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Supplementary information

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SUMOylation enhances DNMT1 function to repress mega-intergenic RNAs and viral mimicry

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Supplementary Information

Figure Legends/Captions (for Extended Data)

Extended Data Figure 1. Cellular phenotyping and assessment of DNA hypomethylation of DNMT1i treatment.
a) Cumulative population doublings of MV-4-11 cells treated with DNMT1i. Flow cytometry analysis of b) viability using AnnexinV and 7AAD and c) cell cycle using propidium Iodide (PI) in MV-4-11 cells treated with DNMT1i for 7 days. d) Scatter plots depicting average methylation over 500-bp bins from RRBS data of MV-4-11 cells (left) and MCF7 cells (right). e) Characterization of DNA methylation changes induced by DNMT1 inhibition. Left, density plot showing the distribution of net methylation change across MV-4-11 cells treated with 100nM DNMT1 inhibitor (DNMT1i) for 7 days relative to DMSO control. Net methylation change (%) quantifies the difference in DNA methylation between DNMT1i-treated and control samples across corresponding genomic bins (b), where the methylation fraction is defined as the proportion of methylated cytosines (5mC) relative to total cytosine counts (5mC + C). The summation term (Σ) indicates that counts are aggregated across all CpG sites within each bin (b). The density distribution was partitioned into three categories for downstream analysis: marked methylation loss (90-100%), average methylation loss (45-55%), and minimal change (−5 to +5%). Bottom, insets display the proportional composition of genomic features within each category, illustrating contributions from promoters, exons, introns, untranslated regions (UTRs), and distal intergenic elements. Right, box-and-whisker plots show the steady-state methylation levels (DMSO condition) of each category, calculated as the average proportion of methylated cytosines per bin. Regions showing minimal change (blue) exhibit low baseline methylation and are disproportionally promoter regions, whereas regions undergoing marked loss (red) correspond to highly methylated loci under steady-state conditions. Collectively, loci that undergo pronounced DNMT1i-dependent demethylation are those with high initial methylation and diverse genomic annotations, spanning promoter-proximal to distal intergenic regions. Box-and-whisker plots show the median (centre line), interquartile range (box; 25th-75th percentiles), and whiskers extending to 1.5× the interquartile range (IQR); points beyond this range are plotted as outliers.

Extended Data Figure 2. DNMT1 redistributes to silenced genes following DNA hypomethylation.
a-e) RNA-seq and ChIP-seq from MV-4-11 cells treated with 100nM DNMT1i for 7 days. a) Heatmaps of normalised transcriptome (left) and H3K27ac ChIP-seq (right) over genomic regions that were kmeans clustered by DNMT1 binding. b) Heatmaps of DNMT1 ChIP-seq. Q1-Q4 represent quartiles ranked by transcription within vehicle cells, with Q1 being the lowest expressed genes. c) Statistics of genomic features at DNMT1 binding sites. d) DNMT1 binding by genomic features as a total (top) and proportion (bottom). e) Gene ontology biological process enriched in cluster 1 (left) and cluster 2 (right) genes. Raw p-values are displayed from Fisher's exact test (one-sided).

Extended Data Figure 3. PxP Assay, chromatin-loading of DNMT1 and generation of dTAG-DNMT1 cells.
a) Representative immunoblot from the Purification of X-linked Proteins (PxP) assay showing DNA-protein crosslink (DPC) formation induced by 5-aza-2′-deoxycytidine (5-aza-dC) and formaldehyde. b) Schematic depiction of the CRISPR-Cas9-HDR strategy used to generate dTAG-DNMT1 cells. The donor construct contains ~100 bp homology arms flanking an insertion cassette comprising mScarlet3, a dual P2A-T2A sequence for efficient ribosomal skipping, a V5 epitope tag, and a flexible linker. The donor template was delivered via AAV infection, and the Cas9 RNP complex was electroporated using the Neon NxT system (Thermo Fisher). c) Immunoblot analysis of lysates from three independent clones homozygous for the dTAG knock-in, demonstrating complete degradation of DNMT1 following treatment with dTAG ligand (500nM, 18 h). d) Schematic of the depletion-reconstitution workflow. dTAG-DNMT1 cells were stably transduced with lentiviral constructs encoding eGFP-tagged DNMT1 wild-type or a catalytic-dead (C1226W) mutant. Upon dTAGV-1 treatment, endogenous DNMT1 is rapidly degraded, leaving only the ectopically expressed eGFP-DNMT1 variant. e) Immunoblot confirming expression of endogenous and ectopic DNMT1 proteins in the depletion-reconstitution system. dTAGV-1 treatment for 24h. f) CellTiter-Glo viability assay showing relative cell growth after 7 days of the indicated treatments. UTD = untransduced. g) Dot blot analysis of ChIP-derived chromatin demonstrating that the DNMT1 C1226W mutant lacks catalytic methyltransferase activity.

Extended Data Figure 4. Enrichment of RNF4-deficiency following DNMT1i treatment
a) Rank plots from genome wide CRISPR screen performed in MV-4-11 cells. Doses and time points are indicated. Data depicted has been filtered for genes that are expressed in MV-4-11 cells based on our RNA-seq data. b) Reanalysis of CRISPR screen data from previously published data12, which depicts gRNA enrichment in azacitidine and decitabine treated OCI-AML2 cells. c) IC50 assay of RNF4WT (gNT, black) and RNF4KO (gRNF4, red) MV-4-11 cells treated with azacitidine (left) or decitabine (right) for 10 days. Data are mean±SD, sigmoidal four parameter logistic curve, n=4 biological replicates. d) Immunoblot confirming successful targeting of RNF4 with sgRNA used throughout study. e) Schematic of competition assay. gNT cells were marked with BFP and gRNF4 cells were marked with GFP, then mixed at a 4:1 ratio prior to treatment. f) Competition assay. RNF4KO cells (gRNF4, labelled with GFP) were seeded at a 1:2 to 1:4 ratio with RNF4WT cells (gNT, labelled with BFP) and treated with DNMT1i at indicated time and doses. Frequency depicts proportion of indicated genotype per condition. Data are mean±SD, n=3 biological replicates. g) cell counts of MCF7 (left) and HEK293T (right) cells treated with DNMT1i for 10 days. Data are mean±SD, n=3 biological replicates. h) Analysis of flow cytometry data depicting the proportion of dead (AnnexinV+PI+) MV-4-11 cells after 10 days of treatment with 1 μM and 300nM DNMT1i respectively. Data are mean±SD, n=3 biological replicates.

Extended Data Figure 5. DNMT1i induces SUMOylation of DNMT1

a) Immunoblot of DNMT1 and VINCULIN loading control from lysates of HEK293T cells expressing exogenous wild type (WT), codon optimised (CO), SUMO interacting motif mutant (mSIM), and catalytic mutant (mCAT2 and mCAT3) RNF4. Endogenous RNF4 was depleted via CRISPR/Cas9. Cells were treated with 300nM DNMT1i for 3 days.

b) DNMT1 was immunoprecipitated after the indicated treatments and sequentially digested with LysC and AspN to improve sequence coverage, particularly around lysine residues that are sterically protected when SUMOylated. c) SUMOylated peptides were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) using remnant-based detection and precursor ion mass shifts indicative of SUMO modification. MS/MS spectrum of DNMT1 peptide modified at Lysine-194 (K194, top) and Lysine-1609 (K1609, bottom) by SUMO2/3, post LysC and ASP-N digestion. Maxquant Andromeda search engine identification scores are also shown. SUMO remnant fragment ions (blue) and DNMT fragment ions (red) are highlighted (+P indicating SUMO containing DNTM1 peptide) and confirming the sequence assignments. d) DNMT1 immunoprecipitation (IP) from lysates of HEK293T treated with 300nM DNMT1i for 3 days. DNMT1, SUMO2/3, and HSP60 antibodies were used to probe immunoblots of IP and input products.

Extended Data Figure 6. SUMOylation does not impact homeostatic functions of DNMT1.

a) Collective summary of SUMOylation sites identified in this study and by Hendriks et al. (2018)22. b) Extracted ion chromatograms (XICs) and LC-MS/MS spectra confirming SUMO2/3 modification of DNMT1 at Lysine-1609 (K1609). DNMT1 was immunoprecipitated from MV-4-11 cells expressing DNMT1WT or the SUMO-deficient DNMT110K→R mutant following the indicated treatments and subjected to LC-MS/MS analysis. XIC traces demonstrate enrichment of the SUMOylated K1609-containing peptide specifically in DNMT1WT cells following DNMT1i treatment and loss of the corresponding signal in DNMT110K→R cells. Representative MS/MS spectrum of the SUMO2/3-modified K1609 peptide is shown. c) Cell counts of HEK293T cells expressing exogenous V5-DNMT1WT or V5- DNMT110K→R treated with 300nM DNMT1i for 10 days. Data are mean±SD, n=4 biological replicates. d) Immunoblot using indicated antibodies on lysates from HEK293T cells expressing exogenous V5 (empty vector), V5-DNMT1WT, or V5-DNMT110K→R treated with 300nM DNMT1i for 3 days. e) Schematic of the depletion-reconstitution workflow. dTAG-DNMT1 cells were stably transduced with lentiviral constructs encoding eGFP-tagged DNMT1WT (WT) or DNMT110K→R (10KR) mutant. Upon dTAGV-1 treatment, endogenous DNMT1 is rapidly degraded, leaving only the ectopically expressed eGFP-DNMT1 10KR variant. f) Cells are chased over a period of 7 days and subject to CellTitre-Glo assay to approximate growth (n=3 independent experiments).

Extended Data Figure 7. Discovery of mintRNAs driven from TINATs.
a) Immunofluorescence analysis of MCF7 cells treated with DMSO vehicle or 1 μM DNMT1i for 7 days with J2 monoclonal antibody (red), which detects dsRNA. Nuclei are stained with DAPI (blue). b-d) RNA-seq and ATAC-seq data from RNF4WT and RNF4KO MV-4-11 cells treated with 100nM DNMTi for 7 days. b) Violin plots quantitation depicting genes upregulated by DNMT1i treatment across the coding genome (left) and non-annotated genome (right). c-d) Heatmaps of c) ATAC-seq, d) H3K27me3 ChIP-seq, and e) RNA-seq read density over LTRs that are significantly enriched ($p<0.05$) in RNF4WT 100nM DNMT1i treated samples relative to DMSO control for both ATAC-seq and RNA-seq (360 LTRs in total). Heatmaps are ranked in the same order based on RNF4WT 100nM DNMT1i RNA-seq data with highest expressed at the top. d) Genome browser viewer generated by SparK depicting a representative mintRNA and its predicted secondary structure generated by RNA-fold. Sequence that was inputted to RNA-fold is highlighted in red. Coordinates for RNA-fold region are chr15:70,345,254-70,352,092. Genome browser views showing Oxford Nanopore Technologies (ONT) long-read cDNA coverage across representative (e) chromosome 8 and (f) chromosome 15 mintRNA loci, with genomic coordinates as indicated. Left, coverage (counts per million, CPM) in MV-4-11 cells treated with DMSO or 100nM DNMT1i for 7 days. Right, read-quality control plots showing percent identity versus aligned read length, read mapping quality versus read length, and average read quality versus read length for each dataset. The increased long-read coverage and contiguously mapped transcripts observed upon DNMT1 inhibition support the presence of de novo, full-length mintRNA species.

Extended Data Figure 8. Unbiased characterisation of endogenous DNMT1 interactome.
IP-MS analysis of MCF7 cell treated with 1 μM DNMT1i for 3 days. Volcano plots depict enrichment in each sample relative to IgG control. FDR <0.05 indicated by horizontal grey line and fold enrichment >50% indicated by vertical grey lines.

Extended Data Figure 9. Viral mimicry is not responsible for DNA hypomethylation-induced cancer cell death whilst knockdown of viral RNA sensors abolishes viral mimicry without affecting mintRNA expression.
a) Competition assay. ADARKO MV-4-11 cells (gADAR, blue) were seeded at a 1:3 ratio with ADARWT cells (gNT, grey) and treated with DNMT1i at indicated time and doses. Frequency depicts proportion of indicated genotype per condition. Each graph depicts two independent experiments with two separate gRNAs. Data are mean±SD, n=3 biological replicates per experiment. b) Western blot analysis of ADAR protein expression from lysates of MV-4-11 cells. c) CPM counts for six dsRNA sensing genes from RNA-seq data of MV-4-11 cells treated with 100nM DNMT1i for 7 days. d) Western blot analysis of MV-4-11 cells depleted for all four expressed dsRNA sensing genes (4KD) treated with 20 ng/mL interferon alpha (IFNa) or 500nM ruxolitinib for two days. Control (4KD) expressed four non-targeting gRNAs. All samples were run on the same immunoblot for each protein assayed. e) IC50 assays of MV-4-11 cells treated with DNMT1i for 10 days. Data are mean±SD, sigmoidal four parameter logistic curve, n=3 biological replicates. f-g) Genome browser tracks generated by Spark of RNA-seq data from MV-4-11 cells treated with indicated dose of DNMT1i for 7 days. f) All four genes that were knocked-down in 4KD cells and g exemplar of a mintRNA (left) and interferon stimulated gene (ISG, right).

Extended Data Figure 10. SUMOylation mobilises DNMT1 to maintain DNA methylation under hypomethylating conditions

(a-c) Structural modelling of DNMT1 SUMOylation. a) An inhibitory state of DNMT1 via the CXXC domain and K697-associated loop occupying the DNA binding interface. b) In the presence of hemi-methylated CpG substrate DNA, the CXXC domain and the K697-assocated inhibitory loop (which becomes disordered) reposition away from the DNA binding surface. c) The sumoylated K697 (in yellow) prevents the CXXC domain occupying the DNA binding interface. In all models shown, the dynamic KG-containing linker and part of the C-terminal, K1609-containing helix are modelled based on AlphaFold prediction. d) Scatter plots depicting average methylation over 100-bp bins from RRBS data of MV-4-11 cells treated with DNMT1i for 7 days, n=3 per treatment. e) DNMT1 binding by genomic features as a total (left) and proportion (right) in MV-4-11 cells. f) Schematic and immunoblot of crude cellular fractionation. Nuclear and chromatin fractions were solubilized in equal buffer volumes, allowing relative protein abundance to reflect proportional loading between fractions.

g-h) Single molecule tracking analysis of MCF7 cells treated with 1 μM DNMT1i for 3 days. g) Representative nuclei of high frame rate (20ms exposure) with traces of all DNMT1 molecules tracked over 6,000 frames. h) Density plots of data from g).

Supplementary Notes

Supplementary Note 1

To quantify the changes in methylation after DNMT1 inhibition more precisely, we modelled methylation at 500 bp resolution using replicate RRBS datasets (Extended Data Fig. 1e). Methylation fractions were compared on a bin-by-bin basis between DNMT1i-treated and control samples to directly measure local methylation loss. This revealed that over half of all genomic intervals underwent a net loss of at least 50% methylation, with affected sites broadly distributed across promoter, intragenic, and intergenic regions. Regions that were recalcitrant to methylation loss were those that already harboured low, but detectable levels of methylation at baseline.

Supplementary Note 2

To systematically define the binding profiles of DNMT1, we performed unsupervised k-means clustering of the DNMT1 ChIP-seq signal, which identified three major clusters as distinguished by the position and distribution of DNMT1 occupancy across gene bodies. We then integrated genomic annotations, chromatin accessibility, and RNA-seq data to further characterise each cluster (Fig. 1d-e and Extended Data Fig 2a-d). Cluster 1 (orange) comprises genes that show a pronounced gain in DNMT1 binding extending from the transcription start site (TSS) through the gene body (TSS → TES) following DNMT1i treatment and resultant hypomethylation. Interestingly, these loci display the highest density of CGI suggesting that DNMT1 is drawn to CGI-rich promoters that become newly hypomethylated upon catalytic inhibition. Gene ontology (Extended Data Fig. 2e) revealed that these genes play critical roles in early embryonic development such as SOX17. Cluster 2 genes (purple) tend to be more highly expressed, show greater H3K27 acetylation (H3K27ac), and are ontologically enriched for housekeeping functions. Within this group, DNMT1 occupancy changes are largely bimodal: some loci, exemplified by FGF8, gain DNMT1 binding, whereas others, such as NPM3, lose signal, specifically at the transcription start site (TSS) (Fig. 1f, right panel). Cluster 3 genes (green), by contrast, represent DNMT1-bound sites that are largely agnostic to the effects of DNMT1 inhibition, showing minimal changes in occupancy.

Supplementary Note 3

To substantiate the observation that mintRNAs are unusually long and complex transcripts, we performed long-read sequencing (Fig. 5l-m and Extended Data Fig. 7e-f). Given the anticipated extensive secondary structure of these RNAs, which could hinder direct RNA capture workflows, we employed a PCR-free cDNA sequencing strategy with a set of tiling primers targeting a representative mintRNA locus on chromosome 8 to ensure robust coverage. Alignments revealed long, contiguous, and overlapping reads extending up to ~20 kb, confirming that these mintRNAs are exceptionally long and continuous transcripts (Fig. 5l-m, Extended Data Fig. 7e-f). Also see methods.

Supplementary Note 4

As altered protein–protein interactions could not explain the increased catalytic activity observed following RNF4 loss during DNMT1i treatment, we next modelled the structural consequences of DNMT1 SUMOylation (Extended Data Fig. 10a-c). The lysines modified on endogenous DNMT1 are distributed across the enzyme (Fig. 4e, Extended Data Fig. 6a). The DNMT1i binds the enzyme preferentially in complex with hemi-methylated DNA. In this state, the active-site loop remains in an open conformation, and the inhibitor occupies the space normally filled by loop residues Gly1228–Arg1234 during catalysis. Catalysis is therefore blocked because the loop cannot close over the target cytosine, locking DNMT1 into a stable,

inactive configuration. Our structural modelling suggests that SUMOylation at key regulatory residues such as K697 could allosterically alter DNMT1's conformational dynamics in ways that could transiently oppose DNMT1i-induced inhibition. Modification at K697 within the CXXC domain would impair the dynamic mobility of the CXXC domain and consequent autoinhibitory contacts, enabling greater chromatin search behaviour and unrestrained catalytic activity (Extended Data Fig. 10a-c).

Supplementary Tables

Supplementary Table 1. DNMT1i CRISPR screen analysis
Supplementary Table 2. Global proteomics raw data and analysis
Supplementary Table 3. mintRNA characterisation by RNA sequencing and MDA5 protection assay
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Supplementary Videos

Supplementary video 1.

Representative videos of vehicle-treated DNMT1$^{10K\rightarrow R}$;RNF4^{WT} nuclei. High frame rate (20 ms exposure) acquisition over 2,200 frames with real-time playback speed. Nucleus (NucBlue) image was acquired prior to SMT then overlayed onto each SMT frame.

Supplementary video 2.

Representative videos of DNMT1i-treated DNMT1$^{10K\rightarrow R}$;RNF4^{WT} nuclei. High frame rate (20 ms exposure) acquisition over 2,200 frames with real-time playback speed. Nucleus (NucBlue) image was acquired prior to SMT then overlayed onto each SMT frame.