

Supplemental Material

Targeting the AR-UBE2C axis unleashes autophagy-associated cell death to overcome intrinsic resistance to PI3K Inhibition in AR-overexpressed breast and prostate cancers

Leilei Fu¹, Hongyao Li², Zixiang Li¹, Wei Liu^{1,2,*}, Lan Zhang^{1,*}, Xiaojun Man^{3,*}

¹Sichuan Engineering Research Center for Biomimetic Synthesis of Natural Drugs, School of Life Science and Engineering, Southwest Jiaotong University, Chengdu 610031, China

²Department of Biotherapy, Cancer Center and State Key Laboratory of Biotherapy, West China Hospital, Sichuan University, Chengdu 610041, China

³Department of Urology, Institute of Urology, The First Hospital of China Medical University, Shenyang 110001, China

*Corresponding author: Xiaojun Man, manxj1983@163.com; Lan Zhang, E-mail: zhanglanx_9@126.com; Wei Liu, E-mail: lwwl1995@163.com

Supplemental Methods

UBE2C expression analysis

Transcriptomic data and clinical annotations were obtained from the TCGA breast invasive carcinoma cohort (TCGA-TNBC). UBE2C expression values were extracted as FPKM data and log2-transformed before analysis. PAM50 molecular subtype information was obtained from TCGA annotations. UBE2C expression was compared between Basal and Normal-like samples using the Wilcoxon rank-sum test. All analyses and visualization were performed using R software (version 4.5.3) and the ggplot2 package. $P < 0.05$ was considered statistically significant.

PI3K pathway activity analysis

Transcriptomic data and clinical information were obtained from the FUSCC-TNBC cohort ($n = 360$). TNBC molecular subtypes were defined according to the previously established FUSCC classification system. Given that the LAR subtype is characterized by activated androgen receptor signaling, whereas the MES subtype is enriched for epithelial–mesenchymal transition and stem-like features, we focused on the comparison between LAR and MES tumors. PI3K/AKT/mTOR pathway activity was inferred using the PROGENy algorithm and normalized as Z-scores. PI3K pathway activity was compared between LAR and MES subtypes using the Wilcoxon rank-sum test. Statistical analyses were performed using R software (version 4.5.3), and plots were generated with ggplot2. $P < 0.05$ was considered statistically significant.

Supplemental Figures

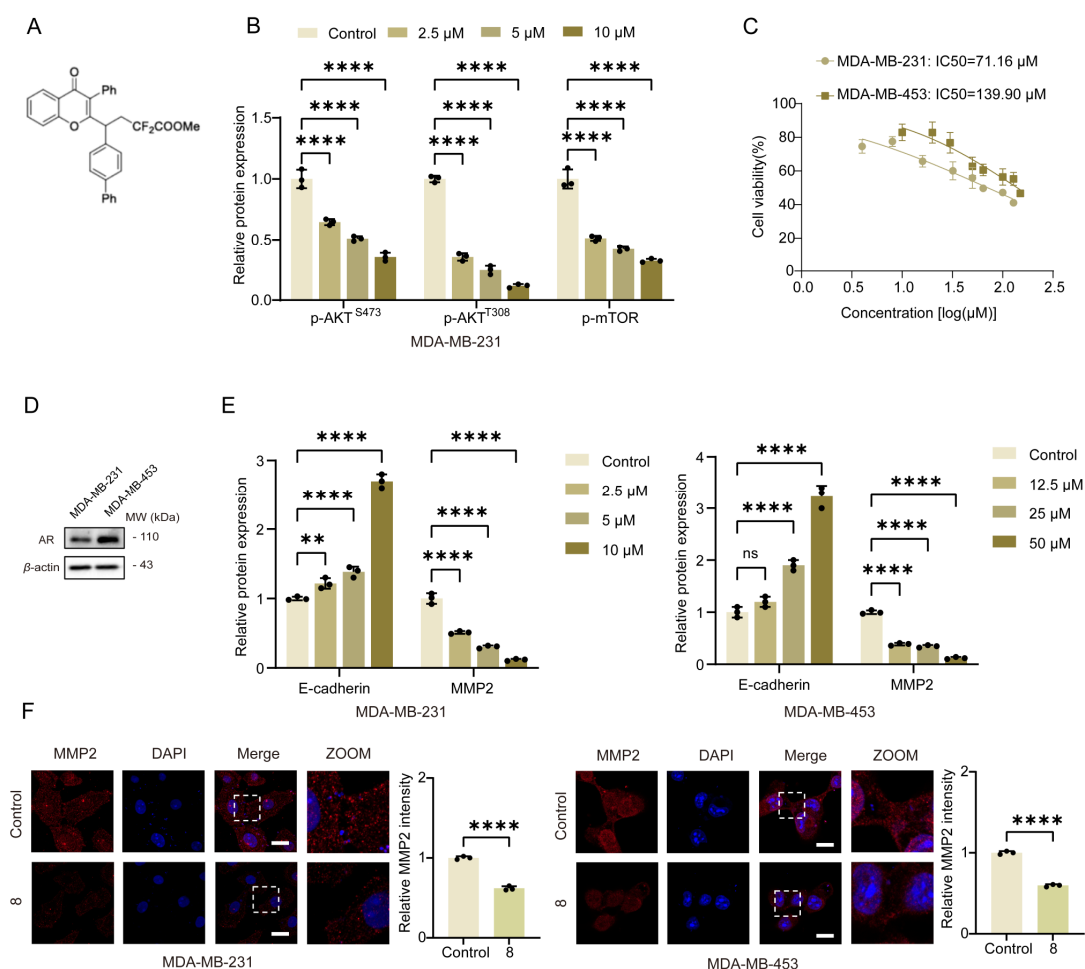


Figure S1. (A) Chemical structure of compound 8. **(B)** Immunoblot analysis of the relative protein expression levels of p-AKT and p-mTOR in MDA-MB-231 cells treated with the indicated concentrations of compound 8. **(C)** The cytotoxicity of Alpelisib for 24 h in AR-low MDA-MB-231 and AR-high MDA-MB-453 cells was assessed by MTT assay. **(D)** Immunoblot analysis of AR protein levels in MDA-MB-231 and MDA-MB-453 cell lines, confirming the AR-low and AR-high expressed phenotypes, respectively. **(E)** Quantification of E-cadherin and MMP2 protein levels from Western blot analyses. **(F)** Immunofluorescence images of MMP2 in MDA-MB-231 and MDA-MB-453 cells treated with

compound 8. Nuclei were stained with DAPI. Scale bar = 5 μ m. Data are presented as mean $\pm$ SEM. ns, not significant, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared with the indicated groups.

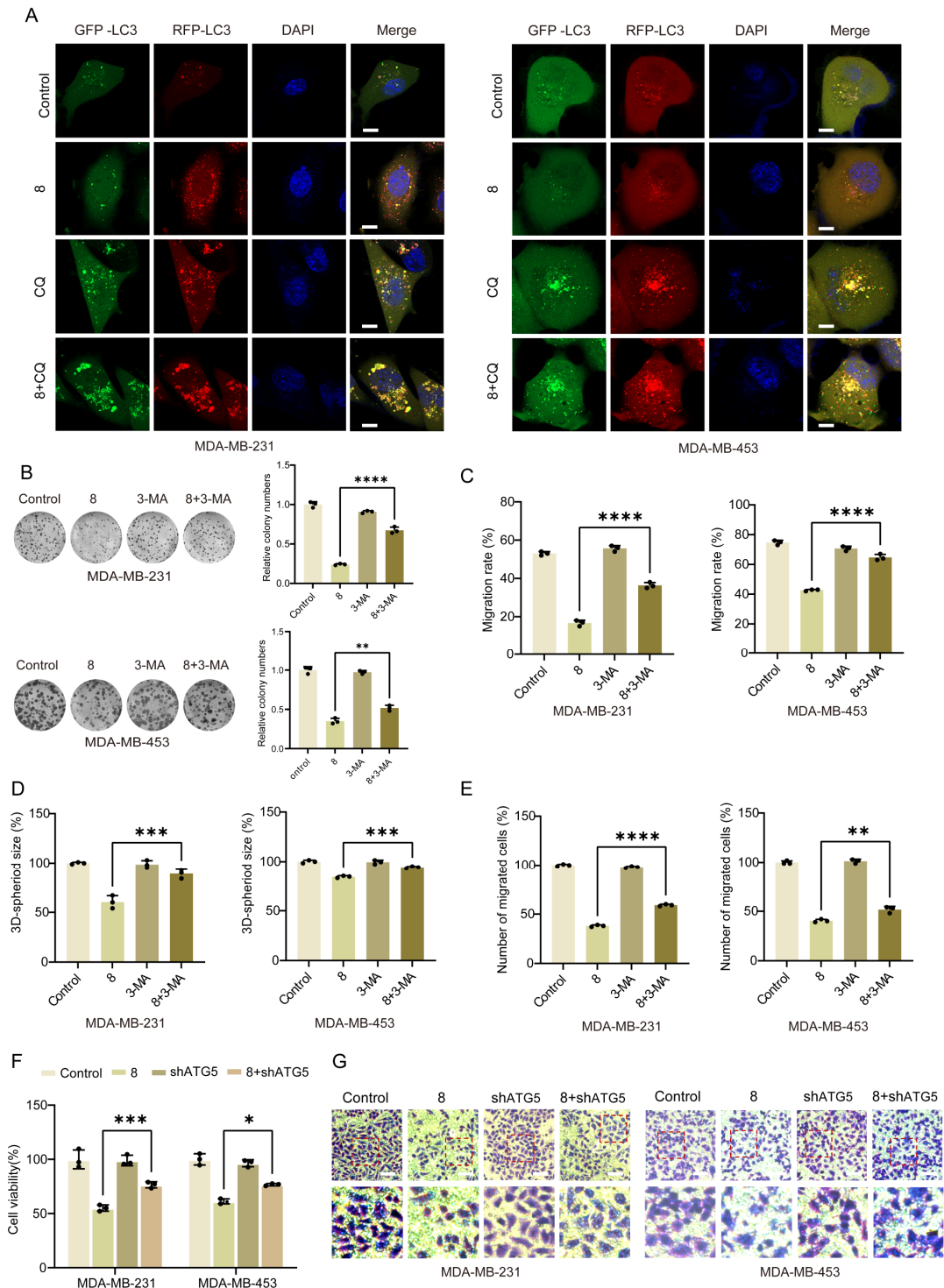


Figure S2. (A) Autophagic flux was evaluated using the tandem mRFP-GFP-LC3 reporter assay. Cells were treated with compound 8 in the presence or absence of CQ. Yellow puncta (GFP+/RFP+) indicate autophagosomes, while

red puncta (GFP-/RFP+) indicate autolysosomes. Scale bar = 5 μ m. **(B)** Colony formation rescue assays showed that the autophagy inhibitor 3-MA significantly reverses the suppression of clonogenic survival induced by compound 8. **(C)** Quantification of cell migration rates from wound healing assays in the presence of compound 8 and/or 3-MA. **(D)** Quantification of 3D tumor spheroid size in the presence of compound 8 and/or 3-MA. **(E)** Quantification of the number of invaded cells from Transwell assays in the presence of compound 8 and/or 3-MA. **(F)** MTT assays showed that ATG5 knockdown partially rescued compound 8-induced cytotoxicity after 24 h of treatment. **(G)** Transwell invasion assays indicated that sh-ATG5 rescues the invasive potential of cells treated with compound 8 for 24 h. Scale bar = 50 μ m. Data are presented as mean $\pm$ SEM. ns, not significant, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared with the indicated groups.

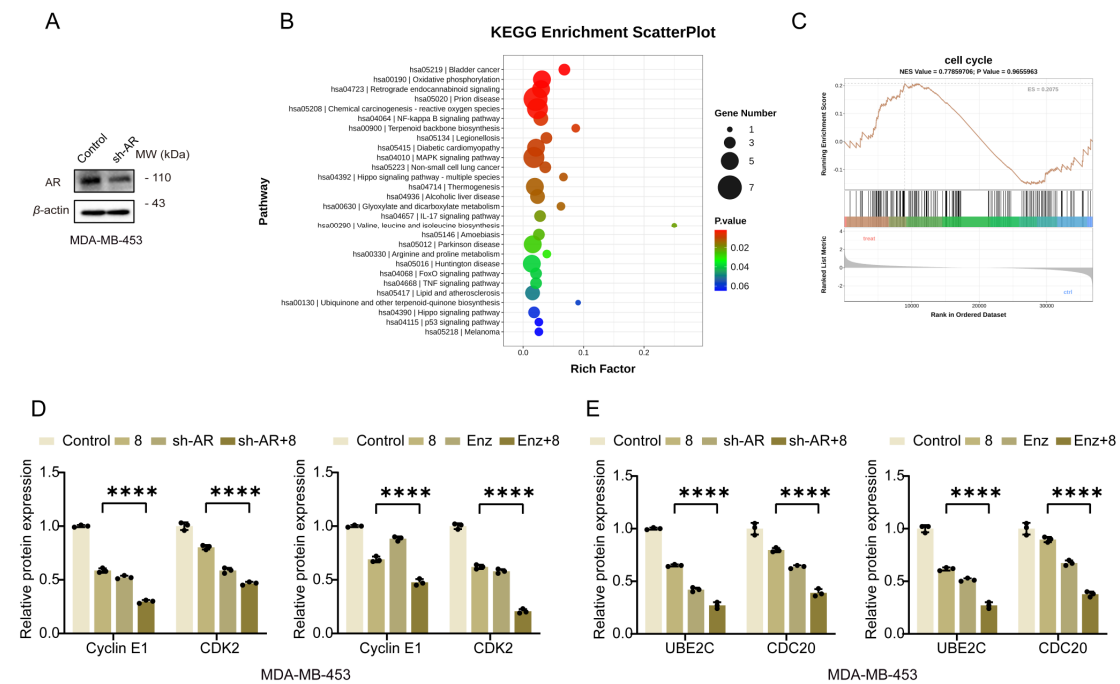


Figure S3. (A) Immunoblot validation of AR knockdown efficiency in MDA-MB-453 cells. **(B)** The KEGG pathway enrichment analysis scatter plot showed the top enriched signaling pathways for the drug-responsive gene signature in the AR-low context(Set B). **(C)** GSEA plot of the cell-cycle pathway in the AR-low drug-responsive gene signature(Set B). **(D)** Immunoblot quantification of the relative Cyclin E1 and CDK2 protein expression levels. **(E)** Immunoblot quantification of the relative protein expression levels of UBE2C and its downstream target CDC20. Data are presented as mean $\pm$ SEM. ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ compared with the indicated groups.

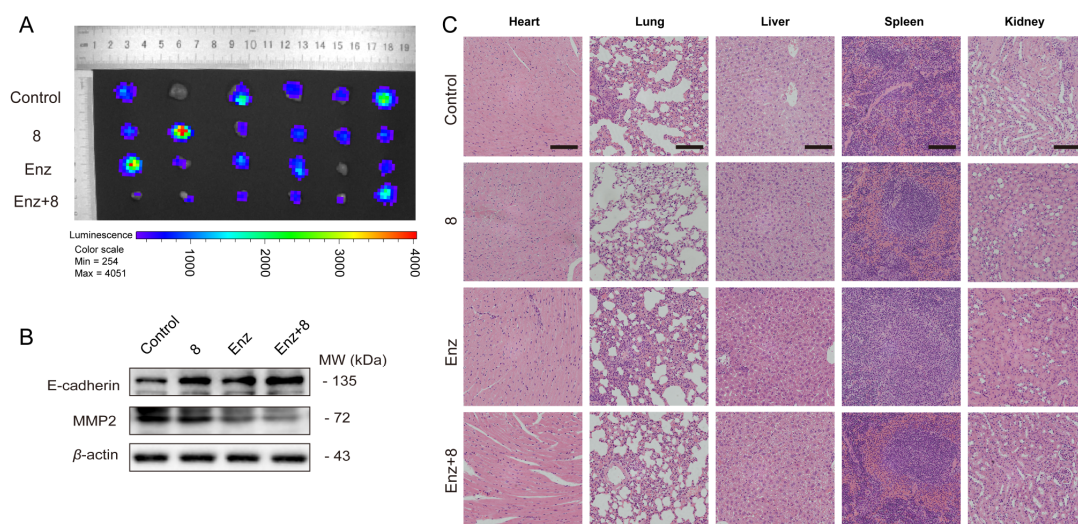


Figure S4. (A) Ex vivo bioluminescence imaging of excised tumors from mice treated with vehicle (Control), compound 8, Enzalutamide (Enz), or Enz + 8 at the experimental endpoint. **(B)** Immunoblot analysis of E-cadherin and MMP2 expression in tumor tissues from different treatment groups. **(C)** H&E staining of major organs (heart, lung, liver, spleen, and kidney) collected at the endpoint, showing no obvious histopathological abnormalities among treatment groups. Scale bar = 40 μ m. Data are presented as mean $\pm$ SEM. ns, not significant, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared with the indicated groups.

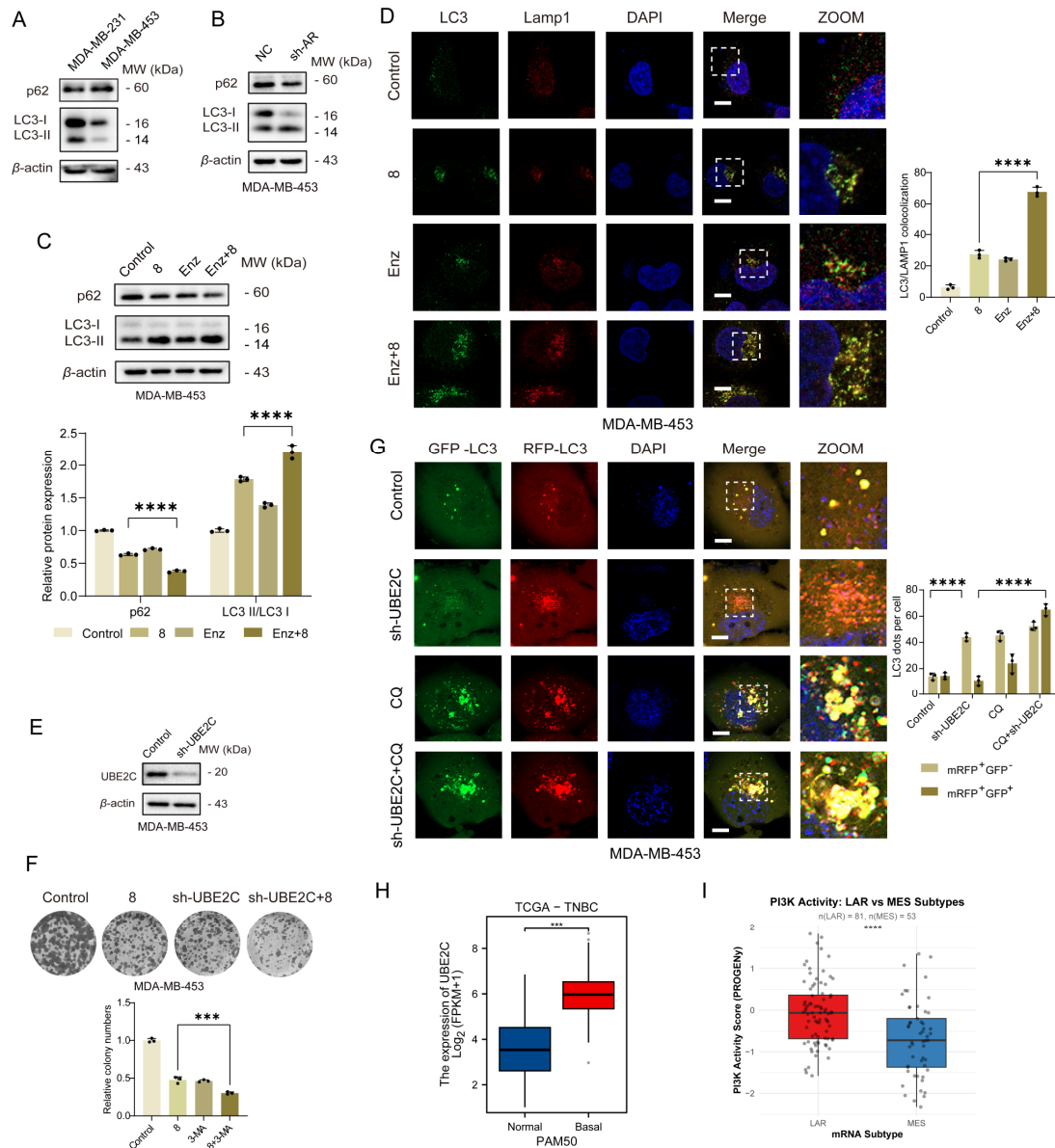


Figure S5. (A) Western blot analysis of basal autophagy levels (p62, LC3-II/LC3-I) in MDA-MB-231 and MDA-MB-453 cells. MDA-MB-453 cells exhibit lower basal autophagy. **(B)** Western blot analysis showed that AR knockdown in MDA-MB-453 cells increases basal autophagy levels. **(C)** Western blot analysis of autophagy levels (p62, LC3-II/LC3-I) in compound 8 combined with Enz. **(D)** Immunofluorescence images of LC3 (green) and LAMP1 (red) co-localization in cells treated with compound 8 and Enz. Yellow signals in merged

images indicate LC3-LAMP1 co-localization. Scale bar = 5 μ m. **(E)** Western blot validation of UBE2C knockdown efficiency in MDA-MB-453 cells. **(F)** Colony formation rescue assay showed that 3-MA reverses the growth suppression induced by compound 8 in sh-UBE2C cells. **(G)** Autophagic flux was evaluated using the mRFP-GFP-LC3 reporter assay. Yellow puncta (GFP+/RFP+) indicate autophagosomes, while red puncta (GFP-/RFP+) indicate autolysosomes. Cells were transduced with sh-UBE2C or control plasmid and treated with CQ where indicated. Scale bar = 5 μ m. **(H)** Distribution of UBE2C mRNA expression levels in patients with the Basal and Normal subtypes in the TCGA-TNBC dataset. **(I)** Distribution of PI3K pathway activity scores in patients with the LAR (n = 81) and MES (n = 53) subtypes in the FUSCC-TNBC cohort. PI3K activity scores were calculated using the PROGENy algorithm based on transcriptomic data. Higher scores indicate stronger transcriptional activity of the PI3K/AKT/mTOR signaling pathway. Data are presented as mean $\pm$ SEM. ns, not significant, * p < 0.05, ** p < 0.01, *** p < 0.001, **** p < 0.0001 compared with the indicated groups.

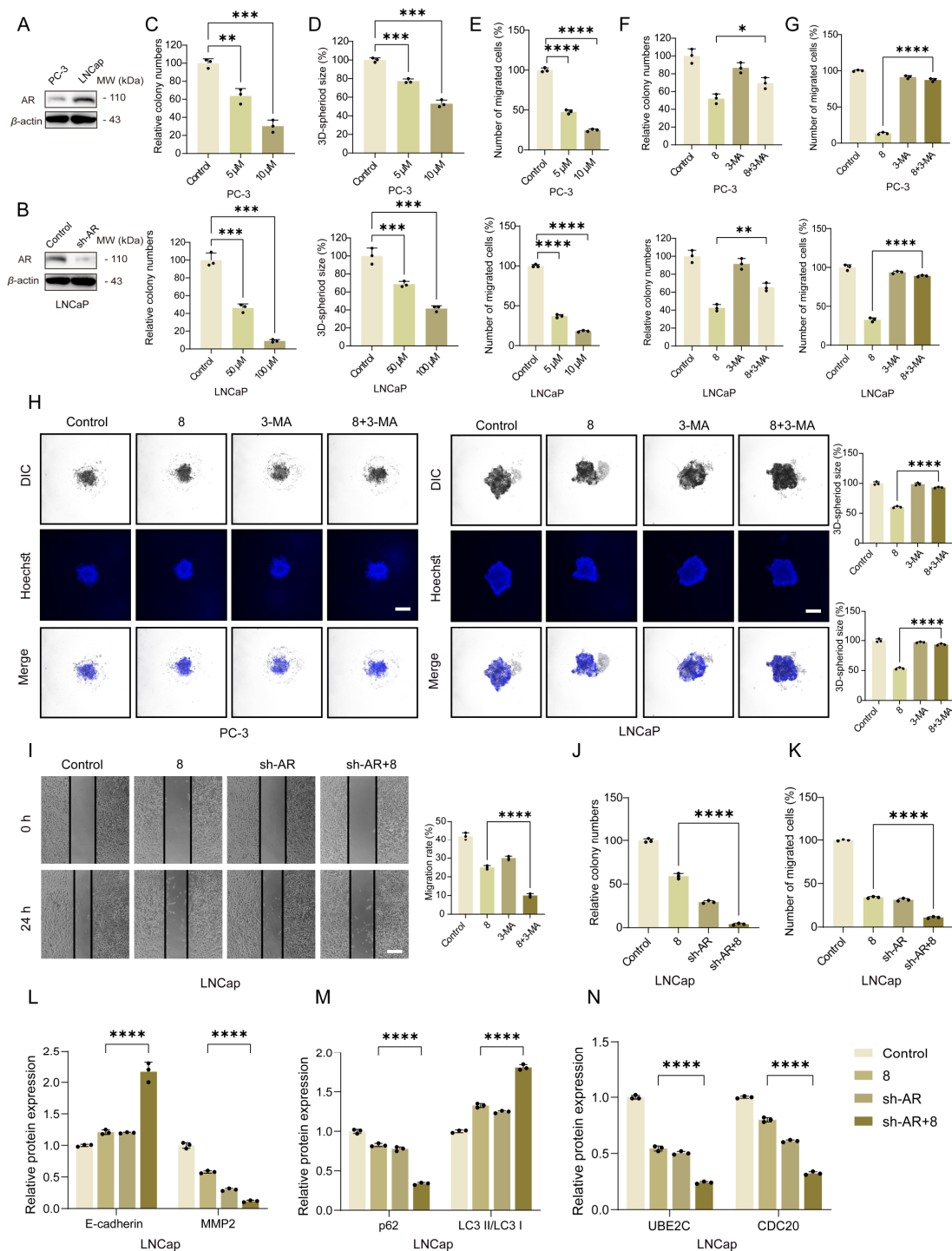


Figure S6. (A) Immunoblot analysis of AR expression in PC-3 and LNCaP cells. **(B)** Immunoblot validation of shRNA-mediated AR knockdown in LNCaP cells. **(C-G)** Quantification of cell viability, clonogenic growth, migration, and spheroid size following compound 8 treatment at the indicated concentrations, with or

without 3-MA. **(H)** Effects of compound 8 and/or 3-MA on spheroid growth in PC-3 and LNCaP cells. Scale bar = 100 μm . **(I)** Wound-healing assays showed migration changes upon AR knockdown and compound 8 treatment. Scale bar = 50 μm . **(J, K)** Quantitative analysis of colony numbers and migrated cells under the indicated conditions. **(L-N)** Quantification of E-cadherin, MMP2, p62, LC3-II/LC3-I, UBE2C, and CDC20 protein levels. Data are presented as mean $\pm$ SEM. ns, not significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, $****p < 0.0001$ compared with the indicated groups.