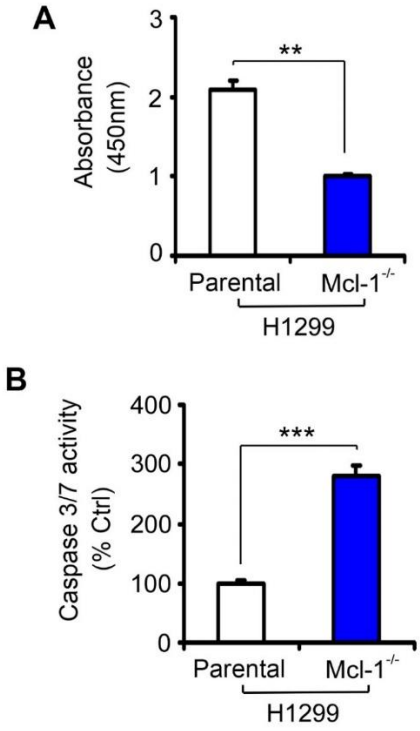
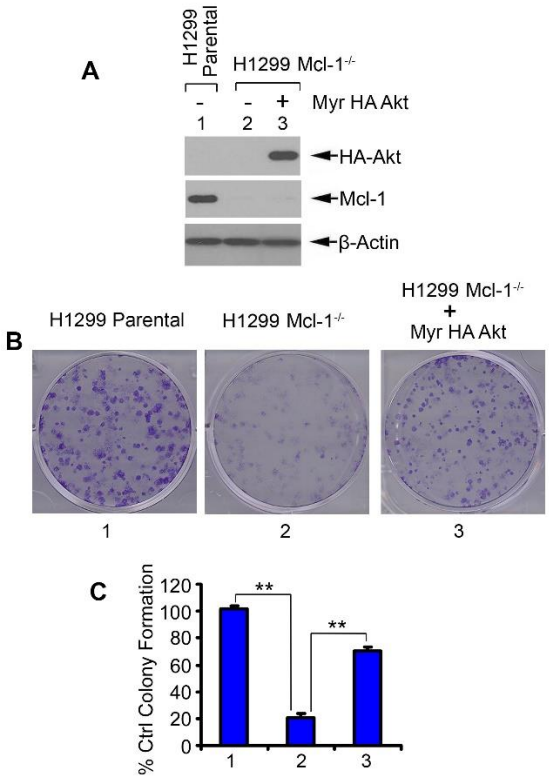
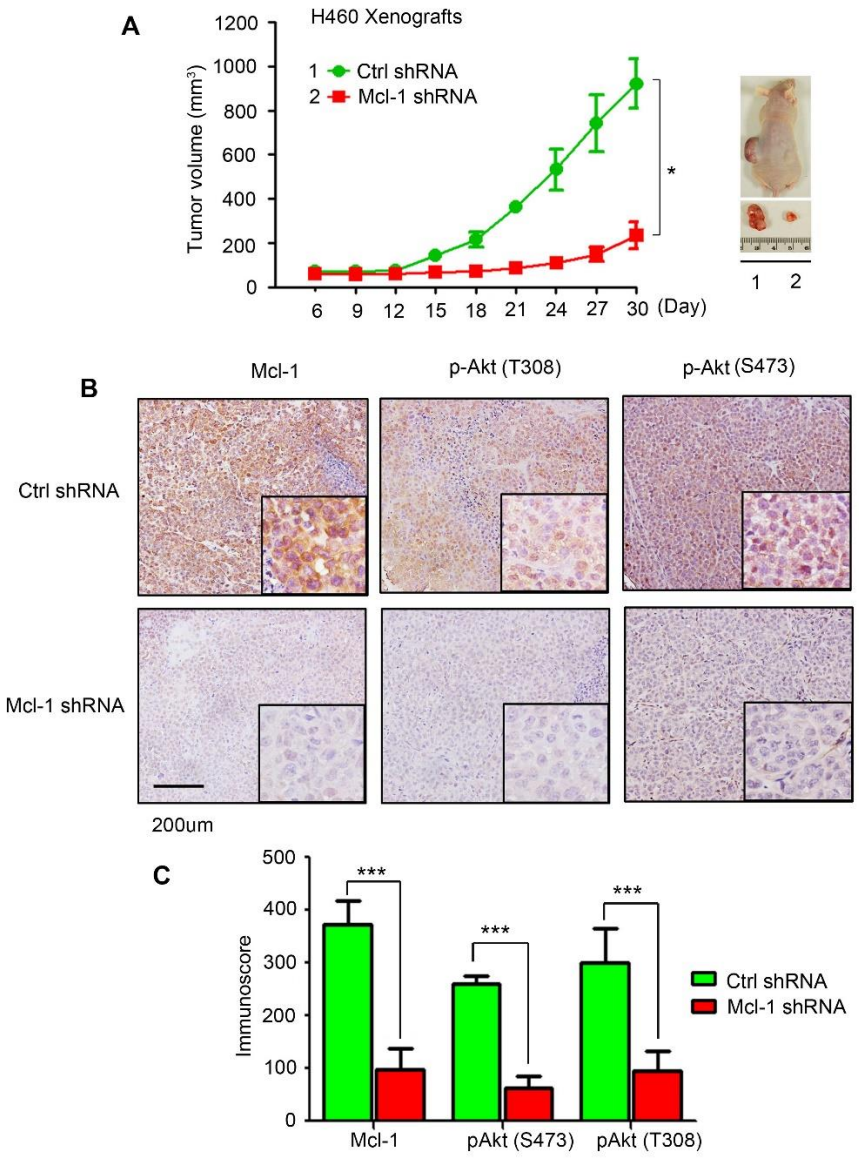
Supplementary Data



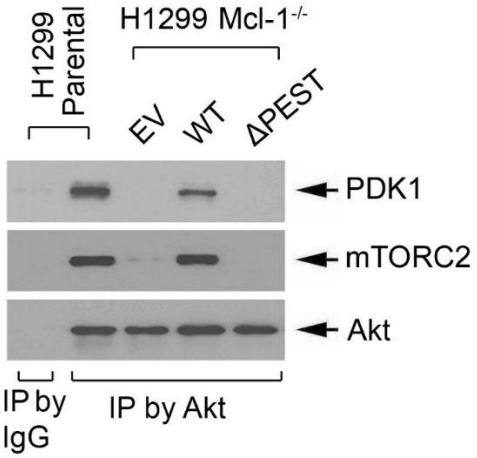
Supplementary Figure 1. Knockout of Mcl-1 from H1299 by CRISPR/Cas9 system results in decreased cell proliferation and increased caspase 3/7 activity. **A**, 2× 10^3 H1299 or Mcl-$1^{-/-}$ H1299 cells /well were plated in a 96-well plate for 72h, followed by incubation with WST-1 at 37°C. The absorbance of each sample was measured at 450 nm using a microplate reader. **B**, 2× 10^3 H1299 or Mcl-$1^{-/-}$ H1299 cells /well were plated in a 96-well plate for 72h, followed by incubation with Caspase-Glo® 3/7 reagent. Samples were analyzed for luminescence using a microplate reader. Data represent the mean ± SD. $^{**}P < 0.01$, $^{***}P < 0.001$, by 2-tailed *t* test.



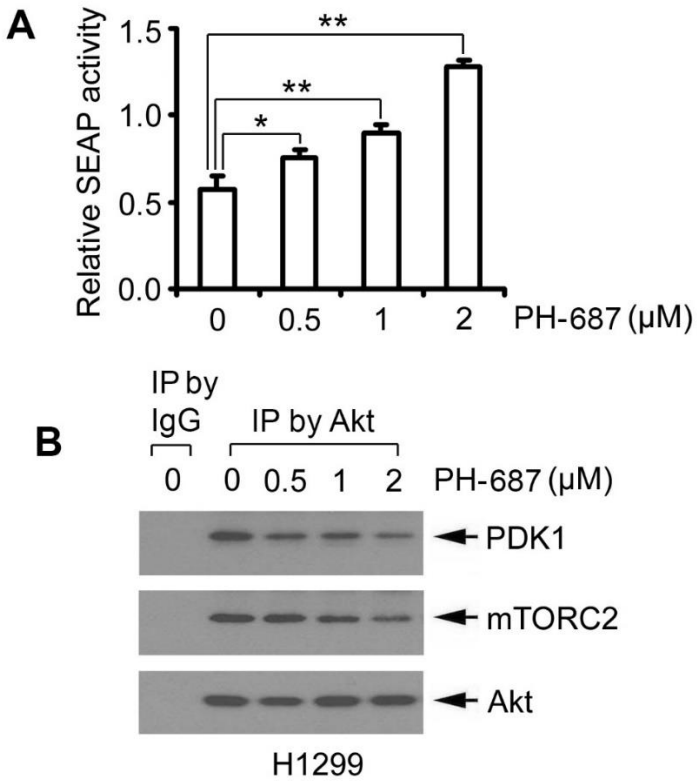
Supplementary Figure 2. Expression of constitutive active form of Akt in H1299 Mcl-1 knockout cells restores cell growth. **A**, The pcDNA3 Myr HA Akt1 or empty vector was transfected into H1299 Mcl-1$^{-/-}$ cells, followed by Western blot using HA antibody or Mcl-1 antibody, respectively **B**, Colony formation assay was performed in H1299 parental cells, H1299 Mcl-1$^{-/-}$ cells and H1299 Mcl-1$^{-/-}$ cells expressing exogenous constitutive active form of Akt. Data represent the mean ± SD. $^{**}P < 0.01$, by 2-tailed *t* test.



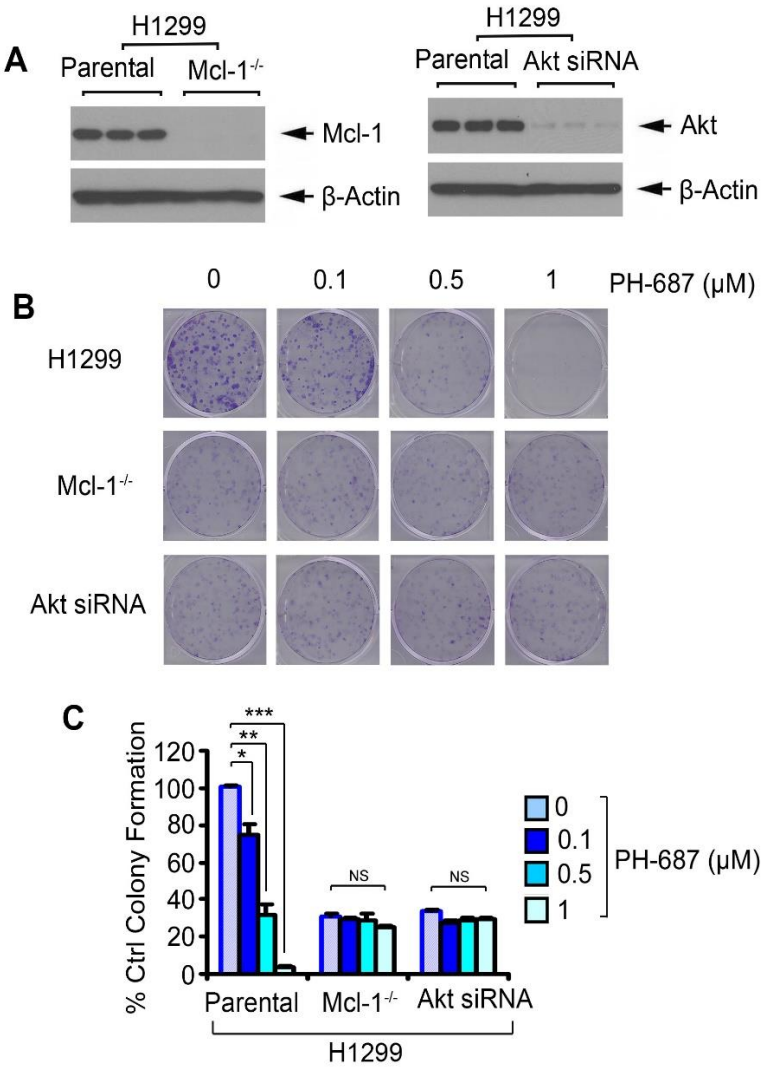
Supplementary Figure 3. Depletion of Mcl-1 by shRNA retards the growth of H460 lung xenografts. **A**, The same number (3×10^6) of H460 cells expressing control (Ctrl) shRNA or human Mcl-1 shRNA were injected into subcutaneous tissue in the flank region of nude mice to generate lung cancer xenografts (n=6 mice each group). Tumor volume was measured once every 3 days. After 30 days, the mice were sacrificed and the tumors were removed, photographed, and analyzed. Data represent the mean ± SD, n=6 per group. *$P < 0.05$, by 2-tailed *t* test. **B** and **C**, IHC staining of Mcl-1, pAkt (S473) and pAkt (T308) was performed in tumor tissues at the end of experiments. Data represent the mean ± SD, n=6 per group. ***$P < 0.001$, by 2-tailed *t* test.



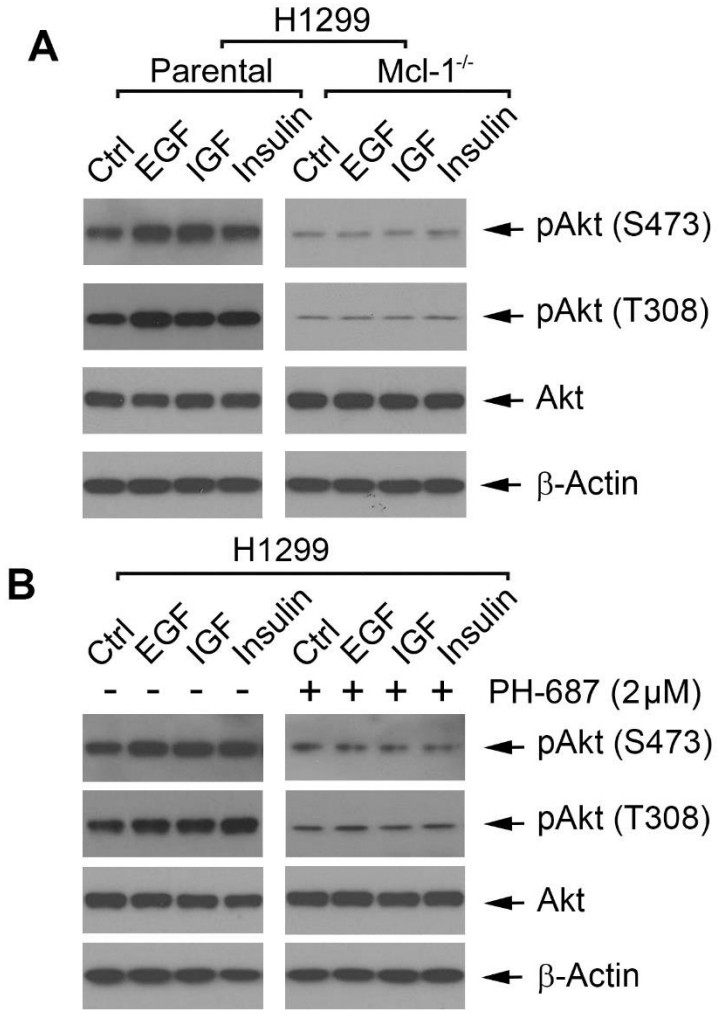
Supplementary Figure 4. Expression of WT Mcl-1 but not the PEST deletion mutant Mcl-1 (ΔPEST) in H1299 Mcl-1$^{-/-}$ cells restores Akt/PDK1 and Akt/mTORC2 interactions. The co-IP experiments using Akt antibody were performed in H1299 parental cells or H1299 Mcl-1$^{-/-}$ cells expressing WT Mcl-1, ΔPEST Mcl-1 mutant or empty vector, followed by Western blot using PDK1, mTORC2 or Akt antibody, respectively.



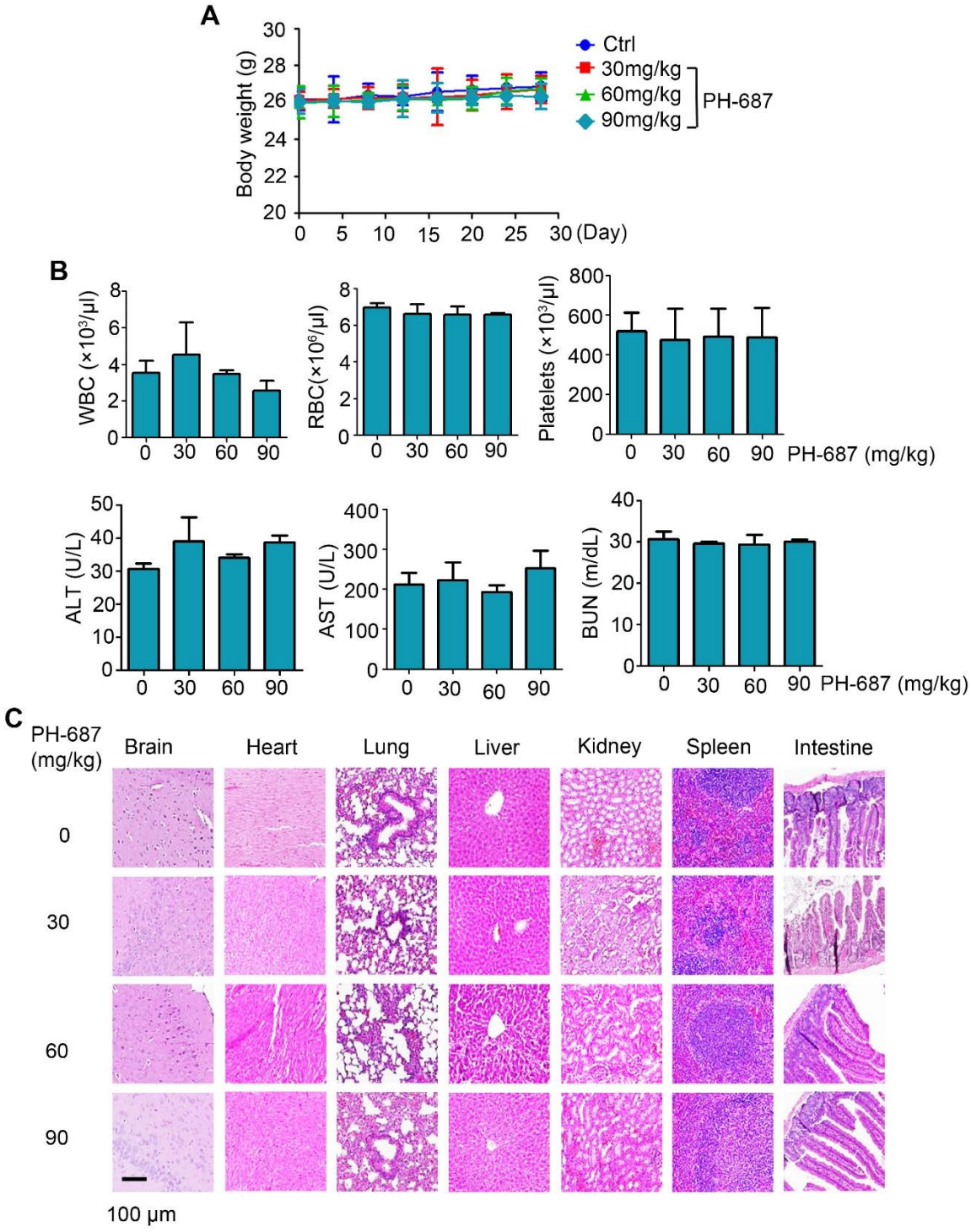
Supplementary Figure 5. Treatment of H1299 cells with PH-687 results in increased intramolecular PH/KD interactions in Akt and decreased interaction of Akt with PDK1 or mTORC2. **A**, H1299 cells were treated with increasing concentrations of PH-687 for 24h, followed by analysis of the intramolecular interaction between PH domain and KD domain using two-hybrid system. Data represent the mean ± SD. *$P < 0.05$, **$P < 0.01$, by 2-tailed *t* test. **B**, H1299 cells were treated with increasing concentrations of PH-687 for 24h, followed by co-IP using Akt antibody and Western blot using PDK1, mTORC2 or Akt antibody, respectively.



Supplementary Figure 6. Mcl-1/Akt signaling pathway is essential for PH-687 inhibition of cancer cell growth. **A**, Mcl-1 was knocked out by CRISPR/Cas 9, or Akt was knocked down using Akt siRNA, followed by Western blot using Mcl-1 antibody or Akt antibody, respectively. **B** and **C**, H1299 parental, H1299 Mcl-$1^{-/-}$ and H1299 cells expressing Akt siRNA were treated with increasing concentrations of PH-687, followed by colony formation assay. Data represent the mean ± SD. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, NS: no significant, by 2-tailed *t* test.



Supplementary Figure 7. Knockout of Mcl-1 or treatment of cells with PH-687 inhibits growth factor (s)-stimulated Akt activation. **A**, H1299 parental and H1299 Mcl-$1^{-/-}$ cells were treated with EGF (100ng/ml), IGF (10ng/ml) or insulin (1μg/ml) for 1h, followed by Western blot for analysis of Akt phosphorylation. **B**, H1299 cells were treated with EGF (100ng/ml), IGF (10ng/ml) or insulin (1μg/ml) in the absence or presence of PH-687 (2μM) for 1h, followed by Western blot for analysis of Akt phosphorylation.



Supplementary Figure 8. *In vivo* toxicity analysis. **A, B** and **C**, After treatment with increasing doses of PH-687 as indicated, body weight (**A**), blood parameters (**B**) and H&E histology of various organs (**C**) were analyzed. Data represent the mean ± SD, n=5 per group..

Supplementary Methods

Colony formation assay

Cells were plated into 6-well plates at 800 cells per well. Cells were cultured in presence or absence of PH-687 as indicated and the medium was replaced every 3 days. After 10 days, cells were then fixed and stained with 0.1% crystal violet in 20% methanol. Surviving colonies were counted and the surviving percentage was calculated and normalized with parental cells or non-treated control cells.

Western blot, immunoprecipitation and GST pull-down assay

Cells were washed with cold 1×PBS and resuspended in ice-cold EBC buffer (0.5% Nonidet P-40, 50mM Tris, pH 7.6, 120mM NaCl, 1mM EDTA, and 1mM-β-mercaptoethanol) containing protease inhibitor mixture set I. Following cell lysis by sonication and centrifugation at 14,000 x g for 15 min at 4 °C, the resulting supernatant was collected as the total cell lysate. As previously described (1), Western blot was performed by loading 50μg of protein per lane on an 8-12% SDS-PAGE, followed by protein transfer to nitrocellulose membrane for analysis of specific protein(s). For immunoprecipitation, total cell lysate was incubated with indicated antibody and agarose beads. The beads were then washed, boiled and subjected to SDS-PAGE and analyzed by Western blot. For GST pull-down assay, cell lysate was incubated with glutathione sepharose 4B beads (GE healthcare) in TBS buffer (50 mM Tris-Cl, pH 7.5, 150 mM NaCl) with protease inhibitor cocktail at 4℃ for 4 h. After washing, the immunoprecipitation complex was subjected to SDS-PAGE and analyzed by Western blot.

Knockdown of Mcl-1 by shRNA

Mcl-1 shRNA was obtained from Santa Cruz Biotechnology ((Dallas, Texas). Hairpin sequence of Mcl-1 shRNA: 5'-GAT CCG AAG ACC ATA AAC CAA GAA TTC AAG AGA TTC TTG GTT TAT GGT CTT CTT TTT-3'. For pseudovirus production, Mcl-1 shRNA was cotransfected into 293FT cells with a lentivirus packaging plasmid mixture (pCMV-dR8.2 dvpr and pCMV-VSV-G) (System Biosciences, CA) using the NanoJuice transfection kit (EMD Chemical, Inc.). After 48h, virus-containing medium supernatant was harvested by centrifugation at 20,000 × g. H460 cells were infected with virus-containing media in the presence of polybrene (8 μg/ml) for 24h. Stable positive clones were selected using 1μg/ml puromycin. Specific silencing of the targeted Mcl-1 gene was confirmed by at least three independent experiments.

Cell proliferation assay

Cell proliferation was measured using WST-1 kit according to the manufacturer's instructions. Briefly, 2×10^3 H1299 or Mcl-$1^{-/-}$ H1299 cells/well were plated in a 96-well plate for 72h, followed by incubation with WST-1 at 37°C. The absorbance of each sample was measured at 450 nm using a microplate reader (BioTek Instruments Inc.).

Caspase 3/7 activity assay

Caspase 3/7 activity was analyzed using Caspase-Glo® 3/7 assay kit according to manufacturer's instructions. Briefly, 2×10^3 H1299 or Mcl-$1^{-/-}$ H1299 cells /well were plated in a 96-well plate for 72h, followed by incubation with Caspase-Glo® 3/7 reagent at room temperature. All samples were analyzed for luminescence using a microplate reader (BioTek Instruments Inc.).

Generation of recombinant protein

The full length Akt, PH deletion mutant (ΔPH) or PH domain–only Akt mutant were cloned into pGEX-4T-1 vector (GE Healthcare, UK) between the BamH I and Sal I sites. Recombinant proteins were expressed and purified from *Escherichia coli* Rosetta (DE3) (EMD Millipore, MA). Briefly, *Escherichia coli* were grown in Luria-Bertani broth at 37℃ with shaking at 250 rpm and IPTG (0.2mM) was added to induce the protein expression when the OD600 reached 0.4. Cells were then incubated at 18℃ and harvested at 18h post IPTG addition before lysis in buffer G (20 mM Tris-HCl pH 8.0, 150 mM NaCl and 1 mM EDTA) by sonication. After centrifugation at 13000 rpm for 10 min, the supernatant was applied to a glutathione sepharose column (GE Healthcare, UK). After washing the column with 10 column volumes of buffer G, the binding proteins were eluted with elution buffer (50 mM Tris-HCl pH 8.0, 10 mM glutathione) and collected and stored at -80℃.

Knockdown of Akt by Akt siRNA

Akt siRNA duplex was transfected into H1299 cells using NanoJuice. After 48h, knockdown of Akt was confirmed by Western blot. The sequence of Akt targeting RNA: 5'-UGC CCU UCU ACA ACC AGG ATT-3'.

Thermal shift assay

Thermal shift assay was performed using the Protein Thermal Shift Dye Kit (Thermo Fisher Scientific, Rockford, IL) as described (2). Purified WT and mutant Akt proteins (2 2 μg each) were incubated with increasing concentrations of PH-687 in Protein Thermal Shift buffer containing Thermal Shift Dye at room temperature, followed by measurement of the fluorescence release using Real-Time PCR Systems (Applied Biosystems). Data were analyzed using Protein Thermal Shift Software v1.0 (Life Technologies) (2).

Mouse blood analysis

Whole blood and serum were collected through cardiac puncture of anesthetized mice. Specimens were analyzed for white blood cells (WBC), red blood cells (RBC), platelets (PLT), alanine aminotransferase (ALT), aspartate aminotransferase (AST) and blood urea nitrogen (BUN) in the Clinical Pathology Laboratory at the University of Georgia (Athens, GA) as described (3).

References

1. Ito T, Deng X, Carr B, May WS. Bcl-2 phosphorylation required for anti-apoptosis function. J Biol Chem. 1997;272:11671-3.
2. Jin L, Li D, Alesi GN, Fan J, Kang HB, Lu Z, et al. Glutamate dehydrogenase 1 signals through antioxidant glutathione peroxidase 1 to regulate redox homeostasis and tumor growth. Cancer Cell. 2015;27:257-70.
3. Han B, Park D, Li R, Xie M, Owonikoko TK, Zhang G, et al. Small-Molecule Bcl2 BH4 Antagonist for Lung Cancer Therapy. Cancer cell. 2015;27:852-63.