Supplementary Information

Pim kinase inhibitors sensitize prostate cancer cells to apoptosis triggered by Bcl-2 family inhibitor ABT-737

Jin H. Song[1, 3] and Andrew S. Kraft[2, 3]

Departments of Biochemistry and Molecular Biology[1], Medicine[2] and the Hollings Cancer Center[3], the Medical University of South Carolina, 86 Jonathan Lucas Street, Charleston, SC 29425

Supplemental Materials and Methods

Cell lysis preparation, immunoblotting and immunoprecipitation

At the end of each experiment, cells were scraped into ice-cold NP-40 lysis buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 1 mM Na_3VO_4) or 0.3 % CHAPS buffer (for immunoprecipitation of mTOR) (1). The lysates were then clarified by centrifugation (16,000 *g*, 15 min) and then the resulting supernatant was used for immunoblotting or immunoprecipitation assays. Tumor tissues from xenografted animals were homogenized and lysed with ice-cold RIPA buffer as reported previously (2). Whole cell lysates were mixed with Laemmli sample buffer and boiled. Aliquots containing equal amounts of protein were subjected to SDS-PAGE. Subsequently, proteins were transferred to nitrocellulose membranes and the membrane was blocked by incubation with 5% milk in TBS-T buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.05% Tween 20), for 1 hour at room temperature. The membrane was then incubated overnight at 4°C with the indicated antibody in 3% BSA in TBS-T buffer, washed three times in the same buffer and

incubated for 1 hour with HRP-conjugated secondary antibody. The membrane was then washed three times with TBS-T buffer and visualized by enhanced chemiluminescence (ECL) Western blotting kit according to the manufacturer's instructions (GE Lifesciences, Piscataway, NJ). Antibodies used to carry out these studies were as follows: goat anti-mouse antibody crosslinked with horseradish peroxidase was purchased from GE Healthsciences, monoclonal anti-HA, GAPDH and β-actin antibodies were obtained from Sigma-Aldrich, anti-Mcl-1, anti-Pim-1 and anti-Pim-2 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA), while antibodies against phospho-4E-BP1, 4EB-P1, phospho-AMPK, AMPK, Raptor, mTOR and Pim-3 were from Cell Signaling Technology (Danvers, MA). The Noxa antibody was purchased from EMD Chemicals, and the antibodies against other Bcl-2 family members, anti-PARP-1 and anti-caspase-3 were obtained as previously reported (3).

7-Methyl-GTP cap binding assay

After treatment, 2×10^7 cells were washed in phosphate-buffered saline then resuspended in 400 μl of 20 mM Tris pH 7.5, 100 mM KCl, 20 mM β-glycerophosphate, 1 mM dithiothreitol, 250 μM Na_3VO_4, 10 mM NaF, 1 mM ethylenediaminetetraacetic acid, 1 mM ethylene glycol tetraacetic acid, 1 mM phenylmethylsulfonyl fluoride and lysed with four successive freeze-thaw cycles. After centrifugation (16,000 *g* for 10 min at 4 °C), 200 μg of protein was applied to 40 μl of m^7-GTP-sepharose 4B beads (GE Lifesciences) and incubated for 2h at 4 °C. The beads were then washed lysis buffer 5X followed by boiling in Laemmli sample buffer. After

SDS–PAGE, the levels of 4E-BP1 and eIF4E were detected by immunoblotting with anti-4E-BP1 and anti-eIF4E antibodies (Cell Signaling Technology).

^{35}S labeling of protein

Cells were methionine starved for 30 min in methionine-free RPMI 1640 media (Invitrogen), containing 10 % methionine-free FBS (Invitrogen). Following starvation, 100 μCi of ^{35}S-labeled methionine (Perkin Elmer, San Jose, CA) was added to each samples and incubation continued for 1 hour. Cells were harvested at the indicated times and lysed. To assess global protein synthesis, lysed proteins were precipitated using 10 % trichloroacetic acid (TCA), washed, dried, and radioactivity measured in a scintillation counter (Beckman Coulter). To determine the ^{35}S incorporation rate to Mcl-1 protein, cell extracts (500 μg of protein) were brought to a volume of 1ml with immunoprecipitation (IP) buffer (50mM Tris-Hcl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA) and incubated with 2 μg of rabbit polyclonal antibody against human Mcl-1 (Sigma) or mouse Mcl-1 (Rockland Immunochemical, Gilbertsville, PA). Immune complexes were precipitated by incubation with protein G-coated Agarose beads (GE Lifesciences) previously equilibrated with IP buffer, and washed 5X with 1 ml of IP buffer.

Detection of XBP-1 splicing by RT-PCR

Total RNA was isolated at various time points and cDNA constructed using Invitrogen SuperScript III reverse transcriptase as we previously reported (4). XBP1 mRNA splicing was assessed by RT-PCR analysis. After synthesis of cDNA with first-stranded cDNA kit (Invitrogen), Human *XBP-1* mRNA was amplified using the forward

primer 5′- CTGGAACAGCAAGTGGTAGA-3′ and the reverse primer 5′-CTGGGTCCTTCTGGGTAGAC-3′. The resulting PCR product was digested with *Pst*I, and separated on 2 % agarose gel. Following primers are used for loading control experiments: Human *β-actin* forward, 5'-TCGTGCGTGACATTAAGGAG-3'; *β-actin* reverse, 5'-AGCACTGTGTTGGCGTACAG-3'. Mouse *XBP-1* was amplified using the forward primer 5′- AAACAGAGTAGCAGCGCAGACTGC-3′ and the reverse primer 5′-TCCTTCTGGGTAGACCTCTGGGAG-3′. Mouse *β-actin* primers are used for a loading control, mouse *β-actin* forward, 5'-GTGGGCCGCTCTAGGCACCAA-3'; mouse *β-actin* reverse, 5'-CTCTTTGATGTCACGCACGATTTC-3'. The resulting PCR product was purified, digested with *Pst*I, and separated on 2 % agarose gel. The PCR product was visualized by ethidium bromide staining under ultraviolet light using the Kodak Image Station 4000R Pro digital imaging system.

Quantitative real-time PCR (qT-PCR) and primers

Relative quantification of gene expression was assessed by quantitative real-time PCR (iQ5 Multicolor Real-Time PCR detection system, BioRad Laboratories) using iQ5 optical system software. The expression levels were normalized to 18S. The primers used for qT- PCR are listed below.

18S forward, 5'-GTAACCCGTTGAACCCCATT-3'; *18S* reverse, 5'-CCATCCAATCGGTAGTAGCG-3'; *Pim-1* forward 5'-CCCGAGCTATTGAAGTCTGA-3', *Pim-1* reverse 5'-CTGTGCAGATGGATCTCAGA-3'; *Pim-2* forward, 5'-CCATTCCCGTGGAGTTGT-3', *Pim-2* reverse, 5'-GAAGCAGGGCACCAGAAC-3'; *Pim-3* forward, 5'-

GACATCCCCTTCGAGCAG-3’; *Pim-3* reverse, 5’-ATGGGCCGCAATCTGATC-3’; *XBP-1* forward, 5’-TGAGCTGGAACAGCAAGTGGT-3’; *XBP-1* reverse, 5’-CCCAAGCGCTGTCTTAACTCC-3’; *sXBP-1* forward, 5’-GGTCTGCTGAGTCCGCAGCAGG-3’; *sXBP-1* reverse, 5’-GGGCTTGGTATATATGTGG-3’; *CHOP* forward, 5’-GGAGAACCAGGAAACGGAAAC-3’; *CHOP* reverse, 5’-TCTCCTTCATGCGCTGCTTT-3’; *NOXA* forward, 5’-GCTGGAAGTCGAGTGTGCTA-3′; *NOXA* reverse, 5′-CCTGAGCAGAAGAGTTTGGA-3′; *MCL-1* forward, 5′-CCAAGGCATGCTTCGGAAA-3’; *MCL-1* reverse, 5′-TCACAATCCTGCCCCAGTTT-3’; *Grp78* forward, 5’-CGACCTGGGGACCACCTACT-3’; *Grp78* reverse, 5’-TTGGAGGTGAGCTGGTTCTT-3’; *Grp78va* forward, 5’-GGTGCTGATGTCCCTCTGTC-3’; *Grp78va* reverse, 5’-TTGGAGGTGAGCTGGTTCTT-3’; *ATF4* forward, 5'-TGGCCAAGCACTTCAAACCT-3'; *ATF4* reverse, 5'-GTTGTTGGAGGGACTGACCAA-3' *ATF6* forward, 5'-AGACTGAAGAGCAGGTGAGCAAA-3'; *ATF6* reverse, 5'-GATGATGAAAAATGGAGCAGCTT-3'; *GADD34* Forward, 5'-CCCAGAAACCCCTACTCATGATC-3'; *GADD34* Reverse, 5'-GCCCAGACAGCCAGGAAAT-3'.

Supplemental Figure Legends

Supplementary Figure S1. The effects of SMI-4a on prostate cancer cell survival. Human prostate cancer LNCaP, PC3 and 22Rv1 cells were treated for 48 h with the indicated doses of SMI-4a, n=4 +/- the standard deviation (SD). Cell viability was determined by MTT assay.

Supplementary Figure S2. Generation of PIM-1, -2, and -3 knock down cells.

A, To generate Pim deficient cell lines, LNCAP cells were infected with lentiviruses carrying short hairpin microRNAs targeting PIM-1, -2, or -3. Non-silencing control shRNA were used as control (shC). mRNA was isolated from these cell pools and qRT-PCR carried out. The result yield from the shC treated cells was set at one and the changes in the knock-down of individual genes compared. **B,** LNCaP cells were plated and the cell proliferation rate in individual knock-down cells was determined by counting cell numbers 72h after plating.

Supplementary Figure S3. The effects of Pim inhibitors and ABT-737 on prostate cancer cell viability.

A, PC-3 cells were treated with Pimi-14j at the indicated doses with or without of the addition of ABT-737. After 24 h treatment, cell viability (n=4) was assessed by MTT assay. Chou and Talalay's combination index (CI) was calculated by using CalcuSyn (Biosoft, Perguson, MO) based on the cell viability data. **B,** LNCaP cells were treated with Pim inhibitors at the indicated doses with or without the addition of ABT-737. After

24h treatment, cell viability was determined by MTT assay. The mean of these measurements +/- the standard deviation is shown.

Supplementary Figure S4. Regulation of Mcl-1 protein by Pim kinase inhibition. **A,** Western blot detection of Mcl-1 protein in PC-3 cells treated with DMSO or SMI-4a (10 μM) for 16h. **B,** PC-3 cells treated with DMSO, K00135 (10 μM) or Pimi-14j (10 μM) for 16h. **C,** LNCaP cells were treated with DMSO, Pimi-14j (10 μM), or SMI-4a (10 μM) for 16h and total RNA subjected to qT-PCR analysis of *MCL-1* transcript level. The normalized expression of *MCL-1* mRNA when compared to *18S* mRNA levels is shown (triplicate experiments+/-S.D.). **D,** Western blot detection of Mcl-1 protein in wild type (WT) MEF or triple Pim kinases knock out (TKO) MEF cells (left). WT and TKO MEF cells were starved of methionine, labeled with ^{35}S-methionine, Mcl-1 immunoprecipitated, followed by SDS-PAGE gels, and autoradiography carried out (right).

Supplementary Figure S5. P**hosphorylation of 4E-BP1 is reduced by Pim inhibition.** **A,** LNCaP cells stably expressing shRNA constructs against Pim-1, 2 and 3 (shPims) or nonsilencing control (shC) or treated with SMI-4a (10 μM) for 16 h were lysed and cell extracts immunoblotted with the antibodies shown. **B,** Western blot analysis of Pim-1, -2 and -3 protein expression in LNCaP cells expressing stable shRNAs to Pim isoforms. **C,** Total RNA was isolated from shC cells and shPims cells and then subjected to qT-PCR analysis. Pim-1, Pim-2 or Pim-3 mRNA levels were normalized to the levels of the 18S mRNA. The mean of triplicate determinations are shown +/- the standard deviation.

Supplementary Figure S6. Inhibition of Pim kinases increases Noxa expression through the induction of endoplasmic reticulum stress response.

A, LNCaP cells stably expressing shRNA targeting Pim-1, 2, 3 (shPims) or nonsilencing control (shC) were lysed and the resulting cell extracts were analyzed by immunblotting with the antibodies shown. LNCaP cells were pretreated with PS-341 (100 nM) for 2 h and then treated with DMSO or SMI-4a (10 μM) for 16h. Cell extracts were analyzed by immunblotting with the antibodies shown. **B,** LNCaP cells were treated with DMSO, 10 μM SMI-4a (4a), 10 μM 10058-F4 (F4), 1 μg/mL Tunicamycin (Tm) or 0.5 μM Thapsigargin (Tg) for 16 h. Total RNA was isolated and then subjected to qT-PCR analysis. CHOP or GADD34 mRNA levels were normalized to the levels of 18S mRNA. The mean of triplicate determinations are shown +/- the standard deviation.

Supplementary Figure S7. Induction of XBP-1 splicing by Pim kinase inhibition.

A, LNCaP or PC-3 cells were treated with 0 (lanes 1, 4), 5 (lanes 2, 5), or 10 (lanes 3, 6) μM SMI-4a for 16 h and then subjected to RT-PCR analysis. The products were run on a 2% agarose gel and XBP-1 unspliced (u) and spliced (s) forms are labeled. **B,** Additional prostate cancer cells were treated with SMI-4a (10 μM) and then subjected to RT-PCR analysis for the detection of XBP-1 splicing. **C,** LNCaP cells were treated with K00135 or F4 (10 μM) and RNA isolated was then subjected to RT-PCR for detection of XBP-1 splicing (top panel). The ratio of the spliced (S) to the unspliced (U) XBP-1 mRNAs was assessed by qT-PCR analysis (bottom panel). The mean of triplicate determinations are shown +/- the standard deviation. **D,** WT MEF or TKO MEF cells were incubated with

Pim kinase inhibitors (10 μM) and thapsigarin (Tg) for 16 h and analysis of XBP-1 splicing analyzed. **E,** LNCaP cells were transfected with shRNAs to Pim or scrambled control (shC) and XBP-1 splicing evaluated.

Supplementary References

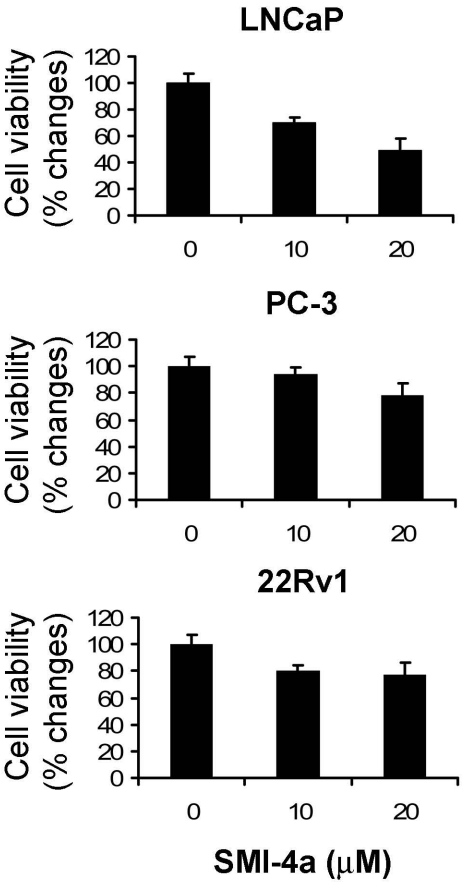
1. Kim DH, Sarbassov DD, Ali SM*, et al.* mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell 2002;110:163-75.

2. Hao C, Song JH, Hsi B*, et al.* TRAIL inhibits tumor growth but is nontoxic to human hepatocytes in chimeric mice. Cancer Res 2004;64:8502-6.

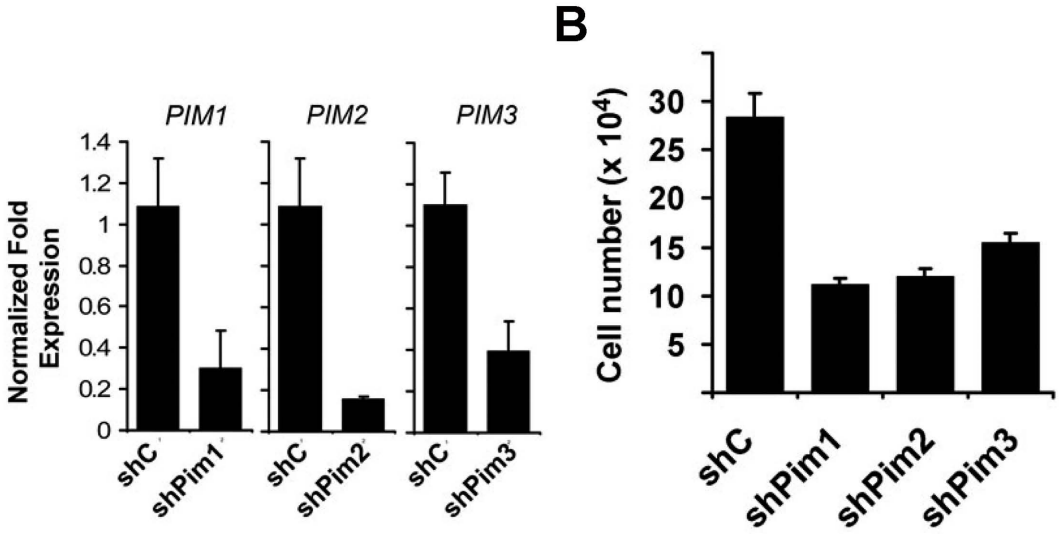
3. Song JH, Kandasamy K, Kraft AS. ABT-737 induces expression of the death receptor 5 and sensitizes human cancer cells to TRAIL-induced apoptosis. J Biol Chem 2008;283:25003-13.

4. Song JH, Kandasamy K, Zemskova M, Lin YW, Kraft AS. The BH3 mimetic ABT-737 induces cancer cell senescence. Cancer Res 2011;71:506-15.

Supplementary Figure S1



Supplementary Figure S2

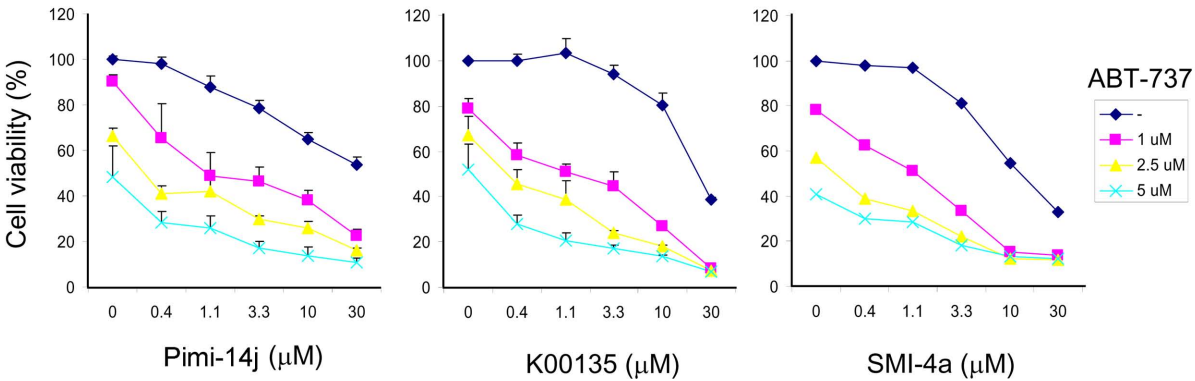


Supplementary Figure S3

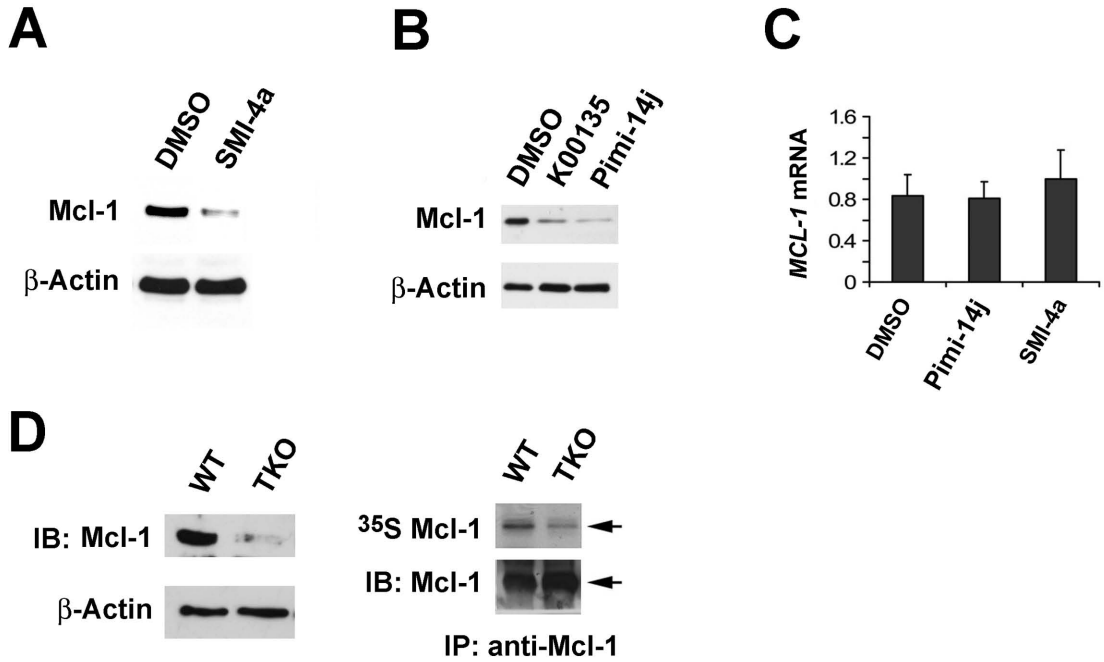
A

Pimi-14j (μM)	ABT-737 (μM)	CI
0.3	1	10.26
1	1	0.318
3	1	0.351
10	1	0.311
30	1	0.259

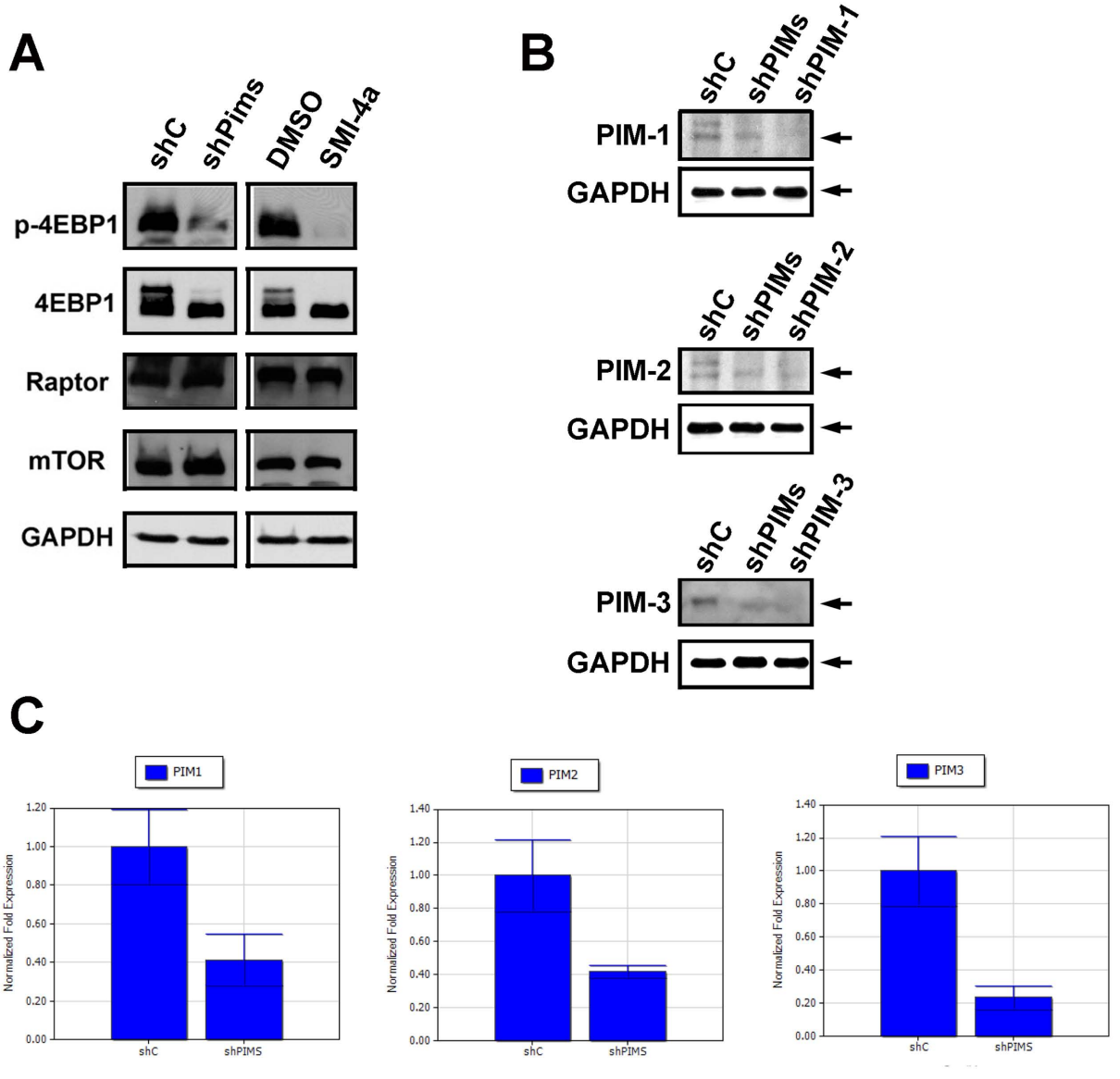
B



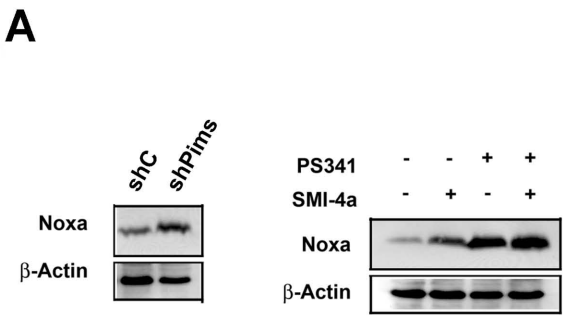
Supplementary Figure S4



Supplementary Figure S5



Supplementary Fig. S6



Supplementary Figure S7