Supplementary Appendix

SIRT5 stabilizes mitochondrial glutaminase and supports breast cancer tumorigenesis

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Supporting Materials and Methods

Western blot analysis

Whole cell lysates from cultured cells were prepared with lysis buffer (20 mM HEPES, pH 8.0, 150 mM NaCl, 1% NP40, 1 mM Na_3VO_4, 20 mM NaF, 20 mM β-glycerophosphate, 30 μg/ml leupeptin, 5 μg/ml aprotinin). Cell debris was pelleted by centrifugation (16,000 *g* for 15 minutes) and removed. Tumor lysates were prepared by grinding tumor samples in liquid N_2 using a pestle and mortar, and then adding lysis buffer to the freeze-dried material. Protein concentrations were determined by Bradford assay (Bio-Rad). All lysates were boiled for 5 minutes in reducing SDS sample buffer to denature proteins. Lysate proteins were resolved on Tris-glycine protein gels (Life Technologies) and then transferred to PVDF membranes (PerkinElmer). Membranes were blocked with 5% milk in TBST (20 mM Tris, 135 mM NaCl, and 0.02% Tween 20) for at least one hour and incubated with primary antibodies diluted in TBST overnight at 4°C. Horseradish-peroxidase-conjugated secondary antibodies were used to detect primary antibodies, followed by imaging using Western Lightning Plus-ECL (PerkinElmer) and HyBlot ES autoradiography film (Denville Scientific).

Immunoprecipitation

To immunoprecipitate endogenous GLS, or ubiquitinated proteins, cells were transduced with control shRNA or SIRT5 shRNA virus and then incubated for 48 hours prior to harvesting. Whole cell lysates were prepared as described above, and pre-cleaned by the addition of 15 μl of Protein G agarose slurry (Invitrogen) followed by rotation at 4°C for 30 minutes. Pre-cleaned supernatant containing 2 mg protein was then rotated at 4°C with either anti-GLS antibody (33 μg) or anti-ubiquitin antibody (10 μg) in 1 ml volume for 2 hours. Next, 25 μl of Protein G agarose slurry was added, and samples rotated at 4°C overnight. Beads were then collected by centrifugation at 100 *g* for 30 s at 4°C, washed three times with 1 ml lysis buffer, and samples prepared for western blot analysis by boiling the beads for

5 minutes in SDS sample buffer. To immunoprecipitate ectopically-expressed HA-tagged GLS, cells expressing the relevant constructs were transduced with either control shRNA or SIRT5 shRNA virus and then incubated for 48 hours. HA-tagged GLS was immunoprecipitated using 15 μl Anti-HA Magnetic Beads (Pierce) following the manufacture's protocol, and samples were prepared for western blot analysis by boiling the washed beads for 10 minutes in SDS sample buffer.

Cell proliferation and anchorage-independent growth assays

Cells (1×10^4 or 2×10^4 per well) were seeded in 12-well plates on Day 0, and on Day 1 the cells were transduced with shRNA in triplicate. Cells were then counted on Days 2, 4, and 6. Data were normalized to the control, and the mean and standard deviation were calculated from the triplicates. For anchorage-independent growth assays, cells (8×10^3 per well) were seeded in 6-well plates in the appropriate growth medium (see above) supplemented with 0.3% agarose, on a base layer composed of growth medium supplemented with 0.6% agarose. The soft agar cultures were subsequently supplied with 1 ml of fresh medium every third day. After 14 days the total number of colonies was counted. Each assay was performed a minimum of three times, and the mean and standard deviation calculated.

Quantitative real-time PCR analysis

Total RNA was isolated from cells using the RNeasy mini kit (Qiagen), and a cDNA library prepared by reverse transcription using the SuperScript III first-strand synthesis system (Life Technologies). Quantitative real-time PCR analysis was carried out using cDNA as a template, specific primers and iTaq Universal SYBR Green Supermix (Bio-Rad). Reactions were performed using the 7500 fast real-time PCR system (Applied Biosystems). Primer sets used were (5'→3'):

- GLS-F (tgtcacgatcttgtttctctgtg) and GLS-R (tcatagtccaatggtccaaag)
- SIRT5-F (ttggagaaaacctggatcctg) and SIRT5-R (ctgaatctgtcggtggctg)
- onco-Dbl-F (gactgtgaaggatctgctctg) and onco-Dbl-R (acctttcttgtgccctatcc)
- GAPDH-F (acagtccatgccatcactgcc) and GAPDH-R (gcctgcttcaccaccttcttg)

Lentivirus system for SIRT5 knockdown and ectopic expression GAC

Short hairpin RNA (shRNA)-encoding plasmids for knocking down SIRT3, SIRT4 and SIRT5 were from Sigma (TRCN0000038892 and TRCN0000038893 for human SIRT3, TRCN0000018946 and TRCN0000232895 for human SIRT4, TRCN0000018544 and TRCN0000018545 for human SIRT5, TRCN0000092833 and TRCN0000092834 for mouse SIRT5), TRCN0000045443 and TRCN0000045445 for human CPS1. For ectopic expression, GAC cDNA was subcloned into plasmid pSin-EF2-Oct4-Pur. GAC mutations were generated by site-directed mutagenesis of this construct (pSin-EF2-Oct4-Pur-GAC-K164R, pSin-EF2-Oct4-Pur-GAC-K1158R, pSin-EF2-Oct4-Pur-GAC-K320R). To generate viruses, the desired plasmid construct (6 μg) was mixed with virus package plasmids pCMV.d8.2 (4 μg) and pMDG (2 μg) in 400 μl DMEM medium, followed by the addition of 30 μl of 1 mg/ml PEI (polyethylenimine MW25K, Polysciences Inc.). The mix was incubated at room temperature for 15 minutes and then added to HEK 293T cells at 80% confluence in a 10 cm dish, containing 8 ml of fresh DMEM medium with 10% FBS. After overnight incubation, the mix was removed and 10 ml fresh DMEM medium with 10% FBS was added. Virus-containing medium was collected at 24 hours, and fresh medium added and then collected after another 24 hours. The virus-containing medium was centrifuged at 150 *g* for 5 minutes, aliquoted and stored at -80°C.

Glutamine consumption and ammonium secretion measurements

MDA-MB-231 cells at 50% confluence in 6-well plates, with or without RNAi-resistant SIRT5 expression, were transduced with constructs for expressing a control shRNA or SIRT5-targeted shRNAs (each sample in biological triplicate). After 24 h, cells (now at 85% confluence) were rinsed once with sterile PBS, and then 1 ml of phenol red-free RPMI medium (Sigma-Aldrich) containing 2 mM glutamine was added. As a control, 1 ml of medium was also incubated in wells containing no cells. After 12 h, the medium was collected and centrifuged at 1,000 *g* for 5 minutes to pellet debris, and the supernatant retained for analysis. Meanwhile,

cells attached to wells were lysed and the protein concentration measured as described above.

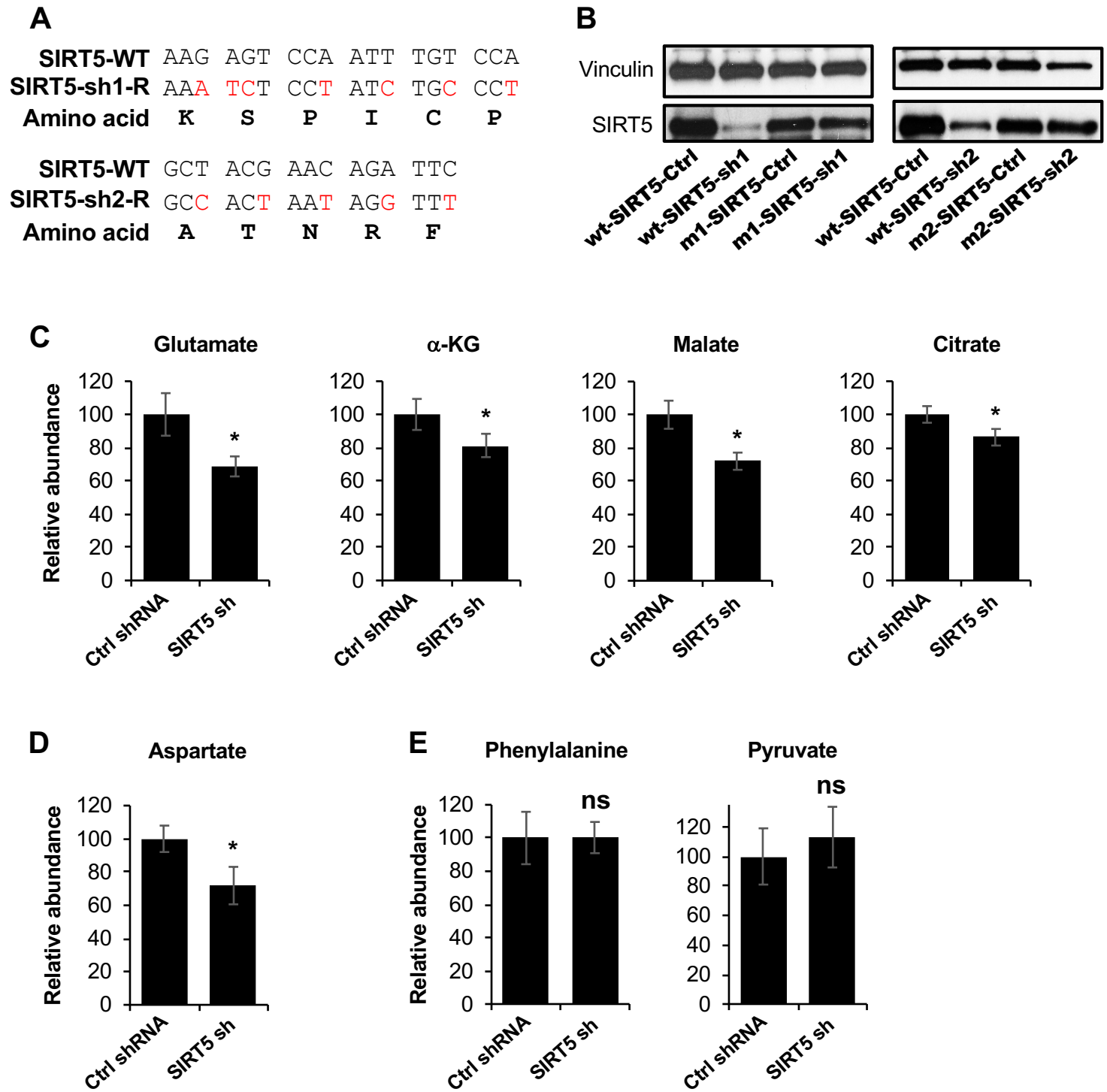
Glutamine and ammonia concentrations were measured using the L-Glutamine/Ammonia Assay Kit (Rapid) (Megazyme) following the manufacturer's instructions, with glutamine and ammonia assays run in parallel on each sample. Briefly, to measure glutamine, 50 μl sample was mixed with 100 μl Assay Buffer 1 and 10 μl Glutaminase Suspension and incubated at room temperature for 5 min, and for ammonia measurements 50 μl of sample only was incubated for 5 min. For all reactions, a blank containing 50 μl H_2O was run in parallel. Then, to both samples, 150 μl Assay Buffer 2, 100 μl NADPH Solution, and H_2O to bring the final volume to 1160 μl was added, followed by incubation at room temperature for 4 min. Absorbance A_1 was then measured at 340 nm. Next, 10 μl Glutamate Dehydrogenase Suspension was added, samples were mixed and incubated at room temperature for 5 min, and absorbance A_2 was measured at 340 nm. Concentrations of glutamine and ammonia were calculated using the extinction coefficient of NADPH at 340 nm.

Metabolite extraction and measurements

MDA-MB-231 cells expressing either a control shRNA or SIRT5-targeted shRNA were harvested 48 hours after transduction, at 80% confluence in 6-well plates (each sample in biological triplicate). Culture medium was aspirated and cells were washed with cold PBS on ice. Then, 1 ml of extraction solvent (80% methanol/water) cooled to -80°C was added to each well, and dishes were transferred to -80°C for 15 min. Cells were then scraped into the extraction solvent on dry ice. All metabolite extracts were centrifuged at 20,000 × *g* at 4°C for 10 min. Liquid chromatography and measurements of metabolite abundances were carried out at the Cornell University Institute of Biotechnology Metabolomics Facility. Solvent in the samples, prepared as described above, was evaporated in a Speed

Vacuum. The cell extracts were then dissolved in 150 μl 60% acetonitrile. As internal standards, sulfadimethoxine and ^{13}C-pyruvic acid were added to all samples at 5 ppm. A hydrophilic interaction liquid chromatography method (HILIC) with a SeQuant ZIC pHILIC column (150 × 2.1 mm, 5 μm) was employed on a Thermo Vanquish UHPLC system for compound separation and detection at room temperature. The mobile phase A was 10 mM ammonium acetate in water, pH 9.8, and the mobile phase B was acetonitrile. The linear gradient was as follows: 0 min, 90% B; 1 min, 90% B, 15 min, 30% B; 18 min, 30% B, 19 min, 90% B, 29 min, 90% B. The flow rate was 0.25 ml/min. The Q Exactive MS (Thermo Scientific) is equipped with a heated electrospray ionization probe (HESI), and the relevant parameters are as listed: ESI voltage, 3.5 kV (negative mode); sheath gas, 50; auxiliary gas, 10; sweep gas, 1. Capillary temperature was set at 275°C, and S-lens was 55. Raw data collected from LC-Q Exactive MS were processed on Compound Discover 2.1, with an input file of theoretical m/z from the databases Chemspider, bioCyc, HMDB, Food metabolome, Phenol Explorer, Mzcloud and the Facility MassList.

Figure S1


A
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SIRT5-sh1-R AAA TCT CCT ATC TGC CCT
Amino acid K S P I C P
SIRT5-WT GCT ACG AAC AGA TTC
SIRT5-sh2-R GCC ACT AAT AGG TTT
Amino acid A T N R F
B
Vinculin
SIRT5
wt-SIRT5-Ctrl
wt-SIRT5-sh1
m1-SIRT5-Ctrl
m1-SIRT5-sh1
wt-SIRT5-Ctrl
wt-SIRT5-sh2
m2-SIRT5-Ctrl
m2-SIRT5-sh2
C
Glutamate
α-KG
Malate
Citrate
Relative abundance
120
100
80
60
40
20
0
*
Ctrl shRNA
SIRT5 sh
D
Aspartate
E
Phenylalanine
Pyruvate
ns

Figure S1. RNAi-resistant SIRT5 constructs and metabolite levels following SIRT5 knockdown. ***(A)*** Partial DNA sequences for SIRT5 showing the mutations made to generate constructs resistant to each of the SIRT5-targeted shRNAs. ***(B)*** Western blots validating that the two mutant SIRT5 DNA constructs (m1-SIRT5 and m2-SIRT5) express SIRT5 protein at levels similar to that of wildtype (wt) SIRT5 from the parental construct, and that they resist the two SIRT5-targeted shRNAs (sh1 and sh2, respectively). ***(C)*** Relative abundances of glutamate and the TCA cycle metabolites α-ketoglutarate (α-KG), malate, and citrate in MDA-MB-231 cells expressing either a control shRNA or SIRT5-targeted shRNA. Mean $\pm$ SD of biological triplicates. ***(D)*** Relative abundance of aspartate in MDA-MB-231 cells expressing a control shRNA or SIRT5-targeted shRNA. Mean $\pm$ SD of biological triplicates. ***(E)*** Relative abundances of phenylalanine and pyruvate in MDA-MB-231 cells expressing a control shRNA or SIRT5-targeted shRNA. Mean $\pm$ SD of biological triplicates.

*$P < 0.05$, ns = not significant.

Figure S2

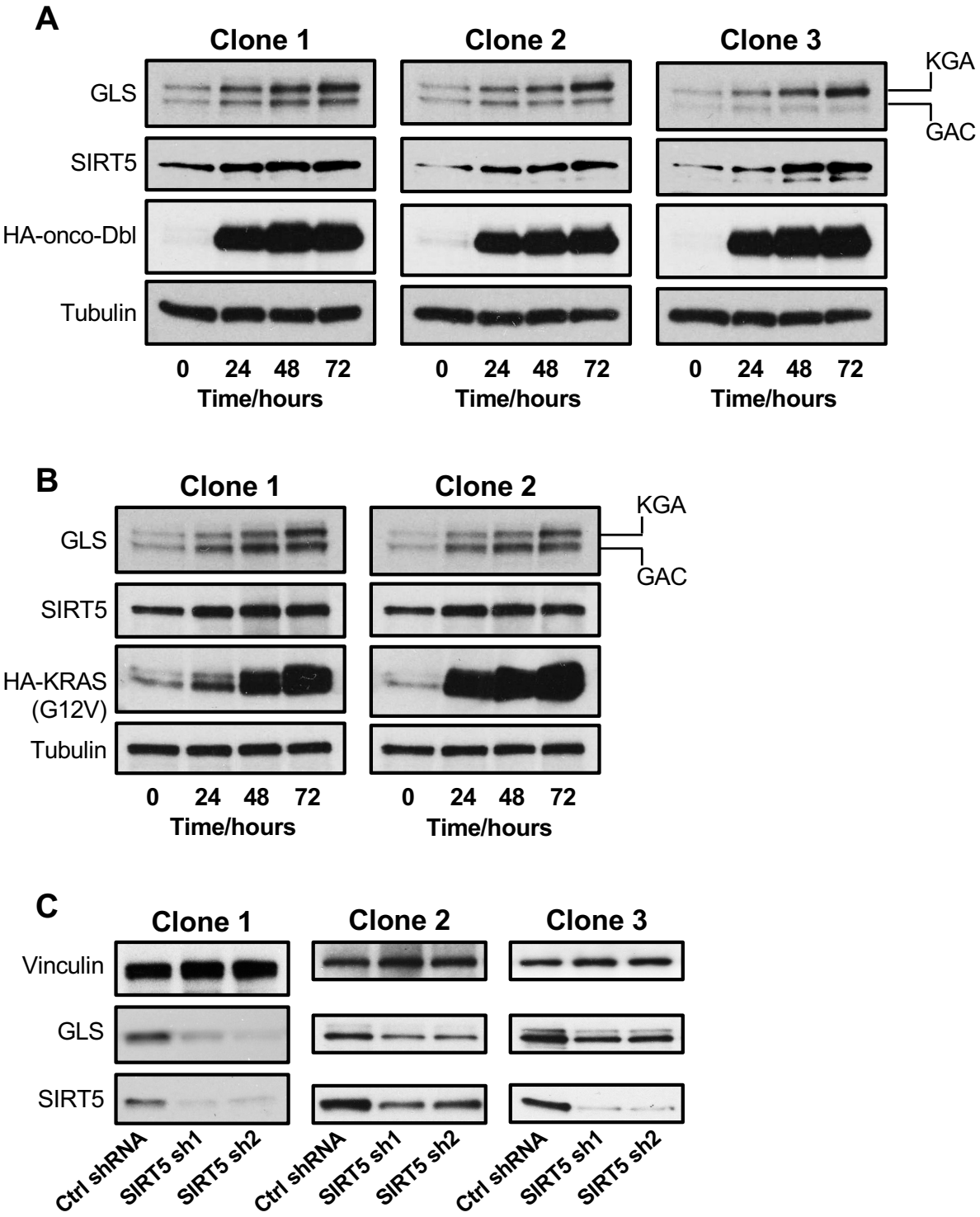

A
Clone 1
Clone 2
Clone 3
GLS
SIRT5
HA-onco-Dbl
Tubulin
KGA
GAC
0 24 48 72
Time/hours
B
Clone 1
Clone 2
GLS
SIRT5
HA-KRAS (G12V)
Tubulin
KGA
GAC
0 24 48 72
Time/hours
C
Clone 1
Clone 2
Clone 3
Vinculin
GLS
SIRT5
Ctrl shRNA
SIRT5 sh1
SIRT5 sh2

Figure S2. Independent replicate clones of the inducible MEF systems. ***(A)*** Western blot analysis of three independent replicate clones of inducible onco-Dbl MEFs, showing levels of HA-onco-Dbl, SIRT5, and GLS at different time-points after induction. Cells were cultured in low-serum (0.5% FBS) medium for the time-course. ***(B)*** Western blot analysis of replicate clones of inducible G12V-KRAS MEFs, showing levels of HA-G12V-KRAS, SIRT5, and GLS at different time-points after induction. Cells were cultured in low-serum (0.5% FBS) medium for the time-course. ***(C)*** Western blots for independent replicate clones of inducible onco-Dbl MEFs, showing that knockdown of SIRT5 results in a depletion of GLS protein. Knockdowns were carried out in high-serum (10% FBS) medium, which favors expression of the GAC splice variant of GLS.

Figure S3

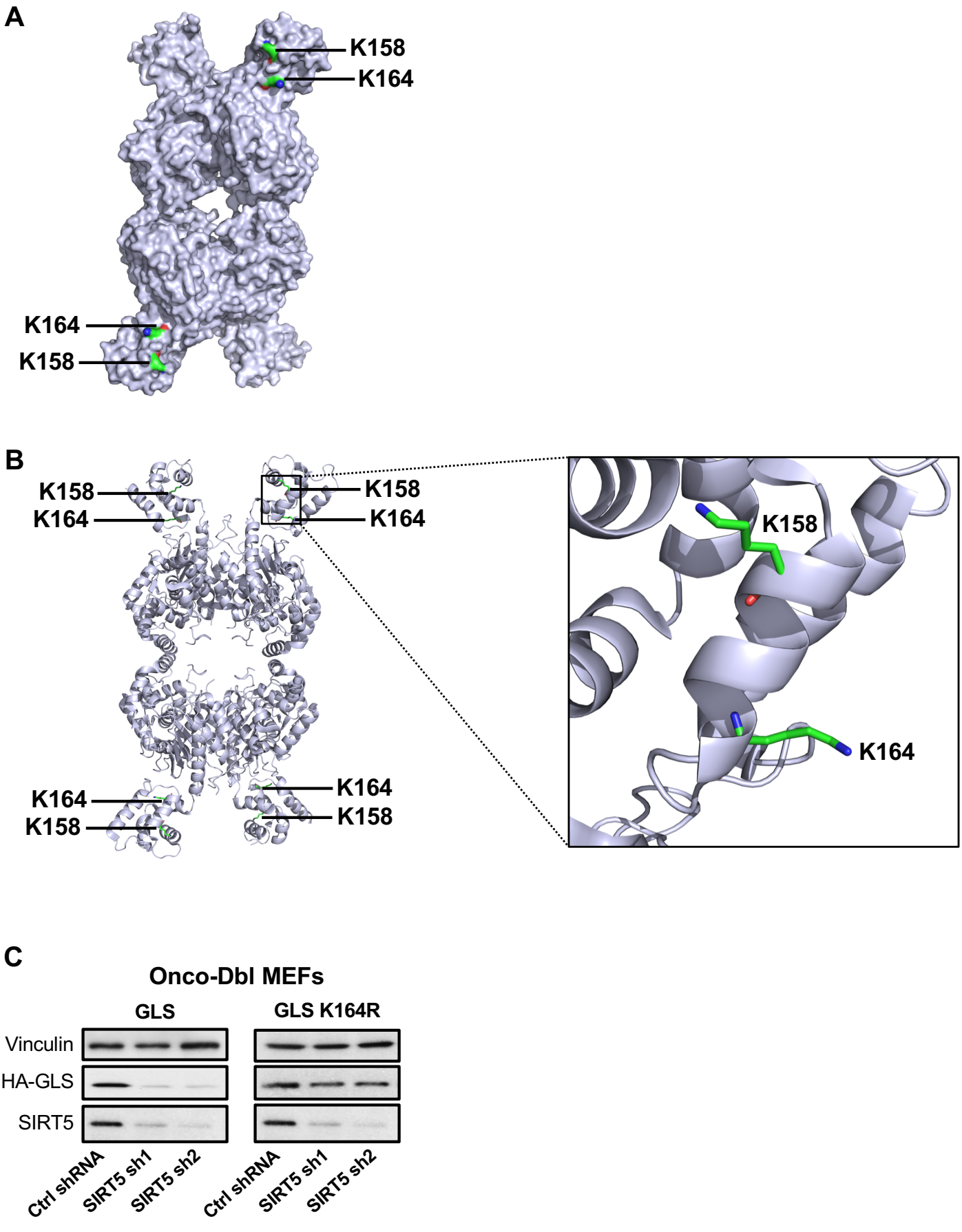

A
K158
K164
K164
K158
B
K158
K164
K158
K164
K164
K158
K164
K158
K164
C
Onco-Dbl MEFs
GLS
GLS K164R
Vinculin
HA-GLS
SIRT5
Ctrl shRNA
SIRT5 sh1
SIRT5 sh2
Ctrl shRNA
SIRT5 sh1
SIRT5 sh2

Figure S3. The location of residues K158 and K164 on GLS. ***(A)*** Surface representation of the human GLS homo-tetramer (1), showing the surface exposure of residues K158 and K164. For two of the monomers these residues are not visible from this perspective but are exposed on the reverse face. ***(B)*** Cartoon representation of the same structure, with K158 and K164 visible on all four monomers. A close-up of the position of these residues is shown for the upper-right monomer. ***(C)*** Western blot analysis of lysates prepared from induced onco-Dbl MEFs ectopically expressing HA-tagged wildtype-GLS or K164R-GLS, along with a control shRNA or SIRT5-targeted shRNA. As is the case in human breast cancer cells, the K164R-GLS variant is protected from degradation following SIRT5 knockdown.

Figure S4

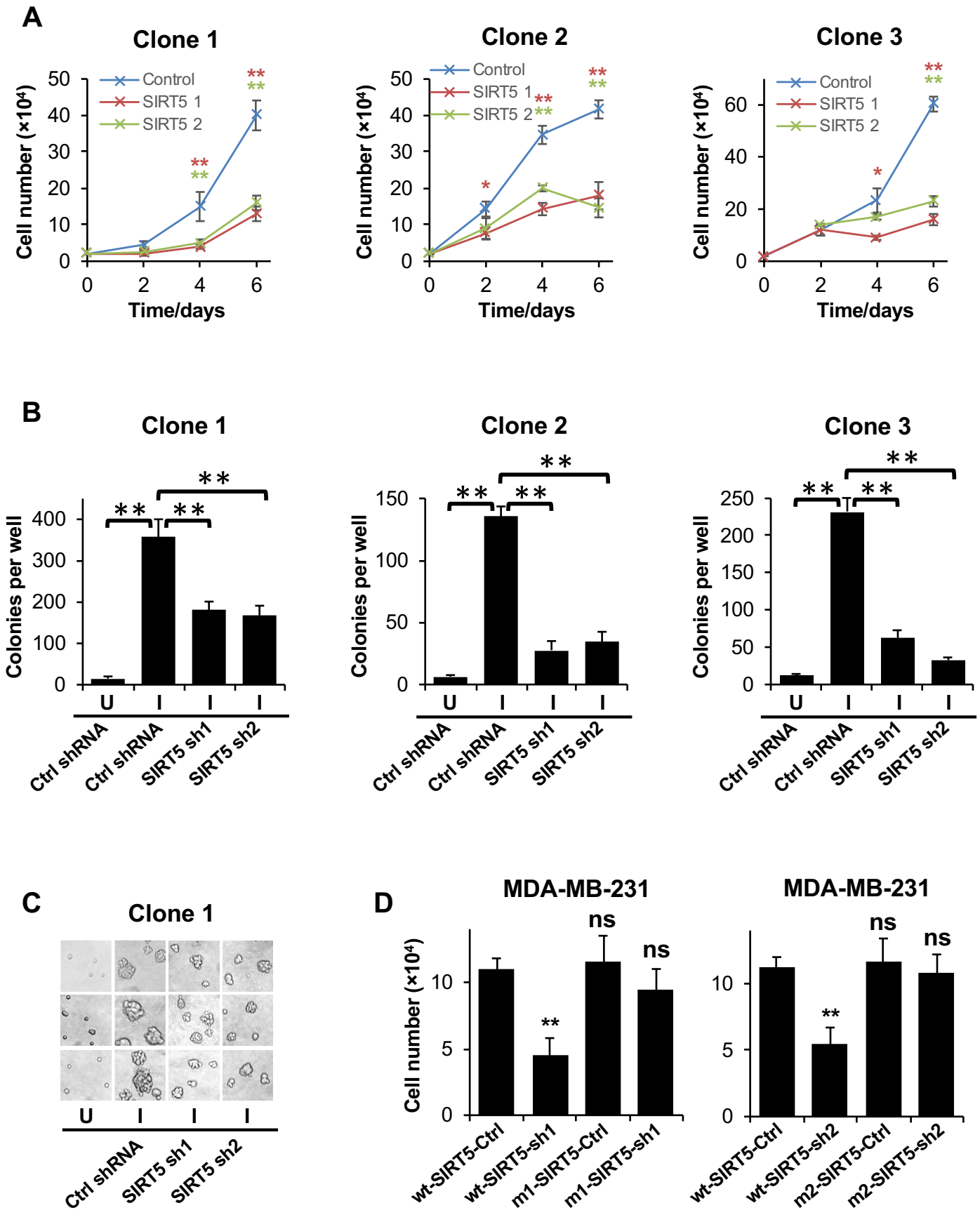
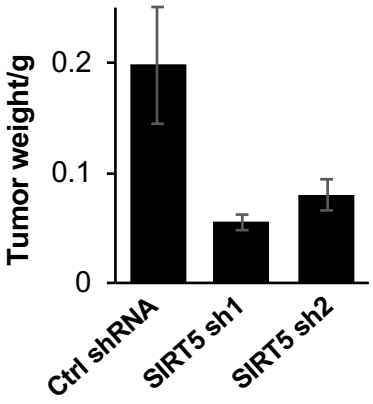

A
Clone 1
Clone 2
Clone 3
Control
SIRT5 1
SIRT5 2
Cell number (×10⁴)
Time/days
B
Clone 1
Clone 2
Clone 3
Colonies per well
U
I
Ctrl shRNA
SIRT5 sh1
SIRT5 sh2
C
Clone 1
D
MDA-MB-231
MDA-MB-231
Cell number (×10⁴)
ns
wt-SIRT5-Ctrl
wt-SIRT5-sh1
m1-SIRT5-Ctrl
m1-SIRT5-sh1
wt-SIRT5-Ctrl
wt-SIRT5-sh2
m2-SIRT5-Ctrl
m2-SIRT5-sh2

Figure S4. SIRT5 supports the proliferation and anchorage-independent growth of transformed MEFs and cancer cells. ***(A)*** Cell proliferation assays for three independent replicate clones of induced onco-Dbl MEFs, expressing either a control shRNA or SIRT5-targeted shRNAs. Mean ± SD of triplicate assays. ***(B)*** Plots showing the number of colonies formed per well in anchorage-independent growth assays of replicate clones of inducible onco-Dbl MEFs, uninduced (U) or induced (I) and expressing either a control shRNA or SIRT5-targeted shRNAs. Mean ± SD of triplicate assays. ***(C)*** Representative image showing relative colony sizes formed by uninduced (U) or induced (I) onco-Dbl MEFs expressing either a control shRNA or SIRT5-targeted shRNAs. ***(D)*** Cell proliferation assays for MDA-MB-231 cells ectopically expressing either wildtype (wt) SIRT5 or RNAi-resistant SIRT5, along with a control shRNA or SIRT5-targeted shRNAs (sh1 and sh2). Mean ± SD of triplicate assays.

$^{**}P < 0.01$, $^{*}P < 0.05$, ns = not significant.

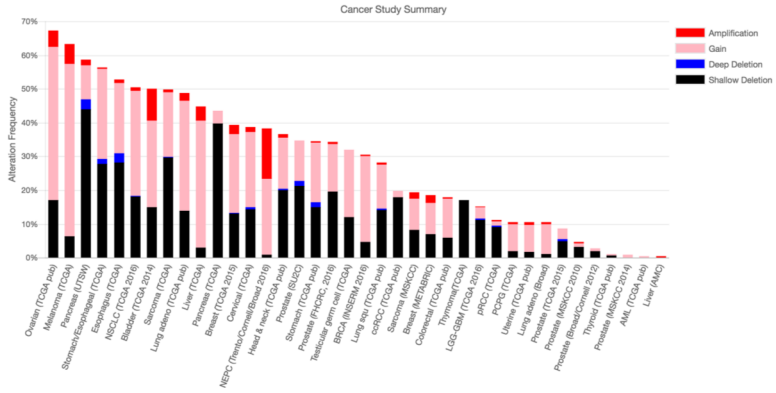
Figure S5

A



B

SIRT5 **copy-number alterations in human tumors**



C

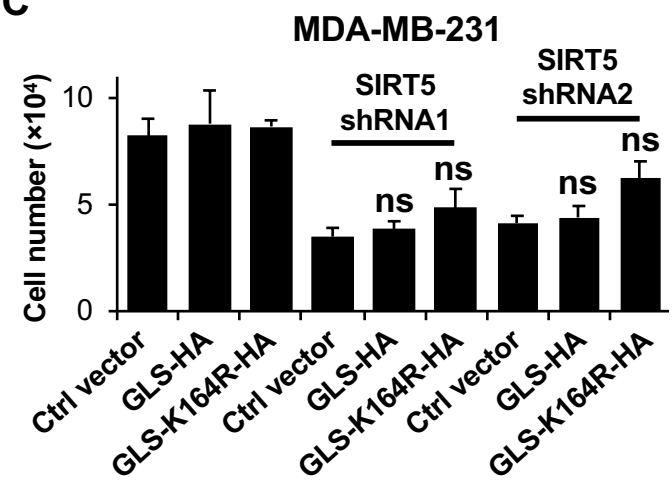


Figure S5. Xenograft tumor weights and *SIRT5* copy-number changes in human tumors. ***(A)*** The weight of xenograft tumors formed by MDA-MB-231 cells expressing either a control shRNA or SIRT5-targeted shRNA and harvested from NSG mice 6 weeks after inoculation. ***(B)*** Data were accessed using cBioPortal (2, 3). Only non-redundant datasets containing $\geq$100 samples were included in the analysis. ***(C)*** Proliferation assays for MDA-MB-231 cells expressing HA-tagged wildtype-GLS or K164R-GLS, along with either a control shRNA or SIRT5-targeted shRNAs. Cells were seeded in 12-well plates (2×10^4 cells/well) and counted after 48 hours. Expression of the degradation-deficient K164R-GLS variant only partially rescues the inhibitory effects of SIRT5 knockdown. Mean $\pm$ SD of triplicate assays.

Figure S6

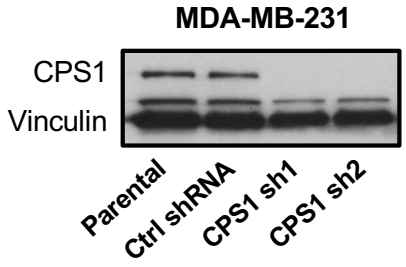


Figure S6. CPS1 expression in MDA-MB-231 cells. Western blots showing that CPS1 is present in MDA-MB-231 cells. To confirm that the band from the anti-CPS1 antibody is on-target, two independent CPS1-targeted shRNAs were used to deplete CPS1 expression.

Supplementary References

1. DeLaBarre B, et al. (2011) Full-length human glutaminase in complex with allosteric inhibitor. *Biochemistry* 50:10764-10770.

2. Gao J, et al. (2013) Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal* 6:pl1.

3. Cerami E, et al. (2012) The cBio cancer genomics portal: an open platform for exploring multidimensional cancer genomics data. *Cancer Discov* 2:401-404.