Supplemental Figures

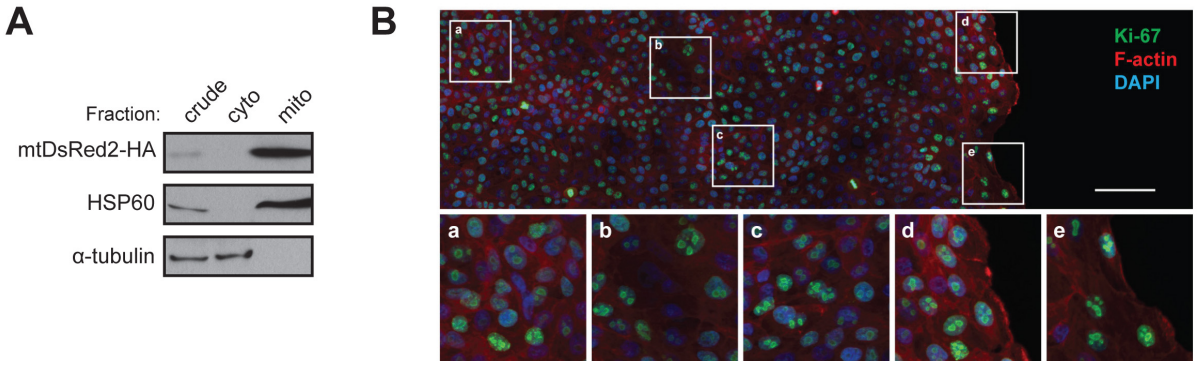

Figure S1, related to Figure 1. (A) Crude, mitochondrial, and cytosolic extracts were probed with HA-tag, HSP60, and α-tubulin antibodies to confirm subcellular localization of mito-DsRed2-HA. (B) Levels of Ki-67 and F-actin (phalloidin) during scratch assay at 18 hours after scratch formation were measured using immunofluorescence. Nuclei were stained with DAPI. Scale bar, 100 μm.

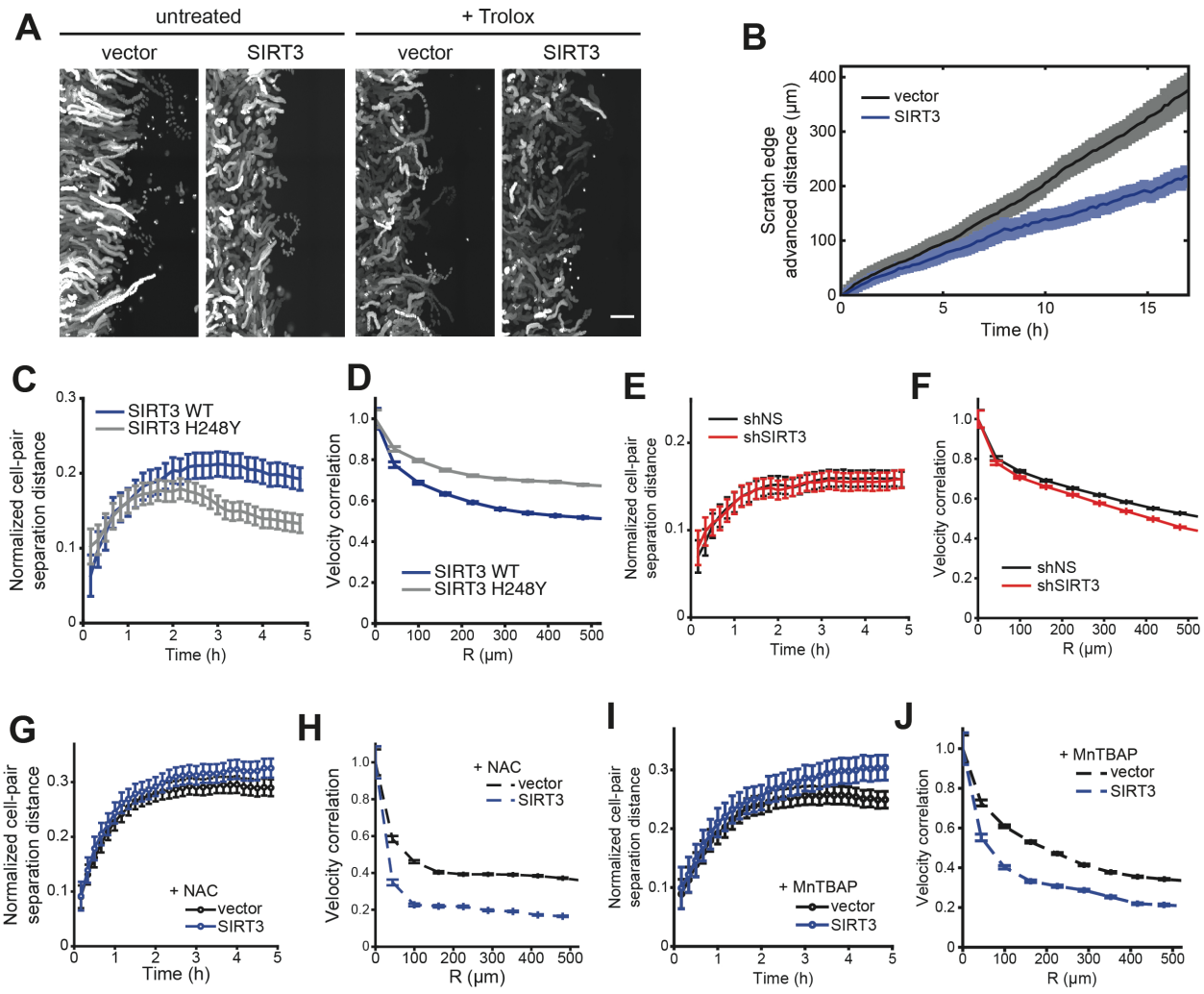

Figure S2, related to Figure 2. (A) Nuclear tracking between 8 and 13 hours after scratch formation in untreated control and SIRT3-overexpressing MCF10A cells and cells treated with 1 mM Trolox. Scale bar, 50 µm. (B) Quantification of scratch edge travel distance in control and SIRT3-overexpressing MCF10A cells from 0 to 17 hours after scratch formation. (C) Normalized cell-pair separation distance from 8 to 13 hours after scratch formation in MCF10A SIRT3 WT and SIRT3 H248Y overexpressing cell. (D) Velocity correlation at 12 hours in MCF10A SIRT3 WT and catalytic mutant SIRT3 H248Y overexpressing cell. (E) Normalized cell-pair separation distance from 8 to 13 hours after scratch formation in non-silencing control and shRNA against SIRT3 MCF10A cells. (F) Velocity correlation at 12 hours in non-silencing control and shRNA against SIRT3 MCF10A cells. (G) Normalized cell-pair separation distance in control and SIRT3-overexpressing cells treated with 5 mM N-acetylcysteine (NAC). (H) Velocity correlation in control and SIRT3-overexpressing cells treated with 5 mM NAC. (I) Normalized cell-pair separation distance from 8 to 13 hours after scratch formation in control and SIRT3-overexpressing MCF10A cells treated with 1 µM MnTBAP. (J) Velocity correlation at 12 hours in control and SIRT3-overexpressing MCF10A cells treated with 1 µM MnTBAP. Data information: For all panels, error bars represent 95%

confidence interval, and non-overlapping bars are statistically significant with $p<0.05$ (n=4-6 wells per group).

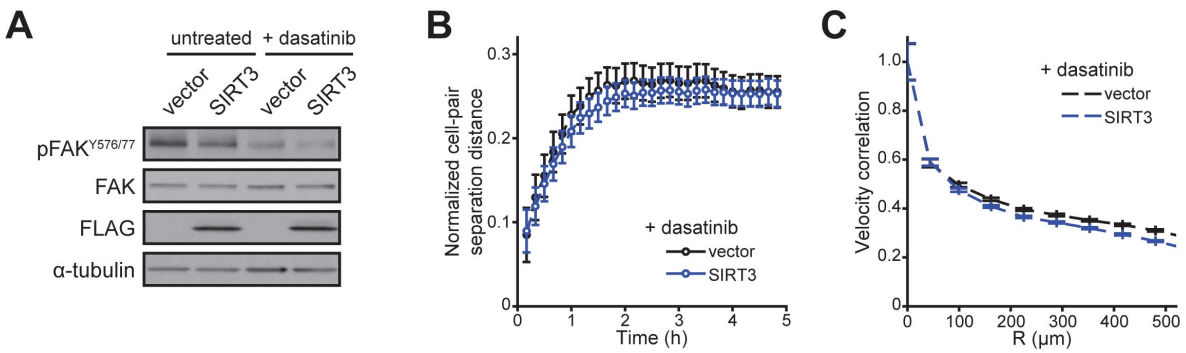

Figure S3, related to Figure 3. (A) Immunoblots of phospho-Tyr576/577 and total FAK in MCF10A cells with SIRT3-FLAG overexpression and treated with dasatinib (2.5 nM). (B) Normalized cell-pair separation distance from 8 to 13 hours in control and SIRT3-overexpressing cells treated with 2.5 nM dasatinib. (C) Velocity correlation at 12 hours in control and SIRT3-overexpressing cells treated with 2.5 nM dasatinib. Error bars represent 95% confidence interval, and non-overlapping bars are statistically significant with $p<0.05$ (n=4-6 wells per group).

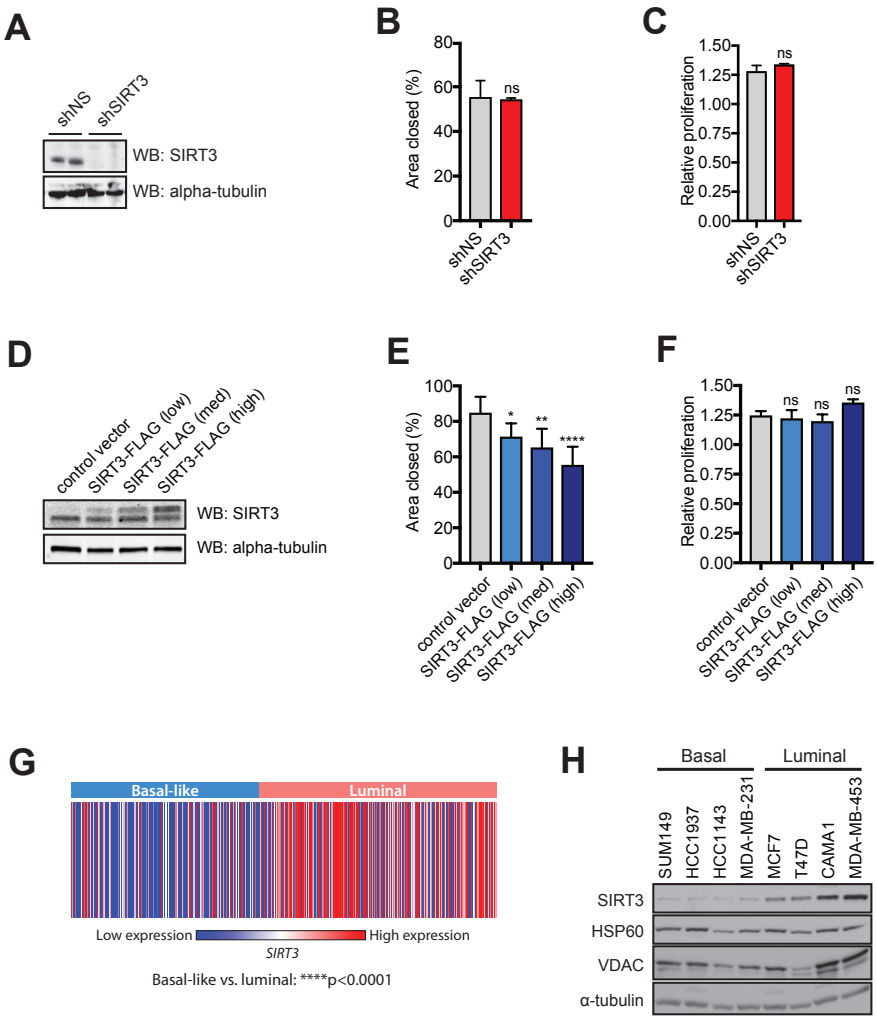

Figure S4, related to Figure 4. (A) Immunoblots of MDA-MB-231 cells with non-targeting shRNA (shNS) and (B) Quantification of migration of MDA-MB-231 with and without SIRT3 knockdown in a scratch assay for 12 hours. (C). Quantification of the proliferation rate of MDA-MB-231 under the same conditions as in B. Cell number was determined at t=0 and t=12 to determine the proliferation rate over the same period as the migration experiment shown in B. (D) Immunoblots of MDA-MB-231 cells expressing empty control vector or increasing levels of SIRT3-FLAG. Lower band shows endogenous SIRT3 whereas the upper band is FLAG-tagged SIRT3. (E) Quantification of migration of the MDA-MB-231 cell lines shown in D in a scratch assay for 10 hours. (F) Quantification of proliferation of the MDA-MB-231 cells shown in D and E over a 10 hour period. Statistical significance was determined using one-way ANOVA corrected for multiple comparisons to the control vector group. *<p=0.05, **<p=0.01, ***<p=0.001, ****<p=0.0001. (G) SIRT3 mRNA levels in basal and luminal breast cancers. Red represents high expression and blue represents low expression. Expression was determined using the Oncomine cancer microarray database (http://www.oncomine.org). (H) SIRT3 protein levels in a panel of basal and luminal breast cancer cells.

A

Dataset	SIRT3 probe	*n*	Log-rank *p* value	Hazard ratio	95% CI
Hatzis	mRNA	127	0.001	2.436	1.433 – 4.068
Zhang	DNA	78	0.0123	1.900	1.150 – 3.140
Chin	DNA	44	0.0188	2.622	1.174 – 5.858
Symmans	mRNA	49	0.026	2.400	1.111 – 5.187
vantVeer	mRNA	24	0.0178	2.577	1.178 – 5.637

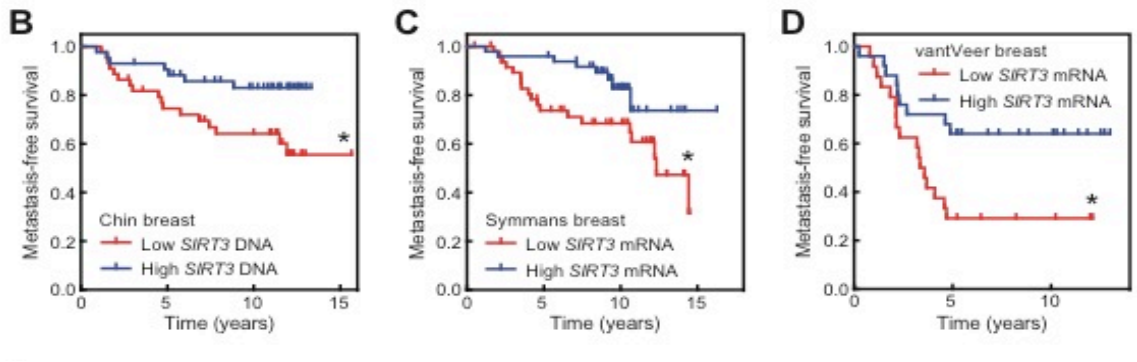

Figure S5, related to Figure 5. (A) Table summarizing the characteristics and metastatic risk outcome in the Oncomine datasets used in this study. *n*, number of samples per group; CI, confidence interval. (B,C and D) Kaplan-Meier curves depicting metastasis-free survival in patients with primary breast cancers of high (top quartile) versus low (bottom quartile) SIRT3 DNA copy number (B) and mRNA expression (C and D) were plotted from Oncomine datasets. Significance was calculated using log-rank test.

Movie S1, related to Figure 1. MCF10A cells were labeled with mito-DsRed2 and used in a scratch assay to visualize mitochondrial dynamics during cell migration. Images were taken every 20 seconds and processed using ImageJ. Scale bar, 10 μm.

Supplemental Experimental Procedures

Cell culture

MCF10A cells were maintained in 1:1 DMEM/F12 media supplemented with 5% horse serum, 20 ng/ml EGF, 0.5 mg/ml hydrocortisone, 100 ng/ml cholera toxin, 10 μg/ml insulin, and penicillin/streptomycin. MDA-MB-231, LM2-4175, and BoM-1833 cells were maintained in DMEM media supplemented with 10% FBS and penicillin/streptomycin. LM2-4175 and BoM-1833 cells were kindly provided by J. Massagué. For SIRT3 overexpression, cells were generated by retroviral infection containing empty pBabe vector or pBabe with human SIRT3 cDNA (FLAG-tagged). To label mitochondria, cells were generated by retroviral infection with pBabe containing mito-DsRed2 cDNA (HA-tagged).

Live cell imaging

Images were acquired every 10 minutes at multiple stage positions with 2 by 2 binning and using the large image setting in NIS-Elements AR software v3 (Nikon), such that the final images at each position were stitched from 4 by 3 or 3 by 3 fields of view. Phase and epifluorescence images capturing H2B-mCherry signals were acquired at every indicated time point. Images of mitochondria during cell migration were collected on a Ti-E inverted motorized microscope with integrated Perfect Focus System (Nikon) equipped with a 60x Plan Apo 1.4 NA DIC optics, CSU-X1 spinning disk confocal head with internal motorized high speed emission filter wheel (Yokogawa) and Borealis modification (Spectral Applied Research) for increased light throughput and illumination homogeneity, custom laser merge module (LMM-7) with AOTF and 100-200 mW solid

state lasers (561 nm for RFP; Spectral Applied Research), dichroic mirrors (405/491/561/642 for RFP; Semrock), ProScan II controller (Prior Scientific), ORCA-AG cooled CCD camera (Hamamatsu Photonics), and a custom built 37°C microscope incubator enclosure with 5% CO_2 delivery. Images were acquired every 20 seconds using MetaMorph software (Molecular Devices). DIC and confocal laser images capturing mito-DsRed2 signals were acquired at every time point.

Western blotting

Cell lysates were collected by scraping cells into radioimmune precipitation assay (RIPA) buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS) supplemented with Complete Mini Protease Inhibitor tablets (Roche) and Phosphatase Inhibitor Cocktails 2 and 3 (Sigma). Protein concentrations were measured using BCA Protein Assay (Pierce) according to manufacturer's instructions. 5-10 μg of protein was loaded into each well of Criterion Tris-HCl gels (Bio-Rad) and transferred onto nitrocellulose membranes. After blocking with 3% BSA, membranes were incubated with primary antibodies overnight. The following antibodies were used: SIRT3, phospho-Tyr397 FAK, phospho-Tyr576/577 FAK, FAK, Src, phospho-Tyr410 p130Cas, p130Cas, HA-tag (all from Cell Signaling), FLAG-tag (Sigma), UQCRFS1 (MitoSciences), HSP60 (abcam), and α-tubulin (Santa Cruz). HRP-linked biotin antibody (Cell Signaling) was used without secondary antibody in Src oxidation assay. HRP-conjugated secondary antibodies (GE Healthcare) and ECL Western blotting substrate (Thermo) were used for chemiluminescence detection.

Crude mitochondrial fractionation

Cells were lysed using ice-cold mitochondrial isolation buffer (225 mM mannitol, 75 mM sucrose, 10 mM MOPS, 1 mM EGTA, and 0.5% BSA supplemented with protease and phosphatase inhibitors, pH adjusted to 7.2) and homogenized using a glass homogenizer. The lysate was centrifuged at 1,000 *g* for 10 min at 4°C and the supernatant, which represented a mixture of mitochondrial and cytosolic fractions ("crude extract"), was centrifuged again at 8,000 *g* for 15 min at 4°C. The mitochondrial pellet, the cytosolic supernatant, and the crude extract from the previous step were mixed with loading buffer for Western blot.

Quantitative real-time PCR

Total RNA was harvested using TRIzol reagent (Invitrogen) according to manufacturer's instructions. RNA purity and concentration were measured using NanoDrop spectrophotometer. cDNA was synthesized from 200 ng of RNA using iScript cDNA synthesis kit (Bio-Rad). Quantitative real-time PCR was performed using SYBR Green I Mastermix on a LightCycler 480 system (Roche). RNA levels for each gene were calculated using ddCt method and normalized using internal reference genes. The following primers were used: SIRT3 F-GGGCTTGAGAGAGTGTCGGGC and R-TCACAACGCCGGTGCAGACC; RPL13A F-CCAAGCGGCTGCCGAAGATGG and R-CTTCCGGCCCAGCAGTACCTGT.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized in 0.1% Triton X-100 for 5 min at room temperature, blocked with 10% normal goat serum for 30 min at 37°C. The cells were then incubated with primary antibodies for 1 h at room temperature and probed with Alexa Fluor 488 or 546 secondary antibodies or Texas Red-X Phalloidin (Life Technologies), as well as DAPI to label nuclei. The following primary antibodies were used: SIRT3, Ki-67 (both from Cell Signaling), and COX1 (MitoSciences). All images were collected with a Ti-E inverted motorized microscope with integregated Perfect Focus System (Nikon) equipped with a 20x Plan Apo 0.75 NA objective lens, a linear-encoded motorized stage (Nikon), halogen trans illuminator with 0.52 NA LWD condenser (Nikon), fast excitation and emission filter wheels (excitation 480/40 nm and emission 525/50 nm for GFP, excitation 560/40 nm and emission 630/75 nm for RFP, excitation 395/25 nm and emission 460/25 nm for DAPI; Chroma Technologies), fast transmitted and epifluorescence light path shutters (SmartShutter; Sutter Instrument), and ORCA-AG cooled CCD camera (Hamamatsu Photonics). Images were acquired using NIS-Elements AR software v3 (Nikon) and analyzed using ImageJ.

Scratch assay

12-well glass-bottom plates (MatTek Corporation) were coated with full DMEM/F12 medium for 24 hours before cells were plated. 350,000 cells were plated per well, and after 24 hours the confluent monolayer was scratched using 1,000 µl pipette tips. Cells were washed and imaged as described below using phenol red-free media. Custom MATLAB software for cell migration analysis was described previously (1). Trolox, N-

acetylcysteine, SU6656, and PF-573228 (all from Sigma) were used at indicated concentrations.

Human tumor lysate array

Qualitative breast cancer tumor lysate array (Protein Biotechnologies) was probed for SIRT3 protein according to manufacturer's instructions. Developed film was scanned and quantified using ImageJ. After probing for SIRT3, the array was stained with Colloidal Gold (Bio-Rad) to measure total protein, which was also scanned and quantified. SIRT3 levels in each spot were normalized using the Colloidal Gold level in the same spot. Each triplicate was averaged, and the ratios of SIRT3 levels between tumor and normal samples were reported as log2 fold change values.

Animal studies

All animal studies were performed in accordance with protocols approved by the Institutional Animal Care and Use Committee, the Standing Committee on Animals at Harvard. For lung metastasis studies, 2 x 10^5 vector- or SIRT3-overexpressing MDA-MB-231 cells in 0.1 ml were injected into the lateral tail vein of 8- to 10-week-old female nude mice (Charles River). Mice were imaged for luciferase activity using Xenogen IVIS system. Mice were anesthetized and 0.15 mg/g of d-luciferin was administered into the peritoneum prior to imaging. Bioluminescence was analyzed using Living Image acquisition and analysis software (Xenogen). Photon flux was calculated from a rectangular region of the thorax of each mouse in prone position. Each value was

scaled to background and normalized to bioluminescence obtained immediately after initial xenograft (day 0).

References

1. Ng, M. R., Besser, A., Danuser, G. & Brugge, J. S. (2012). Substrate stiffness regulates cadherin-dependent collective migration through myosin-II contractility. *The Journal of Cell Biology* 199 (3); 545–563.