Figure S1

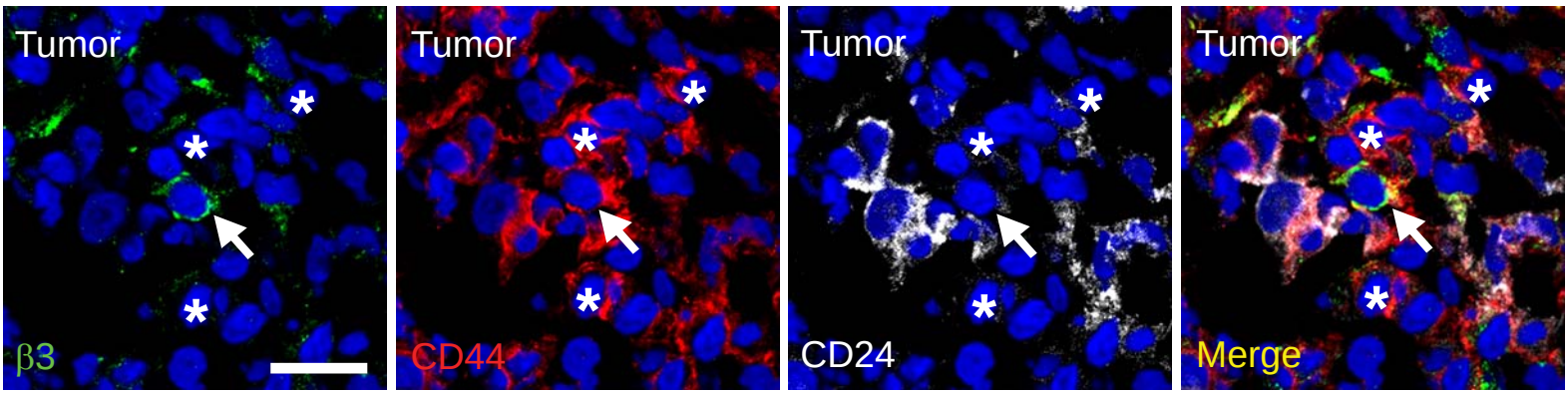


Figure S1. αvβ3 expression is associated with stem markers in primary tumors from breast cancer patients. Related to Figure 1. (**A**) Representative example of immunofluorescent staining for β3 and the cancer stem cell markers CD44 and CD24 in a frozen human breast cancer tissue microarray. Arrows show an example of a β3 expressing tumor cell with the $CD44^{+}/CD24^{low}$ stem marker profile. Asterisks mark $CD44^{+}/CD24^{low}$ cells that lack β3 expression. Nuclei are stained blue in all panels. Scale bars, 20 μm.

Figure S2

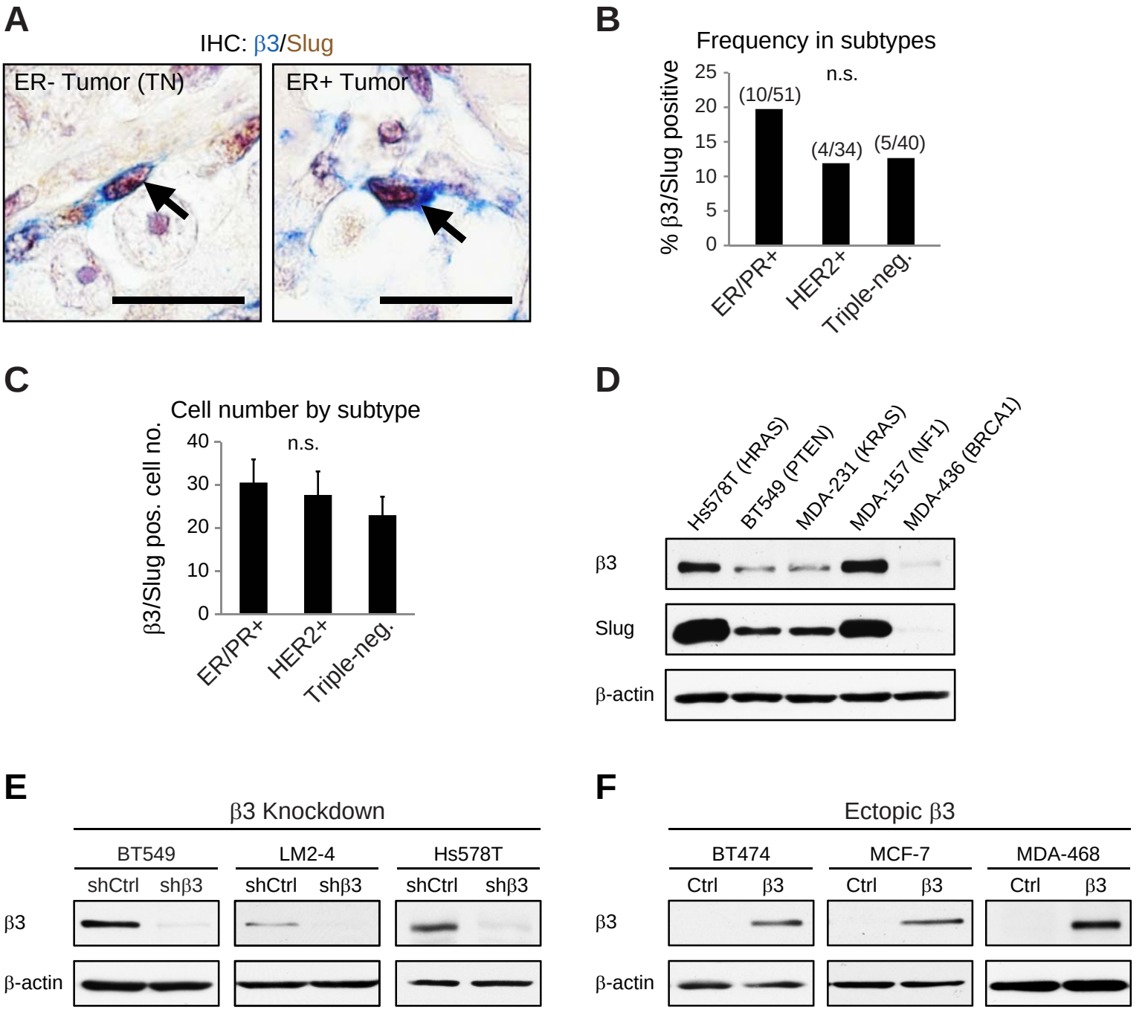


Figure S2. αvβ3 is associated with Slug in patient tumor samples and cell lines. Related to Figure 2. (**A-C**) Analysis of immunohistochemical staining (IHC) for β3 and Slug in breast cancer patient samples from tissue microarrays. (**A**) Representative examples of cells expressing both β3 (blue) and Slug (brown) in patient tumor samples. $\beta3^+/Slug^+$ cells (arrows) are shown for an estrogen receptor-positive (ER^+) and a triple-negative (TN) tumor. n=19/125 ($\beta3^+Slug^+$ tumors/total). $\beta3^+Slug^+$; ER^+=10, $HER2^+$=4, TN=5. Scale bars, 20 μm. (**B**) Frequency of tumors with $\beta3^+/Slug^+$ cells in different subtypes. Numbers over each bar show the $\beta3^+/Slug^+$ tumors/total tumors. Data were analyzed by Fisher's Exact Test. (**C**) Quantitation of the total number of $\beta3^+/Slug^+$ cells per section for each subtype. Data represent the mean ± s.e.m. and were analyzed by one-way ANOVA. (**B** and **C**) n.s. = not significant. (**D**) Western analysis for β3 and Slug in several breast cancer cell lines representing the claudin-low molecular subtype. The dominant oncogenic driver in each cell type is identified in parentheses. (**E** and **F**) Immunoblot analysis for β3 expression in breast cancer cell lines +/- β3 knockdown (**E**) or expression of ectopic β3 (**F**). β3 protein expression was assessed in breast cancer cell lines stably expressing either non-silencing control (shCtrl) or β3 shRNA (shβ3) (**E**), or β3 cDNA (β3) or a vector only control (Ctrl) (**F**). (**D-F**) For all immunoblots data shown is representative of at least 3 independent experiments and β-actin is shown as a loading control.

Figure S3

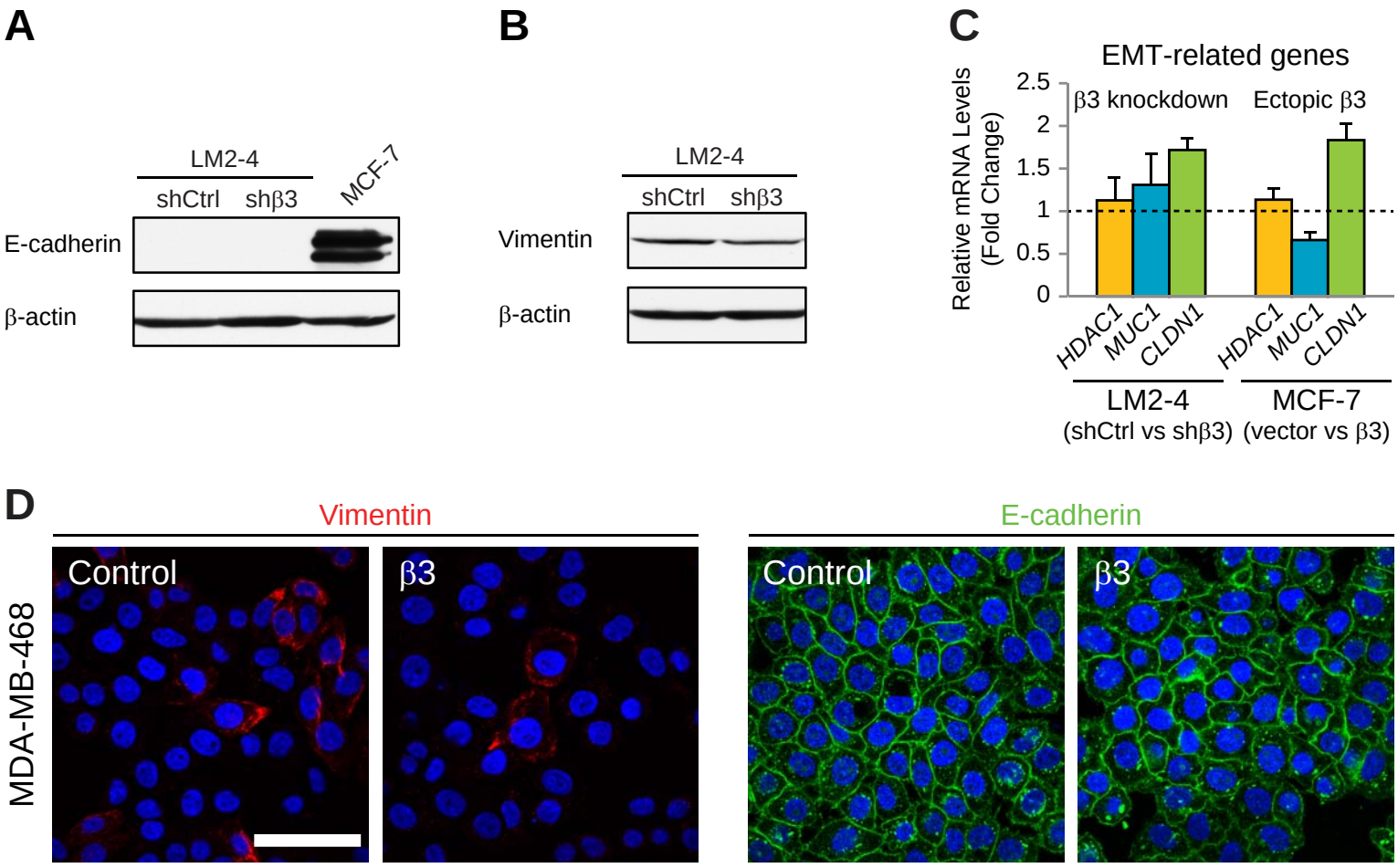


Figure S3. αvβ3 enhances Slug expression, but is dispensable for EMT. Related to Figure 2. (**A** and **B**) Western analysis of EMT markers in β3 knockdown LM2-4 cells. Representative examples are shown from 3 independent experiments. MCF-7 cell lysate was used in **A** as a control for E-cadherin expression. β-actin is shown as a loading control. (**C**) qPCR analysis of additional EMT-related genes in β3 knockdown LM2-4 cells or MCF-7 cells expressing ectopic β3. For each cell type the fold change ($2^{-\Delta\Delta CT}$) is shown relative to the appropriate control cells. β-actin was used as a loading control. Each sample was run in triplicate and data are displayed as the mean ± s.e.m. for 3 independent experiments. (**D**) Representative immunofluorescent images showing Vimentin (red) or E-cadherin (green) in MDA-MB-468 cells expressing ectopic β3 or a vector control. Nuclei are stained blue in all panels. Scale bars, 50 μm.

Figure S4

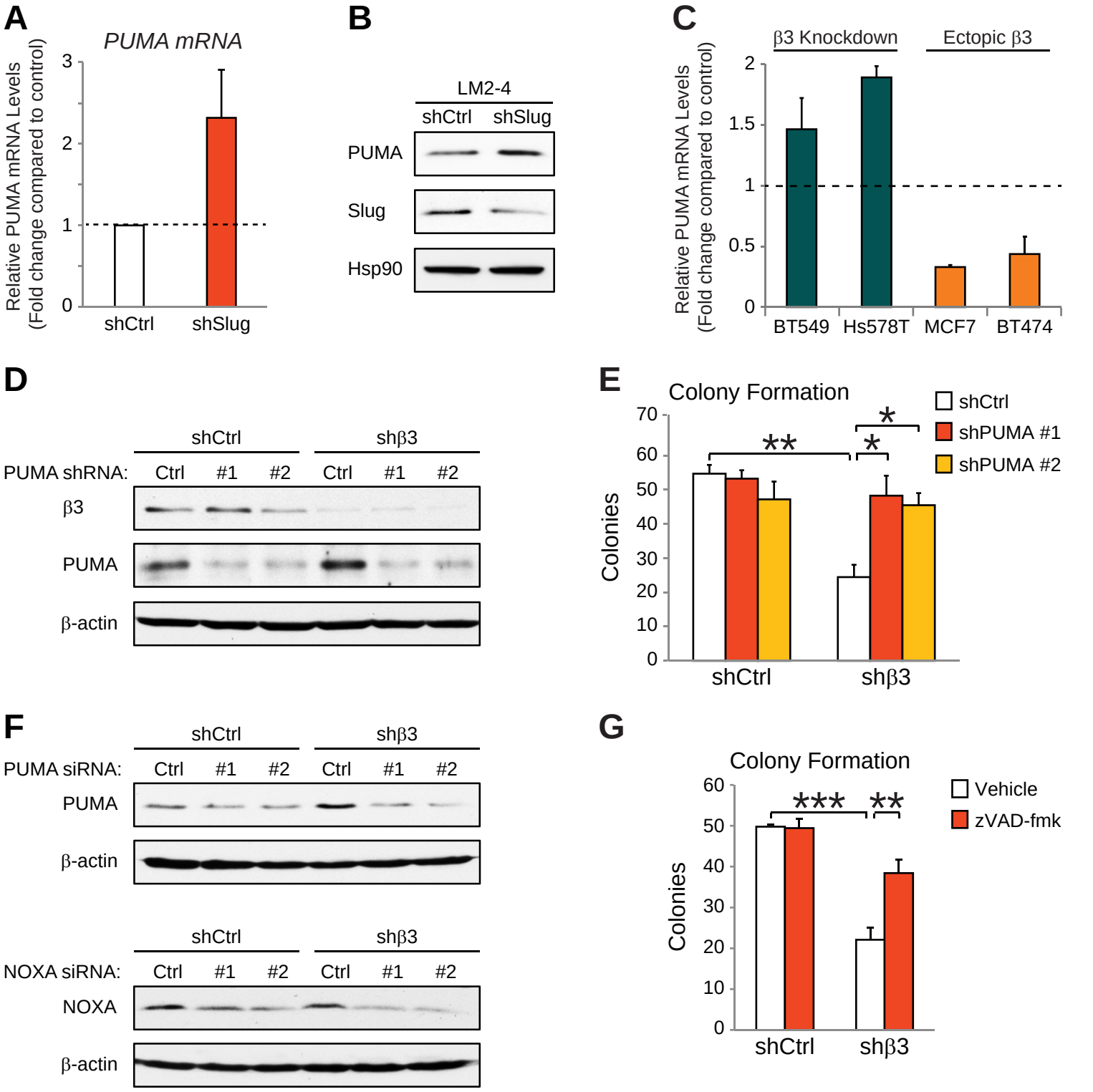


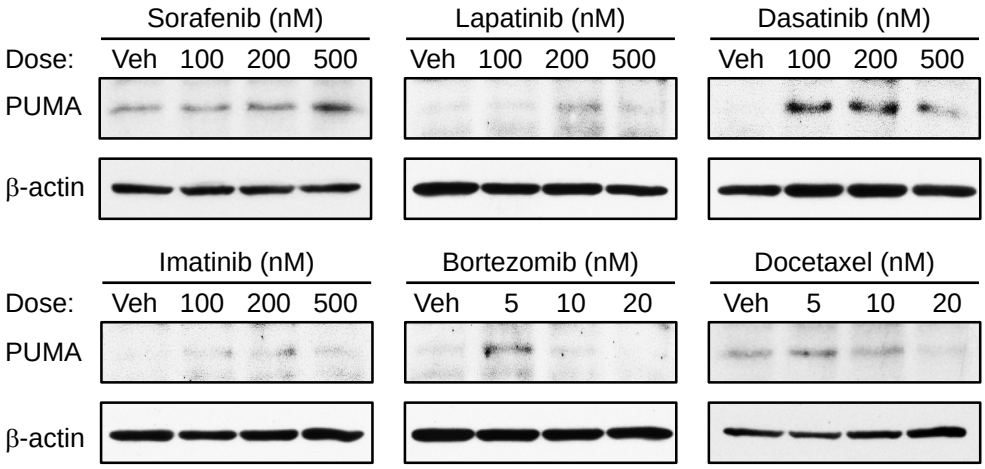
Figure S4. PUMA suppression is critical for αvβ3-driven anchorage-independent colony formation. Related to Figure 3. (**A** and **B**) Analysis of PUMA mRNA and protein levels in Slug knockdown (shSlug) LM2-4 cells by qPCR (**A**) or Western blot (**B**). (**C**) qPCR analysis of PUMA mRNA levels in β3 knockdown breast cancer cells or cell lines expressing ectopic β3. (**A** and **C**) For all qPCR experiments, the fold change ($2^{-\Delta\Delta CT}$) is shown relative to control cells. Each sample was run in triplicate and β-actin was used as a loading control. Data are displayed as the mean ± s.e.m. for four (**A**) or three (**C**) independent experiments. (**B**) Hsp90 is shown as a loading control for total protein. (**D-F**) PUMA knockdown experiments in LM2-4 stable cells expressing non-silencing (shCtrl) or β3 shRNA (shβ3). (**D** and **F**) Immunoblots from cells stably expressing either of two different PUMA shRNA's (**D**) or transiently expressing siRNA's against PUMA (**F**, top panel) or the related protein NOXA (**F**, bottom panel). (**B**, **D** and **F**) Data shown is representative of 3 independent experiments. (**E**) Histogram showing the number of soft agar tumorspheres per field from the indicated cell types after stable PUMA knockdown with either of two different shRNA's (shPUMA). P=0.0021 (shCtrl; shCtrl vs shβ3), P=0.0133 (shβ3; shCtrl vs shPUMA #1), P=0.0303 (shβ3; shCtrl vs shPUMA #2). (**G**) Soft agar tumorsphere assays comparing the effect of the caspase inhibitor zVAD-fmk (20 μM) to cells treated with vehicle control (DMSO). Histograms show the number of colonies per field for the indicated cell types. P=0.0002 (Vehicle; shCtrl vs shβ3), P=0.0069 (shβ3; Vehicle vs zVAD-fmk). (**E** and **G**) n=3 independent experiments performed in triplicate. Data represent the mean ± s.e.m. and statistical analysis was performed by two-way ANOVA with Tukey's multiple comparisons test. *P<0.05, **P<0.01, ***P<0.001.

Figure S5

A

Drug	Major Target(s)
Sorafenib	Raf kinases, PDGFR, VEGFR
Lapatinib	EGFR
Dasatinib	Src family kinases, Abl, PDGFR
Imatinib	Abl kinase, PDGFR
Bortezomib	Proteasome inhibitor
Docetaxel	Chemotherapy

B



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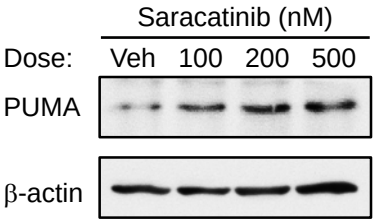


D

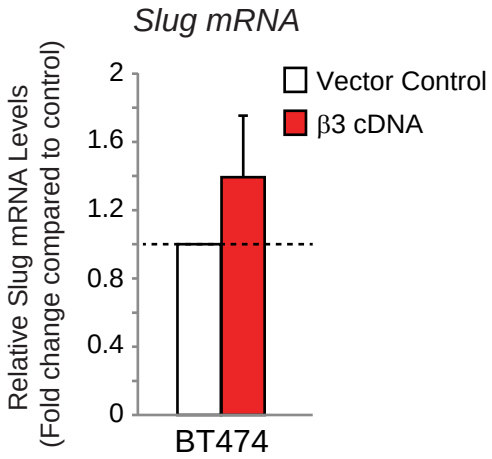


Figure S5. Src inhibitors specifically drive PUMA expression. Related to Figure 5. (**A-C**) Examination of different classes of clinically approved therapies for their ability to induce PUMA expression. (**A**) Table depicting the major targets of the drugs tested in this study. (**B** and **C**) Immunoblot analysis of several clinically-approved drugs with diverse targets for their ability to induce PUMA protein expression in LM2-4 cells. In addition to dasatinib, we also tested the Src inhibitor saracatinib in these assays (**C**). (**B** and **C**) Several doses of each drug were tested at the indicated concentrations and lysates harvested after 24 hr. β-actin is shown as a loading control. (**D**) qPCR analysis showing the relative Slug mRNA expression ($2^{-\Delta\Delta CT}$) in BT474 cells expressing ectopic β3 compared to vector control. Each sample was run in triplicate and β-actin was used as a loading control. Data are displayed as the mean ± s.e.m. for 3 independent experiments.

Figure S6

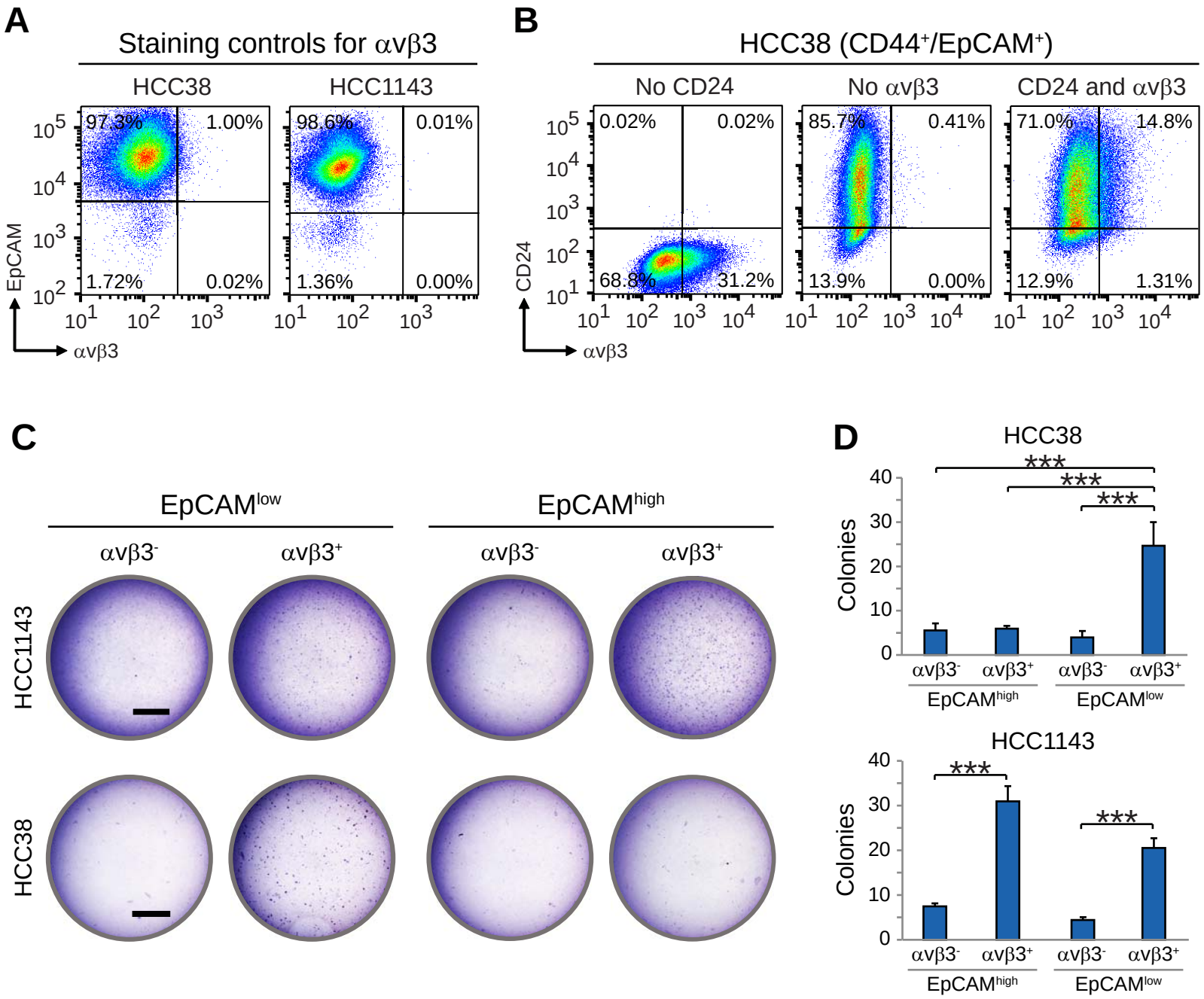
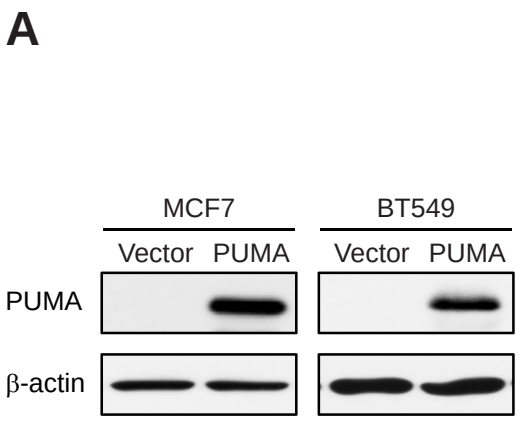
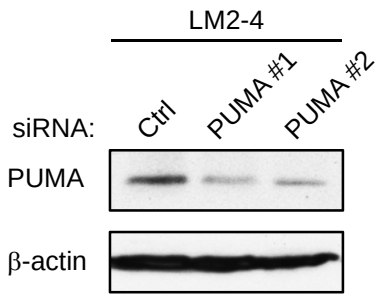
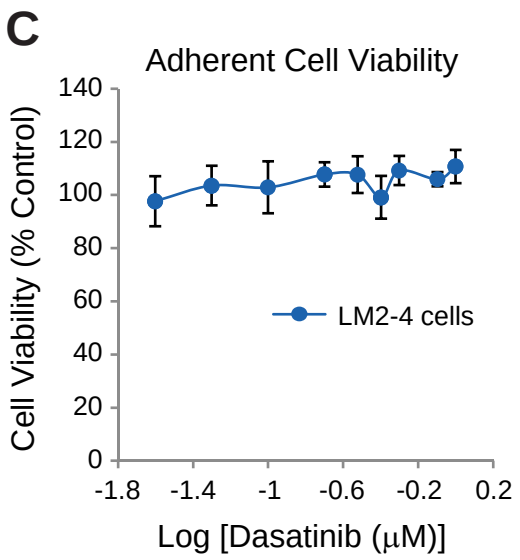


Figure S6. Surface αvβ3 expression identifies a subset of CD49f^{+}/EpCAM^{low} cells with colony initiating capability. Related to Figure 6. (**A**) Representative FACS density plots showing the minus αvβ3 antibody staining controls for HCC38 and HCC1143 cells for the same experiment as shown in Figure 6A. (**B**) Flow cytometry analysis of HCC38 cells that are CD44^{+} and EpCAM^{+} according to their CD24 and αvβ3 status. Shown are the minus CD24 antibody and minus αvβ3 antibody staining controls as well as the data with both antibodies added together. (**C**) Representative example of a soft agar tumorsphere experiment comparing the different FACS-sorted populations from HCC1143 and HCC38 cells and stained with crystal violet. Scale bars, 2 mm. (**D**) Soft agar tumorsphere assays comparing colony number per field in the indicated HCC38 and HCC1143 sorted cell populations. HCC38 (EpCAM^{low}/αvβ3^{+}); P=0.0068 (vs EpCAM^{high}/αvβ3^{-}), P=0.0077 (vs EpCAM^{high}/αvβ3^{+}), P=0.0042 (vs EpCAM^{low}/αvβ3^{-}), HCC1143; P<0.0001 (EpCAM^{high}/αvβ3^{-} vs EpCAM^{high}/αvβ3^{+}), P=0.0009 (EpCAM^{low}/αvβ3^{-} vs EpCAM^{low}/αvβ3^{+}). Data represent the mean ± s.e.m. and statistical analysis performed by one-way ANOVA with Tukey's multiple comparisons test. n=3 independent experiments performed in triplicate. ***P<0.001.

Figure S7







D

	Tumor Incidence			
No. of cells injected	shCtrl		shPUMA	
	Vehicle	Dasatinib	Vehicle	Dasatinib
60,000	2/2	2/2	2/2	2/2
30,000	4/4	4/4	4/4	4/4
15,000	4/4	4/4	3/4	4/4

Figure S7. Src inhibition fails to reduce adherent cell viability or tumor incidence. Related to Figure 7. (**A**) Immunoblots for ectopic PUMA expression after transient transfection of MCF7 or BT549 breast cancer cell lines with PUMA cDNA (PUMA) or empty control vector (Vector). (**B**) Western blot showing PUMA knockdown in LM2-4 cells transfected with two different PUMA siRNA's compared to a control siRNA (Ctrl). (**A** and **B**) Data shown is representative of 3 (**A**) and 5 (**B**) independent experiments and β-actin is shown as a loading control. (**C**) Representative example of a dasatinib dose-response experiment examining LM2-4 cell viability in adherent cells with the XTT assay. Each condition was run in quadruplicate and data represent the mean ± s.d. for each dose of dasatinib tested. (**D**) In vivo tumor initiation assay comparing shCtrl and shPUMA LM2-4 cells injected orthotopically at limiting dilution into adult female mice and treated with 30 mg/kg dasatinib or vehicle (1% citric acid) once daily by oral gavage for the first seven days post-injection. The tumor incidence per number of mice injected is displayed for each condition.

Supplemental Methods

Immunohistochemistry

Immunohistochemical staining for β3 was performed on a formalin-fixed paraffin-embedded (FFPE) breast cancer progression tissue microarray (gift from Michael Karin). Additional FFPE tissue microarrays were purchased from US Biomax for staining with β3 only (BR1502) or for dual staining with β3 and Slug (BR1505b and BR10010d). Dual staining was also performed on FFPE sections from PDX models purchased from The Jackson Laboratory or provided by Alana Welm. De-identified FFPE baseline tumor sections from the WHEL clinical study (1) were purchased from the Moores Cancer Center biorepository. For immunohistochemical staining of FFPE tissues, antigen retrieval was performed in citrate buffer at pH 6.0 and 95°C for 20 min. Sections were blocked in 5% normal goat serum diluted in Tris-buffered saline, pH 7.6/ 0.25% Tween-20 (TBST), incubated in primary antibody 1 h at room temperature (β3) or overnight at 4°C (Slug) followed by biotin-conjugated anti-rabbit IgG and an avidin–biotin peroxidase detection system with 3,3'-diaminobenzidine (DAB) substrate or an alkaline phosphatase system with Vector Blue substrate (Vector), then counterstained with hematoxylin. For dual staining, Slug was performed first, followed by β3 the next day. Stained tumor sections were scored or the number of dual positive cells per section quantified by a blinded observer. All slides were imaged on a Nikon Ti/E inverted microscope using Nikon Elements software. Primary antibodies used for immunohistochemical staining included β3 (D7X3P) and Slug (C19G7) both purchased from Cell Signaling Technology.

Immunofluorescent staining

Staining was performed on cells in culture as well as frozen breast cancer tissue microarrays purchased from US Biomax (β3, CD24 and CD44, BRF401; β3 and pan-cytokeratin, BRF404b). For immunofluorescent staining, frozen sections were first fixed in acetone at -20°C, whereas cells in culture were fixed briefly in 2% paraformaldehyde/PBS for 10 min, and then permeabilized in 0.1% Triton/PBS for 5 min prior to staining. Frozen sections or fixed cells were blocked with 5% normal goat serum (sections), or 3% BSA/2.5% normal goat serum (cells) in PBS at room temperature for 1 h before incubating in primary antibody overnight at 4°C followed by secondary antibody (Alexafluors 488, 568, 647, Invitrogen). 4',6-Diamidino-2-Phenylindole Dihydrochloride (DAPI) was also added to visualize nuclei. Samples were imaged on a Nikon Eclipse C1 confocal microscope using Nikon Elements imaging software. The following primary antibodies were used for staining: β3 (D7X3P), Slug (C19G7), and Vimentin (D21H3) (Cell Signaling Technology), CD44-biotin (Pierce), CD24-Alexafluor 647 (BioLegend), E-cadherin (BD Biosciences) and pan-cytokeratin-Alexafluor 647 (BioLegend).

Cell lines

The following breast cancer cell lines were purchased from ATCC: MCF-7, T47D, BT474, MDA-MB-468, BT-20, HCC1187, Hs578T, BT549, MDA-MB-231, MDA-MB-157, MDA-MB-436, HCC38 and HCC1143. LM2-4 cells, a highly metastatic variant of the MDA-MB-231 cell line (2) was a gift from Robert Kerbel. All cell lines were tested and shown to be free of mycoplasma. Cells used in mice were additionally tested and found to be negative for an extensive panel of mouse pathogens. Cell lines were cultured in complete DMEM medium (DMEM supplemented with 10% fetal bovine serum (FBS) + 1:100 L-glutamine, sodium pyruvate, non-essential amino acids, and antibiotic/antimycotic) except BT-20, which were cultured in αMEM with 10% FBS + 1:100 L-glutamine and antibiotic/antimycotic.

Cell transfection and lentiviral transduction

Transient transfection with β3 cDNA's in the pcDNA3.1 expression plasmid was performed with Lipofectamine 3000 (Invitrogen) as per manufacturer's instructions for 6-well dishes. siRNA transfection was achieved using HiPerFect transfection reagent (Qiagen) as per manufacturer's instructions for a 10 cm dish. AllStars negative control, PUMA (*BBC3*; SIO2655520 and SIO2822820), NOXA (*PMAIP1*; SIO0129430 and SIO0129451) and FBXO11 (*FBXO11*; SIO2639028) FlexiTube siRNAs were purchased from Qiagen. Stable knock-downs were achieved by transducing cells with lentivirus expressing human-specific shRNAs targeting β3 (*ITGB3*; V2LHS_77099), PUMA (*BBC3*; V3LHS_342433 and V3LHS_342436), Slug (*SNAI2*; V3LHS_390965 and V3LHS_413131), c-Src (*SRC*; V2LHS_262793) or a non-silencing control in the pGIPZ vector (Open Biosystems) and pooling puromycin-resistant cells. Ectopic β3 expression was performed by transducing BT474 or MDA-MB-468 cells with lentivirus generated with the FG12 plasmid alone (empty vector) or containing β3 cDNA (gift from Shattil laboratory). Alternatively, MCF-7 or BT474 cells transiently transfected with β3 cDNA/pcDNA3.1, β3 759x cDNA/pcDNA3.1 or pcDNA3.1 alone were treated with 500 μg ml^{-1} G418 for two weeks to select resistant stable clones which were then pooled for use in experiments.

Immunoblotting and immunoprecipitations

Whole cell lysates were prepared from cell lines with RIPA lysis buffer (100 mM Tris pH 7.5, 150 mM sodium chloride, 0.1% deoxycholate, 0.1% SDS, 50 mM NaF, Protease inhibitor cocktail (Roche), 2 mM PMSF, 2mM sodium orthovanadate) combined with scraping and the lysates cleared by centrifugation. Additionally, Slug was pulled-down from BT474 cell lysates with 30 μL of 50% protein A/G beads (Pierce) and 2 μg of mouse anti-Slug antibody (S431259; BD Bioscience) overnight at 4ºC. The beads were then washed 3x with lysis buffer prior to eluting proteins with 2x sample buffer and performing Western analysis. Standard Western blotting procedures were performed. The following antibodies were used for immunoblotting: β3 (D7X3P), Slug (C19G7), ZEB1 (D80D3), Snail (C15D3), Vimentin (D21H3), PUMA (D30C10), NOXA (D8L7U), pY416 Src Family Kinase, Bcl-2, c-Src (L4A1), PTEN (D4.3), and Bim (C34C5) (Cell Signaling Technology), β-catenin (2H4A7; Millipore), E-cadherin (BD Biosciences), FBXO11 (Novus Biologicals), Hsp90 (Santa Cruz) and β-actin (Sigma). For inhibitor studies, the following drugs were used to treat LM2-4 cells and compared to DMSO alone (Vehicle): sorafenib (Chemietek), lapatinib (LC laboratories), dasatinib (Chemietek), imatinib (Chemietek), bortezomib (Chemietek), docetaxel (Chemietek) and saracatinib (Selleckchem). All drug treatments were performed at the specified doses for 24 h prior to harvesting lysates.

Real-time qPCR

qPCR Primers

Primer Name	Primer Sequence (5'-3')
Human	
BBC3-F	GACCTCAACGCACAGTACGAG
BBC3-R	AGGAGTCCCATGATGAGATTGT
CDKN1A-F	TGTCCGTCAGAACCCATGC
CDKN1A-R	AAAGTCGAAGTTCCATCGCTC
MDM2-F	GAATCATCGGACTCAGGTACATC
MDM2-R	TCTGTCTCACTAATTGCTCTCCT
RBL1-F	CTGGACGACTTTACTGCCATC
RBL1-R	TCCAACCGTGGGAATAATGCT
BMI1-F	CCACCTGATGTGTGTGCTTTG
BMI1-R	TTCAGTAGTGGTCTGGTCTTGT
NANOG-F	CAACCAGACCCAGAACATCC
NANOG-R	TTCCAAAGCAGCCTCCAAG
Snail2 (Slug)-F	ATATTCGGACCCACACATTACCT
Snail2 (Slug)-R	GCAAATGCTCTGTTGCAGTGA
HDAC1-F	CTACTACGACGGGGATGTTGG
HDAC1-R	GAGTCATGCGGATTCGGTGAG
MUC1-F	TGCCGCCGAAAGAACTACG
MUC1-R	TGGGGTACTCGCTCATAGGAT
CLDN1-F	CCTCCTGGGAGTGATAGCAAT
CLDN1-R	GGCAACTAAAATAGCCAGACCT
FBXL5-F	AACTCTCCGAGATGCTTAGCC
FBXL5-R	GCAGTGTTGTGCAATCACTTTC
FBXL14-F	TGCGCTCCTGTGACAACATC
FBXL14-R	TGGGCTATGTAAGCCAGACTC
FBXO11-F	GCCGAAAAGAACAGCGTGTC
FBXO11-R	GTTTTGCACGATGACCAAAGTT
BTRC-F	CCAGACTCTGCTTAAACCAAGAA
BTRC-R	GGGCACAATCATACTGGAAGTG
β-actin-F	GGAGGAGCTGGAAGCAGCC
β-actin-R	GCTGTGCTACGTCGCCCTG
CycloA-F	ATGCTGGACCCAACACAAAT
CycloA-R	TCTTTCACTTTGCCAAACACC

Tumorsphere assays

Primary tumorsphere formation was assessed in cells grown under anchorage-independent conditions in either soft agar or methylcellulose. For soft agar assays, cells are suspended in 200 μL 0.3% agar/complete DMEM medium and cultured on top of a bottom layer of 200 μL 1% agar in 48-well dishes. An additional 500 μL of complete medium was then added and cells cultured for 14-21 days prior to fixing and staining colonies with 0.005% crystal violet/20% methanol/PBS and counting colonies consisting of at least 6 cells from 4 fields per well with a 10x objective. Alternatively, sorted HCC38 and HCC1143 cells were cultured in 300 μL of 1% methylcellulose/complete DMEM medium in poly-HEMA coated 48-well dishes and primary tumorspheres assessed by counting colonies consisting of at least 6 cells from an entire well after 14 days. We measured self-renewal by collecting primary tumorspheres by dilution in at least 3 volumes of PBS, dissociating them with trypsin for approximately 10 minutes, and re-seeding in 1% methylcellulose before evaluating secondary colonies after an additional 14 days. The following cell numbers were used per well for the indicated cell types: Soft agar; LM2-4 (2,000), HCC38 (3,000) and HCC1143 (15,000), Methylcellulose; HCC38 (4,500) and HCC1143 (22,500). For the zVAD-fmk and dasatinib treatment studies, the indicated concentrations of drug were added only once, immediately after embedding the cells.

Cell Viability

XTT cell viability assays were performed by first seeding 50,000 LM2-4 cells per well into a 96-well tissue culture plate and allowing them to attach overnight. The indicated concentrations of dasatinib or vehicle alone (DMSO) were then added to the wells in 100 μL phenol-free complete DMEM medium. After 24 h, XTT substrate (Sigma) was added to the wells and incubated for 2 h before reading the A450 nm on a plate reader. Cell viability for each dose of dasatinib was expressed as a percent of the vehicle control wells. Trypan blue staining was performed on cells transfected with PUMA cDNA or empty vector for 48 hours using a ViCell machine as per manufacturer's protocol.

Supplemental References

1. Pierce, J.P., Natarajan, L., Caan, B.J., Parker, B.A., Greenberg, E.R., Flatt, S.W., Rock, C.L., Kealey, S., Al-Delaimy, W.K., Bardwell, W.A., et al. 2007. Influence of a diet very high in vegetables, fruit, and fiber and low in fat on prognosis following treatment for breast cancer: the Women's Healthy Eating and Living (WHEL) randomized trial. *JAMA* 298:289-298.
2. Munoz, R., Man, S., Shaked, Y., Lee, C.R., Wong, J., Francia, G., and Kerbel, R.S. 2006. Highly efficacious nontoxic preclinical treatment for advanced metastatic breast cancer using combination oral UFT-cyclophosphamide metronomic chemotherapy. *Cancer Res* 66:3386-3391.