Supplementary Information Appendix

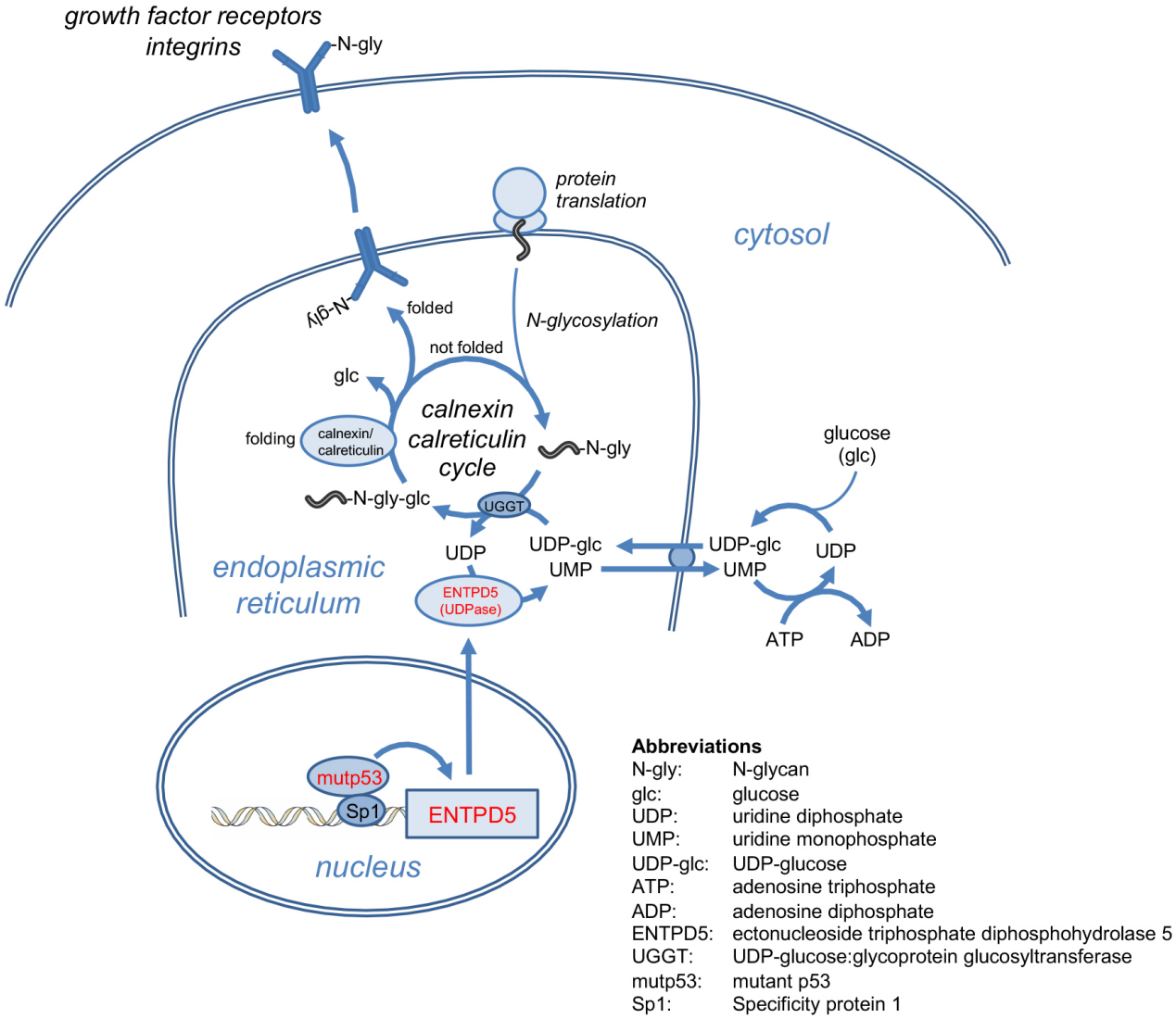

Fig. S1. Function of ENTPD5 in the calnexin/calreticulin cycle.

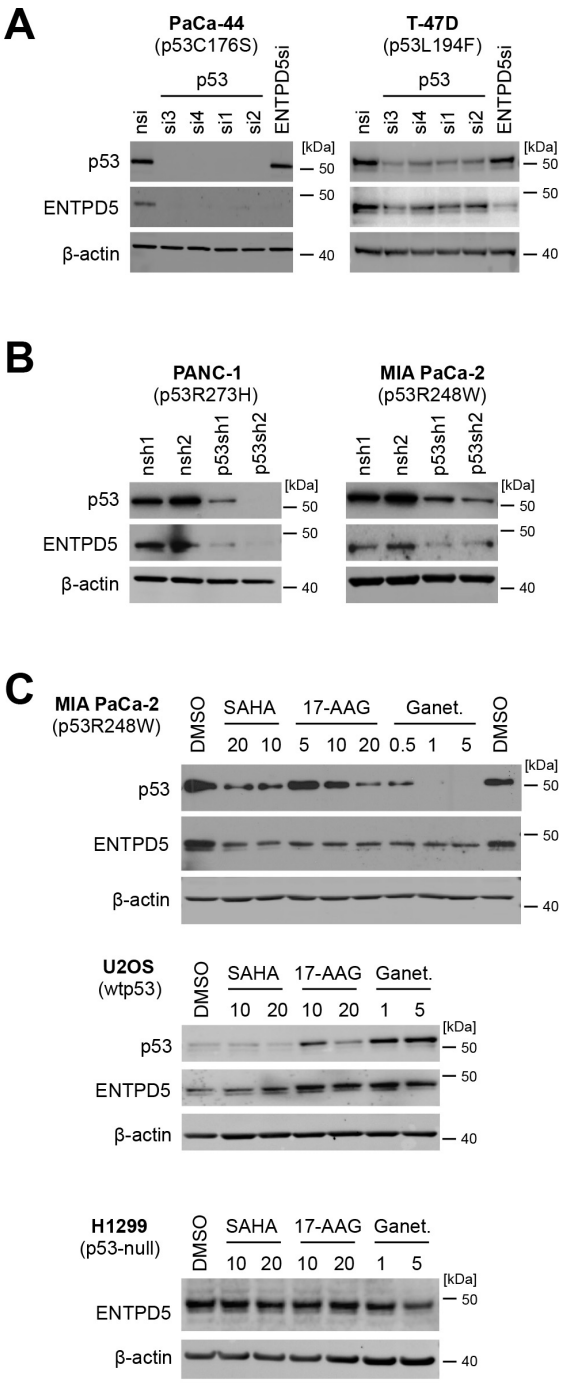

Fig. S2. ENTPD5 as a mutp53 target gene. (*A*) Protein expression of p53 and ENTPD5 in PaCa-44 (pancreas) and T-47D (breast) cancer cells analyzed by western blot after transfection with p53, ENTPD5 and non-targeting control (nsi) siRNAs. β-actin served as a loading control. (*B*) Western blot analysis of p53 and ENTPD5 in PANC-1 and MIA PaCa-2 stably transduced with p53shRNAs (1,2) and non-targeting shRNA controls (nsh1,2). β-actin served as a loading control. (*C*) Protein expression of p53 and ENTPD5 in MIA PaCa-2 (mutp53), U2OS (wtp53) and H1299 (p53-null) treated with increasing doses of the HDAC inhibitor SAHA (suberoylanilide hydroxamic acid) or the Hsp90 inhibitors 17-AAG (Tanespimycin) or Ganetespib for 24 h. All concentrations are given in μM. DMSO served as a solvent control, β-actin as a loading control.

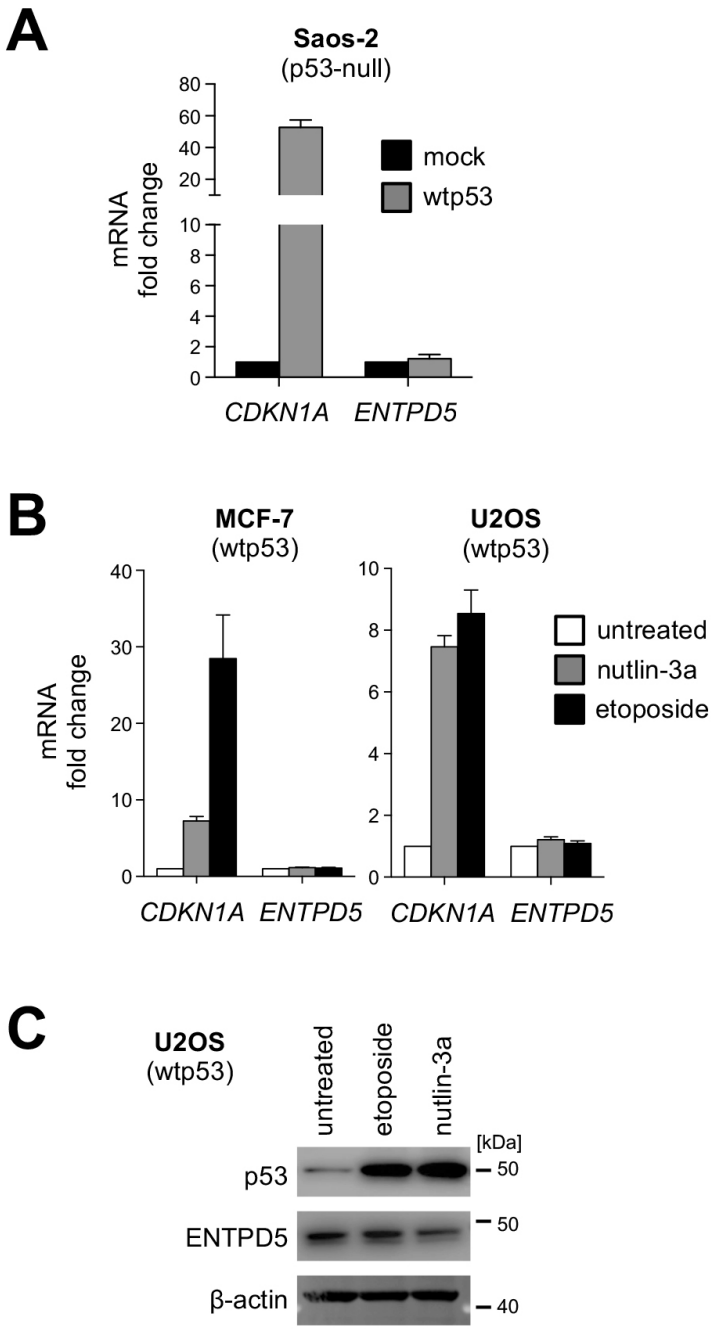

Fig. S3. ENTPD5 is not regulated by wtp53. (*A*) mRNA expression of p21/*CDKN1A* and *ENTPD5* analyzed by RTqPCR in p53-null Saos-2 osteosarcoma cells after adenoviral expression of wtp53 compared to mock. (*B*) mRNA expression of p21/*CDKN1A* and *ENTPD5* analyzed by RTqPCR in p53-wildtype MCF-7 breast cancer and U2OS osteosarcoma cells following p53 activation with 10 µM etoposide or 10 µM nutlin-3a for 24 h compared to solvent control (untreated). (*C*) Protein levels of p53 and ENTPD5 analyzed by western blot in U2OS treated with 10 µM etoposide or 10 µM nutlin-3a. β-actin served as a loading control.

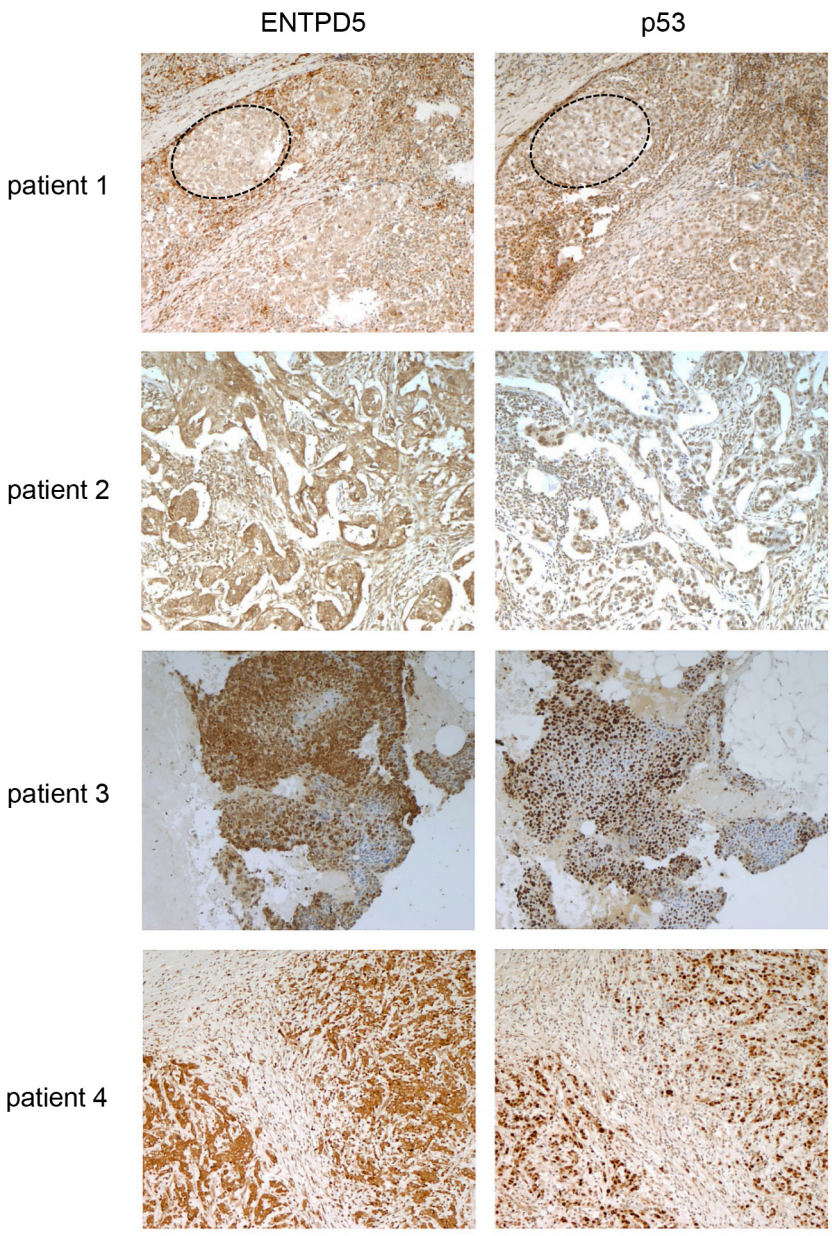

Fig. S4. ENTPD5 expression in triple-negative breast cancer samples. Shown are immunohistological stainings for ENTPD5 and p53 of four representative patient tumors. Patient 1 demonstrates a region of p53-immunonegative tumor cells (dashed line) with weak staining for ENTPD5. Patients 2-4 exhibit tumors with moderate (2) and strong (3,4) p53 immunopositivity with strong cytoplasmic ENTPD5 staining.

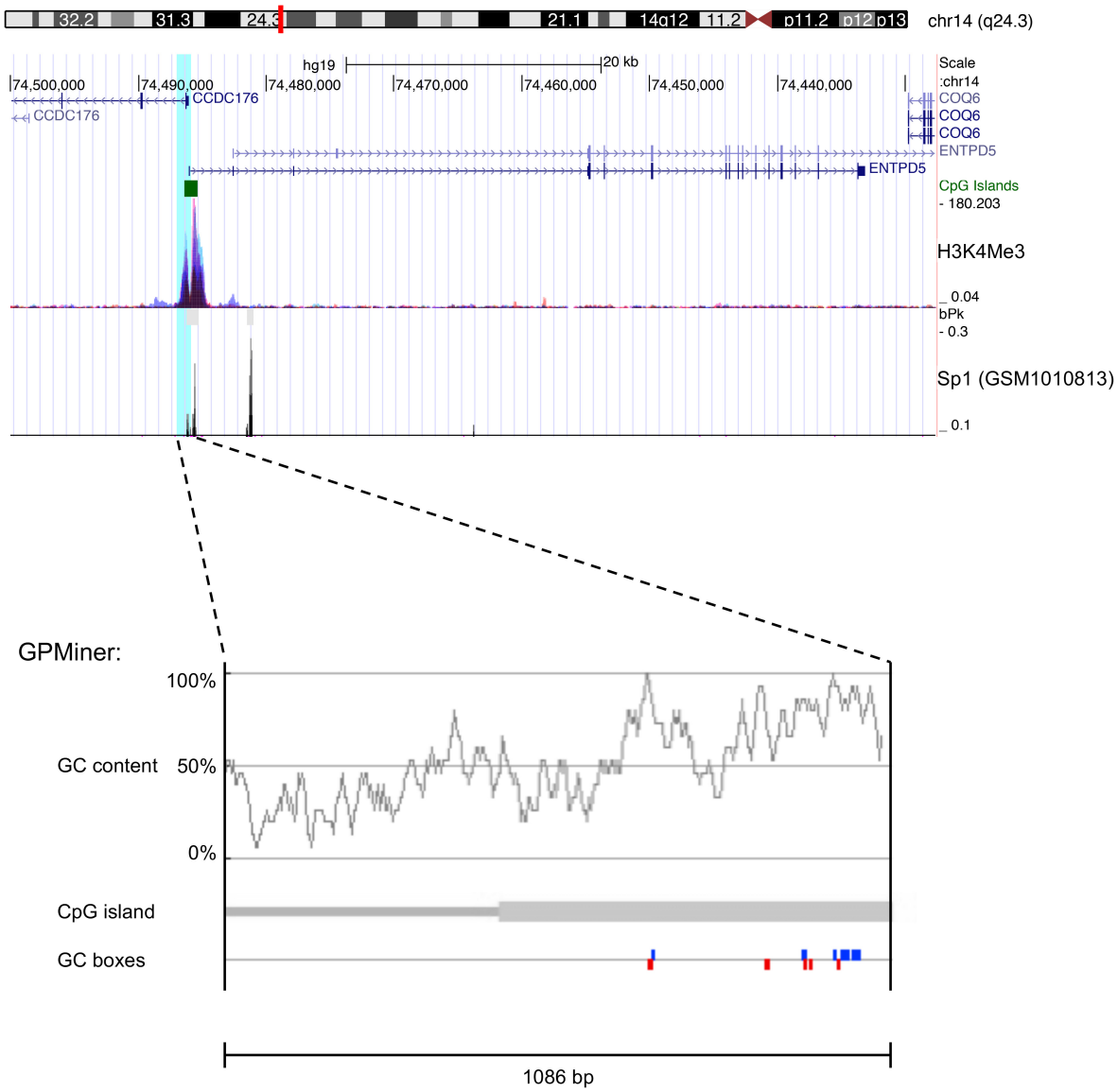

Fig. S5. Analysis of the *ENTPD5* gene promoter. *Top*, UCSC genome browser view of the *ENTPD5* gene. Shown are H3K4me3 peaks as marks for transcription start site regions, CpG islands and ENCODE Sp1 ChIPseq peaks in A549 cells (GSM1010813). The 1086 bp *ENTPD5* promoter region present in the luciferase reporter vector is highlighted with a cyan box. *Bottom*, Results of a GPMiner analysis (1) of the 1086 bp *ENTPD5* promoter region. Indicated is the GC content, location of CpG islands and GC boxes.

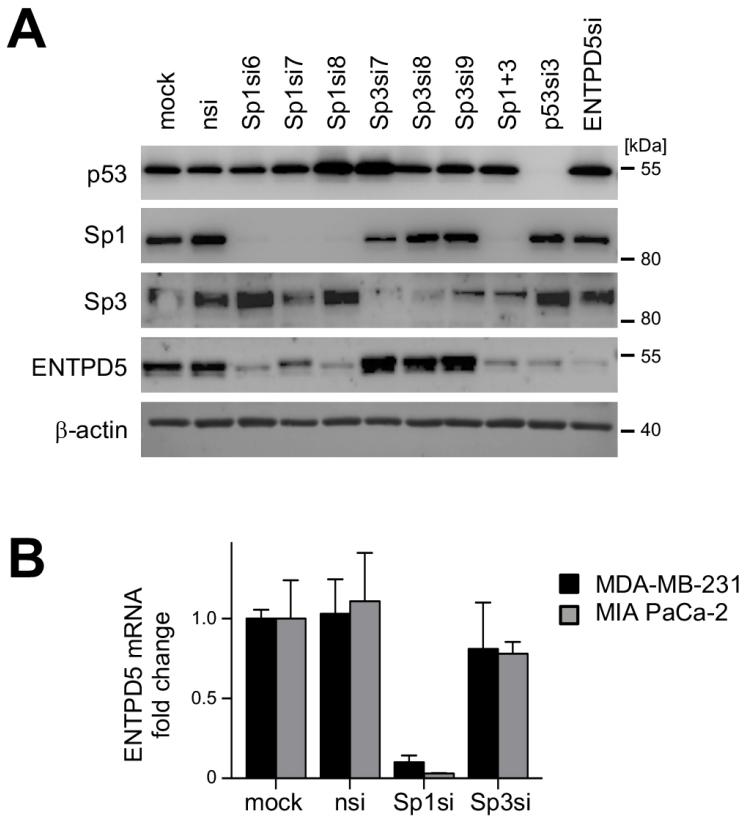

Fig. S6. Regulation of ENTPD5 by Sp1 and Sp3. (*A*) MDA-MB-231 cells were transfected for 72 hours with the indicated siRNAs directed against Sp1, Sp3, p53 or ENTPD5. A non-targeting siRNA (nsi) served as a negative control. Cell protein extracts were subjected to immunoblotting with antibodies against p53, Sp1, Sp3, ENTPD5 and β-actin as a loading control. (*B*) MDA-MB-231 and MIA PaCa-2 cells transfected as in (*A*) were used for RNA isolation. Following reverse transcription ENTPD5 mRNA expression was quantified by RTqPCR. Shown are mean ± SEM (n=3) normalized to GAPDH mRNA expression.

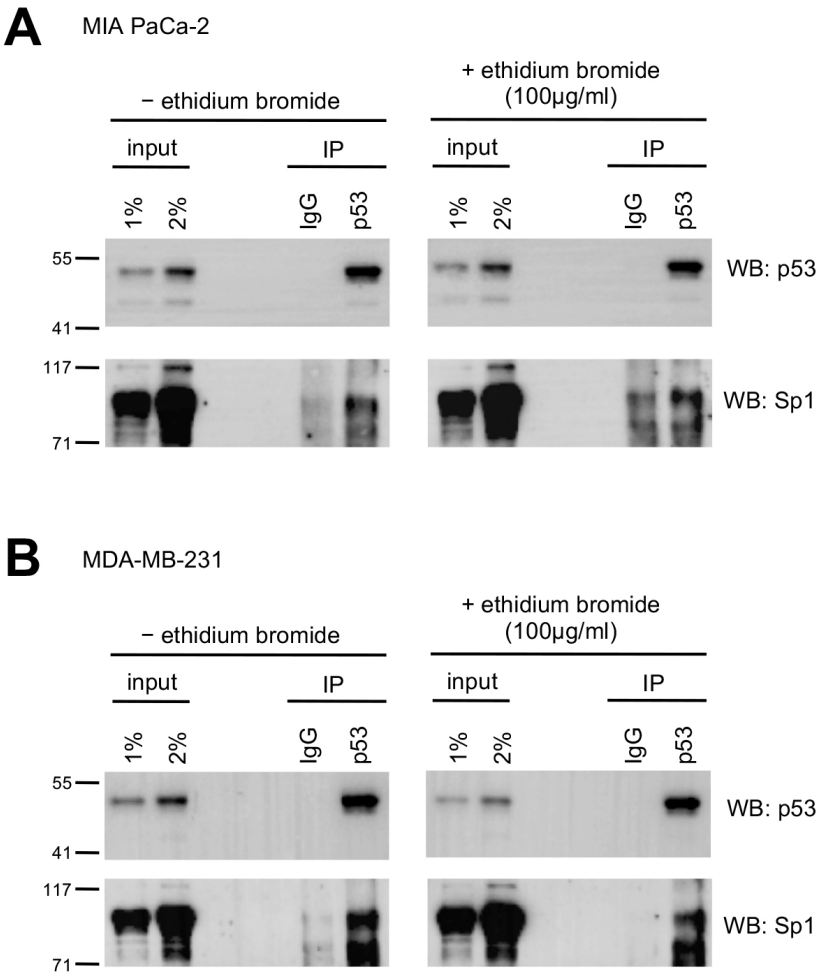

Fig. S7. mutp53 interacts with Sp1 independent of DNA. mutp53 was immunoprecipitated from (*A*) MIA PaCa-2 and (*B*) MDA-MB-231 cells with anti-p53 (FL-393) antibody. IgG served as a control antibody. Immunoprecipitated proteins were subjected to immunoblotting with antibodies against p53 (DO1) and Sp1 (07-645). 1 and 2% of cell input are shown for comparison. To test the interaction of mutp53 with Sp1 for DNA dependence, the experiment was performed both in the absence (left) and presence of 100 µg/ml ethidium bromide (right) (2).

Supplemental Materials and Methods

Reagents

Lentiviral vectors pGLucIPZ and pCLucIPZ for stable, constitutive co-expression of *Gaussia* luciferase (GLuc) and *Cypridina* luciferase (CLuc) together with shRNAs have been described (3). shRNA sequences are listed below. For doxycycline-inducible expression, ENTPD5 cDNA was amplified by PCR from pReceiver plasmid EX-U0055-Lv68 (GeneCopeia) and cloned into pENTR™/D-TOPO® (Thermo Fisher Scientific). pENTR-p53 plasmid has been described (4) and subjected to site-directed mutagenesis to generate mutp53 R248W and R175H. Gateway cloning was used to shuttle ENTPD5 and mutp53 cDNAs into the pInducer20 lentiviral vector (5). A *Renilla* luciferase (RLuc) reporter driven by the ENTPD5 promoter region -990 to +96 relative to the ENTPD5 transcription start site was purchased from BioCat. A cytomegalovirus intermediate early enhancer/promoter-driven *Cypridina* luciferase (CLuc) reporter has been previously described (3). An expression plasmid for HA-tagged endoglin /CD105 has been previously described (6). siRNAs and antibodies are listed below.

Cell cultures

Pancreatic cancer (PANC-1, PaCa-44, MIA PaCa-2), breast cancer (MDA-MB-231, MDA-MB-468, MCF-7), osteosarcoma (U2OS, Saos-2) and lung cancer (H1299) cell lines were cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% (v/v) Penicillin (10.000 U/ml)/Streptomycin (10 mg/ml) and 0.4% Amphotericin B (250 g/ml). Breast cancer cell line T-47D was cultured in RPMI-1640 supplemented with 10% FBS, 1% (v/v) Penicillin (10.000 U/ml)/Streptomycin (10 mg/ml) and 0.4% Amphotericin B (250 g/ml). MCF-10A cells were cultured in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F12) supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 mg/ml Hydrocortison, 100 ng/ml Choleratoxin, 10 µg/ml Insulin and 1% (v/v) Penicillin (10.000 U/ml)/Streptomycin (10 mg/ml). Cells were kept at 37°C with 5% CO2 in humid atmosphere (95%). SAHA, doxycycline and nutlin-3a were obtained from Sigma-Aldrich; 17-AAG, Ganetespib and AZD5363 from Selleckchem; LY294002 from Biomol. siRNA transfection was done using Lipofectamine RNAiMax (#13778075, Thermo Fisher Scientific) according to the manufacturer's protocol. Cells were harvested 72h after transfection with 10 nM of siRNA, unless stated otherwise. Production of lentivirus and transduction of cells using shRNAs were performed as previously described (3). Successfully transduced cells were selected with puromycin (1 µg/ml) and/or neomycin (600 µg/ml), respectively.

RNA analysis

Analysis of mRNA expression by RTqPCR was performed as previously described (7). Primer sequences are listed below. RNA samples from pancreatic cancer cells PANC-1 were subjected to genome-wide gene expression profiling using the Agilent SurePrint G3 Human Gene Expression 8x60K Microarray platform as described (8). Labeling and hybridization was conducted with the Quick-Amp labeling kit (Agilent) according to the manufacturer's instructions. The microarray was analyzed on an Agilent microarray scanner and the G2505C Feature Extraction software v10.5.1.1 (Agilent). Raw microarray data were normalized via within-array normalization using the loess correction method available from the limma package of R/Bioconductor (9). The log2 fold change was calculated for each p53 siRNA compared to a non-targeting control siRNA for probes with a mean intensity > 5. Differential expression was tested using two sided t-tests corrected for multiple hypotheses testing via Benjamini-Hochberg correction. Probes were assigned to genes as described (7) based on Ensembl release 74 (hg19).

TCGA data analysis

RNA sequencing data sets (RNAseqV2) from six different human cancer types (bladder urothelial carcinoma (BLCA), colon adenocarcinoma (COAD), esophageal carcinoma (ESCA), head and neck squamous cell carcinoma (HNSC), brain lower grade glioma (LGG), pancreatic adenocarcinoma (PAAD)) were obtained from The Cancer Genome Atlas (TCGA) (https://tcga-data.nci.nih.gov/tcga/) and grouped into three distinct classes according to their p53 status as previously described (10). Mutation data obtained from whole-exome sequencing deposited in the TCGA allowed classification of samples as (1) p53 wild type, if no p53 mutation was detected, as (2) GOF, if any of the missense

mutations R175H, R248Q, R248W, R249S or R273H was detected, or (3) p53 null, if a p53 nonsense mutation or frameshift truncation was detected. Samples that did not match any of these criteria where discarded. Expression values in terms of FPKM values (fragments per kilobase per million) of several genes were compared between these three groups via box-whisker plots (whiskers indicating the 10th and 90th percentile) and non-parametric Mann-Whitney U tests. Obtained p-values where corrected for multiple hypothesis testing using a Benjamini-Hochberg correction.

Luciferase Reporter Assay
Cells were transiently co-transfected with a *Renilla* and *Cypridina* luciferase reporter plasmids. Cells were lyzed 72 h after transfection using Passive Lysis Buffer (Promega). Luciferase activities were measured in cell lysates on a plate reader luminometer (ORION II, Titertek-Berthold) using coelenterazin and vargulin as substrates, respectively.

Chromatin immunoprecipitation
For ChIP assays MDA-MB-231 and MIA PaCa-2 cells were transfected with siRNA targeting p53, Sp1 or a non-targeting control (nsi). 72h hours after transfection cells were fixed with 1% paraformaldehyde (PFA) for 10 minutes at room temperature (RT). Addition of glycine (125 mM) for five minutes at RT quenched fixation process. After washing two times with ice-cold PBS cells were scraped off the plate with PBS complemented with proteinase-inhibitor (Complete, Roche). Cell pellets were then lysed in SDS lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris pH 8.1) supplemented with protease-inhibitor (cOmplete ULTRA tablets mini, Roche) at a concentration of $2x10^7$ cells/ml. Afterwards cells were sonicated to receive fragments in the size between 500-1000 bp using the X Bioruptor® Sonication System (Diagenode). Efficient sonication was controlled on an agarose gel. After centrifugation sheared chromatin was diluted 1:10 with dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl pH 8.1,167 mM NaCl) and pre-cleared for 1h with Protein G sepharose beads (GE Healthcare, 71-7083-00 AI) at 4 °C. Afterwards 1% input was removed from each sample and proteins were precipitated with antibodies of interest (p53, SP1, IgG) over night at 4°C. The next day Protein G sepharose beads were added to the protein complexes for four hours at 4 °C and washed with Low Salt Immune Complex Wash Buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 150 mM NaCl), once with High Salt Immune Complex Wash Buffer (0.1% SDS, 1%Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8.1, 500 mM NaCl), once with LiCl Immune Complex Wash Buffer (0.25 M LiCl, 1% IGEPAL-CA630, 1% deoxycholic acid (sodium salt), 1 mM EDTA, 10 mM Tris-HCl pH 8.1), and twice with TE (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Protein/DNA crosslinks of ChIP samples and input (stored at 4°C) were resolved by adding 100 µl of 10% Chelex-solution for 10 min at 99°C followed by proteinase K digestion for 30 minutes at 55°C and inactivation of proteinase K at 99°C for 10 minutes. Beads were then recovered by two rounds of elution with 2x100 µl sterile water for 1 minute at 12.000g and 4°C. Binding analysis was done using qPCR and 1µl of template DNA per reaction.

Protein analysis
Cells were lyzed in NP-40 lysis buffer (50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA pH 8.0, 2% NP-40) containing protease inhibitors (cOmplete ULTRA tablets mini, Roche) and subjected to Western blot as described (3). For Immunohistochemistry (IHC) tissues were fixed in buffered formalin and embedded in paraffin, cut and fixed on glass slides overnight at 37°C. Staining was performed as previously described (3).

Co-Immunoprecipitation (Co-IP)
For Co-IPs MDA-MB-231 and MIA PaCa-2 cells were scraped off the plate using NET buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 0,5 % NP40, 10 % Glycerol, 5 mM EDTA) supplemented with protease-inhibitor (cOmplete ULTRA tablets mini, Roche). Protein concentration was determined using Bradford assay (BIO-RAD Protein Assay). 500 µg of total protein were diluted to a total volume of 500 µl (1 µg/µl) with NET buffer followed by pre-clearing using Protein G sepharose beads for 1h at 4°C. Afterwards protein samples were centrifuged for 1.5 min at 3000g at 4°C and 1 to 2% input was removed. For immunoprecipitation protein samples were then supplemented with appropriate antibodies (p53, IgG) and incubated at 4°C overnight. The next day Protein G sepharose beads were added to each sample and rotated for 1h 4°C. Afterwards beads were washed four times with NET

buffer and immunoprecipitated proteins were eluted using 2.5x Roti-Load (Roth, K929.1). Immunoblotting was done as described above. Antibodies are listed below.

Colony formation assay
For clonogenic assays siRNA transfected cells ($1x10^3$) were seeded on 6 cm dishes and colonies were grown for 10 days. Cells were fixed with ice-cold 70% ethanol and colonies were stained with crystal violet. For colony quantification, stained cells were lyzed in 10% acetic acid and absorbance of released dye was measured at 590 nm using a BioTek microplate reader.

3D tissue culture
Three-dimensional culture of MCF-10A cells was done as previously described (11). Briefly, MCF-10A cells (3,000 cells per 8 wells) were seeded in a 3D Life Hydrogel system (Cellendes) and cultured for 14 days with medium changes every 2 days (MCF-10A assay medium containing 5 ng/ml EGF and 333 ng/ml doxycycline). After 14 days cells were fixed with 8% formaldehyde and stained with phalloidin (F-actin) and DAPI. Formation of acini-like spheroids were analyzed using a confocal microscope (Zeiss LSM 700) and at least 60 acini structures per condition were analyzed.

Tumor tissue samples and immunohistochemistry
Tumor samples from women with confirmed triple-negative invasive carcinomas of the breast (ER-, PR-, HER2-) and tumor samples of PDAC as well as samples of normal kidney were obtained in the form of FFPE blocks. All archived FFPE tumor specimens were obtained from University Hospitals Halle/Saale (Germany) and Marburg (Germany) according to institutional guidelines. Individual informed consent was obtained and the use of breast cancer specimens was approved by the institutional review board of Martin-Luther University Halle (Saale). Use of the PDAC samples was in accordance with the rules of the Ethics Committee of the Medical Faculty of the Philipps-University Marburg. Five-micrometer sections of paraffin-embedded tissue were used for the immunohistochemical analysis of p53 und ENTPD5 expression according to standard procedures.

Luciferase measurements in blood and tissue samples
10 µl of blood was obtained by tail vein puncture every 2-3 days and mixed directly with 2 µl of 0.125 IE/ml heparin. Plasma was collected by centrifugation (15 min, 3,600 g, 4 °C) and frozen at -20°C. Animals were sacrificed after 21 days. For induction of dox-regulated vectors, doxycyclin was freshly prepared and given to the mice via the drinking water (1mg/ml dox; 2% sucrose). All blood samples collected over the course of the experiment were thawed and analyzed together with a single batch of reagents. For luciferase activity measurements in the Orion II luminometer (Berthold), plasma was diluted 1:10–1:1,000 with PBS. The GLuc substrate coelenterazine (PJK, Germany) was prepared as a 10 mM stock in acidified ethanol (10 ml EtOH þ 200 ml 6 M HCl). CLuc substrate vargulin (NEB) was prepared according to the manufacturer's protocol. Each diluted plasma sample (5 µl) was measured by injection of 100 µl coelenterazine (stock diluted 1:200 in PBS) or 25 µl vargulin reagent (stock diluted 1:200 in Biolux Cypridina Luciferase Assay Buffer (NEB) prediluted 1:5 in PBS). All plasma samples were measured in duplicates without background correction.
For measurement of luciferase activities in tissue lysates, lungs were excised from dead mice and minced. Approximately 10 mg of lung tissue was lysed in 100 µl passive lysis buffer (Promega) with the TissueLyser LT (Qiagen). Tissue lysate (5 µl) was measured in duplicate for GLuc and CLuc activity without background correction as described above for blood plasma.

Antibodies

name	application	clone/order no.	source
β-actin	WB	AC-15 /ab6276	Abcam
p53	WB,IHC	DO1 / -	Dr. B. Vojtesek
ENTPD5	WB	EPR3783 / ab92542	Abcam
ENTPD5	IHC	EPR3784 / ab108603	Abcam
GLuc	WB,IHC	- / 401P	NanoLight
pAKT (Thr308)	WB	D25E6 / #13038	Cell Signaling
pAKT (Ser473)	WB	D9E / #4060	Cell Signaling
total Akt	WB	C67E7 / #4691	Cell Signaling
AKT substrate	WB	23C8D2 / #10001	Cell Signaling
p53	IP	FL-393 /sc-6243	Santa Cruz
p53	ChIP	FL-393 /sc-6243X	Santa Cruz
Sp1	ChIP, WB	- / 07-645	Merck Millipore
Sp3	WB	D-20 /sc-644	Santa Cruz
mouse IgG-HRP	WB	- / NA9310	GE Healthcare
mouse IgG-Biotin	IHC	- / EO46401	Dako
rabbit IgG-HRP	WB	- / NA9340	GE Healthcare
rabbit IgG-Biotin	IHC	- / EO43201	Dako
HA-tag	IP	HA.11	Covance
HA-tag	WB	C29F4 / #3724	Cell Signaling

PCR primer

gene	application	sense sequence	anti-sense sequence
ENTPD5	RTqPCR	TGATTCTGTGAAGCCAGGAC	ATTGAGTCTTTGGCCACCTC
GAPDH	RTqPCR	CTATAAATTGAGCCCGCAGCC	ACCAAATCCGTTGACTCCGA
p53	RTqPCR	ATCTACTGGGACGGAACAGC	GCGGAGATTCTCTTCCTCTG
ENTPD5-300	ChIP	TTGGTGCCAACTCTGTGTTC	TGGAGTTGCTGCTAGATTGC

shRNAs
All shRNAs were obtained from the ThermoFisher Human pGIPZ shRNAmir library

name/gene	shRNA	target sequence	pGIPZ shRNAmir Library
nsh		ATCTCGCTTGGGCGAGAGTAAG	
p53	sh1	GAGGATTTCATCTCTTGTA	V2LHS_217
	sh2	CGGCGCACAGAGGAAGAGA	V3LHS_333920
ENTPD5	sh1	CCGAGATGGTTGGAAGCAG	V3LHS_306216
	sh2	AGGGTGCTGAGACCGTTCA	V3LHS_306217

siRNAs
All siRNAs were obtained from Dharmacon. Modification: siG, siGenome, OTP, ON-TARGET *plus*
* used as pool of the listed four different siRNAs

name/gene	siRNA	target sequence	modification
nsi control	si1	UGGUUUACAUGUCGACUAA	OTP
	si2	UGGUUUACAUGUUGUGUGA	OTP
	si3	UGGUUUACAUGUUUUCUGA	OTP
	si4	UGGUUUACAUGUUUUCCUA	OTP
p53	si1	GACUCCAGUGGUAAUCUAC	OTP
	si2	GAAAUUUGCGUGUGGAGUA	OTP
	si3	GCAGUCAGAUCCUAGCGUC	OTP
	si4	GGACAUACCAGCUUAGAUUUU	siG
	si5	UGUGAGGGUUAAUGAAAUAUU	siG
ENTPD5	si6	AGACUUGGUUUGAGGGUAU	OTP
	si7	CAGGACAGCUUCCAAUUCU	OTP
	si8	CAUAUUAGCUUGGGUUACU	OTP
	si9	CGAGAUGGUUGGAAGCAGA	OTP
SP1	si6	GAAGGGAGGCCCAGGUGUA	OTP
	si7	GGGCAGACCUUUACAACUC	OTP
	si8	CUACAGAGGCACAAACGUA	OTP
SP3	si7	GGUAUUCACUCUAGCAGUA	OTP
	si8	GAAAUUUGUUUGUCCAGAA	OTP
	si9	GAUAGGAACUGUUAAUACU	OTP
UGGT	si6	CCAAGACUCCUGUGAAAUU	OTP

Supplemental References

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