Supporting Information

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SI Materials and Methods

Cell Culture and Transfection. Breast cancer cell lines were grown in the following media containing 10% (vol/vol) FBS and 1× penicillin/streptomycin: RPMI-1640 for HCC38, HCC1143, HCC1937, and BT549; DMEM for MDA-MB-436, MDA-MB-468, and SKBR3; and EMEM for BT20. MCF10A, MDA-MB-231, and MCF7 were grown as described in ref. 1 and generation of stable MCF10A-TR-MYC cells were described in ref. 2.

Transient knockdowns were performed using siRNAs to inhibitor-2 of protein phosphatase 2A (I2PP2A/SET), SET (M-019586-01), and cancerous inhibitor of PP2A (CIP2A) (M-014135-00), and PP2A C (M-003598-010005) and DharmaFECT1 transfection reagents according to the protocol provided (Dharmacon). Nontargeting (NT) siRNA (NTsiRNA) (D-001206-14) was used as a control. For the xenograft assay, cells were transfected with siRNAs two times over 2 d before transplant. MDA-MB-231-shSET stable clones were made using a shRNA-encoding plasmid obtained from Sigma (TRCN0000063717). Empty vector (SHC001) was used as a control. Briefly, cells were transfected using Lipofectamine 2000 (Invitrogen) and stable clones were generated after selection with puromycin.

Quantitative-PCR. RNA was isolated from breast cancer cell lines using TRIzol reagent (Invitrogen) and cDNA was made as described previously (1). Quantitative PCR (qPCR) was then performed using TaqMan primers for transcription factor c-MYC (Hs00905030_m1), SET (Hs00853870_g1), CIP2A (Hs00405413_m1), ACTIN (Hs99999903_m1), and 18s (Hs03003631_g1).

TissueScan Breast Cancer and Normal Tissue cDNA array (array 4) was obtained from Origene. The array contained dried cDNAs from 48 samples including four normal. SET and CIP2A were measured by using TaqMan probes. 18s was used as an internal control. The relative fold change was measured by the $\Delta\Delta(C_T)$ method between each tumor sample and the average of the normal samples in expression: $\Delta(C_T) = C_T(\text{SET or CIP2A}) - C_T(18S)$; $\Delta\Delta(C_T) = \Delta(C_T) - \text{average}\ (\Delta(C_T)\ \text{of normal samples})$; fold change = $2\text{^}\Delta\Delta\ (C_T)$.

RNA Sequencing and Gene Set Enrichment Analysis. RNA-sequencing (RNA-seq) data for Fig. S1*A* can be accessed at the Gene Expression Omnibus (GEO) (www.ncbi.nlm.nih.gov/geo/, accession no. GSE48216) (3).

For RNA-seq data used in Fig. 5*F* and Fig. S4, *Left*, total RNA was isolated with TRIzol (Invitrogen) from MDA-MB-231 cells following treatment with vehicle (PBS) or 1 μM OP449 for 12 h, or following transfection with control NT, SET, or CIP2A siRNA for 48 h. RNA-seq was performed as described previously (2). Briefly, data were aligned using Bowtie version 0.12.7 to human genome version hg19, and custom R scripts and software were used to count tags that aligned to the exons of University of California, Santa Cruz RefSeq gene models. The χ^2 statistic was calculated for each gene model and the Benjamini–Hochberg false discovery rate (FDR) adjustment was applied. Each sample was normalized based on the total reads that aligned to exons to calculate fold change and expression difference between classes. The c-MYC gene set published in ref. 4 was used to analyze MYC enrichment in the ranked (by expression difference between classes) gene list using Gene Set Enrichment Analysis (GSEA) version 2.0.14.

Antibodies for Western Blot, Chromatin Immunoprecipitation, and Immunofluorescence. Western blot analysis was performed as described previously (5). Immunoblots were visualized using the Odyssey IR imager (LI-COR) that can detect both Fluor 680 and IRDye 800 secondary antibodies (1:10,000). Quantification of Western blots was done using the Odyssey IR software, version 1.2 (LI-COR).

Antibodies used include: MYC Y69 (ab32072, 1:1,000; Abcam), MYC N262 [2 μg for chromatin immunoprecipitation (ChIP); Santa Cruz], monoclonal pS62-MYC (1:500; BioAcademia), pT58-MYC [A00242, 1:50 for immunofluorescence (IF); GenScript], SET (A302-262A, 1:2,000 for Western blot and 1:1,000 for IF; Bethyl), CIP2A [1:50,000 for Western blot and 1:2,000 for IF; a gift from Jukka Westermarck (Finnish Cancer Institute, Turku Centre for Biotechnology, Turku, Finland)], and GAPDH (AM4300, 1:10,000; Ambion). For immunofluorescence we used our pS62-MYC antibody (1:100) we developed as previously described (1) and quantification of IF was done using ImageJ software.

Soft Agar Colony-Forming Assay. A total of 20,000–25,000 cells were plated in 0.35% Nobel agar on top of 0.7% Nobel agar mixed with 2× complete media. For treatments, 2× drug was added to cells at the time of plating and cells were retreated three times per week. Cells were fixed in 0.005% crystal violet overnight at 4 °C after 3–4 wk and colonies were counted from 20 random fields using the EVOS FL cell imaging system (Advanced Microscopy Group).

AnnexinV-7-Aminoactinomycin D Apoptosis Assay. Apoptosis assay was performed according to the BD Pharmingen protocol. MDA-MB-231 cells were treated for 6 h with OP449 or PBS. Following trypsinization, cells were washed twice with cold PBS and then resuspended in 1× binding buffer at a concentration of 1×10^6 cells/mL, 5μL of FITC-AnnexinV, and 5μL of 7-aminoactinomycin D (7AAD) was added to 100 μL of the suspended cells. Cells were incubated at room temperature for 15 min in the dark and then 400 μL of the 1× binding buffer was added to each tube. Cells were analyzed by the FACS Calibur flow cytometer and FCS Express software (De Novo).

Cell Population Expansion and Cell Viability Assays. The cell population expansion assay performed on the IncuCyte (Essen Bioscience) uses captured images to measure live cell content change over time. Cells were transfected with siRNA for 48 h and plated for live cell imaging and Western blotting. The totals of 5,000 MDA-MB-231, 8,000 MDA-MB-436, and 7,000 MDA-MB-468 cells were plated in 96-well plates grown in complete media. Cells were incubated in the IncuCyte for 3 d. Live cell images were taken every 2 h and percent phase object confluence was calculated using IncuCyte Zoom software.

The cell viability assays were done using the 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt assay according to the manufacturer's protocol (Promega).

Growth Inhibition of Primary Breast Tumor Cell Samples. Primary invasive breast carcinoma tissue from two patients was obtained from a needle core biopsy through the Oregon Health & Science University (OHSU) Biolibrary with informed patient consent (Institutional Review Board approval no. 3330). Tissue was dispersed through collagenase/hyaluronidase digestion and plated on collagen-I–coated six-well culture dishes in DMEM/F12 supplemented with 10% FBS, 0.5 mg/mL hydrocortisone, 20 ng/mL EGF, and 10 μg/mL insulin. Cultures were allowed to grow for 24–48 h and then imaged (day 0). Cells were treated with varying

doses of OP449 or PBS for 4 d. Cell colonies were visualized over time on an EVOS Fl inverted digital microscope, and colony area was quantified using ImageJ software.

Xenograft of Human Breast Cancer Cell Lines. Mice were handled in accordance with the OHSU Institutional Animal Care and Use Committee. A total of 1×10^6 (for SET/CIP2A knockdown) or 2×10^6 (for OP449 treatment) of MDA-MB-231, MDA-MB436, and MDA-MB-468 cells in 25% Matrigel + 75% complete media were xenografted into the fourth mammary gland of nonobese diabetic (NOD)/SCID/γ chain null (NSG) mice. For the treatment study, once tumors were palpable, mice were randomly divided into two groups and treated via i.p. injection with 5 mg/kg (for Fig. 6*A*) or 10 mg/kg (for Fig. S5*A*) of OP440 3 d/wk or vehicle control (PBS). Tumors were measured by a digital caliper and tumor volume was calculated by (width × length^2)/2.

TUNEL and Ki67 Analyses. Six xenografted MDA-MB-231 tumors from three control mice and eight tumors from four OP449-treated mice were fixed in formalin and paraffin embedded. Sections from control and OP449-treated tumors were assessed by TUNEL staining using the ApopTag Peroxidase In Situ Apoptosis Detection kit (Millipore) according to the protocol. The mean and SD of total apoptotic cells in 75 random fields for each tumor set are graphed. For Ki67 staining, sections were analyzed by immunofluorescence using Ki67 antibody (1:1,000, NCL-Ki67-MM1; Novocastra).

PP2A Activation in Orthotopic Mammary Tumors. MDA-MB-231 cells were orthotopically xenografted into the fourth mammary gland of NSG mice (Fig. S5*A*). Mice were treated with OP449 (10 mg/kg i.p.) or PBS three times per week for 40 d. Mice were killed and tumors harvested 2 h following the final treatment. Harvested tumors were rapidly dissected, flash frozen in liquid nitrogen, and ground to a fine powder on liquid nitrogen. PP2A assays were performed as described previously (6).

OP449 Detection in Orthotopic Breast Cancer Tumors. Tumor lysates from the PP2A assays above were diluted to 5 mg/mL total protein and prepared for PAGE by diluting 75 μL with Laemmli protein electrophoresis buffer (4×, 25 μL). These protein solutions were heated to 90 °C for 5 min, separated by SDS/PAGE, and transferred onto nitrocellulose membranes (Bio-Rad). The nitrocellulose membranes were blocked using 5% nonfat milk in Tris-buffered saline containing 0.1% Tween 20 (TBST) for 3 h and then washed with TBST. The membrane was incubated overnight at 4 °C in a rabbit anti-OP449 reactive antibody (DU219) diluted in SuperBlock. The membranes were washed with TBST for 1 h and incubated with donkey anti-rabbit IRDye 680 secondary antibodies to detect OP449 in the tumor cell lysates. The membranes were washed thoroughly and protein bands visualized and quantitated Odyssey Infrared scanner; LI-COR).

OP449 Pharmacokinetics. Rats were purchased from Charles River Laboratories with a femoral vein and a jugular vein catheter surgically inserted. Rats were slowly infused with OP449 in lactated Ringer's solution for 1 h at a dose of 2 mg/kg through the femoral vein catheter. At the end of the 60-min infusion period, 200 μL of blood was drawn through the jugular vein catheter for each time point and 20 μL sodium citrate added to prevent coagulation. Anti-coagulated whole blood was centrifuged at 5,000 × *g* for 5 min and plasma was removed. Blood was collected from five rats at 0, 5, 10, 15, 30, 60, 90, and 120 min following the completion of the 60-min infusion. A standard curve for quantitation of OP449 was prepared by spiking OP449 into normal rat plasma to a final concentration of 1.0 μg/mL and performing twofold dilutions. OP449 was quantitated by adding 10 μL of the plasma to 65 μL of PBS and 25 μL of 4× Laemmli protein electrophoresis buffer. Western blot was performed as described above and OP449 was detected by the anti-OP449 reactive antibody (DU219). Stability of OP449 in rat plasma was assessed using plasma from the $T = 0$ time points from each rat. Plasma was divided into aliquots and incubated at 37 °C for 12, 24, or 48 h before being quantified by Western blotting. Residual OP449 for each time point was determined by normalizing each time point to the amount in the starting plasma sample.

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2. Farrell AS, et al. (2013) Pin1 regulates the dynamics of c-Myc DNA binding to facilitate target gene regulation and oncogenesis. *Mol Cell Biol* 33(15):2930–2949.
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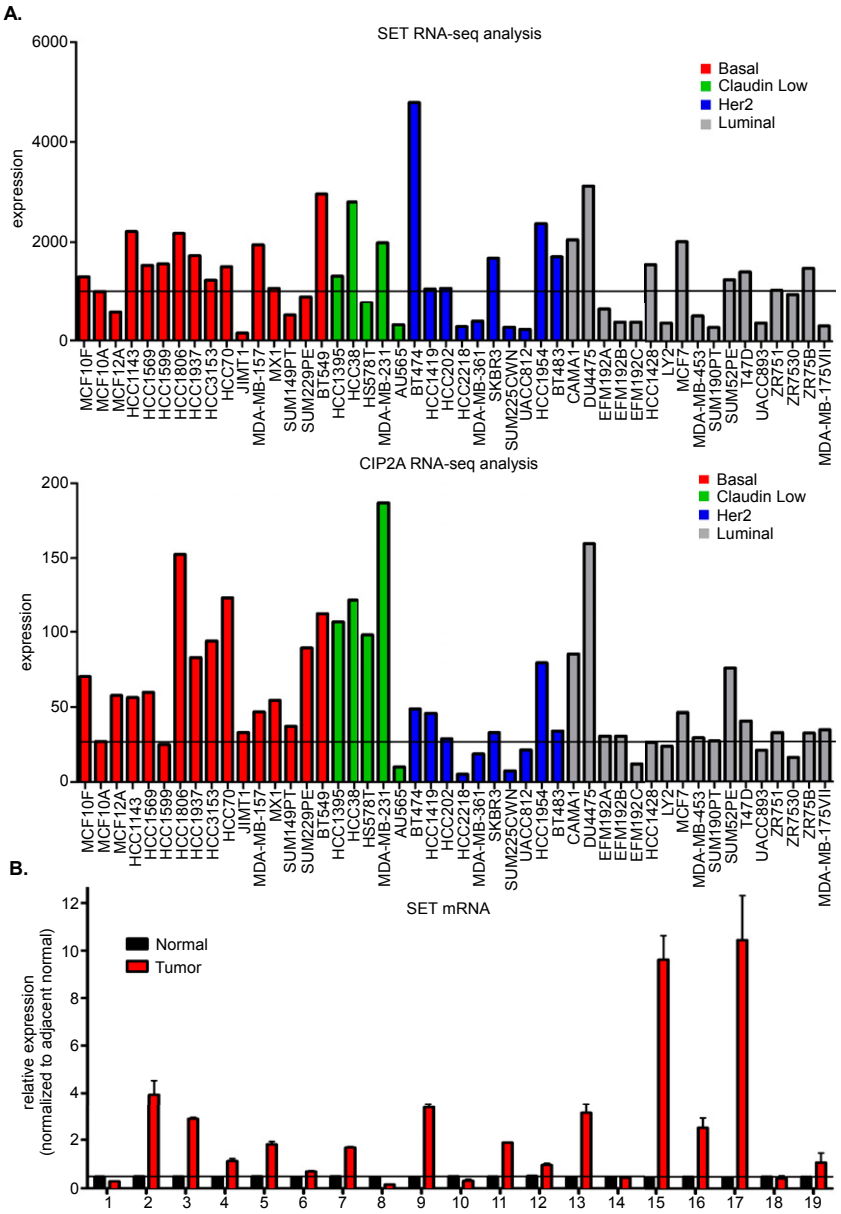

Fig. S1. SET and CIP2A are frequently overexpressed in human breast cancers. (*A*) SET and CIP2A expression from RNA-seq (GSE48216) in breast cancer cell lines grouped by their subtypes using PAM50 (7). Cells were grown in complete serum media. (*B*) qPCR analysis of SET expression, normalized to ACTIN expression, in 19 breast tumor samples relative to their patient-matched normal samples. The error bars represent SD from the triplicate qRT-PCR. Samples have been previously described (1).

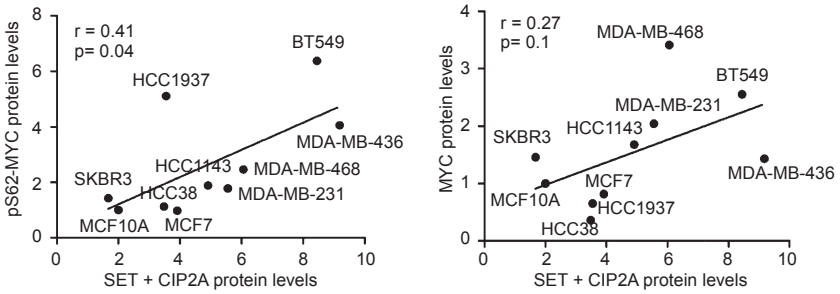

Fig. S2. High SET and CIP2A levels trend with high pS62-MYC and total MYC expression in breast cancer cell lines. Correlation analysis of pS62-MYC (*Left*) and MYC (*Right*) protein with the sum of SET and CIP2A protein levels from Fig. 2*A*. Quantification and normalization as in Fig. 2*A*.

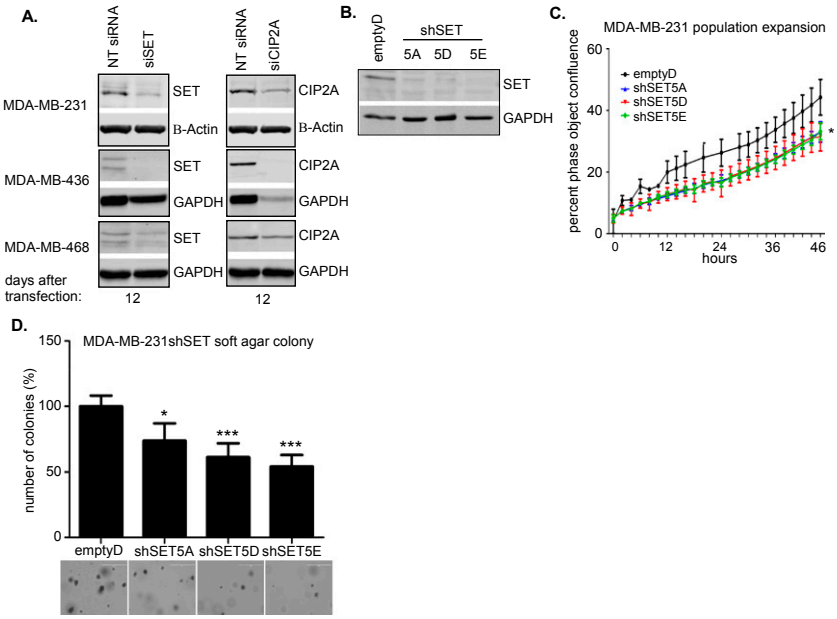

Fig. S3. SET knockdown decreases growth of breast cancer cell lines. (*A*) Western blot analysis of SET and CIP2A in MDA-MB-231, MDA-MB-436, and MDA-MB-468 cells after siRNA-mediated SET or CIP2A knockdown for 12 d. (*B*) Western blot analysis of SET in MDA-MB-231 stable clones with control (clone D transfected with empty vector; emptyD) or SET knockdown using shRNAs. (*C*) Population expansion was analyzed by live cell imaging using IncuCyte technology. (*D*) Soft agar colony-forming assay for MDA-MB-231–shSET clones. The error bars in *C* and *D* represent the SD from three independent experiments.

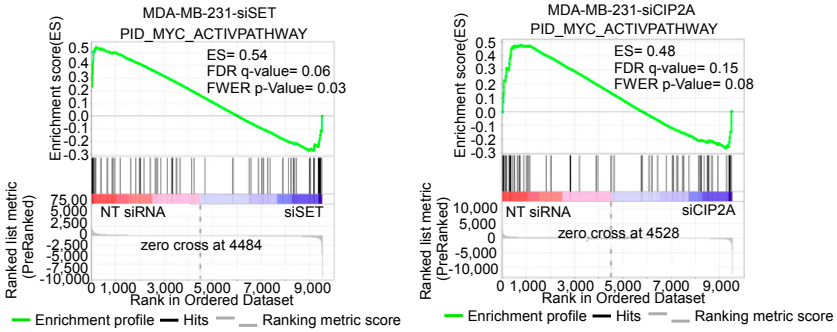

Fig. S4. Global MYC transcriptional activity is decreased after SET or CIP2A knockdown. MYC Gene Set Enrichment Analysis using the MYC gene list published in ref. 4 compares SET or CIP2A knockdown with control siRNA in MDA-MB-231 cells. The positive enrichment scores (ESs), FDR-adjusted *q*-values, and familywise error rate (FWER) *P* values are listed showing the MYC gene signature enrichment in control versus knockdown cells.

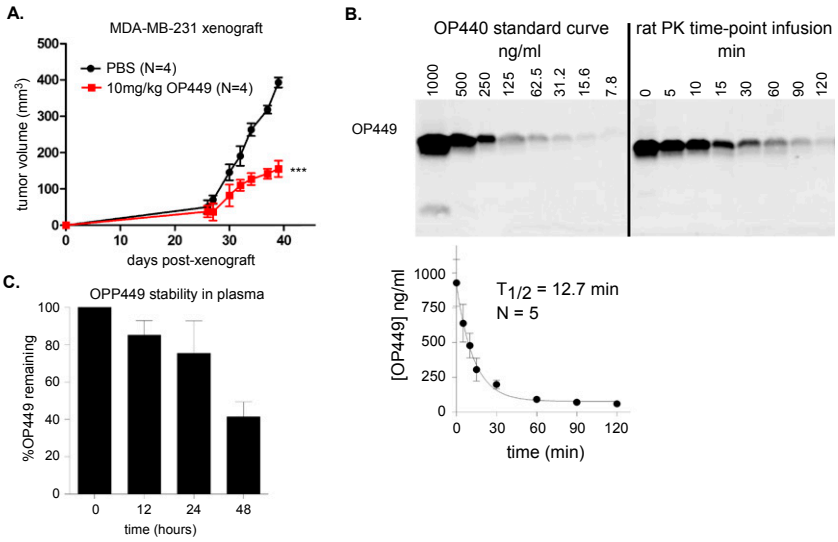

Fig. S5. Xenograft tumor growth and pharmacokinetics of OP449. (*A*) Tumor growth curve for MDA-MB-231 xenografts in the fourth mammary gland of NSG mice following treatment with OP449 or PBS. Mice were injected with 10 mg/kg of OP449 or PBS intraperitoneally (i.p.) three times per week. Tumors were harvested and frozen in liquid nitrogen 2 h after the final injection and used for Western blot analysis in Fig. 6*D* and PP2A activity assay in Fig. 6*E*. (*B*) Pharmacokinetic profile of OP449 following infusion into the femoral vein of nonsedated rats. A total of 2 mg/kg OP449 was infused into the femoral vein of a cannulated rat over a period of 1 h. At the end of the infusion, blood was drawn at the indicated times from a jugular vein cannula, plasma was prepared, run on PAGE, and Western blotted with an anti-OP449 antibody (*Right* blot). A standard curve of OP449 in rat plasma is shown from the same gel (*Left* blot) as the experimental samples for quantification of OP449 in the rat plasma from infused animals. The graph represents the average plasma OP449 levels over time from five rats. (*C*) OP449 stability in plasma. Rat plasma with OP449 was incubated at 37 °C for the indicated times and OP449 levels were quantified by Western blot.