Constitutively Active Androgen Receptor Supports the Metastatic Phenotype of Endocrine-Resistant Hormone Receptor-Positive Breast Cancer

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SUPPLEMENTAL MATERIALS and METHODS

In vitro SUMOylation Assays

Recombinant SAE1/SAE2 (SUMOLink, Active Motif), purified GST-tagged proteins (GST-SUMO-2 and GST-Ubc9), and recombinant Hsp27 (Abcam) were all used in the *in vitro* SUMOylation assays. Standard GST protein purification was performed with Glutathione Spin Columns containing GST-Bind resin (ThermoFisher Scientific). *In vitro* SUMOylation assay was performed as described previously (1). Briefly, AR protein isolated from MCF-7 cells was incubated with SAE1/SAE2, Ubc9, and SUMO2 in presence or absence of Hsp27 in a SUMOylation buffer (50 mM Tris, 10 mM $MgCl_2$, 1mM DTT, 5 mM ATP) for 3 hrs at 30 ^{o}C. SDS loading buffer was added to terminate the SUMOylation reaction and samples were resolved by SDS/PAGE and immunoblotted with specific antibodies.

Proximity Ligation Assay (PLA)

The DuoLink assay kit (Sigma-Aldrich) was used according to manufacturer's instructions. Briefly, MCF-7 cells were grown on coverslips in 6-well plates then transfected. After 48 hrs from transfection, cells were fixed, permeabilized then blocked. Samples were incubated with primary antibodies AR (sc-816, Santa Cruz Biotechnology) and SUMO2/3 (8A2, Kerafast) and subsequently incubated with secondary antibodies linked to PLA probes. Ligation and amplification were followed by DAPI mounting to visualize the nuclei and images were captured by Nikon Eclipse Ti2. The total number of puncta, representing AR/SUMO3 complexes, per nuclei were quantified using Image J (NIH).

Luciferase Reporter Assays

To generate the SUMOylated AR mimetic, AR insert was amplified with the following forward (5'-ATGGAAGTGCAGTTAGGGCTG-3' and 5'-TTTCCCCGGCTTAAGCAGCT-3') and reverse (5'-TCACTGGGTGTGGAAATAGATGGG- 3') primers. Next, the pcDNA3/HA-SUMO3 plasmid, described in (2), was linearized and AR was subsequently cloned into the vector using the In-Fusion HD Cloning Kit (Clontech). To confirm that the AR gene has been inserted correctly, sequencing and restriction digests, with BamHI and XbaI, were conducted. Four different AR-regulated luciferase reporter constructs were used. These include our ARE_3-probasin-, GRE_2E1b-, PSA- and pGL4.26-KLF5-enhancer-luciferase plasmids. Details of these luciferase plasmids have been described in previous publications (3-6). MCF-7 cells were seeded into 24-well plates using phenol red-free media supplemented with 2% charcoal-stripped fetal bovine serum (CSS). After 48 hrs, cells were co-transfected with firefly and renilla luciferase along with ARwt or SUMO3-fused-AR. Equal amounts of transfected plasmids were ensured by the addition of empty vector (EV). On the next day, cells were either unstimulated or treated with 10 nM R1881 for 24 h in the same media described before. Lysates were generated, the dual-luciferase reporter assay system (Promega) was used to measure firefly and renilla activities according to manufacturer's instructions and luminescence levels were read on a microplate reader (VICTOR X4, PerkinElmer). Three independent experiments were conducted, and each experiment was performed in triplicates.

Real-time PCR

Total RNA of biological triplicates was extracted from cells with PureLink RNA Mini Kit (ThermoFisher Scientific) and converted to cDNA with iScript cDNA Synthesis Kit (BioRad)

according to manufacturer's instructions. Expression of target genes with specific primers (Additional file 1: Table S1; IDT) was measured by RT-qPCR using the iTaq Universal SYBR Green Supermix (BioRad) and 7500 Fast Real-Time PCR System (Applied Biosystems). Data was normalized to the reference gene and analyzed by the ΔΔCT method.

Scratch wound healing assays

TamR-7 cells were seeded into 12-well plates at a seeding density of 70,000 cells/well. On the next day, cells became confluent and a scratch was made using a sterile 200 µl pipette tip. After washing with PBS, cells were further cultured in media containing vehicle, 10 µM Enz, 10 µM GA or both. Images were acquired and the gap width of scratch was measured at 0, 24, 48 and 70 hrs, and compared with the initial gap size at 0 hr. The % scratch gap closure was calculated and graphed.

Mammosphere Studies

Single cell suspensions of TamR cells were generated and seeded into ultra-low attachment plates (Corning) at a density of 30,000 cells/ml. Cells were grown in non-adherent conditions for seven days using MammoCult media (Stemcell) supplemented with Heparin (Sigma-Aldrich), hydrocortisone (Sigma-Aldrich), mammoCult proliferation supplement (Stemcell), methylcellulose (Sigma-Aldrich) and penicillin/streptomycin (ThermoFisher Scientific). The media contained treatments of AR antagonists, ginkgolic acid or a combination of both. One milliliter of fresh media was added to each well every two days without removing the old media. To test for self-renewal properties, first generation mammospheres were passaged and

dissociated into single cells then 30,000 cells/ml were reseeded. Images were captured, mammsopheres were counted and their diameters were measured using Image J (NIH).

Table S1: List of primer sequences used for the detection of transcripts.

Gene	Primer Sequence	Reference
SUMO-1	**Forward:** 5'-AGCAGTGAGATTCACTTCAAAGTG-3' **Reverse:** 5'-TCTGACCCTCAAAGAGAAACC-3'	
SUMO-2	**Forward:** 5'-GGATTGTCAATGAGGCAGATCAG-3' **Reverse:** 5'-CCGTCTGCTGTTGGAACACATC-3'	
SUMO-3	**Forward**: 5'-GGCTTGTCAATGAGGCAGATCAG-3' **Reverse:** 5'-CTGCTGGAACACGTCGATGGTG-3'	
SAE1	**Forward:** 5'-AGGACTGACCATGCTGGATCAC-3' **Reverse:** 5'-CTCAGTGTCCACCTTCACATCC-3'	
UBA2 (SAE2)	**Forward:** 5'-AGAGGTGACTGTGCGGCTGAAT-3' **Reverse:** 5'-GGACATCTGGTGCTACCATAGC-3'	
UBE2I (UBC9)	**Forward:** 5'-ATCCAAGACCCAGCTCAAGCAG-3' **Reverse:** 5'-TTGACGATGCCACAAGGTCGCT-3'	
PIAS1	**Forward:** 5'-TAAGGAGGATGGCACTTGGGCA-3' **Reverse:** 5'-TGAGACGCTACCTGATGCTCCA-3'	
HSPB1 (HSP27)	**Forward:** 5'-TCCCTGGATGTCAACAACTTC-3' **Reverse:** 5'- TCTCCACCACGCCATCCT-3'	(7)
SENP1	**Forward:** 5'-ATCAGGCAGTGAAACGTTGGAC-3' **Reverse:** 5'-GCAGGCTTCATTGTTTATCCCA-3'	(8)

β-actin	**Forward:** 5'-TGTACGCCAACACAGTGCTG-3' **Reverse:** 5'-GCTGGAAGGTGGACAGCGA-3'	
Vimentin (VIM)	**Forward:** 5'-TACAGGAAGCTGCTGGAAGGCG-3' **Reverse:** 5'- TGGCAGAGGCAGAGAAATCCTGC-3'	(9)
Fibronectin (FN1)	**Forward:** 5'-CCGCCGAATGTAGGACAAGA-3' **Reverse:** 5'-TGCCAACAGGATGACATGAAA-3'	(9)
ZEB1	**Forward:** 5'-GCACCTGAAGAGGACCAGAG-3' **Reverse:** 5'-TGCATCTGGTGTTCCATTTT-3'	(10)
ZEB2	**Forward:** 5'-TTTCAGGGAGAATTGCTTGA-3' **Reverse:** 5'-CACATGCATACATGCCACTC-3'	(11)
MMP9	**Forward:** 5'-CCGAGCTGACTCGACGGTGATGG-3' **Reverse:** 5'-GAGGTGCCGGATGCCATTCACGTC-3'	(12)
MMP2	**Forward:** 5'-CCCCAAAACGGACAAAGAG-3' **Reverse:** 5'-CACGAGCAAAGGCATCATCC-3'	(13)
E-cadherin (CDH1)	**Forward:** 5'- GAGAGGAATCCAAAGCCTCAGGT-3' **Reverse:** 5'- CTGGTTATCCATGAGCTTGAGAT-3'	(9)
Claudin 8 (CLD8)	**Forward:** 5'-CGTGAGGCAGGCTAACATCA-3' **Reverse:** 5'-AGCAGCACACATCAGTCCTC-3'	(14)
18 S	**Forward:** 5'-AGAAACGGCTACCACATCCA-3' **Reverse:** 5'-CACCAGACTTGCCCTCCA-3'	

SUPPLEMENTAL FIGURES and FIGURE LEGENDS

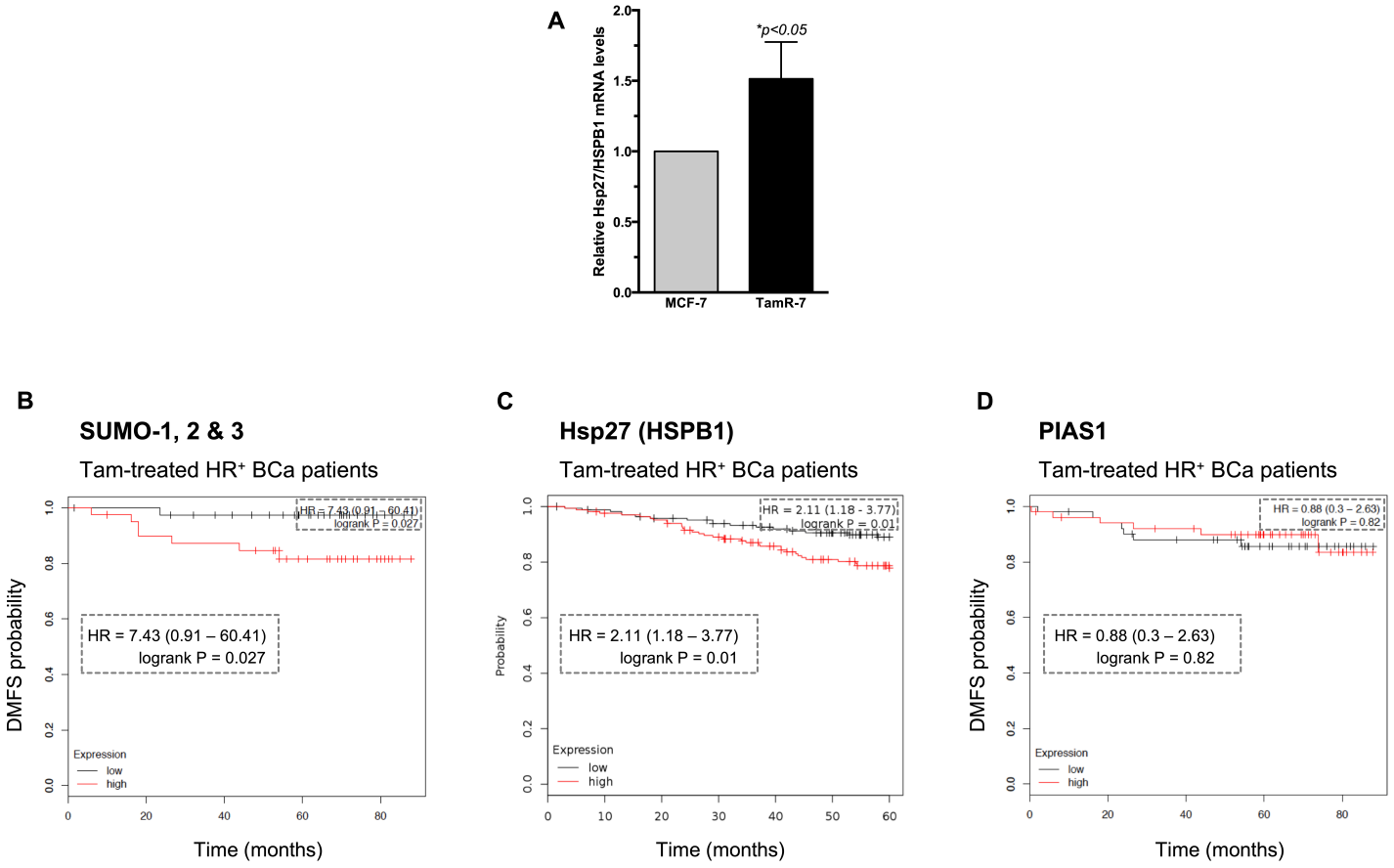

Figure S1. Elevated levels of SUMO isoforms and HSP27 correlate with high probability of metastasis in ET-treated HR+ BCa patients. (A) Transcript levels of Hsp27/HSPB1 are significantly higher in TamR-7 BCa cells. The graph represents fold-change in $2^{\Delta\Delta Ct}$ values of 3 independent experiments and statistical significance in raw ΔCt values using Student's t-test. **(B-D)** Kaplan–Meier distant metastasis free survival curves were generated from the KM Plotter database for Tam-treated HR+-BCa patients and separate high and low gene expressions for: **(B)** all SUMO isoforms; **(C-D)** Hsp27/HSPB1 and PIAS1 (E3 ligases). The hazard ratio at 95% confidence and the logrank P values are enlarged within the plots.

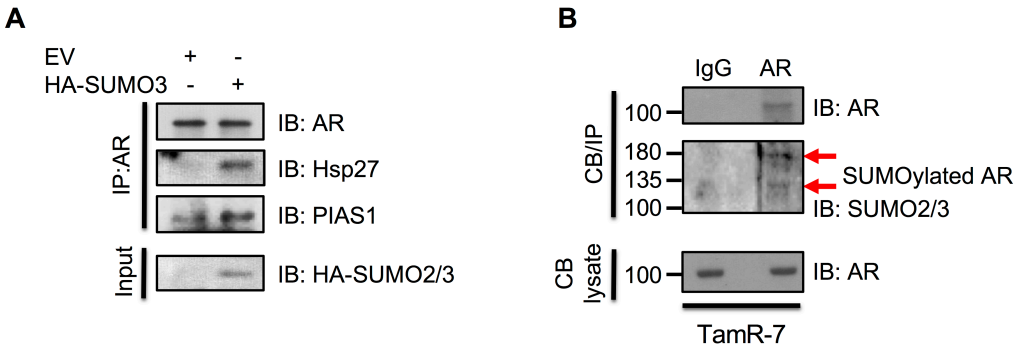

Figure S2. HyperSUMO conditions promotes AR SUMOylation and enhances its interaction with Hsp27. Endogenous AR was immunoprecipitated from: **(A)** MCF-7 cells that were transiently transfected with EV or HA-SUMO3 and **(B)** from chromatin-bound (CB) fractions of TamR-7 cells. Proteins were subsequently resolved by SDS-PAGE and immunoblotted with specific antibodies. Arrows in (B) indicate SUMO-2/3 conjugates of AR.

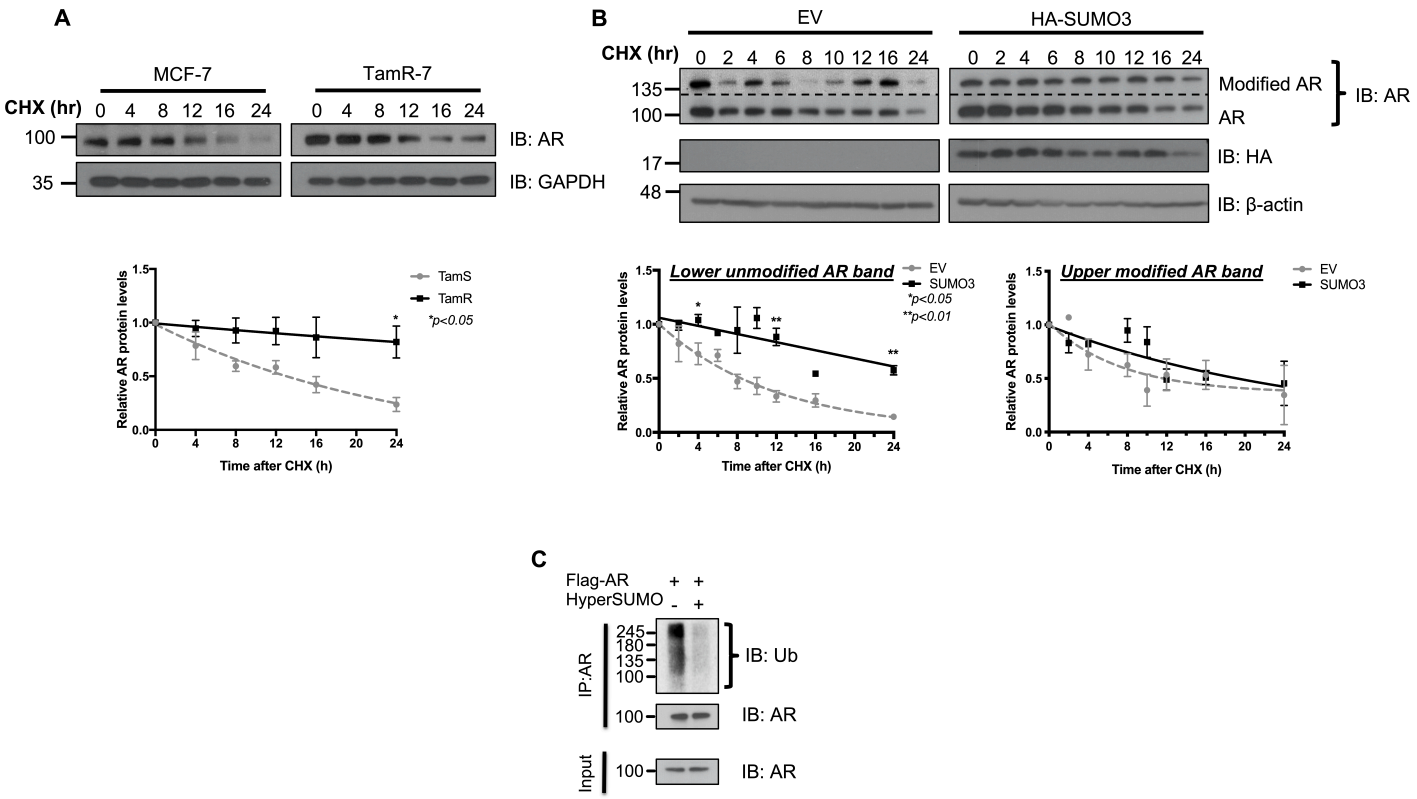

Figure S3. SUMO stabilizes AR and reduces its proteasomal degradation. Cycloheximide (CHX) experiments show that AR's rate of degradation is slower within the hyperSUMO environment of TamR-7 versus parental MCF-7 cells **(A)** and that endogenous AR is more stable in MCF-7 cells that overexpress SUMO-3 **(B)**. Cells were untransfected in (A) or transfected with either empty vector (EV) or HA-SUMO3 (B). On the next day, cells were treated with 10 μM CHX for the indicated time periods and proteins were detected by immunoblotting using anti-AR, anti-HA and anti-GAPDH antibodies. Band intensities were quantified by densitometry using ImageJ. Graphs in panels (A) and (B) illustrate the remaining amounts of AR normalized to the loading control from at least three independent experiments. Statistical significance of *p<0.05 and **p<0.01 were obtained by Student's t-test. **(C)** Mimicking the TamR-7 conditions, by overexpressing AR and SUMO-3 reduces total ubiquitylation of AR in MCF-7 cells. AR pull-down was analyzed by western blot analysis using specific antibodies; the bracket indicates ubiquitylated AR.

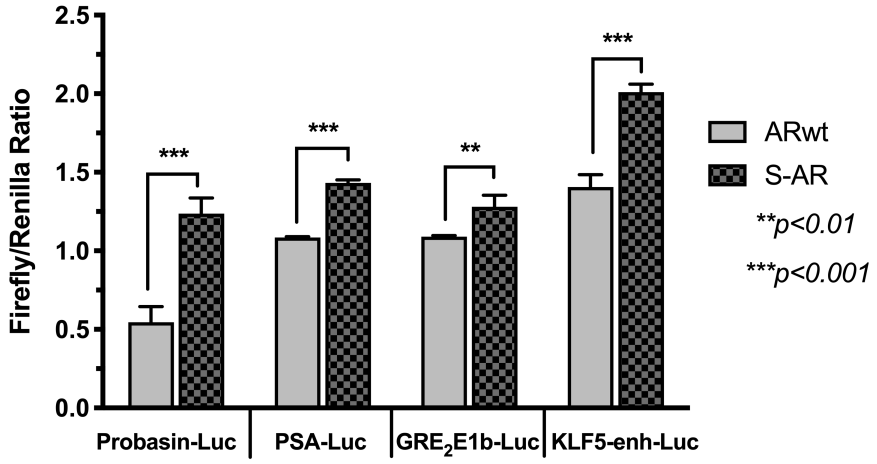

Figure S4. SUMO stimulates basal AR transcriptional activity regardless of the AR-luciferase reporter construct. MCF-7 cells were grown in phenol-red free DMEM media with 2% CSS for 48 hours. Various luciferase reporter constructs (probasin, PSA- or KLF5-enh-Luc) were co-transfected with Renilla luciferase together with either ARwt or SUMO-fused AR (S-AR). After 24 hrs, cells were harvested and assayed for luciferase activity. Data represent fold-change in firefly/renilla ratios ± SEM of three independent experiments, each performed in triplicates. **p<0.01 and ***p<0.001 indicate significant changes from the corresponding ARwt group using Student's *t*-test.

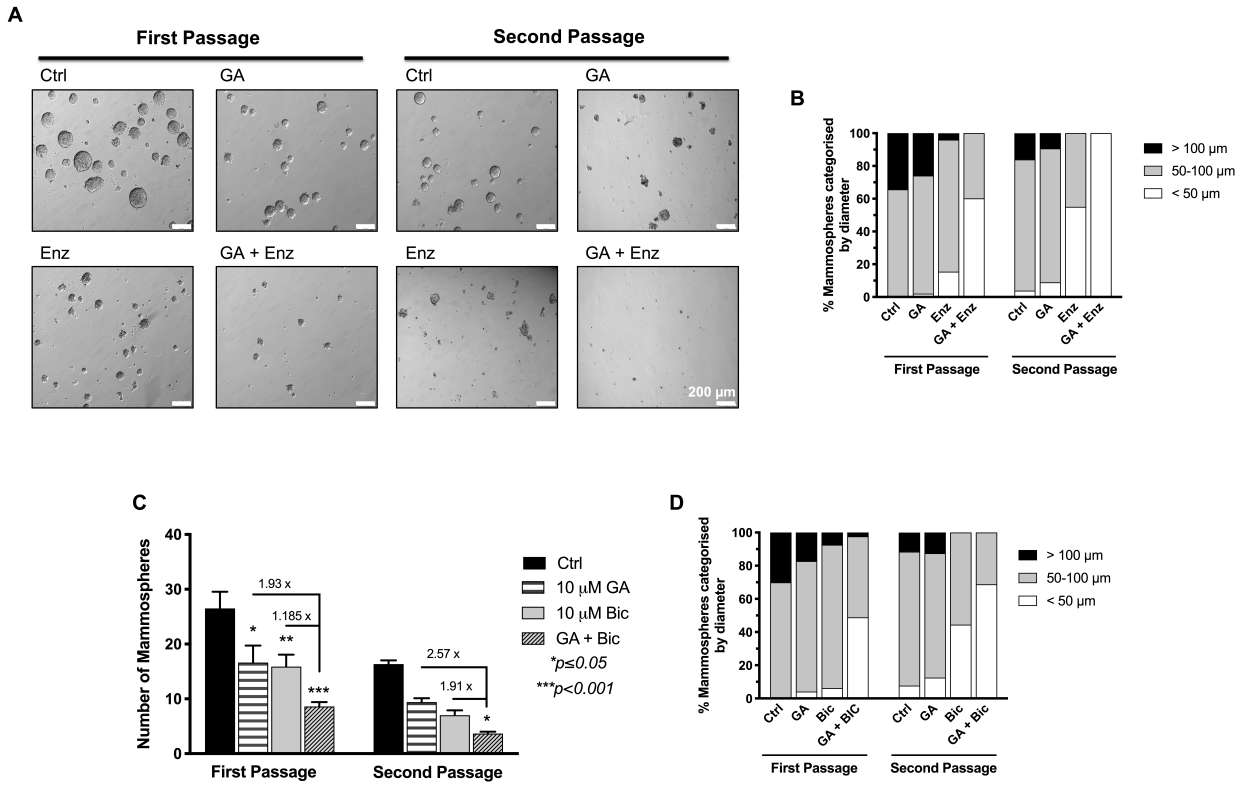

Figure S5. Concurrent targeting of SUMO-modified and unmodified AR decreases TamR BCa growth in 3D cultures. (A-D) First- and second-generation spheroids of TamR-7 were treated with 10 µM of the indicated treatments. **(A)** Representative images of mammosphere counts quantified and graphed in Fig. 6A. Graphs in panels **(B)** and **(D)** represent percentage of mammospheres based on their diameters while **(C)** represents mammosphere counts from two independent experiments. Comparisons with the control group was assessed using one-way ANOVA followed by Tukey's multiple comparison test and statistical significance is shown on graphs.

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