabbit-anti-phospho-IKKα/β$^{S176/S180}$, rabbit anti-phospho-NF-κBp65^{S536}, rabbit anti-phospho-MYPT1^{T696}, rabbit anti-MYPT1, rabbit anti-phospho-MLC2$^{T18/S19}$, rabbit anti-MLC2, rabbit anti-clathrin HC and rabbit anti-hemagglutinin (HA) antibodies were obtained from Cell Signaling Technology (Beverly, MA). Rabbit anti-caveolin-1, rabbit anti-IκB, rabbit anti-NF-κBp65, mouse anti-Ras, rabbit anti-Raf-1, mouse anti-phospho-Raf-1^{S338}, rabbit anti-ERK1/2 and rabbit anti-phospho-ERK1/2$^{T185/Y187}$ antibodies were from purchased Millipore (Temecula, CA). Rabbit anti-phospho-PHB^{T258} and rabbit anti-phospho-PHB^{Y259} antibodies were generated by Abnova, Taiwan [9].

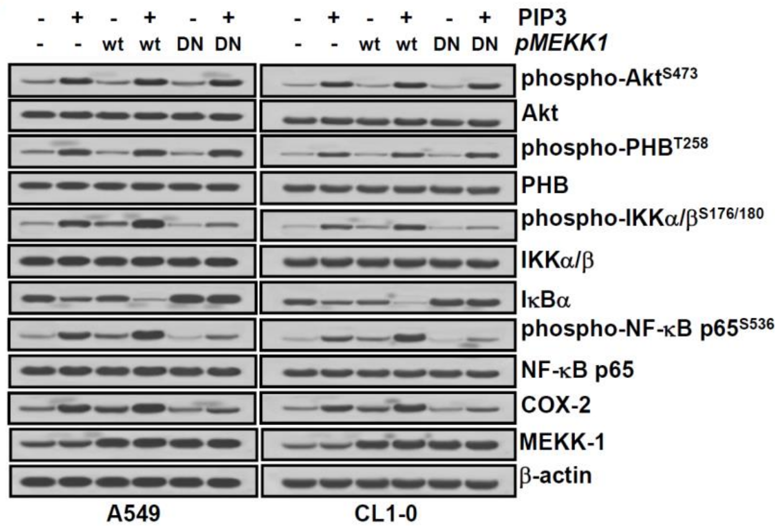


Supplementary Figure 1: EGF, HGF, IGF-1 and PGE2 transiently increase cellular PIP3 level. Lung cancer cells (A549, CL1-0 and CL1-5; 2×10^5/mL) were treated with or without EGF, HGF, IGF-1 and PGE2 respectively for 0-75 min as indicated. Cells were then harvested, and the PIP3 levels in the phospholipid fraction were determined by ELISA. Data represent means ± SD of 3 independent experiments; *, $P < 0.05$ and **, $P < 0.01$ by t test.



Supplementary Figure 2: PIP3 concentration-dependently increases phospho-Akt^{S473}, phospho-Akt^{T308}, phospho-PHB^{T258} and phospho-IKKα/β$^{S176/180}$ in membrane rafts, as well as cytosolic phospho-NF-kB p65^{S536}, COX-2 and PGE2 in an active PHB dependent manner. Lung cancer cells (A549 and CL1-0; 2 $\times$ 10^5/mL) were treated with or without PIP3 (2 and 5 μM) for 24 hours. (A) proteins in the membrane raft or cytosolic plus non-raft membrane (C+M) fraction of lung cancer cells were determined by immunoblotting. The levels of COX-2 and MIG-7 were determined 24 hours after PIP3 treatment while all others were determined 1 hour after PIP3 treatment. Integrin-linked kinase (ILK), a kinase known to be increased by PIP3 [32], is used as an endogenous positive control. Blots are representative of 3 independent experiments. (B) the cellular content of PGE2 was determined by ELISA (right panels) 24 hours after PIP3 treatment. Data represent means ± SD of 3 independent experiments; *, $P < 0.05$ by t test.



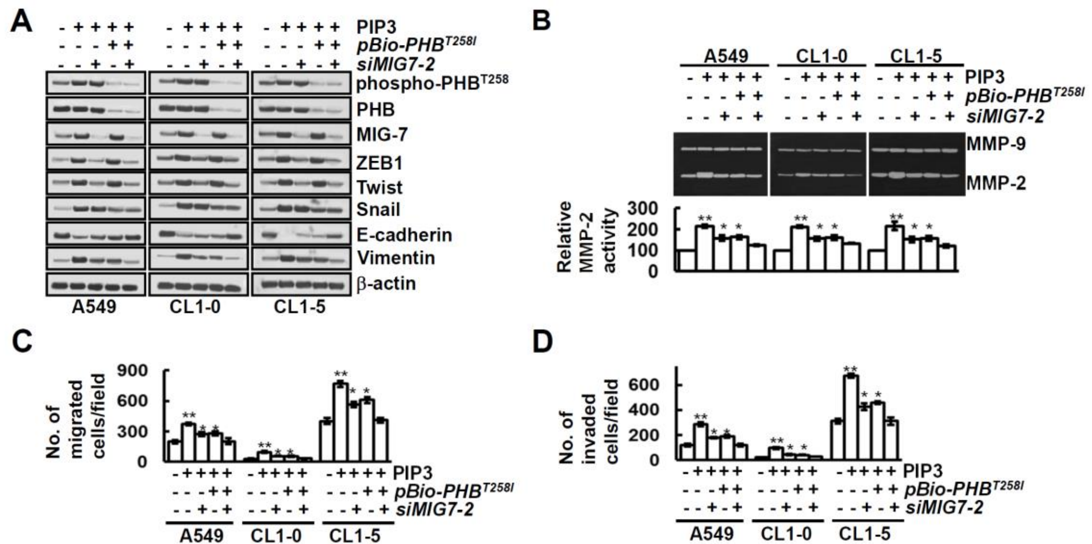


Supplementary Figure 3: Active PHB increases MEKK1 that is pivotal for PIP3-mediated increase in phospho-IKKα/β$^{S176/S180}$, phospho-NF-kB p65^{S536} and COX-2. (A) lung cancer cells (A549 and CL1-0; 2×10^5/mL) were transfected with plasmids *pBio-PHB*, *pD-PHB* or empty vector (*pBio-vector* and *pD-vector*) for 48 hours. Total proteins and proteins in the membrane raft or cytosolic plus non-raft

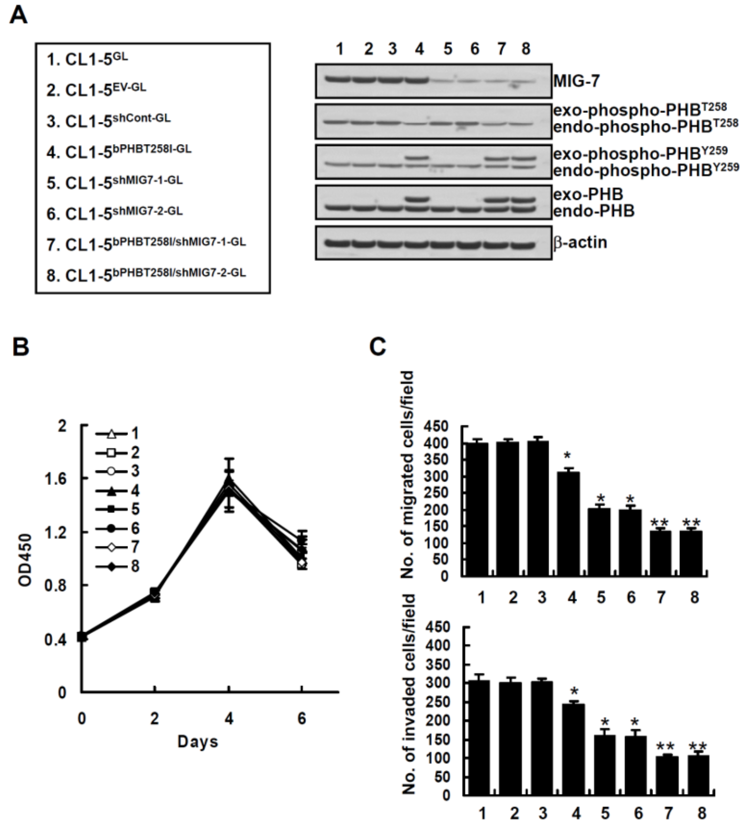
membrane (C+M) fraction were determined by immunoblotting. Caveolin-1 and clathrin heavy chain (HC) protein served as membrane raft and non-raft markers, respectively. (B) lung cancer cells transfected with *pMEKK1-wt*, *pMEKK1-DN* or empty vector (*-pMEKK1*) plasmids for 48 hours were treated with or without 5 μM PIP3 for 24 hours as indicated. The cell lysates were detected by immunoblotting. β-actin was used as a loading control. Blots are representative of 3 independent experiments.

Supplementary Figure 4: Active phospho-PHB is required for the increase of MEKK1 and phospho-IKKα/β$^{S176/180}$ in membrane raft, activation of IKKα/β signaling and increase in phospho-NF-kB p65^{S536} and COX-2. (A) lung cancer cells were transfected with or without *pBio-PHB* (wt, wild-type; T/Y, T258I/Y259F double mutant) and PHB siRNA (*siPHB*) plasmids for 48 hours and then treated with or without PIP3 (5 μM) as indicated. The levels of COX-2 were determined 24 hours after PIP3 treatment while all others were determined 1 hour after PIP3 treatment. Blots are representative of 3 independent experiments. (B) cells (2×10^5/mL) were transfected with empty vector, wild-type *pD-PHB* (wt), or phosphorylation site(s) mutant PHB plasmid constructs (T258I and Y259F) for 48 hours. Proteins in the membrane raft or cytosolic plus non-raft membrane (C+M) fraction of lung cancer cells were determined by immunoblotting. Caveolin-1 and clathrin heavy chain (HC) protein served as

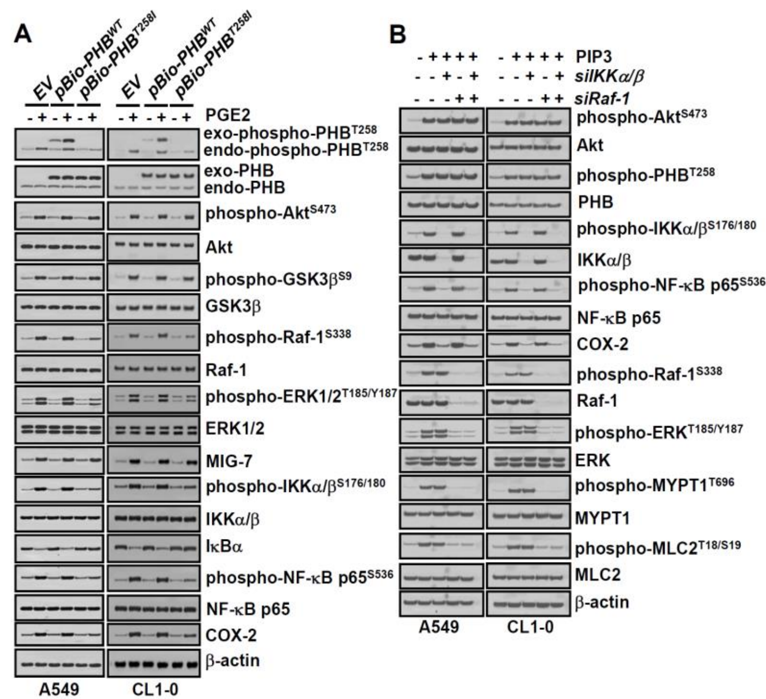
membrane raft and non-raft markers, respectively. (C) D-PHB was immunoprecipitated with anti-HA antibodies and co-immunoprecipitation of phospho-IKKα/β$^{S176/180}$ was detected by immunoblotting (top). Activation of IKKα/β and NF-kB p65 and phosphorylation of PHB at T258 and Y259 were measured by immunoblotting (bottom). Blots are representative of 3 independent experiments.



Supplementary Figure 5: Attenuation of both MIG-7 and phospho-PHB^{T258} shows additive effects in downregulating PIP3-mediated EMT and migration/invasion of lung cancer cells. Lung cancer cells (A549, CL1-0 and CL1-5; 2×10^5/mL) were transfected with control scrambled siRNA (-si*MIG7-2*), *empty vector* (-*pBio-PHB*^{T258I}), *pBio-PHB*^{T258I} or *siMIG7-2* for 48 hours and then treated with PIP3 (5 μM) for 24 hours, as indicated. (A) proteins were examined by immunoblotting. Blots are representative of 3 independent experiments. (B) MMP activities were examined by zymography. (C and D) migration and invasion of the cells were examined by transwell assay as described in Materials and Methods. Data represent means ± SD of 3 independent experiments; *, $P < 0.05$ and **, $P < 0.01$, by t test.



Supplementary Figure 6: Downregulation of both MIG-7 and phosphorylated PHB has an additive effect on inhibition of lung cancer cell invasion *in vitro*. CL1-5^{GL}, CL1-5$^{EV\text{-}GL}$, CL1-5$^{shCont\text{-}GL}$, CL1-5$^{bPHBT258I\text{-}GL}$, CL1-5$^{shMIG7\text{-}1\text{-}GL}$, CL1-5$^{shMIG7\text{-}2\text{-}GL}$, CL1-5$^{bPHBT258I/shMIG7\text{-}1\text{-}GL}$ and CL1-5$^{bPHBT258I/shMIG7\text{-}2\text{-}GL}$ cells were generated as described in Materials and Methods. (A) cells were examined by immunoblotting for protein expression. Blots are representative of 3 independent experiments. (B) growth of cells was examined by cell proliferation assay as described previously [8]. (C) migration and invasion of cells were examined by transwell assay as described in Materials and Methods. Data represent means ± SD of 3 independent experiments; *, $P < 0.05$ and **, $P < 0.01$, by t test.



Supplementary Figure 7: PGE2 shows similar phospho-PHB dependent effects as PIP3 that increases COX-2 via the IKKα/β but not the Raf-1/ERK pathway. (A) lung cancer cells (A549 and CL1-0; 2×10^5/mL) were transfected with empty vector, *pBio-PHB*^{T258I} or *pBio-PHB*^{WT} for 48 hours and then treated with or without PGE2 (20 μg/mL) as indicated. The levels of COX-2 and MIG-7 were determined 24 hours after PGE2 treatment while all others were determined 1 hour after PGE2 treatment. (B) cells were transfected with control scrambled siRNA (-*siIKKα/β* or -*siRaf-1*), *siIKKα/β* (+) or *siRaf-1* (+) for 48 hours and then treated with or without PIP3 (5 μg/mL) for 24 hours, as indicated. The levels of COX-2 were determined 24 hours after PIP3 treatment while all others were determined 1 hour after PIP3 treatment. Proteins were examined by immunoblotting. Blots are representative of 3 independent experiments.