Supporting Information

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SI Text

Antibodies. Flag antibody was obtained from Sigma. PI3K-p85, PI3K-p110 (α, β, and σ), Akt, actin, JNK, ERK, and ASK1 antibodies were purchased from Santa Cruz Biotechnology. *p*-Akt (S473), *p*-ASK1 (T845, S83, S967), *p*-p85 (Y458), *p*-JNK(T183/Y185), and *p*-ERK1/2(T202/Y204) were obtained from Cell Signaling Technology.

Plasmid Constructs and Transfection. Various expression plasmids for DAB2IP were described previously (1, 2). Cells (5×10^5 cells per well) were plated in a six-well plate (Costar) with 70% to 80% confluence before transfection. The transfection was carried out by using Lipofectamin PLUS (Invitrogen) according to the manufacturer's instructions.

Immunoprecipitation, Pull-Down Assay, and Western Blot Analysis. For immunoprecipitation, transfected 293 cells after LY (10 μM) or TNF-α (100 ng/ml) treatment for 30 min were washed twice with cold PBS and lysed in 1.5 mL of cold lysis buffer for 20 min on ice. The immunocomplex was subjected to Western blot analysis as described previously (2).

For GST pull-down assay, cell lysate was prepared by incubating with 0.5 mL of lysis buffer for 20 min on ice, and supernatant was separately incubated with either 30 μL of GST-glutathione-Sepharose or GST-p85-SH3-Sepharose overnight at 4 °C. The pellet was washed twice with lysis buffer, dissolved in sample buffer, and subjected to Western blot analysis.

qRT-PCR. qRT-PCR was performed by using both sets of DAB2IP primer (5′-TCGTGGAAGGACTCATGACC-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′, 2 ng/μL) and 18S RNA primer (5′-GGAATTGACGGAAGGGCACCACC-3′ and 5′-GTGCAGCCCCGGACATCTAAGG-3′, 0.6 ng/μL). All experiments were repeated at least twice in duplicate. The relative level of DAB2IP mRNA from each sample was determined by normalizing the copy number of DAB2IP cDNA with the copy number of 18S RNA cDNA of each sample.

Immunohistochemistry. Tumors and mouse prostate tissue were removed and fixed in 5% formalin and prepared for histological analysis. Consecutive tumor sections were stained with H&E, *p*-Akt (S473, IHC specific; Cell Signaling Technology) and Ki-67 (BD PharMingen) antibodies by using ABC-staining kit (Santa Cruz Biotechnology) with the secondary biotinylated antibody (Invitrogen).

1. Wang Z, et al. (2002) The mechanism of growth-inhibitory effect of DOC-2/DAB2 in prostate cancer. Characterization of a novel GTPase-activating protein associated with N-terminal domain of DOC-2/DAB2. *J Biol Chem* 277:12622–12631.

2. Zhang R, et al. (2003) AIP1 mediates TNF alpha-induced ASK1 activation by facilitating dissociation of ASK1 from its inhibitor 14–3-3. *J Clin Invest* 111:1933–1943.

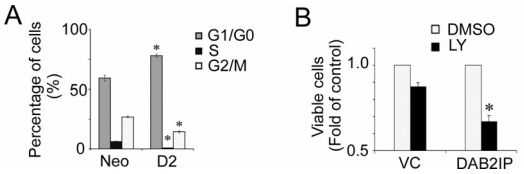

Fig. S1. DAB2IP promoted cell cycle arrest and cell death. (*A*) DAB2IP induced G_0/G_1 cell cycle arrest. Cell cycle distribution of neo and D2 cells was detected by PI staining by using flow cytometry. Asterisk indicates statistical significance in D2 cells compared with neo cells ($P < 0.01$). (*B*) C4–2 cells were transfected with DAB2IP or VC after LY treatment, and viable cells were determined by crystal violet assay. Data (mean ± SEM) were generated from three independent experiments. Asterisk indicates statistical significance in cells transfected with DAB2IP vs. VC ($P < 0.01$).

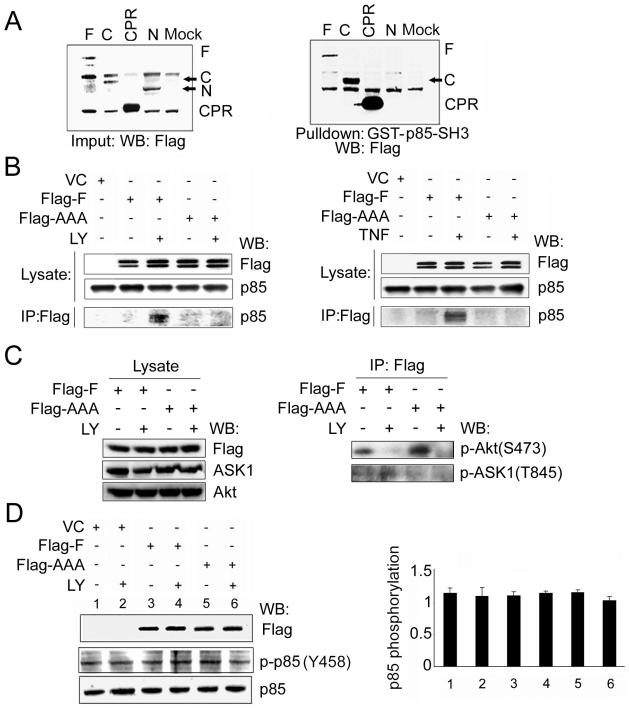

Fig. S2. Interaction of DAB2IP with p85 via PR domain. (*A*) After transfecting with different DAB2IP cDNA constructs, C4-2 cells were subjected to pull-down assay using GST-p85-SH3 fusion protein and resolved on SDS/PAGE and probed with Flag antibody. In these experiments, 5% of each input lysate was used as a loading control. (*B*) Mutation of prolines abrogated DAB2IP binding to p85 in vivo. The 293 cells were transfected with DAB2IP-F or AAA mutant after LY or TNF-α treatment. Cell lysates were IP with Flag and probed with p85 antibody. (*C*) Mutation of prolines impaired Akt inhibition and ASK1 activation in DAB2IP complex. The 293 cells were transfected with DAB2IP-F or VC, and cell lysates were subjected to co-IP with Flag antibody then probed with *p*-ASK1 (T845) or *p*-Akt (S473) antibody. (*D*) DAB2IP did not alter p85 phosphorylation. The 293 cells were transient transfected with DAB2IP-F, AAA mutant, or VC after LY treatment. Cell lysates were subjected to Western blot analysis probed with Flag, *p*-p85 or p85 antibody as indicated. Data (mean ± SEM) were generated from three independent experiments.

Phosphoserine/threonine binding group (pST_bind)				
14-3-3 Mode 1			Gene Card YWHAZ	
Site	Score	Percentile	Sequence	SA
S604	0.3522	0.22%	PSPARSSSYSEANEP	2.006
Src homology 2 group (SH2)				
Fgr SH2			Gene Card FGR	
Site	Score	Percentile	Sequence	SA
Y605	0.4865	0.89%	SPARSSSYSEANEPD	1.512
Src homology 3 group (SH3)				
Cbl-Associated protein C-SH3			Gene Card **N/A**	
Site	Score	Percentile	Sequence	SA
P599	0.7109	0.58%	SSGVQPSPARSSSYS	1.947
Cbl-Associated protein C-SH3			Gene Card **N/A**	
Site	Score	Percentile	Sequence	SA
P696	0.7344	0.81%	SEGAPGRPQLLAPLS	0.809
Basophilic serine/threonine kinase group (Baso_ST_kin)				
PKC alpha/beta/gamma			Gene Card PRKCA	
Site	Score	Percentile	Sequence	SA
T675	0.4597	0.70%	VNLAGLATVRRAGQT	0.785
Akt Kinase			Gene Card AKT1	
Site	Score	Percentile	Sequence	SA
S604	0.5561	0.56%	PSPARSSSYSEANEP	2.006
Acidophilic serine/threonine kinase group (Acid_ST_kin)				
GSK3 Kinase			Gene Card GSK3A	
Site	Score	Percentile	Sequence	SA
S602	0.4827	0.21%	VQPSPARSSSYSEAN	1.71
Proline-dependent serine/threonine kinase group (Pro_ST_kin)				
Cdk5 Kinase			Gene Card CDK5	
Site	Score	Percentile	Sequence	SA
S598	0.4929	0.83%	RSSGVQPSPARSSSY	2.516
Cdc2 Kinase			Gene Card CDC2	
Site	Score	Percentile	Sequence	SA
S598	0.4944	0.49%	RSSGVQPSPARSSSY	2.516
Phosphotyrosine binding group (PTB)				
Shc PTB			Gene Card SHC1	
Site	Score	Percentile	Sequence	SA
Y709	0.5485	0.43%	LSFQNPVYQMAAGLP	0.714

Fig. S3. Akt as a candidate binding protein to the PER domain in DAB2IP. Amino acid sequence of DAB2IP-PER domain (amino acids 591–719) was scanned by using the Scansite program. Akt-binding site at S604 in PER domain is highlighted in red.

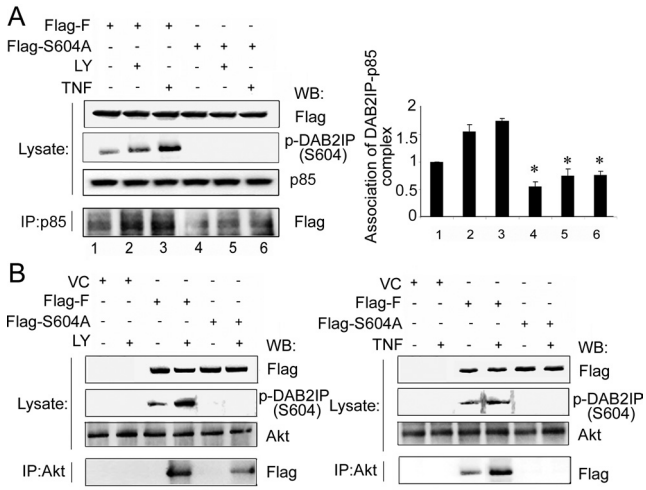

Fig. S4. The role of S604 phosphorylation in DAB2IP activity. (*A*) The 293 cells were transfected with DAB2IP-F or S604A mutant after LY or TNF-α treatment. Cell lysates were subjected to either Western blot analysis probed with *p*-DAB2IP or Flag antibody or co-IP with p85 then probed with Flag antibody. In DAB2IP-F cells without treatment, immunoprecipitated p85 was considered as a basal level (= 1), and the fold of induction was calculated accordingly. Data (mean ± SEM) were generated from two independent experiments. Asterisk indicates statistical significance in cells transfected with DAB2IP-F vs. AAA ($P < 0.01$). (*B*) Cell lysates derived from 293 cells transfected with DAB2IP-F and S604A mutant after LY or TNF-α treatment were subjected to Western blot analysis probed with *p*-DAB2IP or Akt antibody, or co-IP with Akt antibody then probed with Flag antibody.

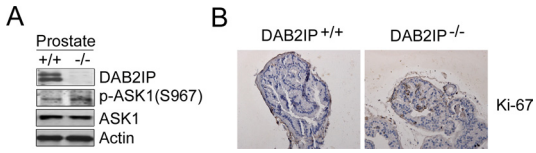

Fig. S5. The role of DAB2IP in prostate hyperplasia. (*A*) Decreased ASK1 activity in the prostate of DAB2IP KO mice. Tissue lysates were analyzed by Western blot using *p*-ASK1 (S967, inactive form) antibody. β-Actin was used as a loading control. (*B*) Elevated cell proliferation marker in the prostate epithelia of DAB2IP KO mice. IHC from the paraffin sections of prostate were determined by Ki-67 antibody. (Magnification: 200×.)

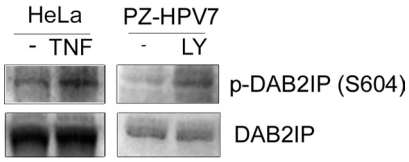

Fig. S6. Elevated S604 phosphorylation of endogenous DAB2IP in HeLa or PZ-HPV-7 after LY or TNF-α treatment. Cell lysates prepared from HeLa and PZ-HPV-7 cells after LY or TNF-α treatment were subjected to Western blot analysis probed with *p*-DAB2IP or DAB2IP antibody.