Role of DAB2IP in modulating epithelial-to-mesenchymal transition and prostate cancer metastasis

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DAB2IP, also known as ASK1-interacting protein-1 (AIP1), a novel member of the Ras GTPase-activating protein family, has been implicated in cell-growth inhibition and apoptosis (10, 11). DAB2IP is down-regulated in various human cancers mainly because of altered epigenetic regulation of its promoter, such as by DNA hypermethylation and/or histone modification (12–17). Thus, it is very likely that DAB2IP functions as a tumor suppressor in cancer development; however, its role and mechanism in cancer metastasis is largely unknown. In the current study, we show that loss of DAB2IP facilitates EMT leading to PCa metastasis.

Results

DAB2IP Modulates Glycogen Synthase Kinase (GSK)-3β-Catenin Signaling Pathway. To dissect the possible mechanism of DAB2IP in EMT responses, we examined the effect of DAB2IP
on the GSK-3β-β-catenin signaling pathway. In canonical Wnt pathways, GSK-3β-mediated β-catenin degradation is inhibited, leading to accumulation of β-catenin in the nucleus that further transactivates β-catenin (TCF) target genes. Thus, a hallmark of β-catenin signaling in both normal and neoplastic tissue is nuclear translocation (19). By knocking down endogenous DAB2IP levels with siRNA, we observed both accumulation of cytoplasmic β-catenin and nuclear translocation of β-catenin as well as reduced membrane-associated β-catenin (Fig. 2A). Although DAB2IP did not alter the steady-state levels of β-catenin mRNA (Fig. S3A Left) in D2 cells, cytoplasmic β-catenin levels substantially decreased, because DAB2IP can increase proteasomal degradation of β-catenin (Fig. S3A Right). Moreover, in DAB2IP-expressing cells, GSK-3β activity was significantly elevated based on Ser9 (S9, negative regulatory site) phosphorylation levels, β-catenin/TCF transcriptional activity (TOP/FOP), and GSK-3β kinase activity, respectively (Fig. 2B–D and Fig. S3 B and C). Furthermore, DAB2IP diminished Wnt-elicited β-catenin/TCF transcriptional activity (Fig. S3D). Consistently, knocking down endogenous DAB2IP in PZ-HPV-7 cells by transient transfection of DAB2IP-siRNA increased GSK-3β phosphorylation (S9), β-catenin levels, and β-catenin/TCF transcriptional activity, which were further potentiated by Wnt treatment (Fig. 2E). Thus, DAB2IP modulates GSK-3β-β-catenin signaling through activation of GSK-3β by reducing S9 phosphorylation.

Wnt signaling is a key inducer of EMT during embryonic development and cancer progression (20–22). We tested whether or not manipulating DAB2IP levels in various cell lines could modulate Wnt-induced EMT. Whereas Wnt only slightly elicited EMT (Fig. 2E, lanes 1 and 2) in PZ-HPV-7 cells, its effect on EMT increased significantly after endogenous DAB2IP was knocked down by DAB2IP-siRNA (Fig. 2E, lanes 3 and 4). In contrast, restoring DAB2IP expression in 293 cells (DAB2IP-negative cell) prevented Wnt-induced EMT (Fig. S3E), strongly suggesting that DAB2IP was an antagonist of Wnt-mediated EMT.

**DAB2IP Recruits PP2A to Active GSK-3β Through Its C2 Domain.** Based on a screening using the Scansite program (http://scansite.mit.edu/) and coimmunoprecipitation (co-IP) (Fig. 3A), GSK-3β appears to directly associate with DAB2IP. Because DAB2IP is not a phosphatase, the mechanism of GSK-3β activation by DAB2IP is likely mediated by a separate phosphatase associated with this complex. We have previously shown that PP2A can form a complex with DAB2IP (23). PP2A is a heterotrimeric complex containing a catalytic subunit (C), a structural subunit (A), and a variable regulatory subunit (B) (24). Although some studies suggest GSK-3β (S9) as a potential substrate for PP2A (25–27), the functional role of PP2A in the Wnt-β-catenin pathway is still controversial (28). The co-IP data (Fig. 3A and Fig. S4 A and D) indicated that DAB2IP could form a complex with GSK-3β and PP2A as well as Axin. Structurally, DAB2IP contains several potential functional domains including an N-terminal pleckstrin-homology (PH) domain as well as C2, GTPase activating (GAP), C-terminal period-like (PER), proline-rich (PR), and leucine zipper (LZ) domains (Fig. 3B). By transfecting Flag-tagged DAB2IP deletion vectors (F, N, C, PHC2, and PH) into 293 cells, the data showed that C2 was the key domain that interacted with both GSK-3β and PP2A (Fig. S4B). Consistently, full-length DAB2IP, N, and PHC2 domains, rather than C and PH domains, inhibited β-catenin/TCF transcriptional activity (Fig. 3C). In addition, other mutant constructs, such as GAPm (GAP mutant) (10), S604A (S604 mutant) (29), and AAA (PR mutant) (29), did not inhibit β-catenin-mediated transcriptional activity (Fig. 3C), indicating...
that these functional domains were not involved in DAB2IP-modulated GSK-3β-β-catenin signaling.

To further assess the direct effect of PP2A on GSK-3β-β-catenin activity, 293 cells were cotransfected with several expression vectors: DAB2IP (F), GSK-3β [wild type (WT) or S9A, constitutive active], and PP2A [WT or catalytic inactive (LP)]. In the presence of both DAB2IP and GSK-3β-WT, PP2A-LP, rather than PP2A-LP, further decreased β-catenin/TCP transcriptional activity (Fig. S4C). We also examined the role of endogenous PP2A in DAB2IP-modulating GSK-3β-β-catenin signaling and MET responses. DAB2IP-expressing cells were treated with the PP2A inhibitor, Okadaic acid (OA), or PP2A-siRNA. Both OA and PP2A-siRNA treatments abolished DAB2IP-mediated dephosphorylation of GSK-3β on S9 (Fig. 3D). Consistently, either OA (Fig. S4E) or PP2A-siRNA (Fig. 3E) treatments alone could block DAB2IP-mediated MET responses. Similar results were also observed in PC-3 cells expressing endogenous DAB2IP (Fig. S4F). These data clearly indicate that PP2A is critical for DAB2IP-mediated GSK-3β activation and MET responses.

β-Catenin Overexpression Reverses DAB2IP-Mediated MET. Because DAB2IP can activate GSK-3β and then lead to decreased cytosolic β-catenin protein levels and nuclear β-catenin transcriptional activity (Fig. 2A and Fig. S5 A-D), we examined whether or not the inhibitory effect of DAB2IP could be reversed by overexpressing β-catenin. The elevated expression of β-catenin in PC-3 cells also induced EMT in a dose-dependent manner (Fig. S5E). Similarly, in both D2 and DAB2IP transfected cells, increasing dosage of β-catenin DNA restored EMT based on marker and morphology (Fig. S6).

DAB2IP Down-Regulation Promotes Tumor Progression and Metastasis. Because PC-3 cells have low metastatic potential (30, 31) and the decreased DAB2IP expression in these cells can initiate EMT (Fig. 1E), we examined the metastatic potential of KD1- versus Con-expressing PC-3 cells using an orthotopic mouse model. Stable CMV-luciferase activity was confirmed in each subline to ensure the equal level before injection. Bioluminescent imaging (BLI) and small-animal positron emission tomography and computed tomography (PET–CT) were used to monitor tumor growth and onset of metastases. One week after injection, both BLI and 2-[18F]fluoro-2-deoxy-D-glucose (18F-FDG)–PET–CT (Fig. 4A and Fig. S7A) clearly detected multiple metastatic lesions in various organ sites in animals injected with PC-3-KD1 cells, and 3D imaging further revealed lymph-node metastases (Video S1). In contrast, control mice only exhibited small primary tumors 5 weeks postinjection, and none of them showed any signs of metastases (Fig. S7 A and D). Nearly all mice bearing DAB2IP-knockdown cells died within 2 weeks (Fig. S7E). From autopsy (Fig. 4B and Fig. S7 B and C), all mice bearing KD1 cells developed macroscopic evidence of enlarged mesenteric lymph nodes as well as additional lymph nodes in various organs, including the liver, kidney, and spleen; occasionally, some mice developed metastatic nodules in visceral organs including liver and stomach (Fig. S7B, green arrows). H&E data showed a definitive distant spread of PCa cells where poorly differentiated tumor cells were present within apparent lymph nodes (Fig. 4C). Immunohistochemistry (IHC) showed that the majority of tumor cells strongly expressed vimentin and p-GSK-3β (S9) (Fig. S7F), but they exhibited weak staining of E-cadherin and cytokeratin (Fig. 4C).

In addition, we also employed another PCa model (i.e., LAPC4) expressing androgen receptor (AR) and prostate-specific antigen (PSA) (32) to examine the effect of DAB2IP on cancer metastasis. By knocking down the endogenous DAB2IP, LAPC4 cells elicited similar EMT changes as those in PC-3 cells in vitro as well as high incidence of lymph-node metastases using orthotopic KD1 tumor models (Fig. S8). These data provide a strong evidence for the inhibitory role of DAB2IP in PCa metastases.

DAB2IP Knockout Mice Exhibit Mesenchymal Characteristics in the Prostate Gland. To further determine the impact of DAB2IP on phenotypic changes in normal prostate glands, DAB2IP knockout (KO; DAB2IP<−/>) mice were employed (33). In DAB2IP WT (DAB2IP+−/) mice, DAB2IP expression was associated with
prostate glandular epithelial cells (Fig. 5A). Consistent with in vitro data (Figs. 2 and 3), S9 phosphorylation of GSK-3β was higher in DAB2IP−/− than that in DAB2IP+/+ mice (Fig. 5B), suggesting that GSK-3β activity was suppressed in DAB2IP−/− mice. Also, the prostate glandular epithelia in DAB2IP−/− mice exhibit decreased E-cadherin, elevated vimentin expression, and strong nuclear β-catenin staining (Fig. 5A). Although no spontaneous PCa has been detected in DAB2IP−/− mice, hyperplastic lesions were often detected in DAB2IP−/− mice older than 6 months of age (Fig. S9A).

We further examined the relationship between DAB2IP expression and EMT markers using different stages of human prostate specimens including normal tissue, benign prostatic hyperplasia (BPH), and PCa with moderate and poorly differentiated tumor. Loss of DAB2IP and E-cadherin as well as increased vimentin and p-GSK-3β were clearly detected in tissues from prostate-cancer patients (Fig. 5C and Fig. S9B). There is a significant correlation between the levels of DAB2IP and E-cadherin (r = 0.8390) and an inverse correlation between the levels of DAB2IP and vimentin (r = 0.7255) levels in all of the

Fig. 4. DAB2IP down-regulation promotes tumor growth and metastasis. (A) Representative BLI imaging of mice bearing PC-3-KD1 tumors with multiple metastatic lesions. Mice (n = 9) were imaged 10 days later to determine local tumor growth and metastasis. (B) Numbers of metastatic nodules in individual mouse bearing Con or KD1 tumors at the time of sacrifice. (C) Representative H&E and IHC staining patterns. H&E staining showed primary tumor without detectable metastases in control mice and the lymph node metastases in mice bearing KD1 tumors 2 weeks postinjection (magnification: 100×). IHC showed the majority of KD1 tumors with a strong positive vimentin staining but weak E-cadherin and Cytokeratin staining (magnification: 200×). The dashed line separates between the area of mouse prostate (P) and PC-3 tumor (T).
samples tested (Fig. S9C). Taken together, our human and mouse in vivo data are consistent with in vitro data from various normal epithelial or cancer-cell lines.

Discussion

In this study, we clearly show that DAB2IP functions as a scaffold protein in modulating GSK-3β-catenin signaling and EMT. In the presence of DAB2IP, interaction of its C2 domain with both PP2A and GSK-3β facilitates GSK-3β activation through S9 dephosphorylation. Then, it decreases nuclear β-catenin accumulation and its transcriptional activity, indicating the potent inhibitory function of DAB2IP in Wnt-β-catenin signaling. Within the DAB2IP-PP2A-GSK-3β complex, GSK-3β seems to be a direct substrate for PP2A, a known negative regulator in Wnt-β-catenin signaling (28, 34), which is the underlying mechanism for the DAB2IP function. The role of S9 phosphorylation of GSK-3β in Wnt-β-catenin signaling is still controversial. For example, the S9 phosphorylation of GSK-3β is not correlated with Wnt-mediated GSK-3β activity in certain cell types (35, 36). However, other studies have shown that many growth factors, such as insulin growth factor, transforming growth factor-β, and epidermal growth factor etc., can increase β-catenin accumulation through S9 phosphorylation of GSK-3β (37–40). Inactivation of GSK-3β through S9 phosphorylation is involved in hepatitis B virus-x protein (HBX)-mediated β-catenin stabilization in hepatocellular carcinoma cells (41). From several prostate-cell lines (e.g., C4-2 and PZ-HPV-7) tested, we showed that S9 phosphorylation of GSK-3β was clearly involved in DAB2IP-mediated β-catenin stability and transcriptional activity, suggesting that the effect of S9 phosphorylation on β-catenin signaling is cell-type dependent.

In general, β-catenin has a dual role in EMT: it not only enhances cell–cell adhesion by associating with cadherin complexes in adheren junctions of cell membrane, but it also functions as a transcriptional coactivator after interacting with TCF transcription factor complexes in the nucleus (21, 42). The induction of EMT by nuclear β-catenin has been explored during development in cell lines and tumors (2). Several studies suggest that β-catenin–mediated transcription can induce Slug (43) or Twist1 (44) gene expression that further represses E-cadherin, thereby contributing to the EMT. Our data show that loss of DAB2IP in cells can lead to the accumulation of nuclear β-catenin (Fig. 2A and Fig. S5C), because DAB2IP facilitates cytoplasmic β-catenin degradation (Fig. S3A). Thus, we believe that DAB2IP can modulate the dynamic switching between membrane- and nuclear-associated β-catenin through PP2A and GSK-3β (Fig. 2 and Fig. S5), which determines EMT (4). In addition, we observed that ZEB1 and ZEB2/SIP1, transcriptional factors associated with EMT (3, 4), were highly elevated in DAB2IP-depleted cells (Fig. S2D). Taken together, these data indicate that DAB2IP is a key regulator in preventing EMT.

The majority of human visceral tumors derived from carcinomas exhibit an epithelial phenotype. To break away from neighboring cells and invade adjacent tissue layers or peripheral lymph nodes, carcinoma cells often lose cell–cell adhesion and acquire motility. In general, 25–30% of patients newly diagnosed with PCa will have local invasive cancer, and almost all of these patients will eventually develop metastatic disease, accounting for most cancer deaths (1). Needless to say, the detection of metastatic lesions at an early stage or during treatment should lead to an increase in disease-free survival rates. From the clinical outcome of PCa progression, the presence of lymph-node invasion has the lowest rate of 10-year progression-free survival rate (45). Our orthotopic PCa animal model (Fig. 4) shows that mice bearing DAB2IP-knockdown cells have a dramatic increase in the incidence of lymph-node metastases as well as the number of metastatic sites where tissues clearly exhibit mesenchymal characteristics. Wnt signaling has been identified as a determinant of lung-cancer metastasis to brain and bone (46). Similarly, our data indicated that down-regulation of DAB2IP can increase the propensity of PCa cells to metastasize to lymph nodes (Fig. 4 and Fig. S8), which was associated with the hyperactivation of Wnt/β-catenin/Tcf pathway (Fig. 2E and Fig. S5D). Consistently, DAB2IP+/− mice exhibit similar changes of EMT markers in the prostate gland (Fig. 5). In addition, prostate hyperplasia was observed in DAB2IP−/− mice after 6 months of age. A previous study has also shown the prostate hyperplasia in adenomatous polyposis coli knock-out (APC−/−) mice after 4.5 weeks of age (47). In both models, the accumulation of nuclear β-catenin was observed; however, unlike PCa, which was detected in APC−/− mice after 6 months of age, no tumor has been detected in our model at this age. Because APC is known to regulate many pathways other than the Wnt/β-catenin pathway (48), it is possible that other downstream effects in addition to nuclear β-catenin activation may be involved in PCa formation in APC−/− mice. In DAB2IP−/− prostate, APC levels remained the same as in DAB2IP+/− mice (Fig. 5B) and thus, might also exert an inhibitory effect on tumor initiation.

In summary, this study delineates the functional role of DAB2IP in EMT, which also explains how loss of DAB2IP in PCa underlies the onset of aggressive metastatic PCa. We believe that the assessment of DAB2IP expression in PCa specimens can be a valuable prognostic biomarker for risk of PCa metastasis and the delineation of DAB2IP function could provide a potential intervention strategy for PCa metastasis.

Materials and Methods

Plasmid Constructs, Conditioned Medium, Antibodies, siRNA Oligonucleotides, and Immunoprecipitation. See SI Materials and Methods.

Cell Culture and Clinical Specimens. C4-2, DAB2IP-transfected C4-2 sublines (i.e., D1 and D2), the vector control subline (Neo), and PC-3 subline (Con, KD1) cells were maintained in T medium (Invitrogen) supplemented with 5% FBS. PZ-HPV-7 was maintained in PRGM medium (Lonza). RWPE-1 cells were maintained in Keratinocyte medium (Invitrogen) containing 10% FBS, PNT-1A and human embryonic kidney 293 cells were maintained in Dulbecco's Modified Eagle’s Medium (DMEM; Invitrogen) containing 10% FBS. MDA-MB-231 was maintained in RPMI medium (Invitrogen) containing 10% FBS.
The Institutional Review Board approved the tissue procurement protocol in this study, and appropriate informed consent was obtained from all patients. Benign prostatic hyperplasia specimens were obtained from transurethral prostatic resection, and primary cancer specimens (Gleason score of 6–9) were obtained from prostatectomy performed in our institute. Cell lysates were subjected to Western blot analysis or immunohistochemical staining (SI Materials and Methods).

In Vitro Migration Assay. For migration assays, 5 × 10^5 cells were plated in the top chamber of a Transwell (24-well insert; pore size = 8 μm; Corning) and incubated with serum-free medium placed in the lower chamber. After incubation for 48 h, cells that did not migrate or invade through the pores were removed by a cotton swab, and cells on the lower surface of the membrane were stained with Cell Stain (Chemicon) and quantified by measuring OD_{560} with 96-well plate.

Analyses of Wnt Signaling Pathway. Cells were treated with Wnt-CM and L-CM for 24 h, and Wnt signaling activities were determined by various assays such as Western blot, qRT-PCR, GSK-3β kinase assay, Luciferase reporter gene assay, and fluorescence confocal microscopy (SI Materials and Methods).

Orthotopic Animal Model and Imaging. All experimental procedures have been approved by the Institutional Animal Care and Use Committee. The ventral prostate of male nruhu mice (6–8 weeks of age) were exposed by midventral incision under anesthesia with 2% isoflurane (in 70% N2O/30% O2) induction of anesthesia after injection, surgical staples were removed, and the tumor growth and local metastasis were monitored using BLI and PET (SI Materials and Methods).

Statistical Analysis. All error bars in graphical data represent mean ± SEM. Student’s two-tailed t test was used for the determination of statistical relevance between groups, and P < 0.05 was considered significant.

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Supporting Information

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SI Materials and Methods

Plasmid Constructs, Conditioned Medium, and Antibodies. Various expression plasmids for DAB2IP, PP2A (WT), PP2A (LP), GSK-3β (WT), or GSK-3β-S9A were described previously (1). For cDNA transfection, cells (5 × 10^6 cells/well) were seeded in a six-well plate (Costar) with 70–80% confluence before transfection. The transfection was carried out using Lipofectamin PLUS (Invitrogen) according to the manufacturer’s instructions.

WNT3A- and control-conditioned medium (Wnt-CM and L-CM) were collected according to the directions from ATCC and treated with cells for 24 h during the experiments.

Anti-DAB2IP polyclonal antibody was described previously (2). Anti-FLAG and anti-vimentin were obtained from Sigma. Anti-GSK-3β was purchased from Santa Cruz Biotechnology. Anti-phospho-GSK-3β (S9) was obtained from Cell Signaling Technologies. Anti-PP2A, anti-actin, anti-E-cadherin, and anti-β-catenin were obtained from BD Pharmingen. Anti-human specific pan-cytokeratin was purchased from abcam. Chemical inhibitors including Okadaic acid and lithium chloride were purchased from Calbiochem.

siRNA Oligonucleotides and Delivery Methods. Three pairs of siRNA oligonucleotides for human DAB2IP (5′-GGAGCGCAACA-GUACCUGTT-3′ [siRIP1-A]; 5′-GGUGAGGGACUUCU-GACATT-3′ [siRIP1-B]; 5′-GGACUGUUUUUUGUACACATT-3′ [siRIP1-C]) and control siRNA (5′-CTGGACTTCCAGAAA-GAACA-3′) were synthesized by Invitrogen. siRNA oligonucleotides for PP2AC were purchased from Thermo Scientific. siRNA oligonucleotides (20 μM) were transfected into cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. pGIPZ-DAB2IP-lentiviral-shRNAmir and pGIPZ-non-silencing-lentiviral-shRNAmir were purchased from Open Biosystems, and infection was performed using DAB2IP-lentiviral-shRNAmir and pGIPZ-shRNAmir lentivirus. The effective pixel size was 103.03 μm. The total CT acquisition time was 6 minutes. CT images were reconstructed with a down-sampling of 4 and an average frame of 1. Under low magnification, the effective pixel size was 103.03 μm. The total CT acquisition time was ~6 min. CT images were reconstructed with a downsample factor of 2 using Cobra Reconstruction Software. The PET imaging was performed directly after the CT scans at 1 h post-injection of 18F-FDG. PET images were reconstructed using Fourier Rebinning and Ordered Subsets Expectation Maximization 2D (OSEM2D) algorithm. Reconstructed CT and PET images were fused and analyzed using the Siemens Inveon Research Workplace (IRW) software.

Immunoprecipitation and Western Blot Analysis. For immunoprecipitation, transfected 293 cells were washed twice with cold PBS and lysed in 1.5 mL of cold lysis buffer [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Triton X-100, 1 mM sodium orthovanadate, 10 mg/mL aprotinin, 10 mg/mL leupeptin, 2 mM phenylmethylsulfonyl fluoride, and 1 mM EDTA] for 20 min on ice. The immunocomplex was subjected to Western blot analysis as described previously (2).

qRT-PCR Analysis. The total cellular RNA was extracted with RNeasy mini kit (Qiagen) treated with RNase-free DNase I (Qiagen). A total of 1 μg RNA was subjected to a cDNA synthesis kit (Bio-Rad). Ten-fold of the cDNA was subjected to a 25-μL PCR carried out in an iCycler thermal cycler (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) with PCR primers (β-catenin: F:GAACCGCTTCTACAGGC and R:CTGGCCATTATCC ACCAGAGT; DAB2IP: F:TCGTGGAAGGACTCATGACC and R: TCCACACCGTCGTGCTGTA, 2 ng/μL) and 18S RNA primer sets (0.6 ng/μL). All experiments were repeated at least twice to duplicate results. The relative level of DAB2IP mRNA from each sample was determined by normalizing 18S cDNA.

GSK-3β Kinase Assay. A fluorescence peptide substrate-based assay was used to assess the GSK-3β kinase activity (Omnia Ser/Thr Recombinant Kit; Invitrogen). Briefly, the GSK-3β complex was prepared from the same amount of cell lysates by immunoprecipitation and then incubated with 10 μM of S/T Peptide substrate in kinase-reaction buffer (containing 1 mM ATP and 1 mM DTT) for 20 min at 30 °C. Fluorescence intensity was collected by measuring OD 485 with a 96-well plate. Relative GSK-3β activity was calculated using untreated cells (100% activity).

Luciferase Reporter Gene Assay. For the reporter gene assay, cells seeded in 24-well plates were transfected with β-catenin Firefly luciferase reporter gene construct (TOP or FOP; 200 ng) and 1 ng of the prL-SV40 Renilla luciferase construct (as an internal control). Cell extracts were prepared 24 h after transfection, and the luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega).

Fluorescence Confocal Microscopy. B16 melanoma cells were transfected with a control (5′-GGACUGUAAUUUGUACACATT-3′) or DAB2IP (5′-GGACUGUAAUUUGUACACATT-3′) siRNA for 72 h. Cells were fixed in 4% formaldehyde and subjected to indirect immunofluorescence microscope with anti-β-catenin. The fluorescein isothiocyanate (FITC)-conjugated anti-IgG was purchased from Molecular Probes. Confocal immunofluorescence microscopy was performed using an Olympus confocal microscope as described (3). Magnification is 500x.

BLI and PET imaging. BLI was performed using IVIS Imaging System (Xenogen). Images and bioluminescent signals were acquired and analyzed using Living Image and Xenogen software. Brieﬂy, in each imaging, four mice were anesthetized, injected with D-luciferin (150 mg/kg intraperitoneally), and then imaged 10 min after injection for 3 min.

Small-animal PET/CT imaging studies were performed using a Siemens Inveon PET/CT Multimodality System. Tumor-bearing mice were injected with ~3.7 MBq (100 μCi) of 18F-FDG through the tail vein. Ten minutes before imaging, the animals were anesthetized using 3% isoflurane at room temperature until stable vitals were established. After the animal was sedated, the animal was placed onto the imaging bed under 2% isoflurane anesthesia for the duration of the imaging. The CT data were acquired at 80 kV and 500 μA with a focal spot of 58 μm. The total rotation of the gantry was 360° with 360 rotation steps obtained at an exposure time of ~200 ms/frame. The images were attained using a CCD readout of 4096 × 3098 with a bin factor of 4 and an average frame of 1. Under low magnification, the effective pixel size was 103.03 μm. The total CT acquisition time was ~6 min. CT images were reconstructed with a downsample factor of 2 using Cobra Reconstruction Software. The PET imaging was performed directly after the CT scans at 1 h post-injection of 18F-FDG. PET images were reconstructed using Fourier Rebinning and Ordered Subsets Expectation Maximization 2D (OSEM2D) algorithm. Reconstructed CT and PET images were fused and analyzed using the Siemens Inveon Research Workplace (IRW) software.

Histology and Immunohistochemical Staining. Tumors were removed, weighed, fixed in 5% formalin, and prepared for histological analysis. Consecutive tumor sections were stained with H&E, E-cadherin, vimentin, and p-GSK-3β (S9). Immunohistochemical staining was carried out using ABC-staining Kit (Santa Cruz Biotechnology Inc.) and the secondary biotinylated antibody to mouse IgG (Invitrogen), respectively. WT and KO mice were killed, and tissues were perfused in situ with

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PBS and then inflated and fixed with 10% buffered formalin. The sections were paraffin embedded, sectioned in 5 μM, and stained with routine H&E. Tissue sections were also stained with DAB2IP, E-cadherin, vimentin, and β-catenin.


Fig. S1. DAB2IP regulates EMT in vitro. (A) Immunofluorescence staining of E-cadherin and vimentin in the control (Neo) or DAB2IP expressing C4-2 cells (D2) with a DAPI counter staining (magnification: ×100). qRT-PCR analysis of Fibronectin and N-cadherin mRNA levels in Neo, D1, and D2 cells. Data (mean ± SEM) were generated from three independent experiments. (B) Cells were seeded in serum-free medium for 48 h, and the relative cell number was determined by 3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. There is no statistical significance between Neo versus D1 or D2 cells (P > 0.05) at day 2. (C) Expression of epithelial or mesenchymal markers in MDA-MB-231 and 293 cells. β-actin was used as a loading control.
Knockdown of DAB2IP initiates EMT in vitro. (A) PNT1-A and PC-3 cells were infected with control lentivirus or lentivirus-expressing shRNA specific to DAB2IP and then selected with puromycin. The morphology was revealed by phase-contrast microscopy (magnification: ×100). Con, control-shRNA vector; KD, DAB2IP-shRNA knockdown. (B) Increased mesenchymal and reduced epithelial markers in DAB2IP-knockdown PNT1-A cells were analyzed by Western blot. (C and D) Increased mesenchymal markers and transcriptional factors in DAB2IP-knockdown cells. N-cadherin, ZEB1, and ZEB2 mRNA levels were analyzed by qRT-PCR in PZ-HPV-7 (Con, KD1), RWPE-1 (Con, KD1), and PC-3 (Con, KD1) cells. Asterisk indicates statistical significance between Con versus KD1 cells (P < 0.01). (E) Loss of DAB2IP promotes cell migration in vitro. (Left) The relative DAB2IP mRNA levels determined by qRT-PCR in PC-3-Con cells and three different clones of PC-3-KD cells. Con, control-shRNA vector; KD1, KD2, and KD3, DAB2IP-shRNA knockdown. (Center) The PC-3-Con cells and PC-3-KD1 cells were plated in Transwell, and cell migration was determined in serum-free medium 48 h after plating. The each bar represented the mean ± SEM of each sample measured in triplicate. Asterisk indicates statistical significance of migrated cells between Con versus KD1, KD2, or KD3 cells (P < 0.01). (Right) Cells were plated under the same culture condition as cell migration assay. The relative cell number was determined by MTT assay. There is no statistical significance of total cell number between Con versus KD1, KD2, or KD3 cells (P > 0.05) at day 2.
Fig. S3. DAB2IP antagonizes nuclear β-catenin activity. (A) DAB2IP promotes β-catenin protein degradation. qRT-PCR analysis of β-catenin mRNA levels in Neo, D1, and D2 cells. Data (mean ± SEM) were generated from three independent experiments. There is no statistical significance in between Neo versus D1 or D2 cells (P > 0.05). D2 cells were treated with proteasome inhibitor MG132 (10 μM) for 12 h, and cell lysates were subjected to Western blotting and probed with β-catenin antibody. β-actin was used as a loading control. (B) DAB2IP inhibits GSK-3β phosphorylation at S9 and β-catenin/TCF transcriptional activity. Two hundred ninety-three sublines were treated with LiCl (20 mM; 6 h), and cell lysates were blotted with p-GSK-3β (S9) and GSK-3β antibody. β-actin was used as a loading control. Two hundred ninety-three sublines were transfected with TOP-flash or FOP-flash followed by LiCl treatment (20 mM; 6 h), and then, luciferase activity was assessed as previously described. Asterisk indicates statistical significance in 293 cells transfected with VC versus DAB2IP (P < 0.01) as well as in DAB2IP-transfected 293 cells with versus without LiCl treatment (P < 0.01). (C) DAB2IP activates GSK-3β kinase activity. After treating with 293 sublines with L- or Wnt-CM, GSK-3β was immunoprecipitated, and the kinase activity was determined as previously described. Asterisk indicates statistical significance in 293 cells transfected with VC versus DAB2IP (P < 0.01). (D) DAB2IP antagonizes Wnt-induced transcriptional activity. C4-2 sublines and 293 sublines were transfected with TOP or FOP followed by L-CM or Wnt-CM treatment; then, luciferase activity was measured, and data (mean ± SEM) were calculated. Asterisk indicates statistical significance in 293 cells transfected with VC versus DAB2IP (P < 0.01). (E) Elevated expression of DAB2IP prevents Wnt-induced EMT in 293 cells. Expression of E-cadherin and vimentin was examined in cells by either VC or DAB2IP transfection followed by Wnt- or L-CM treatment for 24 h. β-actin was used as a loading control.
DAB2IP interacts with GSK-3β and PP2A through the C2 domain. (A) Analysis of protein components in DAB2IP complex. PC-3 cell (Con or KD1) lysates were immunoprecipitated with the GSK-3β, PP2A, and DAB2IP antibodies and then probed with DAB2IP and PP2A or GSK3β antibodies, respectively. Con, control-shRNA vector; KD, DAB2IP-shRNA knockdown. (B) Analysis of the key domain of DAB2IP associated with GSK-3β and PP2A. Two hundred ninety-three cells were transfected with DAB2IP-F (lane 1), -N (lane 2), -C (lane 3), -PHC2 (lane 4), and -PH (lane 5) domain constructs. Cell lysates were immunoprecipitated by GSK-3β or PP2A antibody and then probed with Flag antibodies. (C) The role of PP2A in DAB2IP-mediated β-catenin/TCF transcription activity. Two hundred ninety-three cells were cotransfected with GSK-3β (WT or CA), PP2A (WT or LP), and TOP, and then, reporter assay was performed. Relative luciferase activity was assessed as previously described. Asterisk indicated statistical significance in 293 cells transfected with both DAB2IP and GSK-3β-WT plus PP2A-WT versus PP2A-LP (P < 0.01). VC, vector control; CA, constitutive active vector; LP, catalytic inactive of PP2A (L199P); WT, wild-type vector. (D) Analysis of interaction between DAB2IP and Axin. Two hundred ninety-three cells were transfected with VC or DAB2IP-F. Cell lysates were immunoprecipitated with Axin or Flag antibody then probed with Flag or Axin antibody respectively. (E) The role of PP2A in DAB2IP-modulated EMT. Two hundred ninety-three cells transfected with either VC or DAB2IP were treated with OA. Cell lysates were subjected to Western blotting and probed with E-cadherin and vimentin antibodies. β-actin was used as a loading control. (F) PC-3 cells were treated with OA or transfected with PP2A-specific siRNA for 24 h, and cell lysates were probed with E-cadherin and vimentin antibodies. β-actin was used as a loading control.
Fig. 55. Overexpression of β-catenin in PC-3 cells initiates EMT. Decreased DAB2IP leads to a redistribution of β-catenin from membrane (insoluble, cytoskeleton-associated) fraction to the cytosolic (soluble) fraction in PZ-HPV-7 (H), RWPE-1 (R), and PC-3 (P) cells. (A) Fractions were analyzed by Western blot using antibodies against β-catenin and actin. (B) Quantification of Western blot of subcellular localization. (C) Immunofluorescence staining of β-catenin by confocal microscopy (magnification: ×500). Con, control-shRNA vector; KD, DAB2IP-shRNA knockdown. (D) Hyperactive β-catenin/TCF transcription activity in PZ-HPV-7-KD1 and PC-3-KD1 cells. Asterisk indicates statistical significance between Con and KD1 cells (P < 0.01). (E) Overexpression of β-catenin in PC-3 cells initiates EMT. Transfection of increasing amount of β-catenin in PC-3 cells occurred for 24 h. Total cell lysates were probed with E-cadherin and vimentin antibodies. Lysates from cytosolic fraction were subjected Western blotting and probed with β-catenin, actin, and Lamin A/C antibodies. β-catenin/TCF transcription activity was performed as previously described.
β-catenin overexpression reverses DAB2IP-mediated MET. Transfection of increasing amounts of β-catenin in C4-2 (A) or 293 (B) cells occurred for 24 h. Total cell lysates were probed with E-cadherin, vimentin, and β-catenin antibodies. β-catenin/TCF transcription activity was performed as previously described. (C) The morphology of cells in B was revealed by phase-contrast microscopy (magnification: 100×).
A

Con

KD1

B

Intestine  Liver  Kidney  Spleen

Con

KD1

C

KD1 metastatic tumors

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E

Percent survival

F

Con  KD1

p-GSK3β(S9)
Fig. S7. Characterization of phenotypes of metastatic PC-3 tumors relates with EMT. (A Left) BLI imaging of mice bearing PC-3-KD1 tumors with multiple metastatic lesions. Mice (n = 9) were imaged 10 days later to determine local tumor growth and metastasis. (Right) Representative 18F-FDG-PET-CT images (left, transaxial; middle, coronal; right, sagittal) of mice bearing PC-3-Con (Upper) and PC-3-KD1 (Lower) tumors at 1 h postinjection of 18F-FDG (~100 μCi) through the tail vein. Red arrows indicate metastatic lesions. (B) Representative photograph of various organs from mice injected with PC-3-KD1 or Con cell 14 days postimplantation. The black arrows represent the mesenteric, hepatic, renal, and spleen lymph nodes. The green arrows represent liver metastatic lesions. (C) The majority of PC-3-KD1 metastatic tumors exhibit a strong positive vimentin staining but weak E-cadherin staining (magnification: 200x). (D) Summary of the incidence and location of metastatic nodules in DAB2IP-knockdown and control mice at the time of sacrifice (n = 9). (E) Kaplan–Meier analysis of decreased survival of mice bearing PC-3-KD1 tumors. (F) The majority of PC-3-KD1 tumors exhibit elevated p-GSK-3β (S9) staining (magnification: 400x).

Fig. S8. DAB2IP down-regulation initiates EMT in LAPC4 cells and promotes tumor metastasis. (A) Knockdown of DAB2IP in LAPC4 cells induces EMT. Morphology of LAPC4-Con and LAPC4-KD1 cells was revealed by phase-contrast microscopy (magnification: 100x). Expression of epithelial or mesenchymal markers was analyzed by Western blotting. (B) Representative BLI imaging of mice bearing LAPC4-Con or Con cell 14 days postinjection to determine local tumor growth and metastasis. (C) Representative H&E staining of lymph-node metastasis in LAPC4-KD1 tumors. H&E staining shows sections of a lymph node metastasis with a high-grade tumor (magnification: 100x and 200x, respectively). (D) Tumor or metastatic incidence of mice bearing Con or LAPC4-KD1 tumors was determined at the time of sacrifice. (E) Kaplan–Meier analysis of decreased survival of mice bearing PC-3-KD1 tumors. (F) The majority of PC-3-KD1 tumors exhibit elevated p-GSK-3β (S9) staining (magnification: 400x).
Fig. S9. Profiling DAB2IP expression in mouse and human prostate specimens. (A) H&E shows hyperplastic lesions in prostate gland of KO mice (magnification: 100x). (B and C) Correlation between DAB2IP and E-cadherin (or vimentin) in human specimens. Asterisk indicates statistical significance between normal and BPH or BPH and PCa tissues ($P < 0.05$).

Video S1. 3D-PET-CT imaging of mice bearing PC-3-Con tumors.
Video S2. 3D-PET-CT imaging of mice bearing PC-3-KD1 tumors.