# Supporting Information

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### SI Materials and Methods

**Expression Plasmids, Cell Culture, and Lentiviral Transductions.** The pEGFP–C2 plasmid was purchased from Clontech Laboratories, the pCGN plasmid was a kind gift from Tony Tiganis (Monash University) and the pHIV-1SDm lentiviral expression plasmid was a kind gift from Donald Anson (Department of Genetic Medicine, Children, Youth and Women's Health Service, North Adelaide, South Australia, Australia) (1). The CMV promoter and woodchuck posttranscriptional regulatory element (wpre) were cloned into pHIV–1SDm to construct pHIV–1SDmCMVpre and used for expression of eGFP or the eGFP–INPP4B fusion gene. Lentiviral particles were generated as described (1). Lentiviral particles containing human *INPP4B*-specific shRNAs or empty vector were purchased from Sigma-Aldrich. MCF-7 and BT-474 human breast cancer cell lines were from the American Type Culture Collection (ATCC) and grown according to ATCC recommendations. MCF-7-luc-F5 (Xenogen), T47D, MDA MB 231, Hs578T, and BT-549 were a kind gift from John Price (Monash University) and were grown in Dulbecco's modified eagle medium (DMEM) (Invitrogen) supplemented with 10% FCS. MCF-7-luc-F5, T47D, Hs578T, and BT-549 were also grown in 10 μg/mL insulin. Stably transduced MCF-7-luc-F5 cells expressing INPP4B shRNAs or vector alone were generated from heterogeneous pools of puromycin-resistant clones. INPP4B protein reconstitution in MDA MB 231 cells was achieved by transduction with lentiviral particles carrying pHIV–1SDmCMV.pre encoding GFP alone or GFP-INPP4B. Heterogeneous pools of stably transduced cells were collected by fluorescence activated cell sorting (FACS).

**Antibodies and Fluorescence Microscopy.** The following antibodies were used in this study: Akt rabbit mAb, pSer473-Akt rabbit mAb, pThr308-Akt rabbit mAb (Cell Signaling), β-tubulin mAb (Invitrogen), ERα (ID5) mouse mAb (Santa Cruz), ERα rabbit mAb (Abcam) and *K*i-67 (ThermoFisher Scientific). For immunohistochemistry (IHC) studies, mouse IgG was used as a negative control (Millipore). INPP4B-specific polyclonal and monoclonal antibodies were produced by immunizing rabbits or mice with purified recombinant human His-INPP4B. INPP4B polyclonal antibodies were purified from whole serum on a ProBond Ni-chelating column (Invitrogen). HRP-conjugated anti-rabbit and anti-mouse secondary antibodies (Chemicon) and fluorescently labeled anti-rabbit and anti-mouse secondary antibodies (Molecular Probes) were also used. All samples analyzed by fluorescence microscopy were mounted in SlowFade Gold (Molecular Probes) and imaged using a Leica SP5 confocal microscope.

**Immunoblot Analysis and One-Step Reverse Transcription PCR.** For EGF-dependent pAkt assays, cells in culture were serum starved for 24 h before stimulation with 10 ng/mL (MDA MB 231) or 100 ng/mL (MCF-7-luc-F5) EGF (BD Biosciences). MCF-7-luc-F5 cells were serum starved in phenol red-free DMEM (Invitrogen). All total cell lysates were obtained by direct cell lysis in SDS/PAGE sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, 0.01% bromophenol blue). For immunoblot analysis of INPP4B protein expression in primary human breast cancer tissues, fresh-frozen tissue specimens were minced, then homogenized in RIPA buffer (50 mM Hepes pH 7.4, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 150 mM NaCl, 10% glycerol, 1.5 mM $MgCl_2$, 1 mM EGTA, 50 mM NaF, 5 μg/mL leupeptin, 1 μg/mL pepstatin A, 1 mM benzamadine, 2 mM phenylmethysulfonyl fluoride, 1 mM sodium vanadate), and subjected to four pulse teatments in a 4 °C sonicating waterbath for 5 min. Lysates were then clarified by serial centrifugation at 4 °C for 30 min and 15 min. All lysates were subjected to SDS/PAGE and immunoblotting and signals were visualized by enhanced chemiluminescence (ECL, Perkin-Elmer). Commercially available antibodies were used at dilutions according to the manufacturer's recommendation. INPP4B Abs were used at 1:100. Total RNAs were isolated from breast cancer cell lines using RNeasy mini kit (Qiagen) and subjected to one-step quantitative reverse transcription PCR (RT-PCR) using QuantiTect SYBR Green system (Qiagen) to detect expression levels of *INPP4B* or *GAPDH*. All primers for RT-PCR were obtained from Qiagen. Reactions were performed in triplicate on a Rotorgene 3000 RT-PCR machine. All data were normalized to endogenous *GAPDH* expression using the ΔΔct method, as described (2).

**Cell Proliferation, Anchorage-Independent Growth, and Xenograft Tumor Assays.** For 5-bromo-2-deoxyuridine (BrdU)-incorporation assays, cells were serum starved for 24–48 h in phenol red-free DMEM. Sixteen hours before fixation, cells were incubated with 10 nM BrdU, before being fixed in ethanol fixative (absolute ethanol, 50 mM glycine) and BrdU-incorporation detected using the BrdU-labeling and detection kit I (Roche) according to the manufacturer's instructions. Cell nuclei were costained with ToPro-3 (Molecular Probes). Five random fields/coverslip were imaged and >200 cells/coverslip were counted. For anchorage-independent growth, $2 \times 10^3$ cells were resuspended in 5 mL DMEM/10% FCS/10 μg/mL insulin/0.3% agar and plated on top of 2 mL DMEM/10% FCS/10 μg/mL insulin/0.7% agar in 6-well dishes in triplicate for each experiment. Cells were incubated for 3 wk at 37 °C then each well was imaged using a Leica M165C stereomicroscope and a Leica DFC295 camera. Images were analyzed for the number of colonies/well with Image J. For xenograft tumor growth assays, all procedures involving mice were approved by the Animal Ethics Committee at the Monash University School of Biomedical Sciences (SOBSB/B/2008/14). Eight- to 10-wk-old female Balbc nu/nu mice (Animal Resources Centre) were implanted with 90-d release 1.7 mg/mL $E_2$ pellets (Innovative Research of America) under anesthesia. A total of $1 \times 10^6$ cells were suspended in 15 μL PBS plus 50% growth factor-reduced Matrigel (BD Biosciences) and injected into the mammary fat pads of eight mice/cell line. Two weeks postinoculation, palpable tumors were digital caliper measured twice a week for 3.5 wk. Tumor volumes were calculated using the formula (length × $(\text{width})^2/2$). Tumor cell bioluminescence was detected by injecting mice with 150 mg/kg luciferin before imaging using a Xenogen IVIS200 system.

**Patients and Tumors.** Ethics approval was obtained from the standing committee on ethics in research involving humans, Monash University (CF08/1142–2008000564) for all human tissues and cognate clinicopathological data used in this study. Primary human breast cancer tissues used for immunoblot analysis of INPP4B protein and associated clinicopathologic data were provided by the Victorian Cancer Biobank with appropriate ethics approval. The Victorian Cancer Biobank is supported by the Victorian government, Australia. The use of human tissue collected as part of the Melbourne Collaborative Cohort Study (MCCS) (Cancer Council Victoria, Australia) was approved by the Human Research Ethic Committee of the Cancer Council of Victoria (HREC0622) and included formalin fixed paraffin-embedded (FFPE) normal human mammary tissues, obtained from reduction mammoplasties

or from normal tissues surrounding tumors and 107 breast cancer cases. For the MCCS dataset, breast carcinomas were independently assessed for ER, PgR, HER2, CK5/6, and epidermal growth factor receptor (EGFR) expression by a single pathologist (C. A. McLean) at the Alfred Hospital Department of Anatomical Pathology, Victoria, Australia. Cases were classified as positive for ER and PgR if staining was observed in >5% of the epithelial content and HER2 positive if staining intensity was ≥2. Tumors were classified as CK5/6 or EGFR positive if any level of staining was observed. Ethics approval for the use of cases drawn from the St. Vincent's Campus Outcome Cohort (SVCOC) was approved by the Human Research Ethics Committee of St Vincent's Hospital (HREC SVH H94/080, HREC 06336 SVH H00 036) and comprised 267 invasive ductal carcinomas of the breast from patients treated at St. Vincent's Hospital, Sydney, Australia. A total of 18 tissue microarrays (TMAs) containing two cores of each tumor sample were constructed from FFPE tumor material from the cohort, as previously reported (3). Assessment of ER, PgR, CK5/6, cyclin E1, p27, PTEN, and pAkt IHC staining was completed by four independent investigators (EL-K, SAO'T, EKAM, MRQ), as previously described (4). *PIK3CA* mutational and copy number analyses were performed by PCR, and HER2 amplification was determined by HER2-FISH, with HER2:chromosome 17 ratio >2.2 classified as amplified HER2, as previously reported (4).

**Immunohistochemistry and Immunofluorescence of Human Tissues.** Normal human breast sections were deparaffinized in three changes of xylene and rehydrated through three changes of ethanol. Antigen retrieval was performed in a pressure cooker at 120 °C for 3 min in Tris-EDTA buffer (pH 8). Nonspecific antibody binding was blocked with 1% BSA before sections were incubated with primary antibodies, or IgG alone, overnight at 4 °C at the following dilutions: INPP4B (3D5) (1:250); ERα (1:10); *K*i-67 (1:100); and IgG (5 μg/mL). For immunofluorescent double-labeling of normal tissue, sections were coincubated with primary antibodies followed by detection with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 594 (Molecular Probes). Nuclei were counterstained with ToPro-3. FFPE normal and breast cancer sections from MCCS were processed for IHC staining as above and INPP4B staining was performed in a Lab Vision Autostainer 360 using the INPP4B (3D5) Ab (1:500). Endogenous peroxidase activity was quenched in 0.3% hydrogen peroxidase and immunoreactivity detected using Envision$^+$ HRP-conjugated antibodies with DAB detection (Dako). Sections were hematoxylin counterstained and INPP4B staining was independently assessed and performed blindly for tumor characteristics. TMAs from SVCOC were retrieved in antigen retrieval solution pH 9 (Dako) for 3 min at 125 °C in an autoclave. TMAs were stained for INPP4B in a Dako autostainer using the INPP4B (3D5) Ab (1:500) and FLEX secondary system with DAB detection (Dako). TMAs were hematoxylin counterstained, dehydrated, and mounted. INPP4B staining in two core biopsies/patient was independently scored and the final INPP4B expression level was taken as the average. For all studies, the cutoff for INPP4B expression was >5% of the epithelial content. All imaging of IHC staining was performed on an Olympus Provis light microscope with attached camera.

**Statistical Analysis.** All statistical analyses were performed using the GraphPad 5.0 graphing program and $P < 0.05$ was considered statistically significant. Unless otherwise stated, $P$ values were determined by an unpaired Student's $t$ test (two tailed). For correlations between INPP4B expression and clinicopathological features in breast cancer, $P$ values were calculated using a two-tailed Fisher's exact test from contingency tables.


1. Koldej R, Cmielewski P, Stocker A, Parsons DW, Anson DS (2005) Optimisation of a multipartite human immunodeficiency virus based vector system; control of virus infectivity and large-scale production. *J Gene Med* 7:1390–1399.
2. Dussault AA, Pouliot M (2006) Rapid and simple comparison of messenger RNA levels using real-time PCR. *Biol Proced Online* 8:1–10.
3. Millar EK, et al. (2009) BAG-1 predicts patient outcome and tamoxifen responsiveness in ER-positive invasive ductal carcinoma of the breast. *Br J Cancer* 100:123–133.
4. López-Knowles E, et al. (2010) PI3K pathway activation in breast cancer is associated with the basal-like phenotype and cancer-specific mortality. *Int J Cancer* 126: 1121–1131.
5. Munday AD, et al. (1999) The inositol polyphosphate 4-phosphatase forms a complex with phosphatidylinositol 3-kinase in human platelet cytosol. *Proc Natl Acad Sci USA* 96:3640–3645.

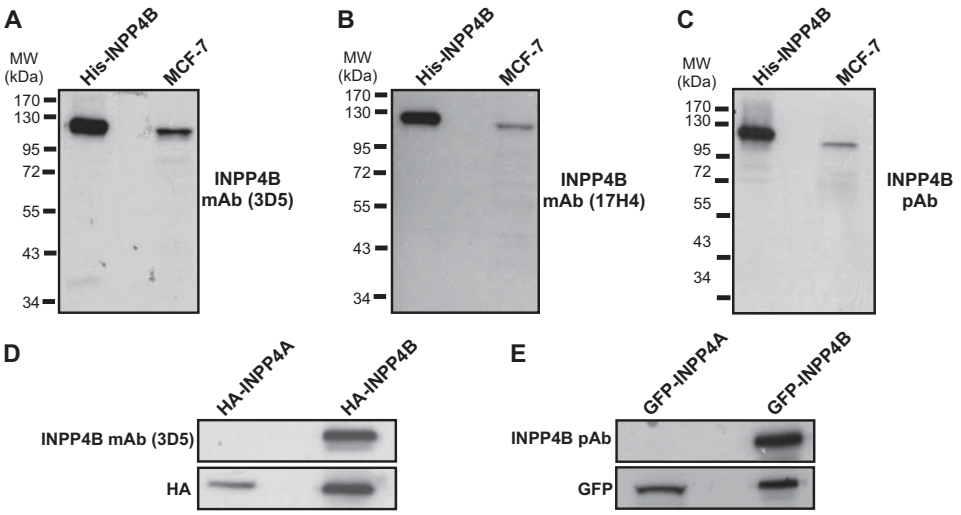


**Fig. S1.** The specificity of INPP4B monoclonal and polyclonal antibodies (Abs). (*A*–*C*) Purified 6xHis-INPP4B and MCF-7 breast cancer cell lysates were immunoblotted with INPP4B monoclonal Ab (mAb) clones 3D5 (*A*) or 17H4 (*B*) or affinity purified polyclonal Ab (pAb) (*C*). All Abs recognized a single immunoreactive protein species corresponding to recombinant INPP4B and endogenous protein in cell lysates, migrating at the predicted molecular weight (MW) for INPP4B (≈104 kDa). (*D* and *E*) Immunoblot analysis of lysates from COS-1 cells expressing recombinant hemagglutinin (HA), or GFP-tagged INPP4B, or the related INPP4A, with INPP4B mAb (3D5) (*D*), or pAb (*E*) confirm the specificity of both Abs for INPP4B only.

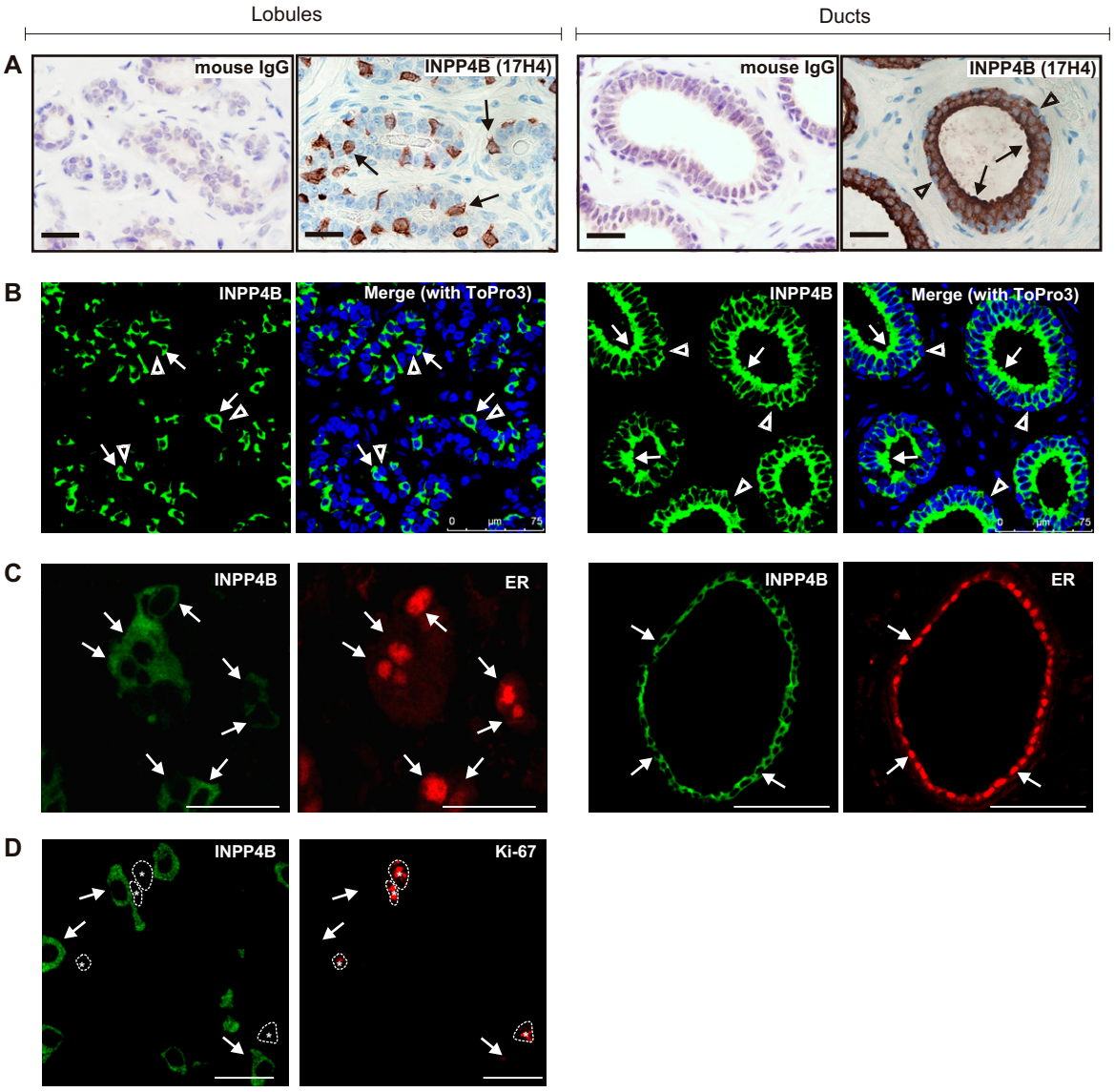


**Fig. S2.** INPP4B is expressed in ER-positive cells in the normal human breast epithelium. (*A*) Normal human breast sections were immunostained using an INPP4B-specific monoclonal antibody (17H4) or mouse IgG. (Scale bars, 50 μm.) In *B*, normal breast tissue was costained for INPP4B (green) and the nuclear marker, ToPro3 iodide (blue). (Scale bar, 75 μm.) In *C*, tissue sections were costained for INPP4B (green) and ER (red). Arrows show INPP4B-positive cells, arrowheads indicate INPP4B-negative cells. (Scale bars, 25 μm and 75 μm.) (*D*) Immunofluorescent costaining of normal human breast lobules for INPP4B (green), *K*i-67 (red), and ToPro3 iodide (blue). Arrows show INPP4B-positive cells. Outlines and asterisks mark *K*i-67–positive nuclei. (Scale bar, 25 μm.)

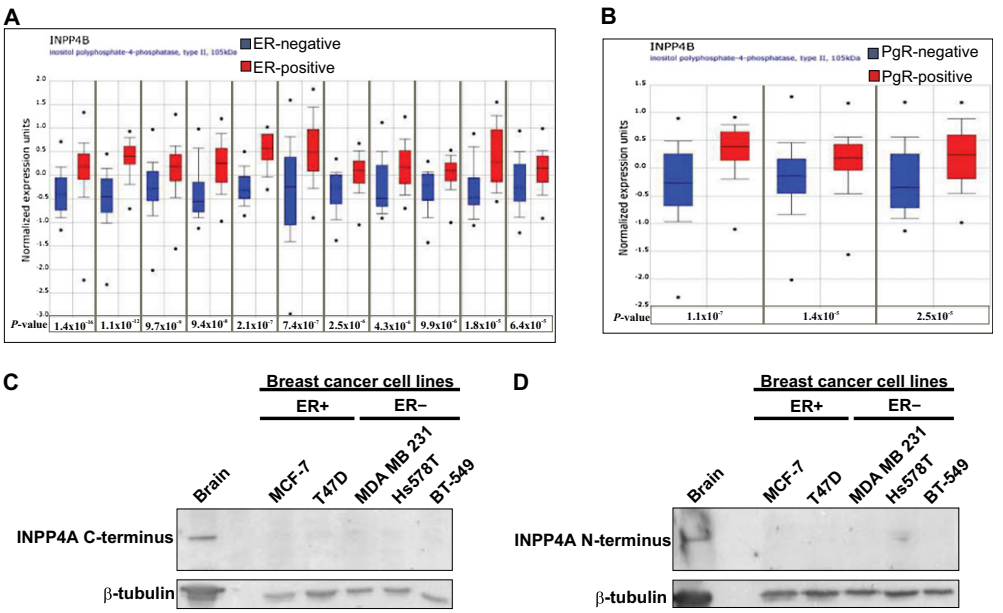


**Fig. S3.** Differential expression of INPP4A and INPP4B in human breast cancer. (*A* and *B*) Metaanalysis of human breast cancer datasets publicly available in the Oncomine Research Program identified a positive correlation between ER (11 independent studies) (*A*) and PgR (three independent studies) (*B*) expression and INPP4B in human breast cancers. INPP4B expression was higher in ER-positive and PgR-positive breast cancers (red bars) compared with ER-negative and PgR-negative breast cancers (blue bars). (*C* and *D*) INPP4A is not expressed in human breast cancer cells. Immunoblot analysis of INPP4A expression in human breast cancer cell lines and mouse brain (positive control) using polyclonal antibodies raised against unique sequences in the INPP4A protein carboxy C terminus (*C*) or amino N terminus (5) (*D*).

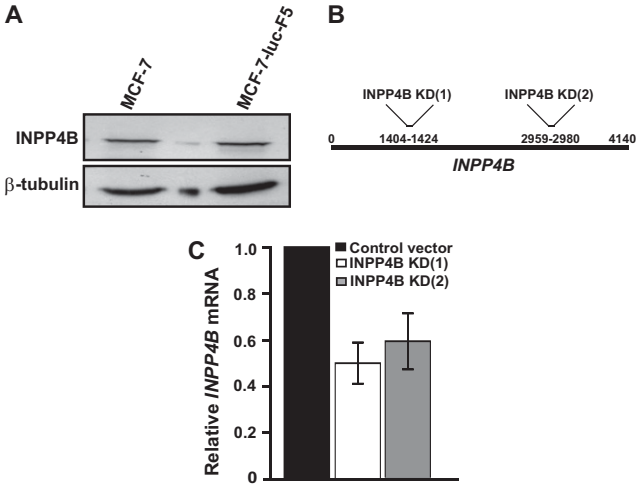


**Fig. S4.** Generation of human breast cancer cell lines with stable INPP4B protein knockdown. (*A*) Immunoblot analysis of MCF-7 and MCF-7-luc-F5 cells with INPP4B pAb, indicating similar INPP4B protein levels in both cell lines. (*B*) Representation of two unique sequences of INPP4B targeted by INPP4B KD(1) and INPP4B KD(2) shRNA sequences. (*C*) Confirmation of INPP4B expression knockdown in INPP4B KD(1) or INPP4B KD(2) MCF-7-luc-F5 cells by qRT-PCR analysis of *INPP4B* mRNA, relative to *GAPDH*. Error bars were calculated as means relative to control vector ± SEM of two experiments.

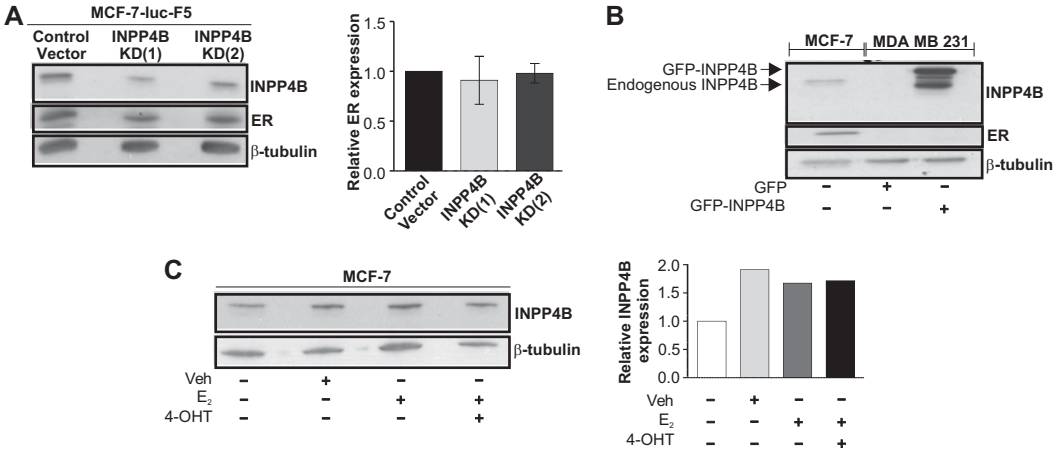


**Fig. S5.** INPP4B and ER do not exhibit reciprocal regulation of the other. (*A*) Immunoblot analysis of ER expression in control, INPP4B KD(1), and INPP4B KD(2) cell populations. The average ER protein expression relative to control ± SEM of three independent experiments is shown. (*B*) Immunoblot analysis of ER expression in MCF-7 or MDA MB 231 cells stably expressing GFP alone or GFP-INPP4B. (*C*) Analysis of INPP4B expression in MCF-7 cells serum starved for 24 h or treated with vehicle alone or 10 nM 17 β-estradiol ($E_2$) in the absence or presence of 1 μM 4-hydroxytamoxifen (4-OHT) for 96 h. The graph shows the average INPP4B expression relative to serum-starved cells from one representative experiment.