

Supplementary Data

Crosstalk between EZH2 and DNA methylation mediates neuroendocrine prostate cancer lineage plasticity

Richa Singh^{1,*}, Varadha Balaji Venkadakrishnan^{2,3,4,*}, Eddie Imada¹, Yasutaka Yamada², Nicholas J. Brady¹, Kate E. Dunmore^{1,5}, Richard Garner^{1,5}, Matthew A. Booker⁶, Brian Hanratty⁷, Michael C. Haffner^{7,8}, Michael Y. Tolstorukov⁶, Luigi Marchionni¹, Brian D. Robinson¹, David S. Rickman^{1,9,10,#}, and Himisha Beltran^{2,3,4,10,#}

¹Department of Pathology and Laboratory Medicine, Weill Cornell Medicine, New York, NY USA

²Department of Medical Oncology, Dana-Farber Cancer Institute, Boston, MA, USA

³Harvard Medical School, Boston, MA, USA

⁴The Broad Institute of MIT and Harvard, Cambridge, MA, USA

⁵Biochemistry, Cellular, and Molecular Biology Graduate Program, Weill Cornell Medicine, New York, NY USA

⁶Department of Informatics and Analytics, Dana-Farber Cancer Institute, Boston, MA, USA

⁷Division of Human Biology, Fred Hutchinson Cancer Center, Seattle, WA, USA.

⁷Divisions of Human Biology and Clinical Research, Fred Hutchinson Cancer Center, Seattle, WA, USA.

⁸Department of Laboratory Medicine and Pathology, University of Washington, Seattle, WA, USA

⁹Meyer Cancer Center, Weill Cornell Medicine, New York, NY USA

¹⁰Senior author

*These authors contributed equally (each has the right to list themselves in first author order on their CV)

#Correspondence:

David S. Rickman, Ph.D.

Department of Pathology and Laboratory Medicine,

413 East 69th Street

Weill Cornell Medicine,

New York, NY 10021 USA

Phone: 646- 962- 3946

Email: dsr2005@med.cornell.edu

Himisha Beltran, M.D.

Dana-Farber Cancer Institute

450 Brookline Avenue, Dana 1420

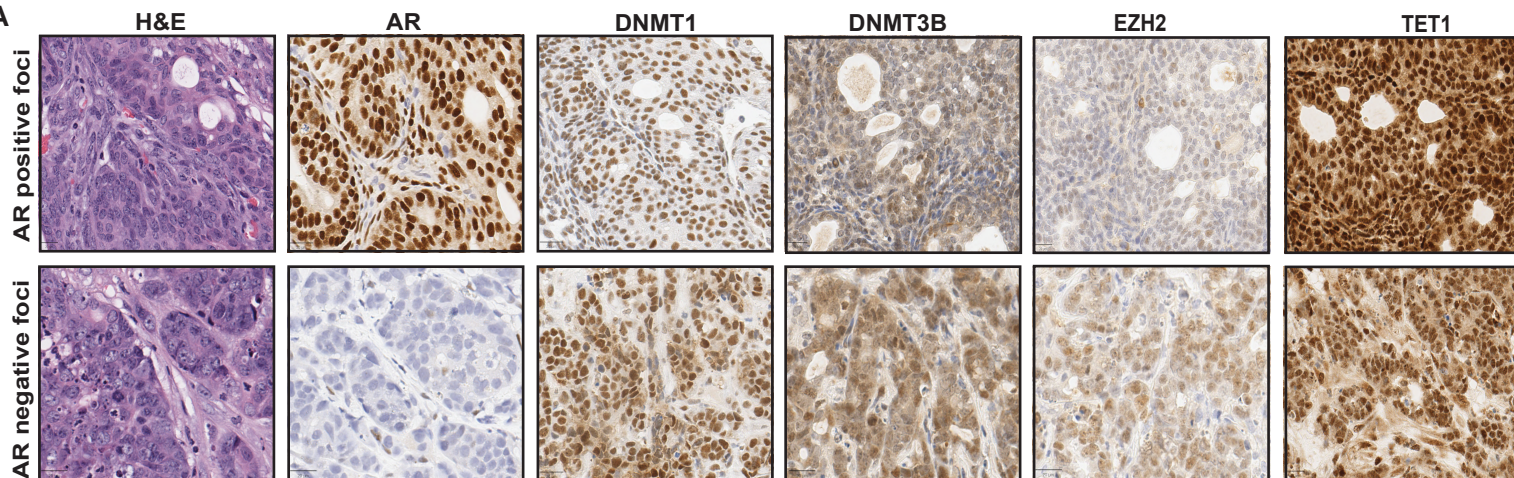
Boston, MA 02115, USA

Phone: 617- 582- 9421

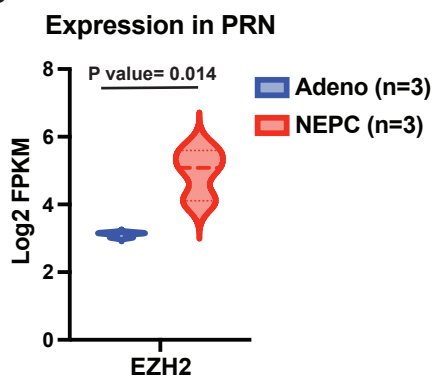
Email: himisha_beltran@dfci.harvard.edu

Figure S1

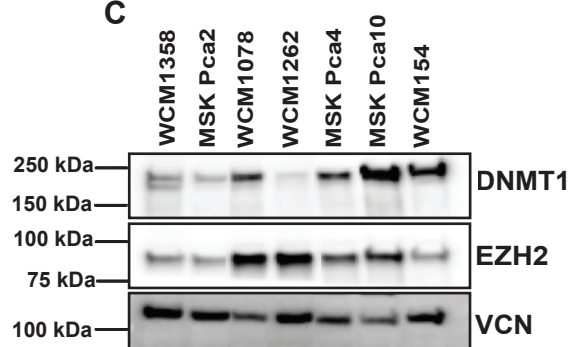
A



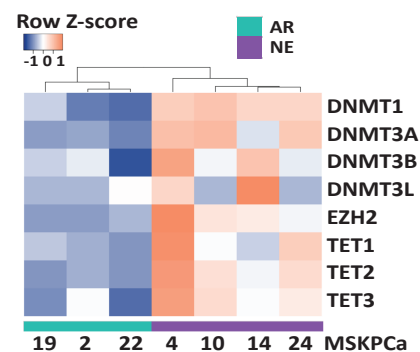
B



C



D



E.

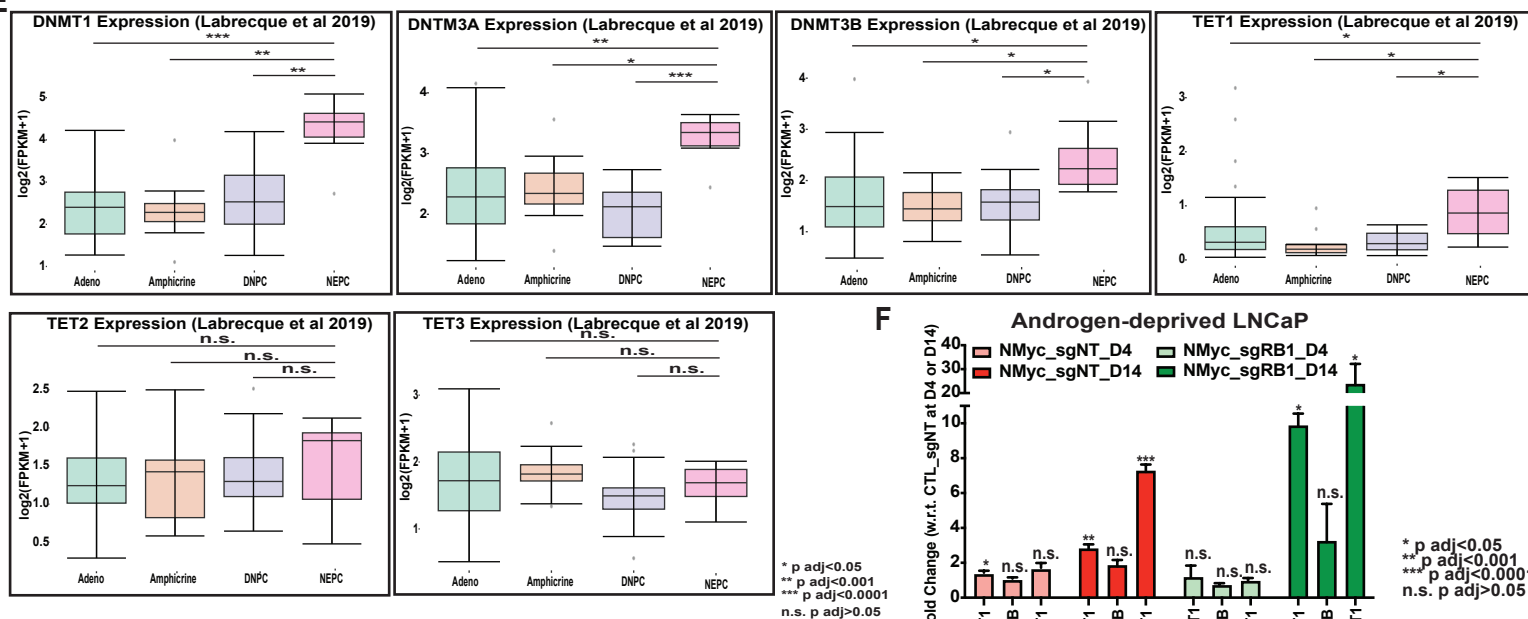


Figure S2



Figure S3

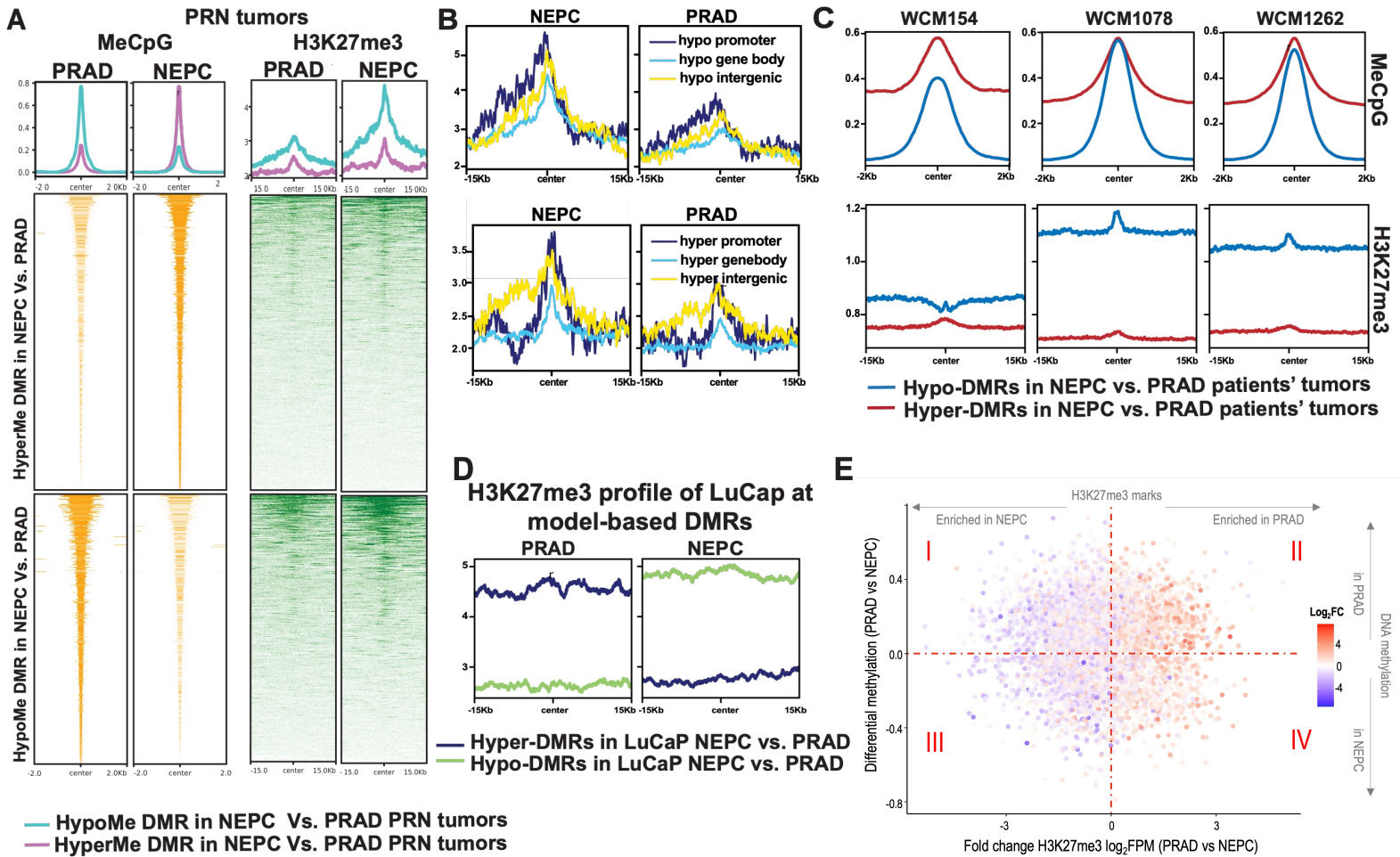


Figure S4

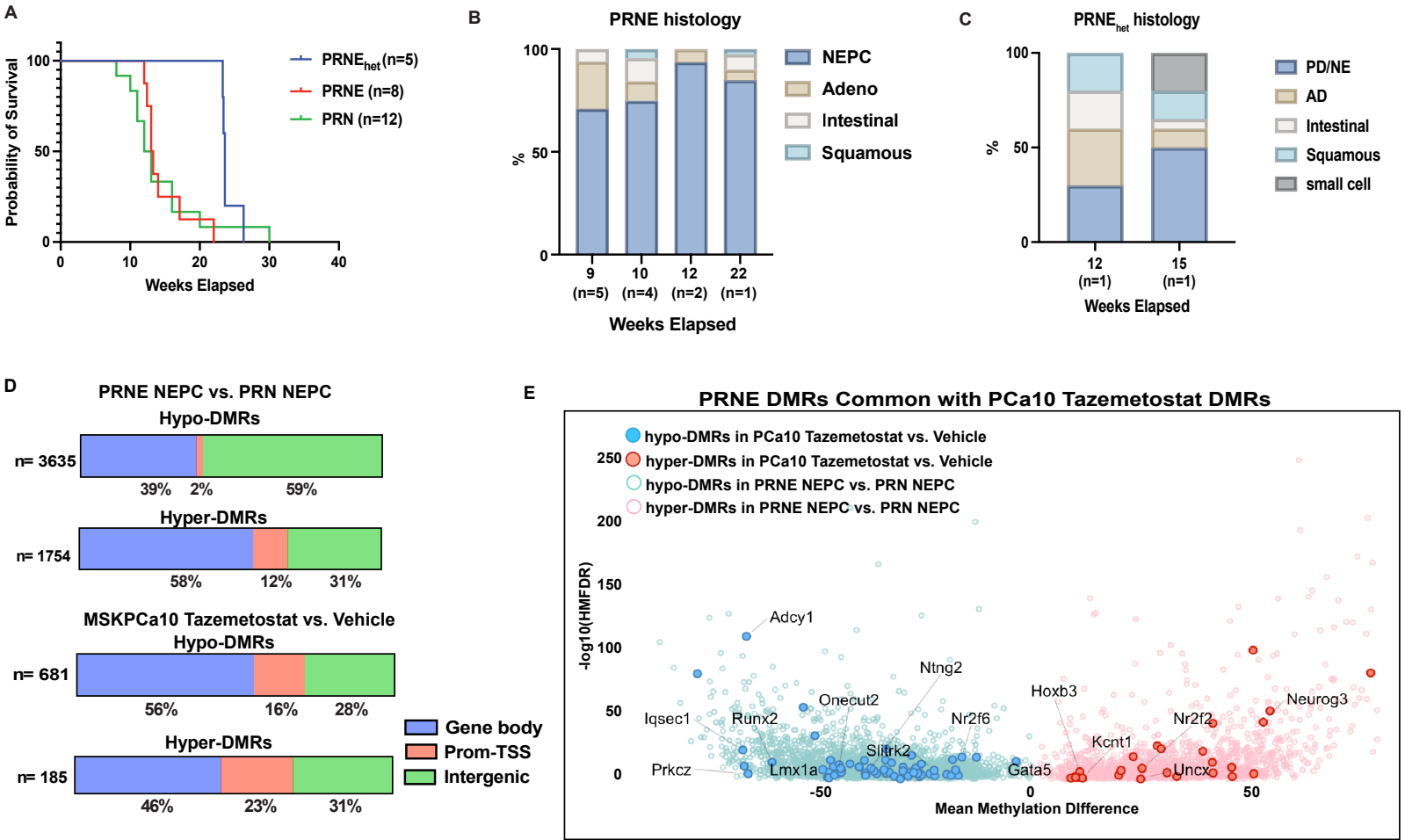
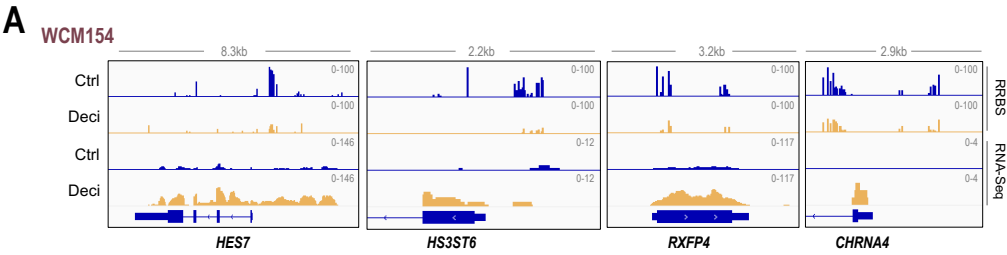
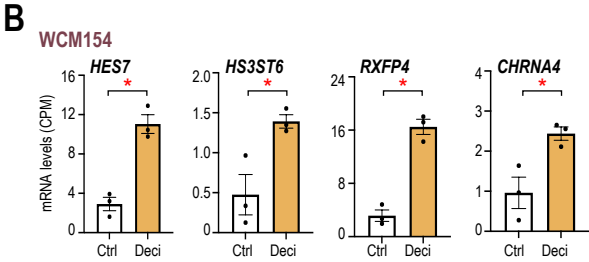


Figure S5

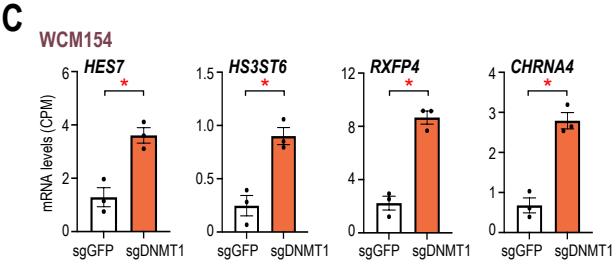
A



B



C



D

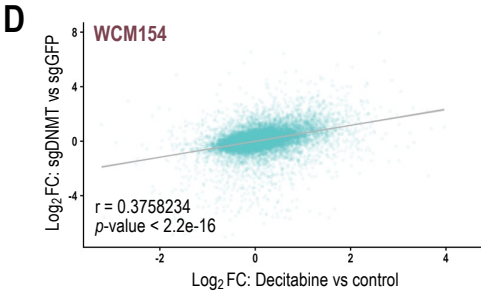


Figure S6

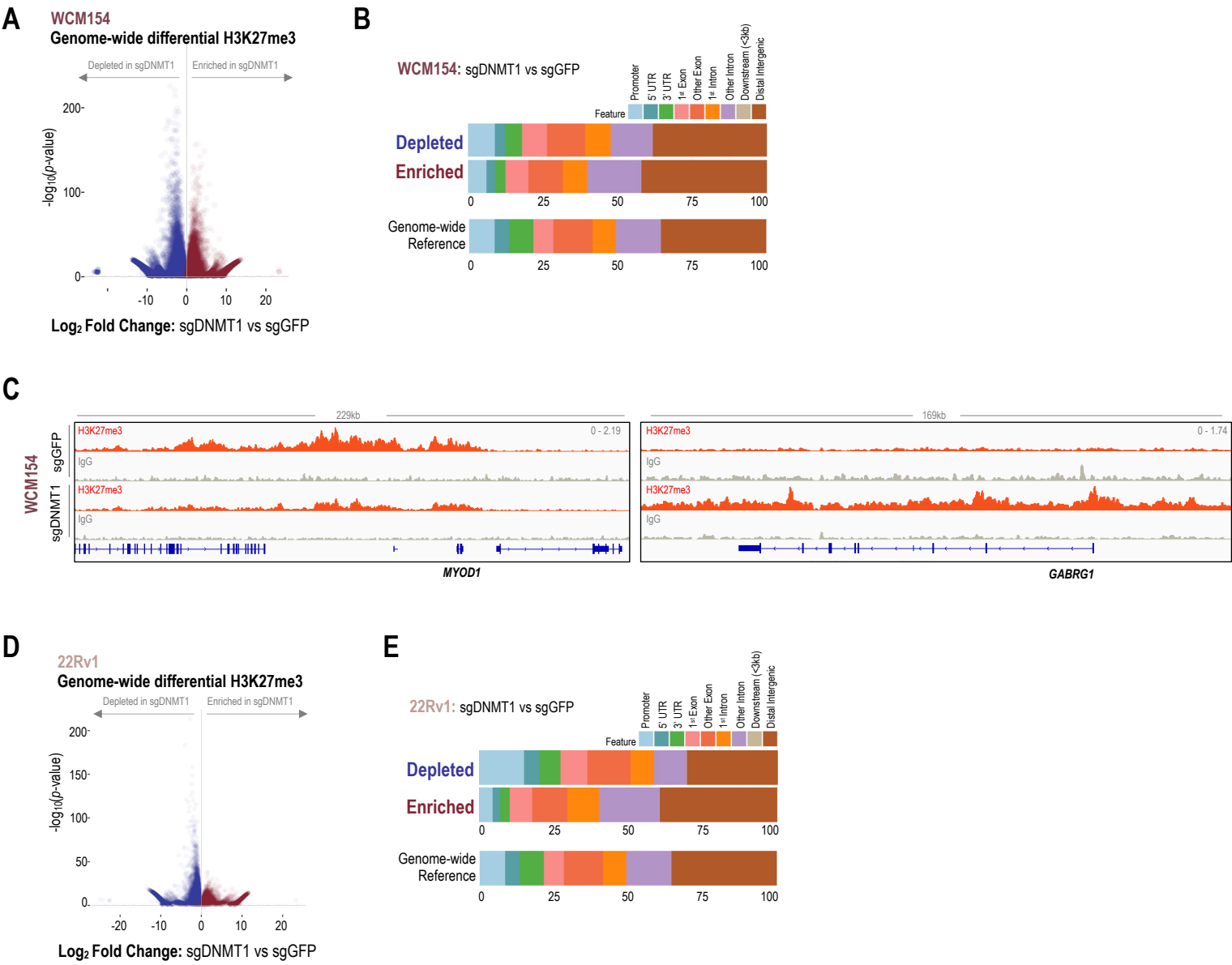


Figure S7

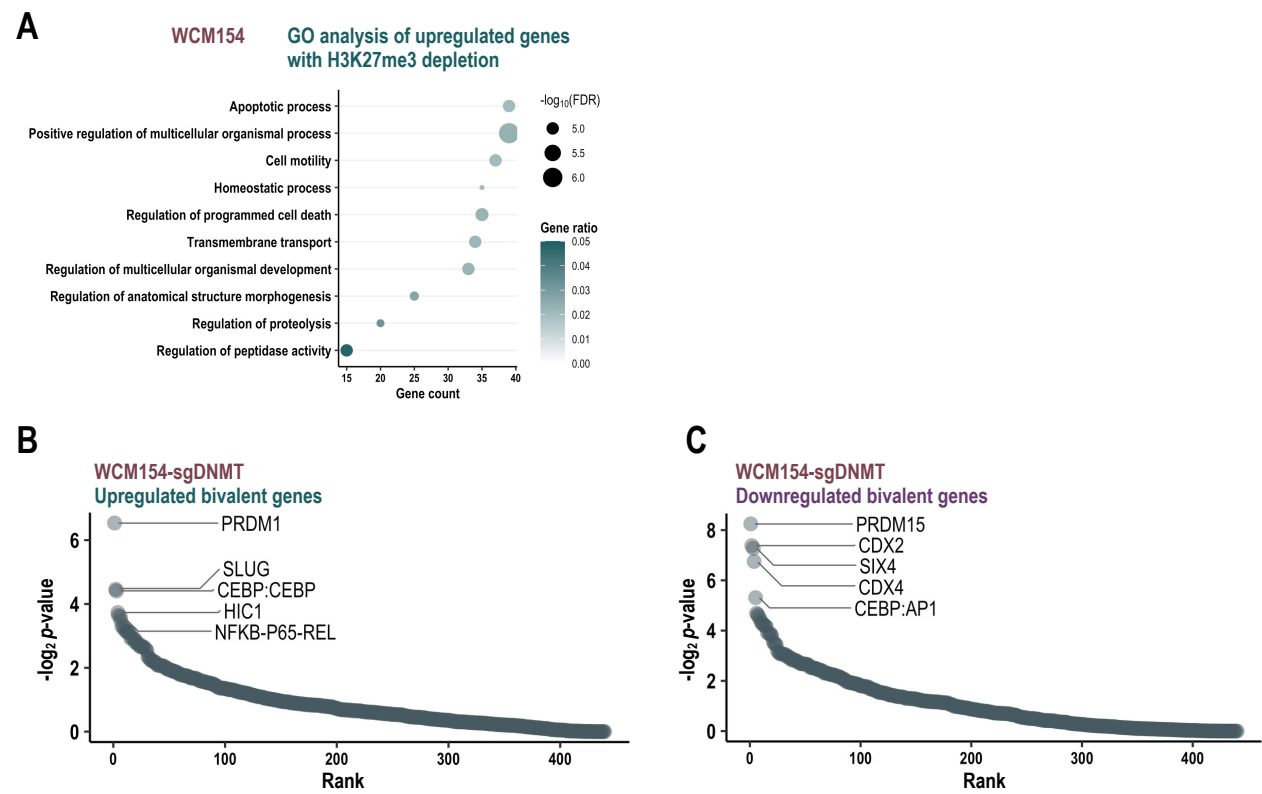
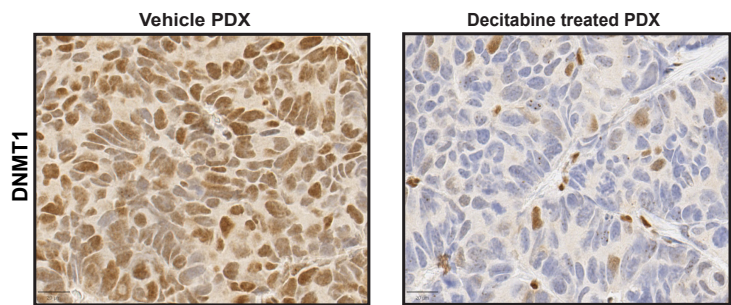


Figure S8

A



B

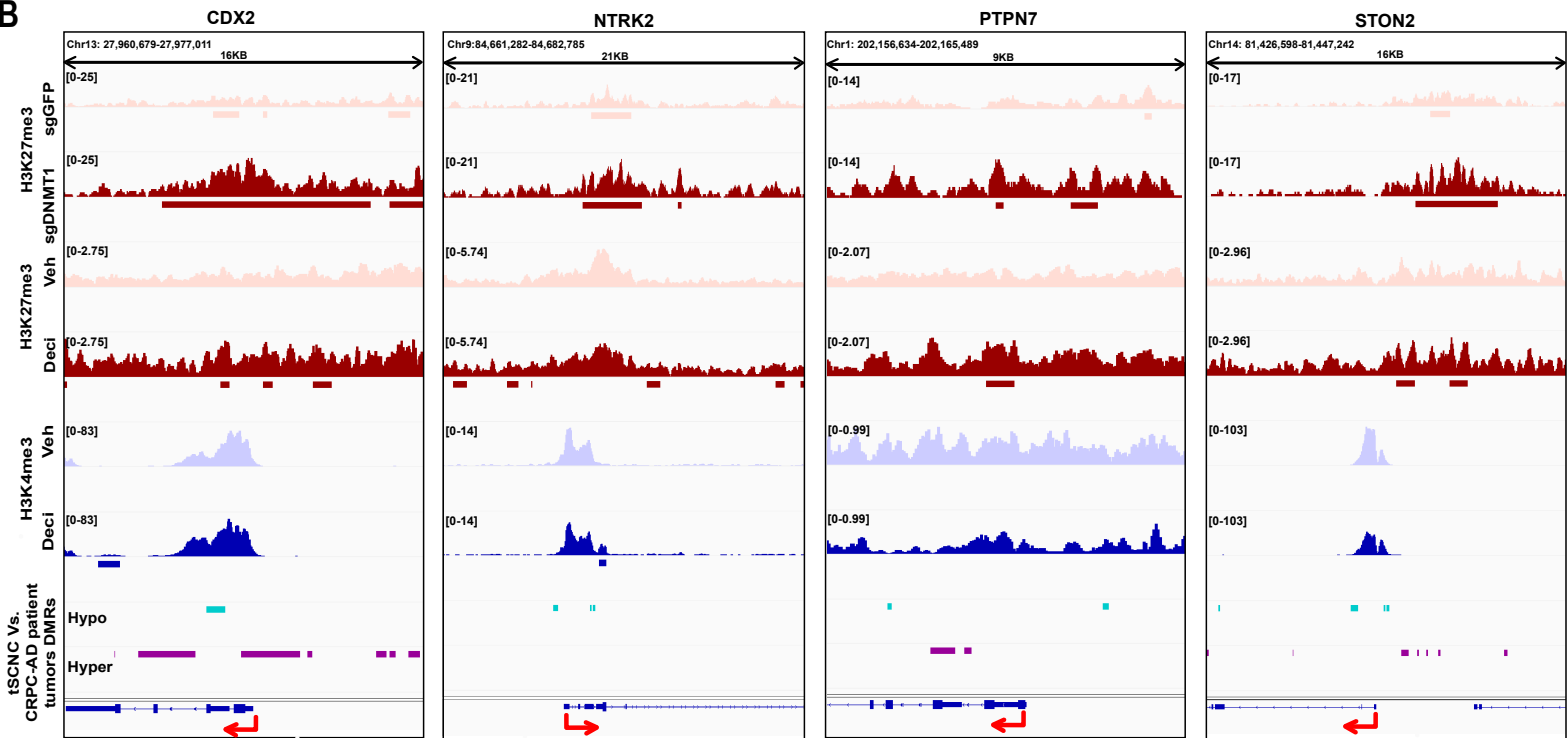
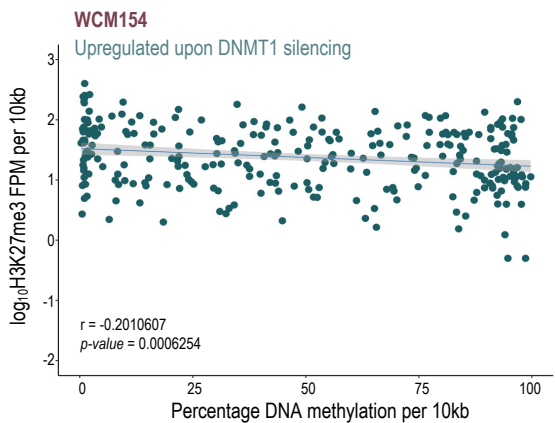
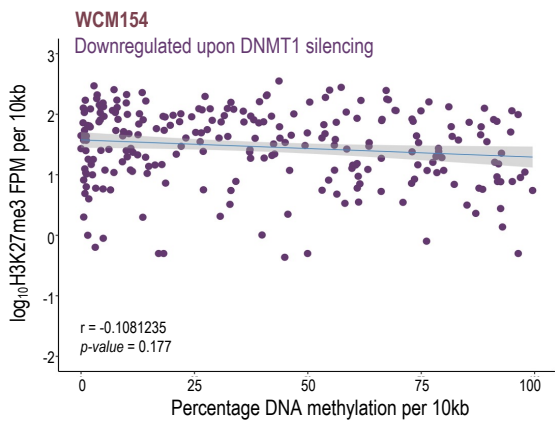
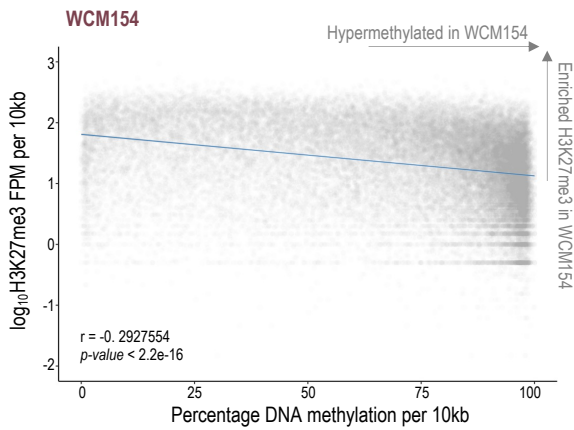


Figure S9

A



B

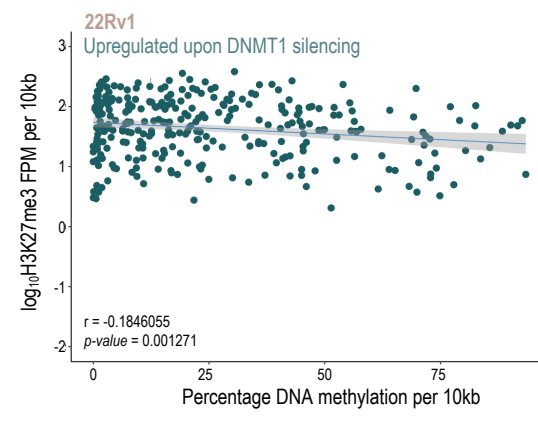
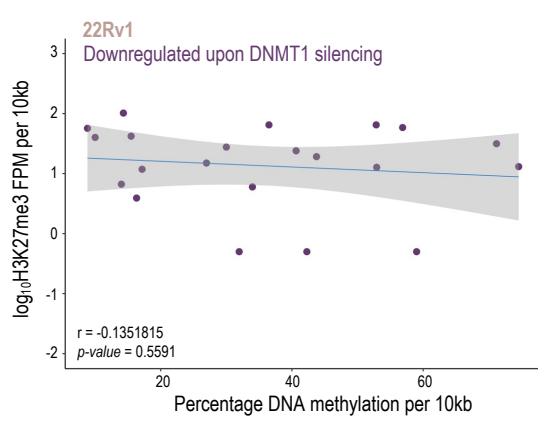
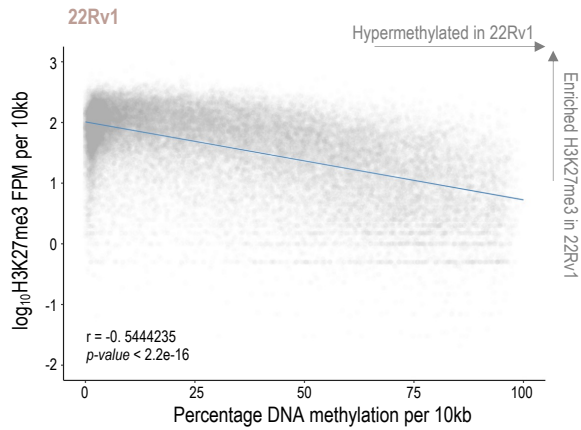


Figure S10

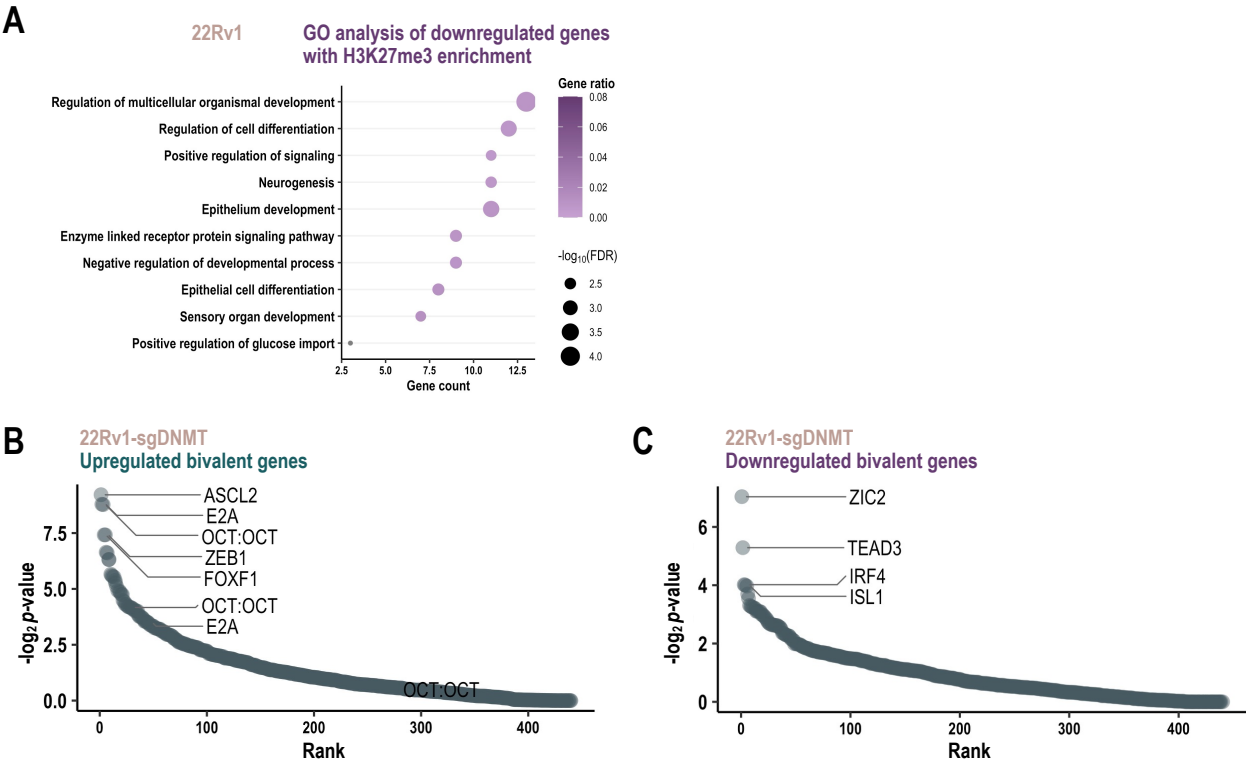
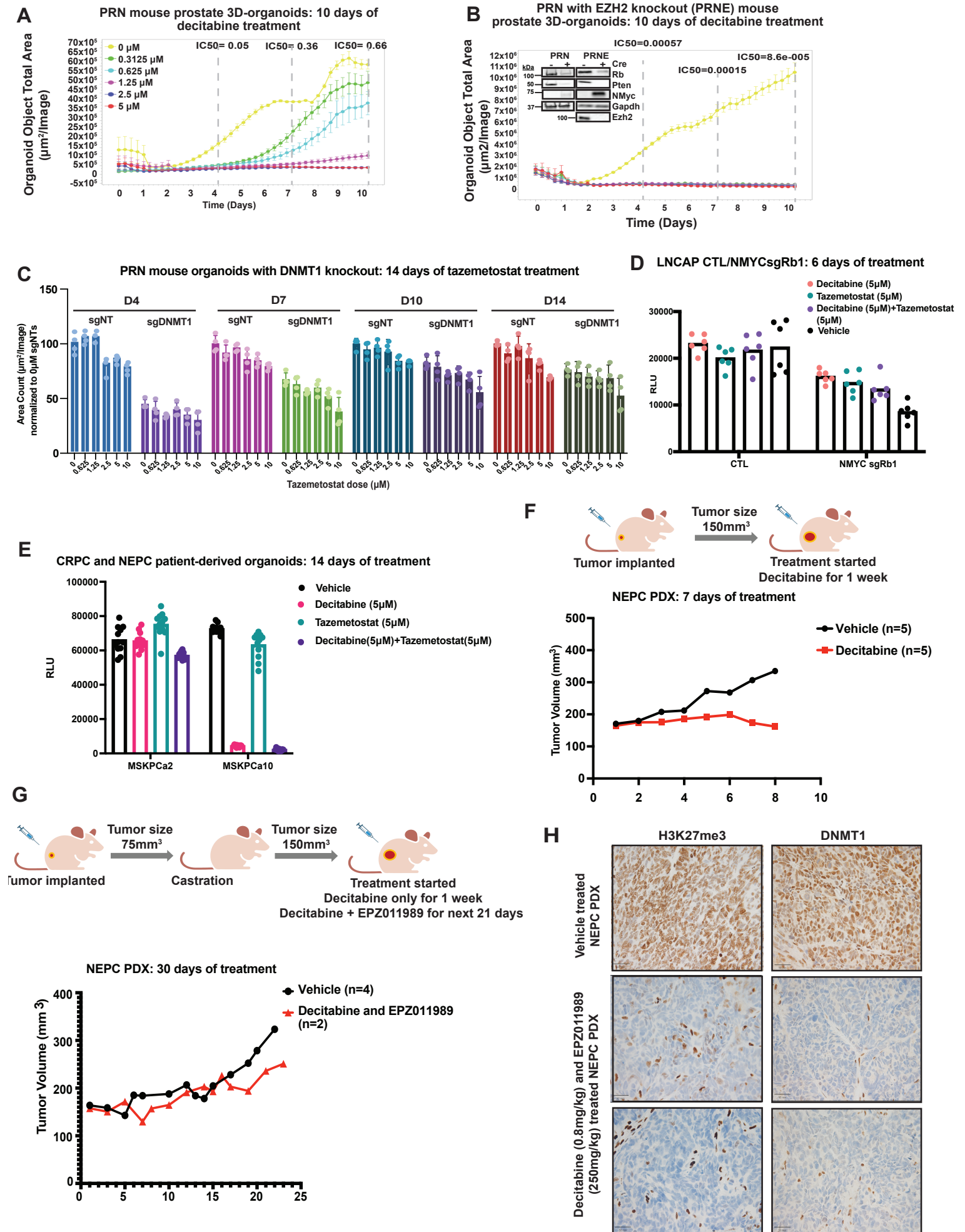
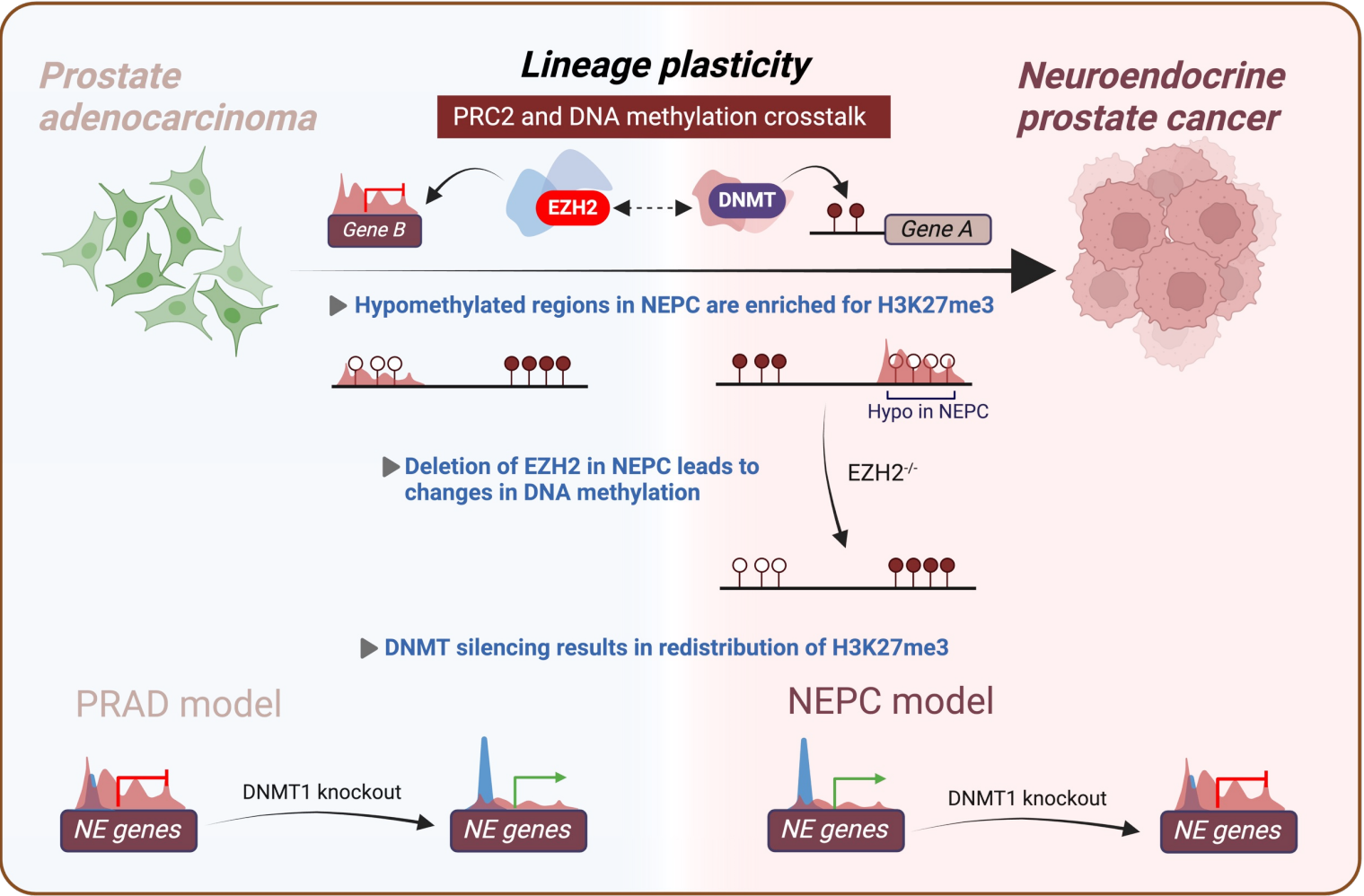


Figure S11



Graphical abstract



Supplementary Figure legends

Figure S1: Expression of DNA methylation regulators in advanced prostate cancer.

- A.** H&E and immunohistochemistry-staining for AR, DNMT1, DNMT3B, EZH2 and TET1 in AR-positive and AR-negative histologically confirmed NEPC primary prostate tumors from PRN murine model. Scale 20 μ M.
- B.** Expression (log2 FPKM) of EZH2 in PRAD and NEPC tumors of PRN mice (n=3 each).
- C.** Western blot of DNMT1 across patient-derived organoids- PRAD (WCM1358 and MSKPCa2) and NEPC (WCM1262, WCM1078, MSKPCa10, MSKPCa4 and WCM154).
- D.** Expression (row Z-score normalized TPMs) of DNMTs, and TETs in NEPC (MSKPCa10, MSKPCa4, MSKPCa24 and MSKPCa14) and PRAD (MSKPCa22, MSKPCa2 and MSKPCa19) patient-derived organoid lines.
- E.** Expression (log2 FPKM) of DNMTs and TETs in patient tumors. P value based on Wilcoxon test.
- F.** Expression (fold change) of *DNMT1/3B* and *TET1* genes in LNCaP N-Myc overexpressed and *RB1* knockout cells normalized to respective LNCaP control non-targeting lines after 4 and 14 days of androgen-deprivation. P value based on unpaired t-test. Distribution was checked using Kolmogorov-Smirnov test.

Figure S2: Clinical relevance of PRN-defined DMRs.

- A.** Bar plot showing genomic distribution of differentially hypo- (Hypo-DMR) and hyper- (Hyper-DMR) methylated regions in neuroendocrine prostate cancer (NEPC) foci (n=2) compared to adenocarcinoma (PRAD) foci (n=2) in the PRN murine model.
- B.** Ideogram showing distribution of PRN-defined hypo- and hyper-methylated DMRs across all chromosomes.

C. PCA analysis showing segregation of AR-negative and NEPC-positive clinical samples from AR-positive and NEPC-negative samples based on the methylation of genes associated with PRN-defined DMRs.

D. GSEA pathway analysis for DMRs at intergenic regions in PRN NEPC tumors (n=2) compared to PRN PRAD tumors (n=2). The False Discovery Rate (FDR) q-value was calculated using the default permutation-based method in GSEA.

E. GSEA pathway analysis for DMRs at intergenic regions in NEPC PDOs (n=3; MSKPCa10, MSKPCa4 and WCM154) compared to PRAD PDOs (n=2; WCM1358 and MSKPCa2) that are common with DMRs from patient CRPC tumors (NEPC and PRAD). The False Discovery Rate (FDR) q-value was calculated using the default permutation-based method in GSEA.

F. GSEA pathway analysis for DMRs at gene body in PRN NEPC tumors (n=2) compared to PRN PRAD tumors (n=2). The False Discovery Rate (FDR) q-value was calculated using the default permutation-based method in GSEA.

G. GSEA pathway analysis for DMRs at gene body in NEPC PDOs PDOs (n=3; MSKPCa10, MSKPCa4 and WCM154) compared to PRAD PDOs (n=2; WCM1358 and MSKPCa2) that are common with DMRs from patient CRPC tumors (NEPC and PRAD) from *Zhao et al.* The False Discovery Rate (FDR) q-value was calculated using the default permutation-based method in GSEA.

Figure S3: Model-specific H3K27me3 deposition in hypomethylated DMRs.

A. Cumulative methylation signal (“MeCpG”, left, yellow) and H3K27me3 deposition (right, green) in PRN NEPC (n=2) and PRAD (n=2) at differentially hypo- and hyper-methylated regions.

B. H3K27me3 deposition of NEPC (n=2) and PRAD tumors (n=2) of PRN mice at differentially hypo- and hyper-methylated regions of PRN NEPC tumors (n=2), separated based on genomic context (promoters, gene body and intergenic regions).

C. Methylation signal (top) and H3K27me3 deposition (bottom) from each NEPC patient-derived organoid, at hypo- and hyper-methylated regions from CRPC patient tumors (NEPC and PRAD).

D. Cumulative H3K27me3 deposition in AR-negative NEPC (n=5) and AR-positive PRAD (n=5) LuCaP patient-derived xenografts at LuCaP-defined DMRs of NEPC (AR-negative NE-positive) versus PRAD (AR-positive NE-negative) PDXs.

E. Scatter plot with differentially enriched 10kb genome-wide bins of H3K27me3 log₂FPM values in NEPC (n=9) and castration resistant PRAD (n=9) samples on the x-axis¹ and fold change of DNA methylation of castration resistant PRAD (n=95) and NEPC (n=5) on the y-axis²; Gene expression (transcript per million log₂FoldChange for NEPC (n=8) vs PRAD (n=9))¹ is represented as a gradient of *blue to red*. *Red* indicates induction in NEPC, and *blue* indicates downregulation in NEPC. Genes with less than 0.25 absolute fold change were removed.

Figure S4: Description of the PRNE model.

A. Overall survival of PRNE mice (n=8) compared to PRN mice (n=12) and PRNE-heterozygous (PRNE_{het}) mice (n=5) using the Kaplan-Meier estimator.

B. Histology distribution of primary tumors of PRNE mice at 9, 10, 12 and 22 weeks of age.

C. Histology distribution of primary tumors of PRNE_{het} mice at 12 and 15 weeks of age.

D. Bar plot showing genomic distribution of differentially hypo- and hyper-methylated regions of NEPC tumors of PRNE (n=2) compared to NEPC tumors of PRN mice (n=2) and tazemetostat (EZH2i)-treated MSKPCa10 NEPC organoids compared to vehicle-treated controls.

E. Volcano plot showing differentially methylated regions (DMRs) in PRNE NEPC tumors (n=2) compared to PRN NEPC tumors (n=2) highlighting the similarly altered DMR-associated genes in tazemetostat (EZH2i)-treated MSKPCa10 NEPC organoids compared to vehicle-treated controls. Hypomethylated neuron-related genes and hypermethylated H3K27me3 target genes are labeled.

Figure S5: Validation of DNMT inhibition or silencing on DNA methylation and gene expression.

A. Screenshots from Integrative Genomics Viewer (IGV) of RRBS data indicating methylated CpG levels in WCM154 treated with vehicle (Ctrl) or Decitabine at the genomic loci of indicated genes and forward transcription levels from parallel RNA-Seq data; blue, Ctrl; yellow, Decitabine.

B. WCM154 treated with vehicle (Ctrl) or decitabine control were processed for RNA-Seq. mRNA levels of indicated genes represented as CPM values normalized to library sizes. Columns, means of values obtained from 3 independent biological replicates; white, Ctrl; yellow, Decitabine; bars, SEM values; *p-value < 0.05, all statistical analyses used Wilcoxon tests.

C. WCM154-sgGFP or sgDNMT1 were processed for RNA-Seq. mRNA levels of indicated genes represented as CPM values normalized to library sizes. Columns, means of values obtained from 3 independent biological replicates; white, sgGFP; orange, sgDNMT1; bars, SEM values; *p-value < 0.05, all statistical analyses used Wilcoxon tests.

D. Pearson's product-moment correlation of Log2FoldChange in mRNA levels of decitabine treated samples (n=3; x-axis) vs sgDNMT1 (n=3; y-axis) in WCM154; teal, individual genes.

Figure S6: Redistribution of H3K27me3 upon DNMT1 silencing in prostate cancer models.

A. Volcano plot with differentially enriched 10kb bins of H3K27me3 FPM values in WCM154 sgDNMT1 or sgGFP; *blue*, H3K27me3 regions depleted upon DNMT1 silencing; *red*, H3K27me3 regions enriched upon DNMT1 silencing.

B. Bar plot showing distribution of subtype specific H3K27me3 depleted or enriched upon DNMT1 silencing in WCM154; *light blue*, promoter; *blue*, 5' untranslated regions (UTR); *green*, 3' UTR;

pale pink, 1st exon; *coral*, other exon; *orange*, 1st exon; *violet*, other intron; *gray*, downstream (< 3kb); *brown*, distal intergenic.

C. Screenshots from Integrative Genomics Viewer of CUT&RUN of IgG or H3K27me3 levels in WCM154 sgGFP and sgDNMT1 at the genomic loci of indicated genes; *gray*, IgG; *orange*, H3K27me3.

D. Volcano plot with differentially enriched 10kb bins of H3K27me3 FPM values in 22Rv1 sgDNMT1 or sgGFP; *blue*, H3K27me3 regions depleted upon DNMT1 silencing; *red*, H3K27me3 regions enriched upon DNMT1 silencing.

E. Bar plot showing distribution of subtype specific H3K27me3 depleted or enriched upon DNMT1 silencing in 22Rv1; *light blue*, promoter; *blue*, 5' untranslated regions (UTR); *green*, 3' UTR; *pale pink*, 1st exon; *coral*, other exon; *orange*, 1st exon; *violet*, other intron; *gray*, downstream (< 3kb); *brown*, distal intergenic.

Figure S7: Analyses of differentially regulated genes upon DNMT1 silencing in WCM154.

A. Gene ontology analysis of genes upregulated with H3K27me3 enrichment in WCM154 upon DNMT1 silencing with *p-value* < 0.05 and Log₂FoldChange > 0; *size*, -log₁₀FDR; *green-black gradient*, gene ratio.

B-C. HOMER analysis was performed setting the promoter region as 1kb upstream or downstream from TSS of differentially regulated bivalent genes in WCM154 upon DNMT1 silencing and top significantly enriched transcription factor motifs are labelled.

Figure S8: Confirmation of DNMT inhibition upon decitabine treatment.

A. Immunohistochemistry showing reduced DNMT1 expression in a representative decitabine treated NEPC xenograft compared to vehicle treated tumor. Scale 20 μ M.

B. IGV tracks showing H3K27me3, H3K4me3 and patient tumor DMRs at *CDX2*, *NTRK2*, *PTPN7*, and *STON2*.

Figure S9: Relationship between H3K27me3 and DNA methylation at baseline in prostate cancer models highlighting differentially expressed genes after DNMT1 silencing.

Scatter plots with 10kb genome-wide bins of H3K27me3 log₂FPM values in WCM154 (**A**) or 22Rv1 (**B**) samples on the x-axis and percent DNA methylation for each 10 kb bin in y-axis; *purple*, promoters that are downregulated in Figures 4 and 5; *green*, promoters that are upregulated in Figures 4 and 5; *r* value corresponding *p*-value calculated from Pearson's product-moment correlation analysis; *gray line*, linear regression; *gray band*, standard error of regression.

Figure S10: Analyses of differentially regulated genes upon DNMT1 silencing in 22Rv1.

A. Gene ontology analysis of genes downregulated with H3K27me3 enrichment in 22Rv1 upon DNMT1 silencing with *p*-value < 0.05 and Log₂FoldChange < 0; *size*, -log₁₀FDR; *violet-black gradient*, gene ratio.

B-C. HOMER analysis was performed setting the promoter region as 1kb upstream or downstream from TSS of differentially regulated bivalent genes in 22Rv1 upon DNMT1 silencing and top significantly enriched transcription factor motifs are labelled.

Figure S11: Co-targeting of DNMT and EZH2 *in vitro* and *in vivo*.

A. Growth response of Adeno Cre-transduced PRN prostate organoids to varying doses of decitabine for 10 days in vitro was studied using incucyte. IC₅₀ values were calculated based on confluence (object total area) at 4, 7 and 10 days of treatment.

B. Growth response of Adeno Cre-transduced PRNE prostate organoids to varying doses of decitabine for 10 days in vitro was studied using incucyte. IC₅₀ values were calculated based on confluence (object total area) at 4, 7 and 10 days of treatment. Inset shows the protein levels of alleles deleted/induced by in vitro adenoviral Cre transduction in PRN and PRNE organoids.

C. Growth response of Adeno Cre-transduced PRN prostate organoids with DNMT1 knockout/non-targeting controls to varying doses of tazemetostat for 14 days in vitro was studied using incucyte.

D. Cell viability of LNCaP cells with *Rb1* deletion and *MYCN* overexpression compared to LNCaP control cells after treating with decitabine (5μM) and/or tazemetostat (5μM) for 6 days was assessed using Cell Titer Glo assay. Vehicle controls were treated with equal volume of DMSO.

E. Cell viability of patient-derived CRPC (MSKPCa2) and NEPC (MSKPCa10) organoids after treating with decitabine (5μM) and/or tazemetostat (5μM) for 14 days was assessed using Cell Titer Glo assay. Vehicle controls were treated with equal volume of DMSO.

F. Schematic shows sub-cutaneously implanted NEPC xenografts (PDXs) were treated with decitabine (0.8mg/kg) once daily for 7 days by intraperitoneal injection. Figure generated using BioRender. Control mice were treated with equal volume of vehicle. Tumor volume was assessed every day using caliper measurements for both vehicle (n=5) and decitabine (n=5) treated mice. Control mice were treated with equal volume of vehicle.

G. Schematic shows sub-cutaneously implanted NEPC xenografts (PDXs) were castrated when tumor size reached 75mm³ and treated with decitabine (0.8mg/kg) and EPZ011989 (250mg/kg), an EZH2 inhibitor when tumor size reached 150mm³. Figure generated using BioRender. Decitabine was administered once daily for a month by intraperitoneal injection while EPZ011989

was administered twice daily starting at day 8 for 21 days by oral gavage. Control mice were treated with equal volume of vehicle. Tumor volume was assessed every other day using caliper measurements for both vehicle (n=4) and decitabine+EPZ011989 (n=2) treated mice.

H. Immunohistochemistry showing reduced DNMT1 expression and H3K27me3 in a decitabine and EPZ011989 treated NEPC xenograft tumors compared to a representative vehicle treated tumor. Scale 50mm.

References cited

1. Venkadakrishnan VB, Presser AG, Singh R, Booker MA, Traphagen NA, Weng K, Voss NCE, Mahadevan NR, Mizuno K, Puca L, Idahor O, Ku SY, Bakht MK, Borah AA, Herbert ZT, Tolstorukov MY, Barbie DA, Rickman DS, Brown M, and Beltran H.
Lineage-specific canonical and non-canonical activity of EZH2 in advanced prostate cancer subtypes.
Nat Commun. 2024;15(1):6779. doi: 10.1038/s41467-024-51156-5.
url: <https://www.ncbi.nlm.nih.gov/pubmed/39117665>.
2. Zhao SG, Chen WS, Li H, Foye A, Zhang M, Sjostrom M, Aggarwal R, Playdle D, Liao A, Alumkal JJ, Das R, Chou J, Hua JT, Barnard TJ, Bailey AM, Chow ED, Perry MD, Dang HX, Yang R, Moussavi-Baygi R, Zhang L, Alshalalfa M, Laura Chang S, Houlahan KE, Shiah YJ, Beer TM, Thomas G, Chi KN, Gleave M, Zoubeidi A, Reiter RE, Rettig MB, Witte O, Yvonne Kim M, Fong L, Spratt DE, Morgan TM, Bose R, Huang FW, Li H, Chesner L, Shenoy T, Goodarzi H, Asangani IA, Sandhu S, Lang JM, Mahajan NP, Lara PN, Evans CP, Febbo P, Batzoglou S, Knudsen KE, He HH, Huang J, Zwart W, Costello JF, Luo J, Tomlins SA, Wyatt AW, Dehm SM, Ashworth A, Gilbert LA, Boutros PC, Farh K, Chinnaiyan AM, Maher CA, Small EJ, Quigley DA, and Feng FY.
The DNA methylation landscape of advanced prostate cancer.
Nat Genet. 2020;52(8):778-89. doi: 10.1038/s41588-020-0648-8.
url: <https://www.ncbi.nlm.nih.gov/pubmed/32661416>.

Table S1: Antibody list

Protein	Vendor	Catalog	Experiment
DNMT1	Cell Signalling Technology	5032S	WB/IHC
EZH2	Cell Signalling Technology	5246S	WB/IHC
H3K27me3	Cell Signalling Technology	9733S	WB
H3K27me3	Abcam	ab6002	ChIP
H3K4me3	Millipore-Sigma	17-614	ChIP
Alpha tubulin	Abcam	ab52866	WB
Vinculin	Abcam	ab129002	WB
TET1	Genetex	GTX125888	IHC
DNMT3B	Novus Biologicals	NB300-516	IHC
H3K4me3	Epicypther	13-0041	CUT&RUN
H3K27me3	Invitrogen	MA511198	CUT&RUN

Table S2: Oligonucleotide sequences

Gene	Oligos	5'>3'
EZH2	guide RNA 1	CGGAAATCTTAAACCAAGAA
	guide RNA 2	ACCAAGAATGGAAACAGCGA
	guide RNA 3	ACAGAAGTCAGGATGTGCAC
non-targeting control	guide RNA 1	GCACTACCAGAGCTAACTCA
	guide RNA 2	GTACGTCGGTATAACT
Rb1	guide RNA 1	AAGTGAACGACATCTCATCT
	guide RNA 2	AGATGAGATGTCGTTCACTT