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Supplemental information

Methylation of the chromatin modifier KMT2D by SMYD2 contributes to therapeutic response in hormone-dependent breast cancer

Ryan Blawski, Bujamin H. Vokshi, Xinyu Guo, Srushti Kittane, Mirna Sallaku, Wanlu Chen, Martina Gjyzari, Tony Cheung, Yuhan Zhang, Christopher Simpkins, Weiqiang Zhou, Amanda Kulick, Peihua Zhao, Meihan Wei, Pranavkrishna Shivashankar, Tatiana Prioleau, Pedram Razavi, Richard Koche, Vito W. Rebecca, Elisa de Stanchina, Pau Castel, Ho Man Chan, Maurizio Scaltriti, Emiliano Cocco, Hongkai Ji, Minkui Luo, and Eneda Toska

Supplemental Figure 1

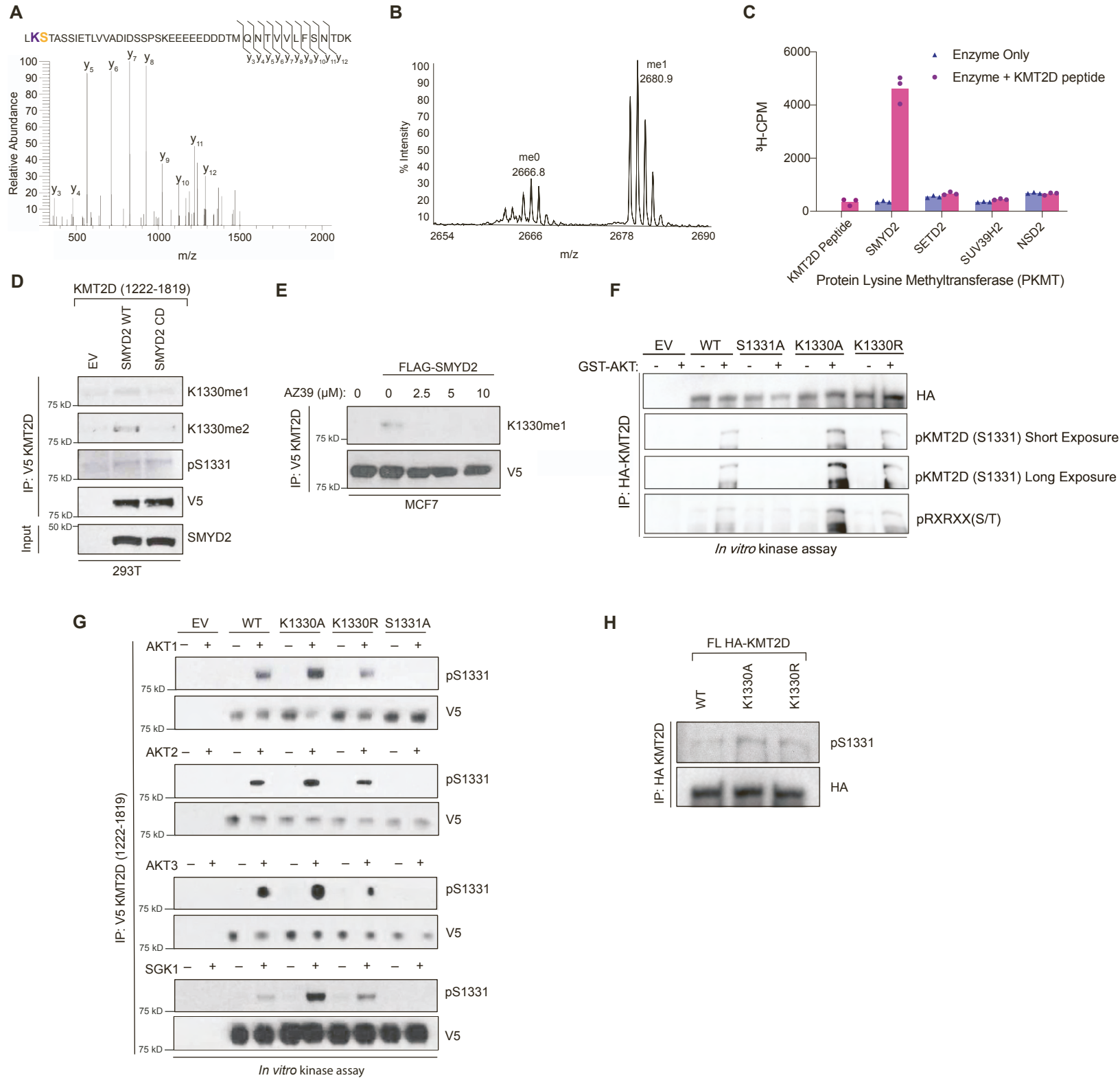

Figure S1. SMYD2 methylates KMT2D

A. MS/MS spectrum of the methylated KMT2D peptide. Full-length KMT2D was expressed and immunoprecipitated from HEK293T cells.

B. *In vitro* methyltransferase assay with recombinant SMYD2 and the wild type KMT2D peptide (aa1321-1343). Unmodified and modified peptides were detected using Matrix Assisted Laser Desorption/Ionization mass spectrometry (MALDI). Monomethylation of the KMT2D peptide is shown, with the corresponding mass shift of +14 Da.

C. Radiometric screen as conducted in Fig.1B including additional recombinant protein lysine methyltransferases. Reactions were conducted with the wild type KMT2D peptide (aa1321-1343), and ^{3}H-SAM and activity was detected using liquid scintillation counting.

D. The wild type V5-tagged construct of KMT2D was co-expressed with either WT SMYD2 or a SMYD2 catalytic dead mutant (SMYD2 Y240A) in HEK293T cells. Following immunoprecipitation, samples were probed for K1330me1/2 and pS1331.

E. The wild type V5-tagged KMT2D fragment construct (aa1222-1819) was co-expressed with wild type SMYD2 in MCF7 cells treated with the indicated doses of SMYD2 inhibitor AZ39 for 48hrs. Following immunoprecipitation samples were subjected to immunoblot with the K1330me1 antibody.

F. *In vitro* kinase assay using recombinant AKT1 and full-length wild type or mutant HA-KMT2D immunoprecipitated from HEK293T cells as a substrate. Activity was assessed on K1330A/R mutants with the S1331A phosphorylation site mutation as a control. Cells were treated with 2μM AKT inhibitor MK2206 for 1 hour before collection. Immunoblots were probed with both the site-specific pS1331 antibody (published previously) and a pan-pRXRXX(S/T) antibody.

G. *In vitro* kinase assay using recombinant AKT1, AKT2, AKT3 and SGK1 with immunoprecipitated wild type V5- fragment KMT2D (aa1222-1819), K1330A/R and S1331A mutants as substrates.

H. WT, K1330A and K1330R full length KMT2D HA-tagged constructs were overexpressed in 293T cells and immunoprecipitated using HA-conjugated agarose. Phosphorylation was detected using the site-specific pS1331 antibody.

Supplemental Figure 2

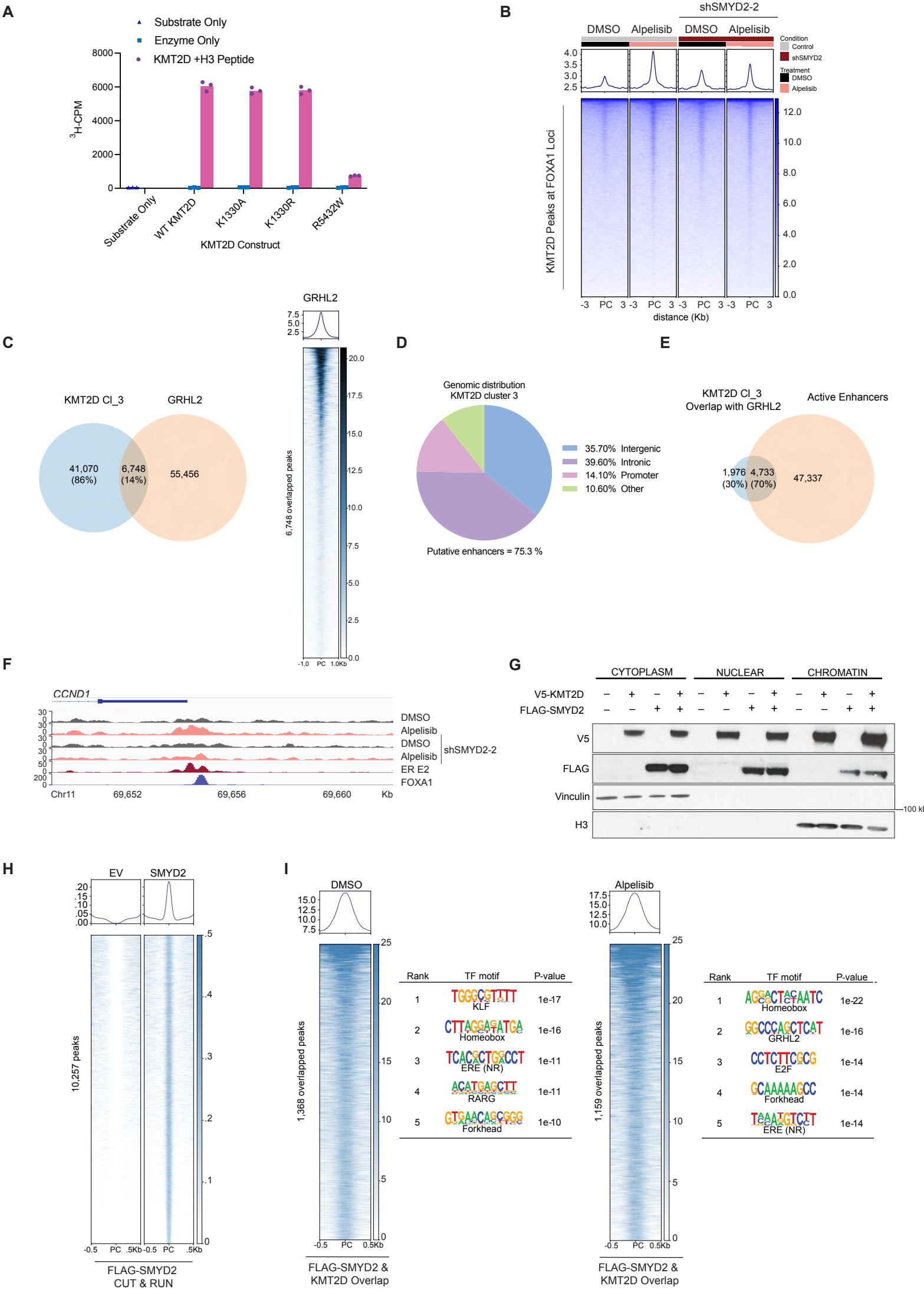

Figure S2. SMYD2 knockdown affects binding of KMT2D genome-wide

A. *In vitro* methylation assay to assess the activity of immunoprecipitated wild type KMT2D, K1330A/R or KMT2D catalytic dead mutants on an H3 peptide substrate (aa1-21). Activity was detected using liquid scintillation counting.

B. KMT2D binding sites that overlap with FOXA1 binding sites in MCF7 cells with SMYD2 knockdown and/or alpelisib treatment (ENCODE: ENCSR126YEB).

C. Venn diagram and tornado plot demonstrating KMT2D binding sites in cluster 3 (Fig. 2E) overlapping with GRHL2 ChIP-seq (GEO: GSE81714).

D. Genomic distribution of KMT2D binding sites in cluster 3 (Fig. 2E).

E. Venn diagram displaying overlap of GRHL2-KMT2D peaks (Fig. S2C) with active enhancers defined by H3K27ac ChIP-seq (GEO: GSE124228) excluding promoter and TSS peaks.

F. KMT2D binding at ER-FOXA1 target gene *CCND1* with SMYD2 knockdown and/or alpelisib treatment.

G. Cellular fractionation assay using MCF7 cells with overexpression of V5-KMT2D and/or FLAG-SMYD2 constructs. V5 and FLAG were used to detect the cellular distribution of KMT2D and SMYD2 respectively, while VINCULIN and HISTONE 3 were used as fractionation controls.

H. Tornado plots showing enrichment of FLAG-SMYD2 chromatin binding relative to the empty vector control in a FLAG CUT & RUN experiment performed in MCF7 cells with overexpression of FLAG-SMYD2. Number of differential peaks (10,257) is shown. (n=3 technical replicates)

I. Tornado plots showing the overlap of FLAG-SMYD2 CUT & RUN peaks with KMT2D ChIP-seq in both dmso and alpelisib-treated sample conditions. HOMER motif analysis for the overlapped peaks in each condition was performed with top significant motifs shown to the right of their respective tornado plots.

Supplemental Figure 3

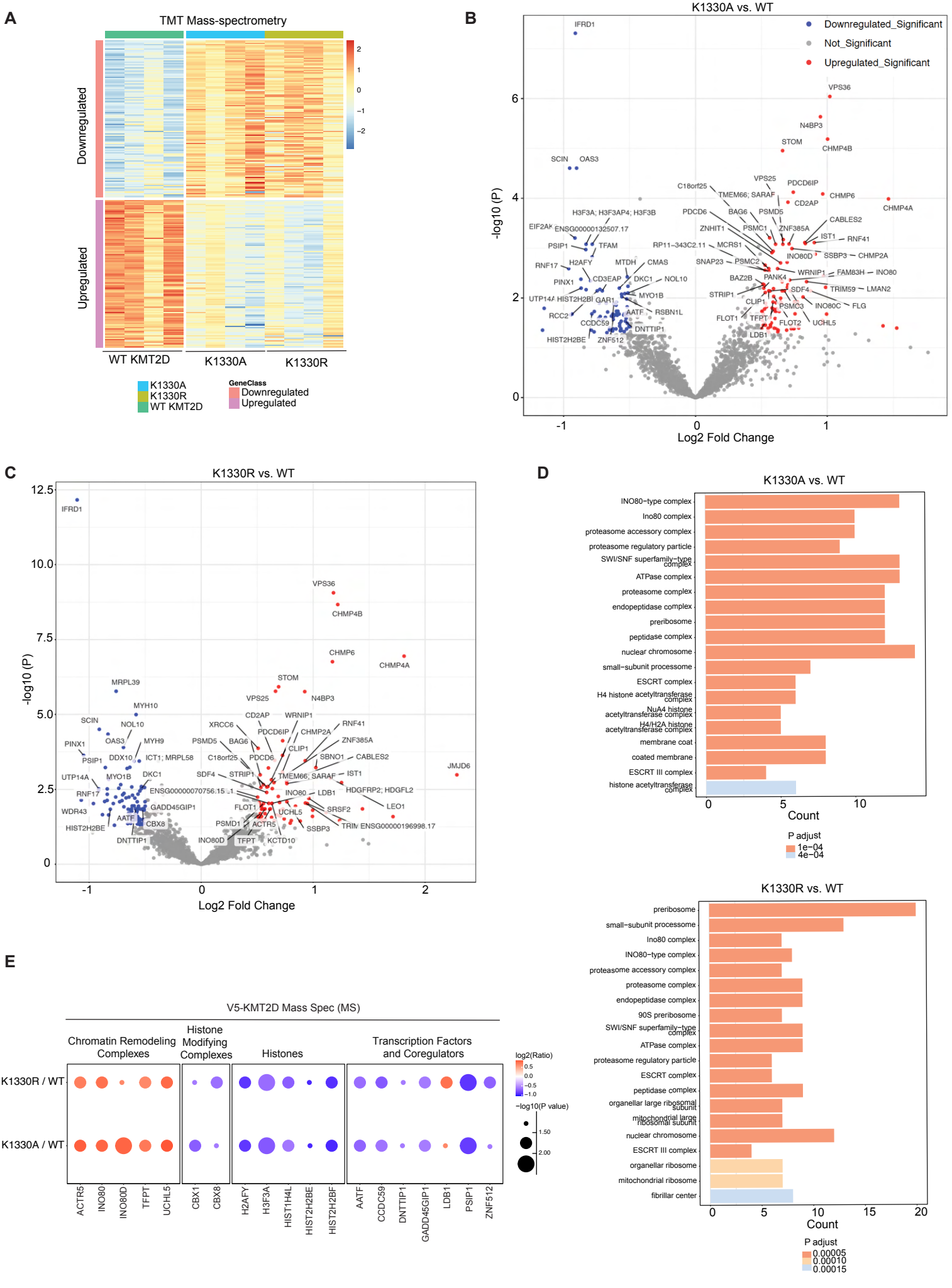

Figure S3: IP-MS/MS identifies differential interactors for WT and methyl-dead KMT2D mutants

A. Heatmap showing differential enrichment of interacting proteins comparing wildtype V5-fragment KMT2D to the K1330A and K1330R mutants. Total of 2,583 proteins with 226 differentially enriched at a p value <.05 and $\log_2$ fold change >.5. (n=4 technical replicates)
B. Volcano plot showing upregulated and downregulated proteins in the KMT2D K1330A mutant sample compared to wildtype KMT2D. Significance is indicated by an adjusted p value of <.05 and $\log_2$ fold change >.5.
C. Volcano plot showing upregulated and downregulated proteins in the KMT2D K1330R mutant sample compared to wildtype KMT2D. Significance is indicated by an adjusted p value of <.05 and $\log_2$ fold change >.5.
D. Plot of significant protein families enriched in the K1330A and K1330R mutants compared to wildtype KMT2D using gene ontology analysis.
E. Epigenetic and transcriptional regulators identified to differentially interact with wildtype versus K1330A/R mutant KMT2D. The color of each dot represents the $\log_2$ protein abundance ratio of the KMT2D mutant versus wildtype and the size corresponds to the $\log_{10}$ p value.

Supplementary Figure 4

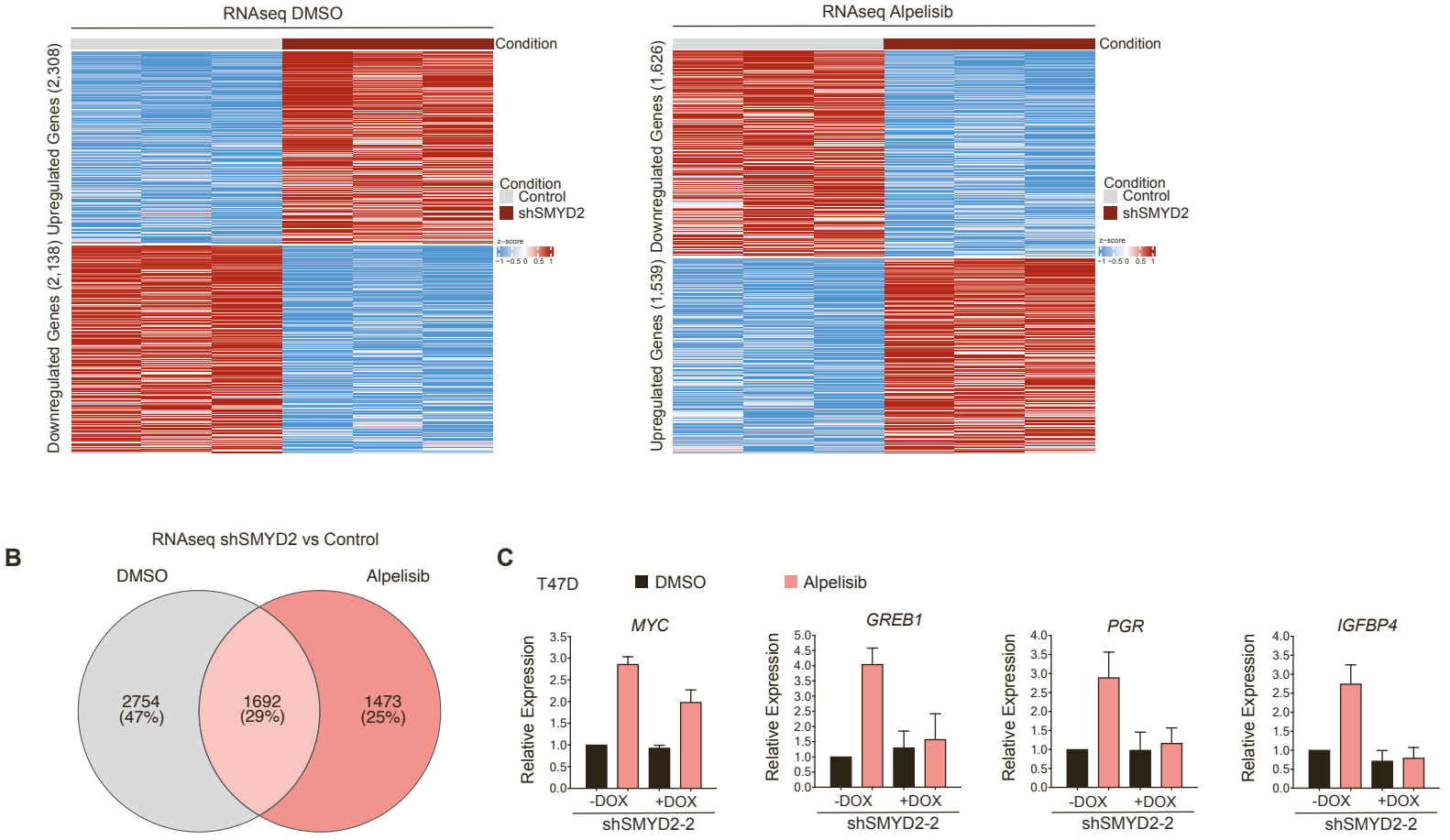

Figure S4. Knockdown of SMYD2 affects alpelisib-induced ER-dependent transcription

A. Heatmap showing the number of upregulated (2,308) and downregulated genes (2,138) with SMYD2 knockdown in the DMSO condition of the MCF7 RNA-seq. (left) Heatmap showing the number of upregulated (1,539) and downregulated genes (1,626) with SMYD2 knockdown in the alpelisib treatment condition of the MCF7 RNA-seq. (right) Heatmaps were generated using k-means clustering of genes filtered by adjusted p value < .1. (n=3 technical replicates)

B. Venn diagram showing differential genes upon SMYD2 knockdown in each drug treatment condition (DMSO or alpelisib) and the overlapped genes between the two treatment conditions. Differential genes were filtered by p value < .1.

C. RT-qPCR analysis of estrogen receptor target genes in T47D cells with doxycycline-inducible SMYD2 knockdown, treated with DMSO or 1μM alpelisib for 24 hours. (n=3 biological replicates, mean, SD).

Supplementary Figure 5

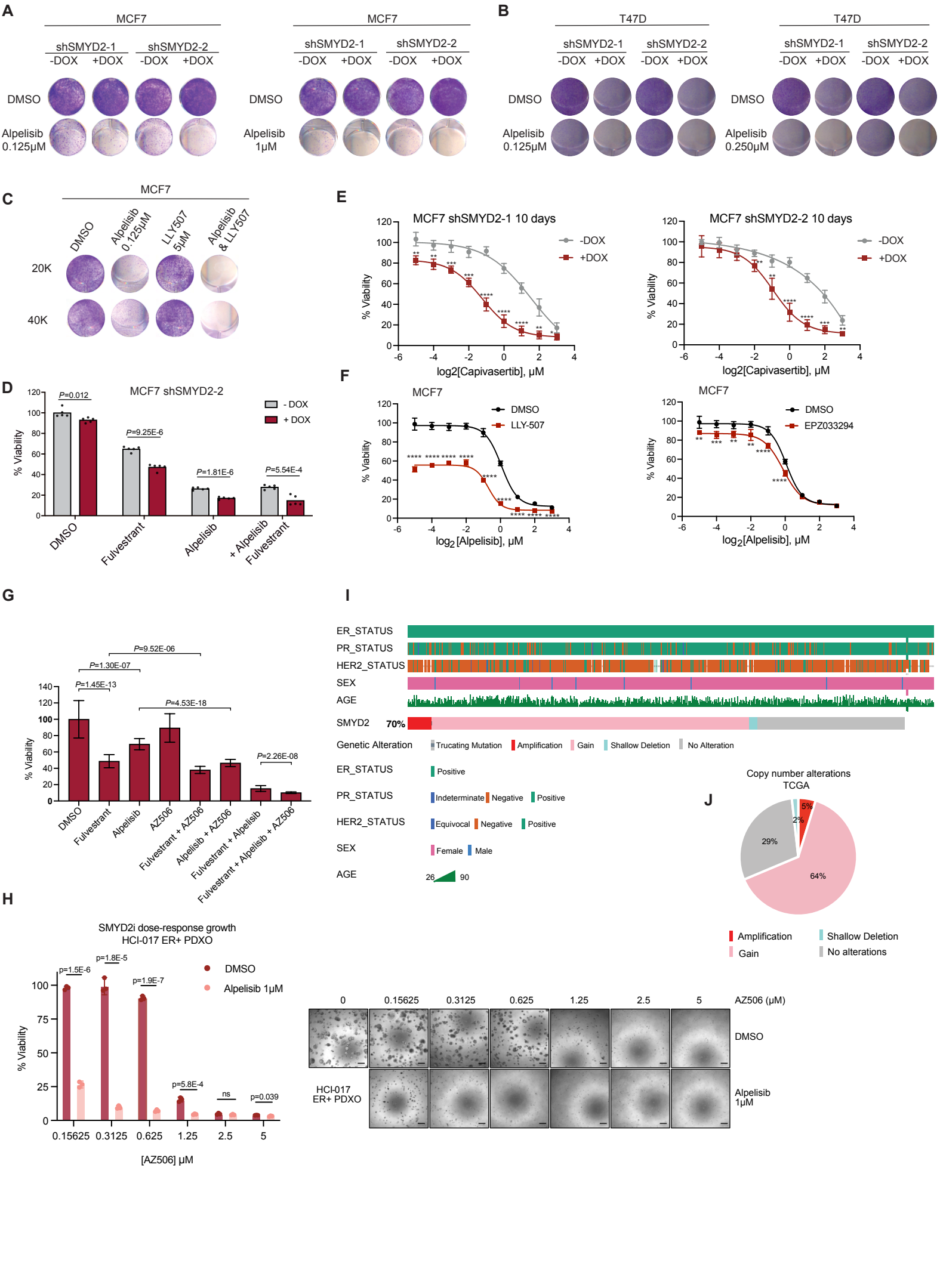

Figure S5. SMYD2 inhibition improves the therapeutic response of breast cancer cells to PI3K/AKT inhibitors

A. Proliferation assays in the shSMYD2 MCF7 cell lines using two doses of alpelisib, .125μM and 1μM, for a treatment period of five days. Results were visualized using crystal violet staining. (n=3 biological replicates, representative replicate shown)

B. Proliferation assays in the shSMYD2 T47D cell lines using two doses of alpelisib, .125μM and .25μM for a treatment period of five days. Results were visualized using crystal violet staining. (n=3 biological replicates, representative replicate shown)

C. Proliferation assays in MCF7 cells to assess the effects of alpelisib (.125μM), SMYD2 inhibitor LLY507 (5 μM) and the combination for a treatment period of five days. Results were visualized using crystal violet staining. (n=3 biological replicates, representative replicate shown)

D. MTT assays with a shSMYD2 MCF7 cell line treated with fulvestrant, alpelisib, or the combination for a treatment period of ten days. Drug concentrations were as follows: fulvestrant (100nM), alpelisib (1μM). Indicated p values were generated with an unpaired, two-tailed Student's t test. (n=3 biological replicates, representative replicate shown)

E. Proliferation assays in the shSMYD2 MCF7 cell lines using a dose-response of the AKT inhibitor capivasertib for a treatment period of ten days. Cell viability was measured using PrestoBlue reagent and significance at individual doses was assessed using an unpaired, two-tailed Student's t test. P values are indicated as follows (*< 0.05,** < 0.01,***< 0.001 and ****< 0.0001). (n=4 biological replicates, mean, SD)

F. MTT proliferation assays to assess the combination of a single dose of SMYD2 inhibitors LLY507 (5μM) and EPZ033294 (10μM) with a dose response of alpelisib for a treatment period of five days. P values are indicated as in Fig. S1E. (n=3 biological replicates, representative shown, mean, SD)

G. Proliferation assays in MCF7 cells treated with fulvestrant, alpelisib, or the SMYD2 inhibitor AZ506 and the combinations. Drug concentrations are as follows: fulvestrant (6.25nM), alpelisib (62.5nM) and AZ506 (5 μM). Viability was measured using PrestoBlue reagent after a treatment period of ten days. P values for individual comparisons were calculated using an unpaired, two-tailed Student's t test. (n=3 biological replicates, representative shown, mean, SD)

H. The HCI-017 ER+/PTEN- organoid model was embedded in Matrigel in 96 well plates and treated for 14 days with increasing doses of AZ506 ranging from 0 to 5 μM and combined with either 1 μM of Alpelisib (PI3K inhibitor) or DMSO vehicle control. Live cell viability was measured

by CellTiterGlo quantification and data were plotted as means +/- SEM. (n=3, two-tailed Student T tests) Images at each dose were taken using 4X magnification. Scale bar: 200μm.

I. Plot of SMYD2 alterations in the ER+ breast cancer patient dataset from TCGA.

J. Pie chart showing percentage of each type of SMYD2 alteration in ER+ TCGA breast cancer patients.

Supplementary Figure 6

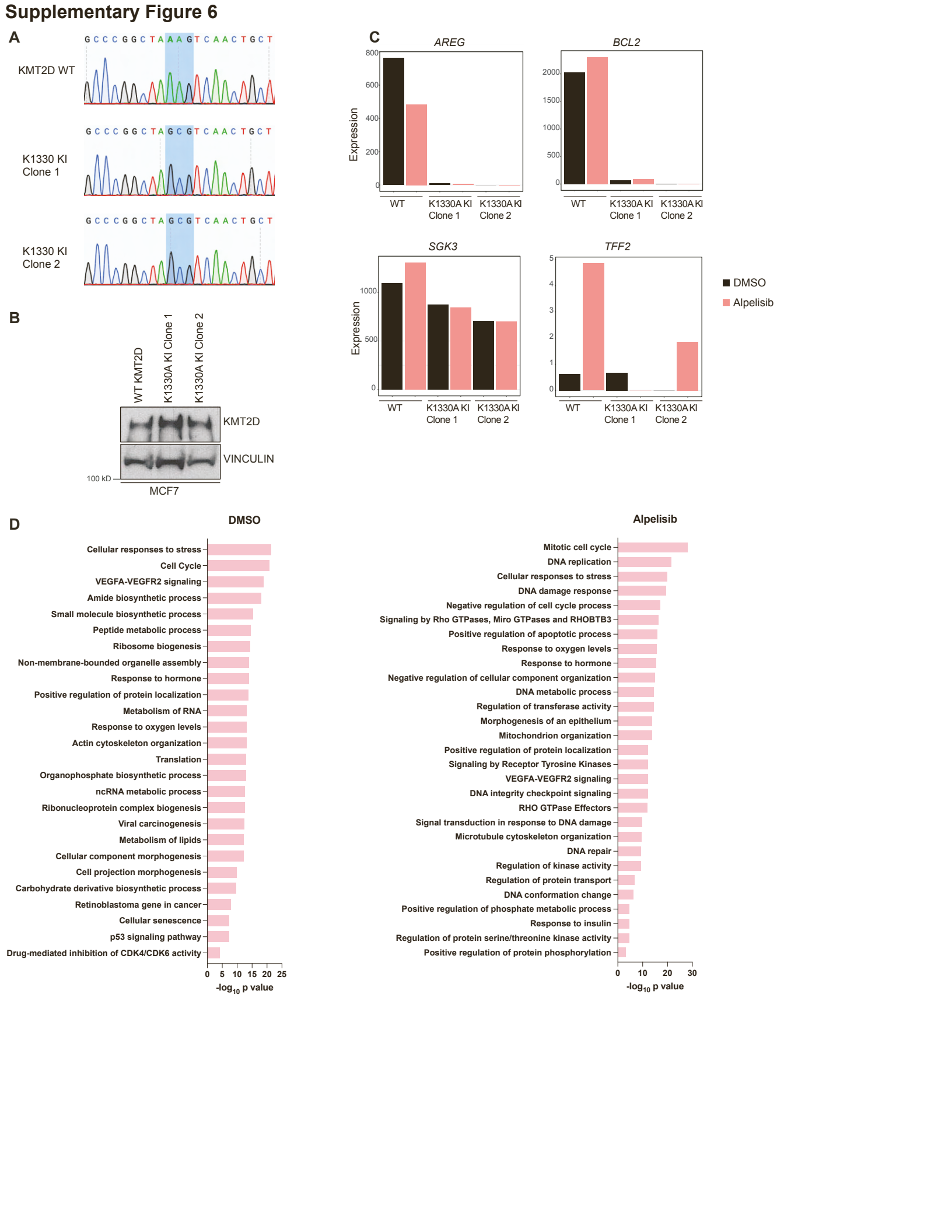

Figure S6. KMT2D K1330A CRISPR knock-in breast cancer cells downregulate estrogen-responsive gene sets

A. Sequencing traces showing the site-specific edit of the KMT2D 1330 lysine (AAG) to alanine (GCG) in the two CRISPR knock-in clones.
B. Western blot to assess expression levels of KMT2D in the K1330 edited clones compared to unedited MCF7 cells. VINCULIN is used as a loading control.
C. Expression of *AREG*, *BCL2*, *SGK3*, and *TFF2* in WT, K1330A clone 1 and K1330A clone 2 cells treated with DMSO or alpelisib (1μM) as established by RNA-seq.
D. Metascape ontology analysis of the overlapped genes between knock-in and knockdown RNA-seq in each treatment condition (DMSO or alpelisib). Top significant pathways are shown with indicated p values.