IMPACTS OF DNA METHYLATION ON H2A.Z DEPOSITION AND NUCLEOSOME STABILITY

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ABSTRACT

In eukaryotes with DNA methylation, the histone variant H2A.Z and DNA methylation are maintained in mutually exclusive sections of the genome. How this antagonism is established, however, remains an open question. Here, we examined the impacts of DNA methylation on both the intrinsic stability of H2A.Z nucleosomes and chaperone-mediated H2A.Z deposition. Cryo-EM and endonuclease accessibility analyses show that H2A.Z nucleosomes with methylated DNA are more open and accessible compared to their unmethylated counterparts. In *Xenopus laevis*, H2A.Z preferentially associates with unmethylated DNA in both the fibroblast cell line XTC-2 and sperm pronuclei formed in the transcriptionally silent egg extract. The proportion of H2A.Z that colocalizes with methylated DNA, however, is higher in sperm pronuclei than in XTC-2. By monitoring nucleosome assembly on synthetic DNA constructs in *Xenopus* egg extracts, we find that the H2A.Z bias for unmethylated substrates is dependent on the SRCAP complex, the major H2A.Z deposition chaperone. Consistently, recruitment of the SRCAP complex to DNA is suppressed by DNA methylation. Altogether, we propose that the SRCAP complex is the major determinant for preferential H2A.Z enrichment on unmethylated DNA, whereas DNA methylation destabilizes DNA wrapping in H2A.Z-containing nucleosomes.

INTRODUCTION

Structural features of the nucleosome, a protein-DNA complex composed of ~146 bp DNA wrapped around a histone octamer, directly affect and regulate genomic accessibility to a diversity of DNA binding proteins, in part, through incorporating histone variants with unique physical properties (1, 2). The highly evolutionarily conserved H2A variant, H2A.Z, is involved in transcriptional regulation with a well described localization at the transcription start sites (TSSs) of active genes (3–5). However, while typically associated with gene activation, H2A.Z has also been connected to a range of functions including DNA repair, chromosome segregation, heterochromatin maintenance, and transcriptional repression (5, 6). Loss of H2A.Z induces lethality in many eukaryotes, such as mice, *Drosophila*, and *Tetrahymena* (7–9), and dysregulation of its localization has been linked to several diseases, including various cancers and the developmental disorder Floating-Harbor syndrome (10, 11). What mechanisms mediate proper H2A.Z localization, and how that connects with its broad-reaching functions, represents an important area of study.

Genomic sequencing analysis of a wide range of eukaryotes have found H2A.Z and DNA methylation, typically occurring as 5-methylcytosine (5mC), to be strikingly anti-correlated across the genome, at both a global and local level (12–18). The high conservation of this antagonism points to the existence of a shared fundamental pathway involved in creating this dichotomy and emphasizes its functional importance. In *Arabidopsis,* H2A.Z mediates

recruitment of the demethylase ROS1, thereby promoting DNA demethylation; however, this pathway uses plant-specific machinery that targets only a particular set of genomic loci (16). More general mechanisms for how the mutually exclusive H2A.Z and DNA methylation compartments exist at large remain to be resolved.

DNA methylation may mediate H2A.Z exclusion via two distinct mechanisms: 1) influencing the intrinsic physical stability of the nucleosome, and/or 2) modulating the activity of nucleosome-remodelers. H2A.Z and H2A diverge in sequence along areas important for stabilizing intra-nucleosome interactions, such as the L1 loop, docking domain, and C-terminus (4). DNA methylation can influence the geometry of DNA, particularly its roll and twist (19). A large body of work has been reported on the physical impacts of either H2A.Z (20–29) or DNA methylation (19, 30–39) on the nucleosome. Whether those effects are stabilizing or destabilizing, however, remains controversial. Nevertheless, it is possible that the combination of H2A.Z and DNA methylation's effects may physically bias against retention of H2A.Z nucleosomes on methylated DNA and contribute to their overall antagonism.

H2A.Z remodeling is primarily carried out by the Ino80 family of nucleosome remodelers. Both the SRCAP complex (SRCAP-C) and Tip60 complex (Tip60-C) mediate exchange of canonical H2A to H2A.Z within a nucleosome (40), while the Ino80 complex (Ino80-C) has been reported to evict H2A.Z (42, 43). Tip60-C can also associate with the H2A.Z chaperone, ANP32e, to evict H2A.Z from nucleosomes (45, 46). Members of this remodeler family are prime candidates for enforcing the separation of H2A.Z and DNA methylation; however, it is unknown whether any of these complexes exhibit DNA methylation sensitivity.

To determine molecular mechanisms contributing to the mutual exclusion of H2A.Z and DNA methylation within the genome, here we investigated the effects of DNA methylation on both the physical stability of H2A.Z nucleosomes and remodeler-mediated deposition of H2A.Z onto synthetic DNA constructs by employing cryo-EM structural analysis and the physiological *Xenopus* egg extract system, respectively.

MATERIALS AND METHODS

Histone purification and refolding

pET3 vectors containing sequences for human histones H2A, H2A.Z, H2B, H3.2, and H4 (gifts from Shixin Liu) were expressed in Rosetta (DE3) cells (Novagene, Cat# 70954). Cells were pelleted 4000 x *g*, 20 min, 4 °C and resuspended in wash/lysis buffer [50 mM Tris-HCL pH 7.5, 100 mM NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, 0.2 mM PMSF] + 1 % Triton-X-100 then sonicated. Inclusion pellets were spun 25,000 x *g*, 20 min, 4 °C, and incubated with DMSO at room temperature (RT) for 30 min. Pellets were then resuspended in unfolding buffer [20 mM Tris-HCl pH 7.5, 7 M guanidine-HCl, 10 mM DTT], sonicated until pellets were fully broken up, and re-pelleted at 25,000 x *g*, 20 min, RT. The supernatant was then collected and dialyzed in urea dialysis buffer overnight at 4 °C [10 mM Tris-HCl pH 7.5, 7 M Urea, 1 mM EDTA, 5 mM β-mercaptoethanol, 100 mM or 200 mM NaCl for H2A/H2B and H3/H4, respectively]. Samples were then pelleted at 25,000 x *g*, 20 min, RT and the supernatant passed through Q Sepharose Fast Flow resin (Cytiva, Cat# 17051010). The flow-through was then collected and added to SP Sepharose Fast Flow resin (Cytiva, Cat# 17072910), washed with urea dialysis buffer, and eluted with elution buffer [10 mM Tris-HCl pH 7.5, 7 M Urea, 1 mM EDTA, 600 mM NaCl, 5 mM β-mercaptoethanol].

Equimolar ratios of histones for either octamer, or dimer and tetramer preparation were then mixed and dialyzed into refolding buffer [10 mM Tris-HCl pH 7.5, 1 mM EDTA, 5 mM β-mercaptoethanol, 2 M or 500 mM NaCl for octamers or dimers/tetramers, respectively]. Refolded histones were then purified via FPLC (ÄKTA pure, Cytiva, Cat# SN2626712) (Superdex 200 10/300 GL increase column, Cytiva, Cat# 28990944).

Generation of DNA arrays

Generation of HSat2 arrays for use in chromatinization assays or arrays of Sat2R-P halves to prepare palindromic sequences for cryo-EM analysis was done as previously described in (47). Briefly, DNA sequences of interest (see **Table 1**) containing one BglII site, one BamHI site, and two EcoRV sites surrounding the sequence were generated and purchased from Integrated DNA Technologies (IDT™) and cloned into the pUC18 vector just downstream of the XbaI site. The plasmid was then digested with either XbaI (NEB Cat# R0145) and BglII (NEB Cat# R0144) (to obtain the vector) or BglII and BamHI (NEB Cat# R3136) (to obtain the insert), and agarose gel purified. Vector and insert DNA were then ligated with T4 DNA ligase (NEB Cat# M0202). The digest and ligation steps were repeated until the desired repeat number was achieved.

Palindromic DNA preparation for Cryo-EM

Arrays of Sat2R-P halves were generated as described above. Plasmid containing an array of 601L halves (601L-145-8X) was a gift from Curt Davey (Addgene plasmid # 158572; http://n2t.net/addgene:158572; RRID: Addgene_158572) (48). Arrays of palindromic DNA halves for Cryo-EM analysis were then digested with EcoRV (NEB Cat# R3195) and purified via PEG precipitation (0.5 M NaCl, 8 % w/v PEG8000). Supernatant were collected and further purified via phenol-chloroform extraction. Insert ends were dephosphorylated with QuickCIP (NEB Cat# M0525) and phenol-chloroform extracted. Samples were then digested with either HinfI (NEB Cat# R0155) (601L DNA) or AvaI (NEB Cat# R0152) (Sat2R-P DNA), phenol-chloroform extracted, and FPLC purified in 1xTE buffer (Superdex 200 10/300 GL increase column, Cytiva, Cat# 28990944). DNA halves were then incubated with T4 DNA ligase (NEB Cat# M0202) per manufacturer's protocol until all halves were completely ligated.

Methylation of DNA substrates

DNA substrates were enzymatically methylated using the *M.SssI* CpG methyltransferase (NEB, Cat# M0226). Approximately 160 U of methyltransferase were used for 50 µg of DNA in 1x NEB 2 buffer with 500 µM SAM. Samples were incubated at 37 °C for at least one hour. Completion of methylation was checked using the methylation sensitive restriction enzyme BstUI (NEB, Cat# R0518). DNA was purified using phenol-chloroform extraction prior to use.

Nucleosome Reconstitution

DNA and either histone octamers or dimers and tetramers were mixed at a final target concentration of 1 µM in 1x TE buffer and 2 M NaCl. Exact ratios of DNA to histones were titrated for each reconstitution batch, starting at equimolar ratios. Samples loaded in dialysis buttons were then added to high salt dialysis buffer [10 mM Tris-HCl pH 7.5 at 4 °C, 1 mM EDTA, 2 M NaCl, 0.01 % Triton X-100, 5 mM β-mercaptoethanol] at 4 °C. The high salt dialysis buffer

was then exchanged into low salt dialysis buffer [10 mM Tris-HCl pH 7.5 at 4°C, 1 mM EDTA, 50 mM NaCl, 0.01 % Triton X-100, 5 mM β-mercaptoethanol] at a rate of ~0.7 ml/min over a period of 30-40 h. Nucleosome quality was assessed on a 5 % 37.5:1 acrylamide/bis 0.5 x TBE gel (120V).

Mass Photometry Analysis of Nucleosomes

Mass photometry analysis was performed on a Refeyn Two^{MP} instrument and analyzed using DiscoverMP v2022R1 (with IScat v1.52.0 and IScat Utils v1.43.0). 24 x 50 mm with 170 ± 5 µm thickness No. 1.5H glass slides (ThorLabs, Cat# CG15KH1) and silicone gaskets (Grace Bio-labs, Cat# 103250) were washed 2x with alternating 100 % isopropanol and deionized water. Clean gaskets were then placed on the cleaned slides to serve as a sample chamber. 5 µl of 1x PBS was placed in the gasket to calibrate each sample collection then 5 µl of nucleosome samples were added for a target final concentration of ~5-10 ng/µl nucleosomes in the well. Measurements were conducted at room temperature.

Cryo-EM Sample Preparation and Data Collection

Human H2A.Z nucleosomes were reconstituted on either 601L or Sat2R-P DNA as described above with a final concentration aim of 1.6 µM in 1 ml. Reconstituted nucleosomes were dialyzed into buffer containing 10 mM HEPES-KOH pH 7.4 and 30 mM KCl then concentrated to a final concentration of ~1 mg/ml using Amicon® Ultra Centrifugal Filters (Cat# UFC5010). Samples were then mixed with 10x cryo-protectant buffer [10 mM HEPES-KOH pH 7.5, 30 mM KCl, 10 % Trehalose, 1 % Hexanediol, 1 mM $MgCl_2$] and 3 µl of each solution was applied to plasma-cleaned) Quantifoil gold R1.2/1.3 400-mesh grids (Quantifoil) either graphene coated (graphene coating was done in-house as specified in (49) or not for the Sat2R-P and 601L samples, respectively. Grids were then vitrified using the Vitrobot Mark IV (FEI) at 100 % humidity, 2 sec blotting time, blot force 1. Data collection was conducted using the Titan Krios (ThermoFisher), equipped with a 300 kV field emission gun and a K3 direct electron detector (Gatan), at a magnification of x81,000 (0.86 Å/pixel). Total micrographs collected for each sample as follows: 4544 for unmethylated Sat2R-P nucleosomes; 6891 for methylated Sat2R-P; 7020 for unmethylated 601L; 5427 for methylated 601L. Data statistics summarized in **Suppl Table 1**.

Cryo-EM Data Analysis

Diagrams of analysis workflows are shown in **SFig 1** and **SFig 5**. Micrographs were motion-corrected using MotionCor2 (50) in Relion v4 (51). Downstream analysis was conducted in CryoSPARC v4.6 (Structura Biotechnology Inc.). Motion-corrected micrographs then underwent patch CTF correction. Particles were picked using Topaz v0.2 (52) trained on a set of 1,000-2,000 manually picked nucleosome-like particles and then extracted to 384 pix (~330 Å) boxes with 4x binning and used for 2D classification into 200-250 classes. Particles from selected classes were then run through a series of heterogeneous refinements with 1-2 nucleosome-like models and 4 decoy models generated through *ab initio* reconstruction to sort out junk particles from those containing nucleosomes. The heterogenous refinements were iterated until greater than 90 % of particles in the round were classified as nucleosome-like. These particles were then further refined either via Refine 3D, CTF refinement, and polishing in Relion v4 (601L structures) or homogeneous and non-uniform refinements in CryoSPARC v4.6 (Sat2R-P). Particles for the

methylated Sat2R-P structure underwent another heterogeneous refinement using a closed nucleosome, open, and hexasome model as starting classes to select for the highest resolved closed nucleosome structure. Final resolutions were determined using the gold standard FSC threshold (FSC = 0.143). Local resolution maps shown in **SFig 3** were generated using the local resolution estimation tool with default parameters in CryoSPARC. The maps were then visualized on density maps filtered using the local filter tool with default parameters. For the *in silico* mixing 3D classification analysis (**SFig 4**): micrographs from both unmethylated and methylated Sat2R-P structures were merged and subjected to the data analysis pipeline described above. Six starting models representing different nucleosome structure types were then constructed using the ab initio 3D reconstruction tool. These models were then used as input volumes, and cleaned particles from the merged analysis were then sorted to each of these models using the heterorefinement tool in CryoSPARC.

Atomic Modeling and RMSD analysis

Initial atomic models were generated using previously published structures of human H2A.Z-containing histone core (PDB ID: 3WA9) (53) and modified versions of either 601L DNA (PDB ID: 3UT9) (54) or Sat2R DNA (PDB ID: 5CPI) (31). Models were docked into the EM density maps using Phenix v1.21, then iteratively refined and assessed in Phenix v1.21 (55) and *Coot* 0.9.8.7 (56). RMSD values were calculated in PyMOL v2.5.7 (Schrödinger) using the cealign function. For the Sat2R-P models, DNA positioning was determined using the EM densities at the dyad of the unmethylated map which allowed for pyrimidine and purine distinction. A two-base pair shift was determined by assessing ambiguity of densities around nucleotides which change from purine to pyrimidine, and *vice versa*, between mirrored DNA models versus those which retain identity. After determining best-fit DNA models for the unmethylated structure, the same DNA positionings were then used for the methylated structure.

DNA Preparation for Restriction Digest Assay

Non-palindromic 1HinfI_Sat2R DNA (**Table 1**) was prepared via PCR using Q5 polymerase (NEB Cat# M0491). Final concentrations of reaction mixture as follows: 1x Q5 reaction buffer; 200 µM dNTPs; 2 µM forward and reverse primers; 0.2 g/µl DNA template; 0.02 U/µl Q5 polymerase. Roughly 60-65 PCR cycles were run following manufacturer's protocol with a melting temperature of 60 °C. PCR products were phenol-chloroform extracted and then FPLC purified in 1xTE buffer (Superdex 200 10/300 GL increase column, Cytiva, Cat# 28990944).

Restriction Digest Assay

Nucleosomes were mixed at 30 ng per timepoint sample in 1x rCutSmart® buffer (NEB Cat# B6004S) [50 mM potassium acetate, 20 mM Tris-acetate pH 7.9 at RT, 10 mM magnesium acetate, 100 µg/ml recombinant albumin] and 0.5 U/µl HinfI restriction enzyme (NEB Cat# R0155S). Samples were incubated at 37 °C and added to stop buffer [final concentrations: 20 mM Tris-HCl pH 8.0 at RT, 20 mM EDTA, 0.1 % SDS, 0.6 mg/ml Proteinase K] at each timepoint. Stopped samples were then incubated at 55 °C for 40 min and run on a 10 % 37.5:1 acrylamide/bis 0.5x TBE gel (55 min at 150 V) and stained with SYBR™ Safe DNA stain (Thermo Fisher Scientific, Cat# S33102). Fluorescent signals were measured on a LI-COR Odyssey M system and quantified using ImageJ via histogram analysis as previously described in (57). The

ratio of Cy5: SYBR™ Safe signals was then determined for each time point and plotted. A two-tailed t-test assuming unequal variance was then performed on data from n = 5 experiments for statistical analysis.

Antibodies

Antibodies used in this study are listed in **Table 2**. Custom antibodies were generated through Cocalico Biologicals, Inc.™ using their standard protocol. Target binding of the H2A.Z antibody used for CnT-BS and SRCAP custom antibody in *Xenopus* egg extract was verified using mass spectrometry analysis (MS) (**Supplementary Data 1 and 2**).

Mass Spectrometry Validation

For the MS analysis, 20 µl of antibody-bound Protein A Dynabeads™ (Invitrogen, Cat# 10008D) (0.8 µg antibody per 1 µl of beads) were incubated at a 1:1 ratio with CSF egg extract (see next section) at 4 °C for 1h. Samples were collected via magnet and washed 3 times with sperm dilution buffer (SDB) [150 mM Sucrose, 10 mM HEPES-KOH pH 8.0, 1 mM $MgCl_2$, 100 mM KCl] then run on a 4-20% gradient SDS-PAGE gel as a 1 cm short-stack. The gel was then stained with Coomassie Brilliant Blue G-250 (Thermo Fisher) and the short stack was cut out. The cut gel was then destained using a solution of 30 % acetonitrile and 100 mM ammonium bicarbonate in water and dehydrated using 100 % acetonitrile. Samples were subjected to heat assisted (50 °C) trypsinization for 1 h. The resulting peptides were extracted three times with a solution of 70 % acetonitrile and 0.1 % formic acid then concentrated using a SpeedVac and resuspended in 10 µl volumes. 3 µl of purified peptides were subsequently analyzed by LC-MS/MS using a Dionex 3000 HPLC system equipped with an EasySPrayer (ES902), coupled to an Orbitrap ASCEND mass spectrometer from Thermo Scientific. Peptides were separated by reversed-phase chromatography using solvent A (0.1 % formic acid in water) and solvent B (80 % acetonitrile, 0.1 % formic acid in water) across a 45-min gradient. Data was processed using ProteomeDiscoverer 1.4 and quantified using ProteomeDiscoverer v1.4/Mascot v3.1. The spectra were then queried against a *Xenopus laevis* database (58) and matched peptides filtered using a Percolator calculated FDR of 1 %. As proxy for protein amount, the average area of the three most abundant peptide signals was used. Relative enrichment of proteins in the sample was then calculated by dividing by known protein concentrations in *Xenopus laevis* eggs and then the enrichment over IgG control was calculated. Common contaminates were marked in red.

Xenopus laevis frogs and preparation of sperm and CSF egg extract

Female *Xenopus laevis* frogs were purchased from Xenopus 1. Male frogs were purchased from either Xenopus 1 (for use in non-sequencing experiments) or the National Xenopus Resource (*XLa.J-Strain*^{NXR}, Cat# NXR_0024; for use in sequencing experiments). Vertebrate animal protocols (20031 and 23020) approved by the Rockefeller University Institutional Animal Care and Use Committee were followed for housing and care.

CSF egg extract and demembranated sperm nuclei were prepared as specified previously (59, 60).

Sperm Pronuclei Preparation
Sperm from J-strain frogs were added to prepared CSF egg extract at a final concentration of 1500 sperm/µl extract with a total extract volume of 200 µl for each sample. 90 ng (~1 % of total DNA) of lambda DNA (Promega Cat# D1521) was also added to assess bisulfite conversion efficiency and the extract solution was then cycled to interphase with the addition of $CaCl_2$ to a final concentration of 0.3 mM and incubated at 20 ºC for 90 min. Samples were then diluted 1:4 with 1xDB3 buffer [10 mM HEPES-KOH pH 8.0, 100mM NaCl, 0.5 mM spermine, 0.5 mM spermidine, 1 mM ASEBF (GoldBio Cat#A-540-1), 1x ROCHE cOmplete™ protease inhibitor cocktail (Cat# 11697498001), 200 mM Sucrose, 0.1 % Tween-20], layered onto 1 ml of 60SDB3 [1xDB3 + 60 % sucrose] and centrifuged at 4ºC, 10,000 rpm for 45 min (Beckman Coulter SX241.5 rotor). Sample pellets were washed 2x with 1xDB3, centrifuging at 4,000 x *g*, 4 ºC for 4 min, then resuspended in 1x CnT-WB [20 mM HEPES-KOH pH 7.8, 150 mM NaCl, 0.5 mM spermidine, 1x ROCHE cOmplete™ protease inhibitor cocktail, 0.1 % Tween-20].

XTC-2 Cell Line
XTC-2 cells (gift from Dr. David Shechter) were cultured in Leibovitz's L-15 Medium (Gibco™, Cat# 11415064) (0.7x amphibian strength, diluted with cell culture grade water (Corning®, Cat# 46-000-CV)) supplemented with 500 U/ml each of penicillin and streptomycin (Gibco™, Cat# 15140122), and 10 % FBS (R&D systems, Cat# S11150). XTC-2 cells were cultured in a 26 °C incubator without CO_2 supply. Cell line identity was verified via mitotic-spread karyotyping.

XTC-2 Nuclei Preparation
XTC-2 cells were harvested at 90-100 % confluency from a 15 cm dish. Nuclei isolation was performed at 4 ºC using the Nuclei EZ Prep kit (Millipore Cat# NUC101-1KT) as per manufacturer's protocol and resuspended in 1x CnT-WB. For each sample, 18 ng (~1 % of total DNA) of unmethylated lambda DNA (Promega Cat# D1521) was added to 300,000 nuclei to assess for bisulfite conversion efficiency.

Purification of Tn5
Tn5 was purified as described in (61). Briefly, VR124 pETv2-10His-pG-Tn5 (E54K, L372P) *E. coli* strain (Addgene #198468) were cultured to log growth phase and Tn5 expression induced with IPTG addition. Cells were harvested at 8,000 x *g*, 30 min at 4 ºC, washed with ice-cold DPBS, re-pelleted and frozen. Frozen pellets were then resuspended at 4 ºC in lysis buffer LysEQ [20 mM HEPES pH 7.8, 800 mM NaCl, 20 mM Imidazole, 10 % Glycerol, 1 mM EDTA, 2 mM TCEP-HCl, 1x EDTA-free cOmplete protease inhibitor] and sonicated. Lysates were pelleted at 20,000 x *g*, 35 min at 4 ºC, then filtered through a 0.45 µm PES filter. Supernatant was then loaded onto a HisTrap HP 5 ml column (Cytiva, Cat# 17-5247-01) pre-equilibrated with LysEQ buffer. The loaded column was then washed with 10 column volumes each of WashB1 [20 mM HEPES pH 7.8, 800 mM NaCl, 30 mM Imidazole, 10 % Glycerol, 1 mM EDTA, 2 mM TCEP-HCl] and WashB2 [20 mM HEPES pH 7.8, 800 mM NaCl, 45 mM Imidazole, 10 % Glycerol, 1 mM EDTA, 2 mM TCEP-HCl]. Protein was then eluted with EluB [20 mM HEPES pH 7.8, 800 mM NaCl, 400 mM Imidazole, 10 % Glycerol, 1 mM EDTA, 2 mM TCEP-HCl]. Tn5-containing fractions were pooled and dialyzed 4 ºC overnight in SECB buffer [50 mM Tris-HCl pH 7.5, 800 mM NaCl, 10 % glycerol, 0.2 mM EDTA, 2 mM DTT] then filtered through a 0.45 µm PES filter and FPLC purified on a HiLoad

16/600 Superdex 200 pg column (Cytiva, Cat#: 28989335). Purified fractions were pooled and concentrated with a 30 MWCO Amicon Ultra Centrifugal filter (final concentration 12 μM) and mixed with UltraPure glycerol to a final concentration of 55 % glycerol. Purified Tn5 was stored at -20 °C.

CUT&Tag-Bisulfite Library Preparation

Sequencing libraries were prepared as previously described in (62). Briefly, prepared nuclei samples were bound to 10 μl of activated concanavalin A beads (EpiCypher Cat# 21-1401) and incubated with specified primary and secondary antibodies (**Table 2**) in 50 μl Antibody Binding Buffer [CnT-WB + 2 mM EDTA + 0.1 % w/v BSA] for 1 h shaking at RT, washing 3x with CnT-WB between each incubation. Samples were then incubated with 50 μl of transposome complex (1:1 pG-Tn5:adaptor primer mix, ~0.1-0.2 μM final to each sample) in CnT-300 [CnT-WB with 300 mM NaCl] for 1 h at RT then washed with CnT-300. Transposome activity was initiated with addition of 300 μl Tagmentation Buffer [20 mM HEPES-KOH pH 7.8, 300 mM NaCl, 0.5 mM spermidine, 10 mM $MgCl_2$, 1x ROCHE cOmplete™ protease inhibitor cocktail, 0.1 % Tween-20] and incubated 37 °C, 1500 rpm on thermomixer, 1 h. Reactions were stopped with addition of stop buffer [15 mM EDTA, 0.1 % SDS, 0.2 mg/mL Proteinase K final concentrations] and incubated at 55 °C overnight, then phenol-chloroform extracted. Purified samples were resuspended in 11 μl of 10 mM Tris-HCl, pH 8.0 and oligonucleotide replacement/gap repaired carried out through addition of 2 μl of 10 μM Tn5mC-ReplO1 DNA oligo (**Table 1**), 2 μl 10x Ampligase Buffer (Lucigen), 2 μl dNTP mix (2.5 mM for each dNTP). Solutions were subject to the following heating protocol: 50 °C 1 min, 45 °C 10 min, cool to 37 °C at -0.1 °C/second. Once at 37 °C, 3U of T4 DNA polymerase and 12.5U of Ampligase (Lucigen) were added and samples incubated for 30 min. Reactions were stopped with 20 mM EDTA final and purified using the MinElute PCR purification kit (Qiagen Cat# 28004). Samples were bisulfite converted using the EZ DNA Methylation-Lightning Kit (Zymo Research Cat# D5030) and libraries amplified (14 cycles) using NEBNext Q5U Master Mix (NEB Cat# M0597) and Illumina Nextra i5/i7 adapter primers.

Sequencing Data Analysis

Adapter trimming, deduplication, and genome alignment. Libraries were sent to Novogene for sequencing on the NovaSeqX Plus system (PE150). XTC-2 data was then downsampled to 30 million reads using seqtk (v1.5-r133) to have all samples at similar read depths. Adapter trimming and dedeuplication of the demultiplexed reads was then done using fastp v0.24.0 (63) [--adapter_sequence CTGTCTCTTATACAC --adapter_sequence_r2 CTGTCTCTTATACAC -g --poly_g_min_len 5 -p -l 20 --cut_tail -y -Y 20 -q 20 –dedup]. Processed reads were then aligned to the *Xenopus laevis* genome (Xenla 10.1) and methylated CpG proportions for all mapped reads calculated using Bismark v0.24.2 (64) [-q --local --score-min G,20,8 --ignore-quals --no-mixed --no-discordant --dovetail --maxins 700].

Count matrix generation and sequencing depth normalization. The *Xenopus laevis* genome was subset into 1000 bp bins. Reads from processed bam files were then tallied according to bins to generate a genome-wide count matrix. Mitochondrial associated reads and bins which contained no reads across all samples were removed. Size factors were then estimated using

DESeq2 v1.42.1 (65) to generate sequence depth normalized bigwig files. Count matrices were also vst transformed in DESeq2 then used to generate the read fragment correlation plot. For other analyses, only genomic bins containing at least 17 reads across at least 2 samples were kept and replicates for each condition were averaged.

DNA Methylation analysis. To get methylated CpG proportions for each sample (**Fig 4A**), H2A.Z peaks were first identified with SEACR (v1.3) (66) using a cutoff of 0.025 by AUC with a stringent threshold. Individual CpG data was then extracted using Bismark with a cutoff of 5 reads and disregarding the first 9 bp of read 2 (due to adapter fill-in). Methylation proportions for H2A.Z samples were then calculated for CpGs overlapping with H2A.Z peaks. For H3, only regions outside of identified H2A.Z peaks were used to assess the CpG methylation status of general genomic background non-coincident with H2A.Z enriched areas. To generate H3-associated methylation 500 bp tiles for use in plotting (**Suppl Fig 9B-D**), methylation data was extracted with a specified cutoff of 1 read and disregarding the first 9 bp of read 2 then imported into a methylRawList object with a minimum read coverage of 2 and counted over 500 bp tiles with a 500 bp step size and a minimum of 2 CpG sites using methylKit v1.28.0 (67).

Generation of plots. Genomic annotations for the top 20 % of filtered bins containing the most reads for each condition were generated and proportions plotted using ChIPseeker v1.38.0 (68) with reference gene annotations gathered from Xenbase (69). Heat maps and plot profiles were created using deepTools v3.5.5 (70). For comparison of H3 methylation sites and H2A.Z, a signal matrix was first generated using the tiled H3 methylation bigwigs against all annotated *Xenopus laevis* genes, removing genes with no methylation data available. The resulting gene list was then used to plot corresponding H2A.Z signals.

in vitro **mRNA Synthesis**

DNA sequences of proteins of interest were cloned into pSP64T plasmids modified to include a Kozak sequence (GCCACC) upstream of the coding sequence and linearized. pSP64T (DM#111) was a gift from Douglas Melton (Addgene Plasmid# 15030). RNA production was carried out using the MEGAscript™ SP6 transcription kit (Invitrogen, Cat# AM1330) as per manufacturer's protocol, with the addition of 4.5 mM CleanCap® Reagent AG (TriLink, Cat# N-7113) and a reduction in GTP concentration down to 1.5 mM final.

Chromatinization in Egg Extract Assays

pAS696 containing a 19-mer array of Widom 601 DNA was a gift from Aaron Straight (71). 601 and HSat2 arrays were digested with XbaI and EcoRI or XbaI and BamHI, respectively, along with DraI and HaeII. DNA fragments containing the array sequence were then purified through a PEG precipitation titration, starting at 4.5 % of PEG (MW8000) and increasing in 0.5 % increments until 8 % (NaCl added to 0.5 M final concentration in the starting solution). Fragment containing fractions were then assessed via agarose gel and purified via phenol-chloroform extraction. Fragment ends were biotinylated with biotin-14-dATP (Jena Bioscience, Cat# NU-835-BIO14-S) using Klenow Fragment (3´→ 5´ exo-) (NEB Cat# M0212) following manufacturer's protocol. Biotinylated DNA was phenol-chloroform extracted and bound to M-280 Streptavidin Dynabeads™ (Invitrogen, Cat# 11206D) at 300 ng DNA per 1 µl of beads. DNA bead binding was

carried out at room temperature for a minimum of 1 h shaking in the following solution: 1x DNA Bead Binding buffer (DBB) [750 mM NaCl, 50 mM Tris-HCl pH 8.0, 0.25 mM EDTA, 0.05 % Triton-X-100]; 2.5 % (w/v) polyvinyl alcohol; 750 mM NaCl. DNA beads were then washed 2x with DBB and 3x with sperm dilution buffer (SDB) [150 mM Sucrose, 10 mM HEPES-KOH pH 8.0, 1 mM $MgCl_2$, 100 mM KCl] and added to egg extract at a ratio of 4 µl DNA beads to 30 µl of egg extract. Experiments that did not use mRNA for exogenous protein expression also included 0.1 mg/ml cycloheximide dissolved in EtOH. $CaCl_2$ was then added to the extracts at a final concentration of 0.3 mM and incubated at 20 ºC for 1 h. Samples were then diluted 10-fold with SDB, and DNA beads were collected on a magnetic rack and washed 3x with SDB + 0.05 % Triton-X-100 before running on SDS-PAGE gel and assessed via western blot.

Western Blot Analysis

Samples were boiled in 1x SDS loading buffer [125 mM Tris-HCl pH 6.8, 0.01 % SDS, 25 % glycerol, 0.03 mg/ml bromo-phenol blue, 350 mM 2-mercaptoethanol] then run on either a 4-20 % SDS-PAGE gel (Bio-Rad, Cat# 4561096) or 3-8 % Tris-acetate gel (for SRCAP detection) (Invitrogen, Cat# EA0375BOX). Samples were then transferred from the gel to a nitrocellulose membrane (Cytiva, 0.1 µm pore size, Cat# 10600000) overnight at 4 ºC in 1x transfer buffer [20 mM Tris, 150 mM Glycine, 20 % Methanol] (14V). Membranes were blocked for 30 min at RT with Intercept® (TBS) Protein-Free Blocking Buffer (LICORbio, Cat# 927-80001), followed by primary antibody incubation (diluted in Intercept® T20 (TBS) Antibody diluent (LICORbio, Cat# 927-65001)) for 1 h at RT. Membranes were then washed 3x, 5 min each wash, in 1xTBS+0.05 % Tween-20 then incubated with appropriate secondary antibodies for 1 h at RT and washed. Blots were then imaged on a LI-COR Odyssey M system and signals quantified using ImageJ via histogram analysis as previously described in (57). Ratios of either H2A.Z or H2A.X-F signals to H4 signals were then determined for normalization purposes in the chromatinization assays.

SRCAP Depletion from Egg Extract

SRCAP antibody was bound to Protein A Dynabeads™ (Invitrogen, Cat# 10008D) at a concentration of 0.8 µg antibody per 1 µl of beads overnight at 4 ºC in wash/coupling (W/C) buffer [10 mM HEPES-KOH pH 8.0, 150 mM NaCl]. Beads were then washed 2x with W/C buffer and 3x with SDB. An equal volume of beads and egg extract (supplemented with 0.1 mg/ml cycloheximide) were then incubated together for 1 h at 4 ºC and beads removed via magnet.

Other Instruments and Software Used

ÄKTA pure SN2626712 (Cytiva) and UNICORN v7.11 (Cytiva) for FPLC purification of histone proteins.
UCSF ChimeraX v1.9 (72) for structure visualization.
LI-COR Odyssey M, LI-COR Acquisition v1.2, and ImageJ v1.54f (73) for western blot and gel imaging, data analysis, and visualization.
QuantStudio™ 5 (Thermo Fisher, Cat# A28574) and QuantStudio™ Design & Analysis v2.7 for QC of sequencing samples.
Inkscape v1.3.2 for figure generation.

RESULTS

Cryo-EM analysis of H2A.Z nucleosomes in the presence or absence of DNA methylation

To assess the physical impacts of DNA methylation on H2A.Z nucleosomes, we solved cryo-EM structures of human H2A.Z nucleosomes reconstituted with either methylated (Me) or unmethylated (UM) Sat2R-P DNA (**Fig 1, Suppl Fig 1, Suppl Table 1**). The Sat2R-P sequence is a 152 bp artificial palindromic sequence containing 9 evenly distributed CpG sites and was engineered from a published canonical nucleosome crystal structure containing a methylated derivative of the human satellite II sequence (termed Sat2R) (31) (**Fig 1A**). This sequence was chosen for two reasons: 1) human satellite II (HSat2) represents a highly abundant methylated sequence which undergoes disease-associated differential methylation (74–78), and 2) the symmetric palindromic sequence was needed to increase final structure resolutions due to the two-fold pseudo symmetry about the nucleosome dyad axis. Prepared nucleosomes were run on a native PAGE gel to assess for quality, where both samples appeared as a singular band with minor comparable populations of unincorporated DNA (**Suppl Fig 1A**).

Cryo-EM structures of UM and Me Sat2R-P H2A.Z nucleosomes were solved at a final resolution of 3.05 Å and 2.78 Å, respectively (**Fig 1B and C, Suppl Fig 1**). We found that the nucleosome dyad was shifted roughly two base pairs from the center of the Sat2R-P palindrome, introducing a sequence asymmetry despite our intended use of the palindromic sequence. The shifted nucleotide positions were determined using the higher resolution densities surrounding the nucleosome dyad, which allowed for pyrimidine and purine distinction (**Suppl Fig 2A-D**). Two atomic models (termed v1 and v2) with mirrored DNA sequences were generated for each structure to account for the resultant sequence asymmetry, with the final EM densities likely reflecting an average of these two models (**Suppl Fig 2E**).

As a whole, both the methylated and unmethylated structures resembled previously published H2A.Z nucleosome structures and exhibited a linker DNA asymmetry where one side of the nucleosome (distal face 2, DF2) contained a highly flexible linker DNA region compared to the other (distal face 1, DF1) (**Fig 1B and C**) (53, 79, 80). As a palindromic DNA sequence was used, it is likely that the asymmetrical faces in the cryo-EM structures arose due to an averaging of closed and open H2A.Z nucleosome particles into the same structure rather than representing an asymmetry found in individual particles.

In a direct comparison of DF2 linker DNA lengths, we found that the Me Sat2R-P map had much weaker linker DNA density than UM Sat2R-P, resulting in a ~7 bp shorter resolved DNA model for the methylated structure (**Fig 1D**). This loss of linker density indicates destabilized histone-DNA interactions and increased DNA flexibility in the presence of DNA methylation. Along similar lines, Root Mean Squared Distance (RMSD) analysis identified the H2A.Z-H2B dimer of DF2, as well as the overall DNA backbone, as regions of considerable difference between the methylated and unmethylated structures, whereas DF1, the closed side of the nucleosome with a highly resolved linker DNA, was largely similar between the two (**Fig 1E**).

DNA methylation creates open H2AZ nucleosome structures

To further characterize areas of change, maps of unmethylated and methylated H2A.Z structures were filtered and resampled to the same resolution (~3 Å) for fair comparison (**Fig**

2A). Three major differences between the maps were identified. First, cryo-EM densities of the H3 N-terminus and H2A.Z C-terminus on DF2 were much weaker in the methylated structure as compared to unmethylated, preventing model construction in these areas (**Fig 2B**). Second, an extra density on the H2A.Z-H2B acidic patch was seen on the methylated map but was not present on the unmethylated map (**Fig 2C**). Third, between the two major orientations the H4 N-terminal tail can adopt (81), the unmethylated map showed a strong preference for the inward orientation whereas the methylated structure exhibited similar populations of inward and outward orientations (**Fig 2D**).

The H3 N-terminus interacts with the H2A/H2A.Z C-terminus and DNA to hold the linker DNA close to the body of the nucleosome (29, 82). Loss of the H3 N-terminal density from the methylated structure suggests destabilized DNA-histone interactions that are consistent with the shorter resolved linker DNA on DF2 of the methylated model compared to unmethylated (**Fig 1D**). The H4 N-terminal tail, on the other hand, mediates inter-nucleosomal interactions through contacts with a neighboring acidic patch (83, 84). The extra density on the Me Sat2R-P acidic patch may then have come from the H4 tail of another nucleosome, in line with the changes also observed in H4 tail orientation. These observations may be consistent with reports that DNA methylation increases condensation of nucleosomes, as well as bare DNA, *in vitro* and *in vivo* (39, 85, 86).

To more quantitatively assess methylation-dependent structural changes, we conducted an *in silico* mixing 3D classification analysis (81, 87) by combining micrographs collected from both methylated and unmethylated nucleosome samples and then sorted particles picked from the combined micrographs into six 3D classes of nucleosomes, each with varying degrees of open or closed linker DNA (**Fig 2E and F, Suppl Fig 3**). Most of the unmethylated particles (>60%) were sorted into the closed linker classes (Class 1 and 2) while the majority of methylated particles (>60%) belonged to the open linker and hexasome classes (Classes 3-6) (**Fig 2F**). Additionally, the DNA backbone of the methylated nucleosome structure had a lower local resolution compared to unmethylated, despite the methylated map having a higher global resolution (**Suppl Fig 4**). This is consistent with the increased population of open linker and translationally shifted nucleosome particles in the methylated structure. Altogether, our cryo-EM structural analysis suggests that the presence of DNA methylation creates open and potentially destabilized H2A.Z nucleosome structures.

Cryo-EM structures of 601L H2A.Z nucleosomes show no difference between methylation status

In addition to the Sat2R-P nucleosomes, we also solved cryo-EM structures of human H2A.Z nucleosomes reconstituted on methylated and unmethylated 601L DNA, a palindromic version of the lefthand portion of the classic Widom 601 sequence (**Suppl Fig 5 and 6, Suppl Table 1**) (88, 89). Final maps of the unmethylated and methylated structures were obtained at 3.09 Å and 3.25 Å, respectively (**Suppl Fig 5B-E**). RMSD analysis of the solved structures showed that both maps were essentially identical to each other, with the few differences present localized to highly flexible regions of the nucleosome (**Suppl Fig 5F**). The lack of clear structural differences when using 601L DNA is likely due to its artificially strong nucleosome positioning ability, which may have prevented detection of subtle changes caused by DNA methylation.

DNA methylation increases accessibility of H2A.Z but not H2A nucleosomes

In order to validate the observed opening of the H2A.Z nucleosome structure by DNA methylation, and to determine if this methylation effect is also present on nucleosomes containing canonical H2A, we measured nucleosome DNA accessibility via restriction enzyme digest (**Fig 3**). We tested both human H2A and H2A.Z nucleosomes reconstituted with methylated and unmethylated versions of 152 bp non-palindromic Sat2R DNA that was modified to include a singular HinfI site 18 bp in from the 5′-end containing a Cy5-fluorophore (**Fig 3A**). Homogeneity of the reconstituted nucleosomes was assessed via native PAGE and mass photometry analysis prior to restriction digest analysis (**Suppl Fig 7**). Digest of bare control DNA confirmed that HinfI behaved similarly on both methylated and unmethylated Sat2R substrates (**Fig 3B**). We found that H2A.Z nucleosomes were more accessible to enzymatic digest than H2A nucleosomes, independent of DNA methylation status (**Fig 3C and D**). When comparing methylation-dependent differences for each type of nucleosome, methylated and unmethylated H2A nucleosomes were largely digested at the same rate. Methylated H2A.Z nucleosomes, on the other hand, exhibited a small but more pronounced increase in accessibility as compared to its unmethylated counterpart (**Fig 3C and D**). These results corroborate our structural findings and also suggest that the effects of DNA methylation may be negligible on an intrinsically stable nucleosome (i.e. containing H2A) but can further destabilize, in a selective manner, the already open and accessible H2A.Z nucleosome.

H2A.Z and DNA methylation localization in *Xenopus laevis* sperm pronuclei and a fibroblast cell line

Results from our structural and biochemical experiments indicate decreased wrapping stability of H2A.Z nucleosomes on methylated DNA. While functional implications likely exist, this subtle effect on the intrinsic physical stability of the H2A.Z nucleosome is unlikely to be the sole driver of the mutually exclusive H2A.Z and DNA methylation compartmentalization in cells. To identify the major biochemical mechanism underlying this dichotomy, we turned to the *Xenopus laevis* egg extract system, a unique cell-free system that allows for characterization of chromatin proteins under physiological conditions (90–95).

To ensure that *Xenopus* egg extract is a valid tool for studying the H2A.Z-DNA methylation antagonism, we conducted CUT&Tag – Bisulfite Sequencing (CnT-BS) (62), a method which allows for the direct assessment of DNA methylation on CUT&Tag reads, against H2A.Z of *Xenopus* sperm nuclei incubated in interphase extract. This condition mimics fertilization and allows for full nucleosome assembly onto sperm (primarily composed of protamines and H3/H4 tetramers) to create functional pronuclei (96). We also collected samples against H3 to serve as a pan-nucleosome control. Since sperm pronuclei in egg extract represents a unique transcriptionally silent state (97–99), we also performed CnT-BS on XTC-2 cells, a *Xenopus laevis* fibroblast cell line (100), to assess changes in DNA methylation and H2A.Z overlap across different nuclei types.

Initial comparisons of CnT-BS reads across two biological replicates of our four conditions demonstrated good reproducibility between replicates, while each set of experimental conditions exhibited distinct characteristics. A greater than 99 % bisulfite conversion efficiency rate, as assessed through our unmethylated lambda DNA spike-in, was achieved, and correlation matrices of both the mapped reads and methylation calls were highly

similar between biological replicates (**Suppl Fig 8, Suppl Table 2 and 3**). When comparing across conditions, for both the H3/H2A.Z mapped reads and DNA methylation sites, the first degree of difference seen was between XTC-2 and sperm samples, and then H3 vs H2A.Z following after, indicating that large-scale changes in the DNA methylation and genomic landscape occurred between the two different nuclear states (**Suppl Fig 8**).

To assess characteristics of the H2A.Z and H3 genomic distributions, we subset the *Xenopus* genome into 1,000 bp bins and classified the top 20 % of bins containing the most mapped reads for each condition based on annotated functionalities (69). We found that a greater proportion of highly mapped H2A.Z bins were located near promoter regions as compared to H3 for both sperm and XTC-2 samples (**Suppl Fig 9A**). Although transcription is suppressed in *Xenopus* egg extracts, H2A.Z in sperm pronuclei were well positioned at TSSs, as generally seen for actively transcribed genes in somatic cells (**Suppl Fig 9B and C**). This mirrors the reported deposition of H3K4me3 at the +1 TSS sites in *Xenopus* sperm nuclei prior to remodeling as well as reports in zebrafish sperm and *Drosophila* that H2A.Z is positioned at promoters prior to zygotic genome activation (ZGA) (13, 96, 101).

H2A.Z is biased towards hypomethylated regions of the genome in both *Xenopus* sperm pronuclei and the XTC-2 fibroblast cell line

We next determined the methylation status of CpGs associated with either H2A.Z or H3 across replicates of XTC-2 and sperm nuclei (**Fig 4A**). One notable drawback of CUT&Tag is a bias towards open and accessible regions (102, 103), which may artificially amplify open chromatin signals and lead to an underestimation of DNA methylation status. To circumvent this issue, we analyzed H3 CpG methylation in areas outside of H2A.Z peaks (which tend to be highly accessible) to compare genomic methylation profiles in H2A.Z low/absent regions (H3) vs H2A.Z enriched peaks (H2A.Z).

Overall, sperm chromatin had a higher degree of methylation than XTC-2 chromatin, with 85.3 ± 0.4 % *s.d.* of H3-associated CpGs methylated in sperm compared to 53.5 ± 0.4 % in XTC nuclei (**Suppl Table 2**). Methylated CpG frequencies of H2A.Z-associated reads were reduced to 43.0 ± 0.7 % in sperm chromatin and even further down to 3.0 ± 0.3 % in XTC-2 cells. Mapping of H3 associated methylation calls to annotated genes of the *Xenopus laevis* genome showed ubiquitous distribution of methylation across the length of genes, except for at the TSSs where there was a marked depletion of methylation (**Suppl Fig 9B and C left**). Nearly all of these H3-marked hypomethylated TSSs exhibited H2A.Z enrichment in both sperm pronuclei and XTC-2 (**Suppl Fig 9B and C right**), indicating that hypomethylation-exclusive positioning of H2A.Z is most prominent at the TSSs (**Suppl Fig 9D**).

DNA methylation-sensitive H2A.Z deposition on synthetic DNA constructs in *Xenopus* egg extract

To directly test whether the presence or absence of DNA methylation can dictate preferential H2A.Z deposition independently of other pre-existing epigenetic marks or DNA sequence, we took advantage of the *Xenopus* egg extract's ability to efficiently assemble nucleosomes *de novo* onto naked DNA templates (104) (**Fig 4B**). We found that, upon incubation of DNA coated magnetic beads in interphase egg extract, H2A.Z accumulated less efficiently on methylated DNA substrates compared to unmethylated (**Fig 4C and D**), whereas

this methylation-sensitive deposition was not seen for H2A.X-F, the predominant H2A histone variant in *Xenopus* egg extract (**Fig 4E and F**) (105). The methylation bias of H2A.Z held true for arrays of either the GC-rich Widom 601 (57 % GC content) or the more AT-rich human HSat2 (38% GC content) sequence. We next asked whether both H2A.Z variants, H2A.Z.1 and H2A.Z.2 (H2A.V), exhibit the same DNA methylation sensitivity. Since our antibody does not distinguish between the two H2A.Z paralogs, we translated ALFA-tagged versions of the variants in egg extract from exogenously added mRNA and found that both paralogs displayed preferential deposition onto unmethylated DNA (**Suppl Fig 10**).

The SRCAP complex mediates H2A.Z's preferential deposition on unmethylated DNA

Since SRCAP-C is known to play a major role in depositing H2A.Z to nucleosomes via exchange of H2A dimers on the nucleosome with H2A.Z (40), we examined if the observed preferential association of H2A.Z with unmethylated DNA in *Xenopus* egg extracts is mediated by SRCAP-C. Depletion of SRCAP-C from extract using a custom antibody against *Xenopus laevis* SRCAP greatly reduced the amount of H2A.Z associated with unmethylated DNA compared to control depletion (ΔIgG), while its binding to methylated DNA was not affected (**Fig 5A-C**). Consequently, in SRCAP-C-depleted (ΔSRCAP) extracts, residual H2A.Z binding to DNA became insensitive to DNA methylation status, while the amount of H2A.X-F on DNA was not affected by SRCAP-C depletion (**Fig 5B and D**). Consistent with the critical role of SRCAP-C in the preferential deposition of H2A.Z to unmethylated DNA, DNA-binding of SRCAP and ZNHIT1, two core subunits of SRCAP-C, was greatly inhibited by methylation in egg extract (**Fig 5E-F**). In contrast, subunits of the other H2A.Z remodeling complexes, Ino80 (Ino80-C) and p400 (Tip60-C), did not exhibit methylation-sensitive DNA binding (**Suppl Fig 11**). From these results, we conclude that SRCAP-C is the major driver biasing H2A.Z deposition against methylated DNA.

DISCUSSION

In this study, we found that DNA methylation can affect H2A.Z deposition and stability via two distinct mechanisms (**Fig 6)**. DNA methylation first directly opposes H2A.Z deposition by inhibiting SRCAP-C DNA binding. SRCAP-C accounts for the majority of H2A.Z deposition on unmethylated DNA and is the primary mediator behind DNA methylation's exclusion of H2A.Z in the transcriptionally inactive *Xenopus* egg extract. A subpopulation of H2A.Z, however, is still deposited onto methylated DNA, driven by methylation insensitive mechanism(s) whose identity remains to be established. Once formed, H2A.Z nucleosomes on methylated DNA are more open and accessible compared to their unmethylated counterparts. This decrease in DNA-histone contact stability of methylated H2A.Z nucleosomes may then further facilitate clearance of H2A.Z from methylated regions of the genome.

Supporting our observation that a SRCAP-independent mechanism(s) exists to deposit H2A.Z on methylated DNA in *Xenopus* egg extract, a substantial fraction of H2A.Z-associated genomic DNA is methylated in sperm pronuclei. In the fibroblast cell line XTC-2, that methylated H2A.Z fraction is greatly reduced. However, due to the accessibility bias inherent within CUT&Tag, our data likely underestimates the true degree of H2A.Z and DNA methylation overlap. A previous study in human myometrium reported a CpG methylation rate of 20 % for H2A.Z (17). Nevertheless, we believe the decreased trend in H2A.Z and DNA methylation overlap of XTC-2 cells holds true and suggests that the high degree of H2A.Z and DNA methylation

coincidence in sperm pronuclei reflects its unique early development and/or transcriptionally silent state. Given the strong preference of SRCAP-C binding to unmethylated DNA in *Xenopus* egg extracts, we predict that additional pathways missing in the egg extract system are necessary to reenforce the antagonism between DNA methylation and H2A.Z enrichment.

Perhaps the greatest difference between XTC-2 cells and *Xenopus* eggs is the presence of transcription, which is one of the main evictors of H2A.Z and necessary for global H2A.Z turnover as well as pruning of randomly deposited H2A.Z within gene bodies (106–109). Traversing of RNA polymerase (Pol) II across nucleosomes, aided by the FACT complex, results in transient histone dimer displacement (110–113), leading to the possibility that the more accessible H2A.Z nucleosome structures formed on methylated DNA may have increased susceptibility to Pol II disruption. Furthermore, FACT and Spt6 do not reincorporate H2A.Z into the reassembled nucleosome post Pol II encounter, reinforcing selective loss of H2A.Z (114, 115). Outside of transcription, the egg extract system also lacks both the DNMT3 enzymes responsible for *de novo* methylation and the TET system responsible for DNA methylation removal in animals (116). In *Arabidopsis*, H2A.Z promotes ROS1-associated DNA demethylation (16) and must be excluded to ensure faithful heterochromatin formation (117). Thus, H2A.Z-driven mechanisms to limit DNA methylation remain relevant pathways to increase the H2A.Z/DNA methylation separation within the genome which may be missing in eggs/early embryonic states.

Exactly how SRCAP-C senses DNA methylation remains a pertinent question. Recent structural work on the yeast SWR1 complex and human SRCAP-C indicate that both the ATPase and the ZNHIT1 subunit (Swc6 in yeast) mediate binding of, and one-dimensional diffusion on, naked DNA substrates (118, 119), marking them as likely candidates responsible for methylation sensing. Additionally, the YEATS domain-containing subunit GAS41, found in both SRCAP-C and Tip60-C, recognizes H3K14ac and H3K27ac (120, 121), and deposition of H3K27ac, itself, is antagonized by DNA methylation at enhancers (122). It is possible that both direct recognition of DNA methylation and changes in the PTM landscape of methylated chromatin play a role in dictating SRCAP-C localization and activity.

While a general consensus on how DNA methylation influences nucleosome stability remains controversial, our results indicate that DNA methylation creates more open nucleosome structures, particularly on H2A.Z nucleosomes, similar to reports from FRET-based and molecular simulation studies on canonical nucleosomes (37–39, 123). Although the ambiguity in DNA positioning of our cryo-EM structures prevents us from discussing the structural coordination of specific CpG sites, we favor the idea that the increase in accessibility is due to effects of DNA methylation on global DNA structure rather than at specific methylated segmants. This is in line with observations that methylation increases overall DNA stiffness, making it harder for the DNA to bend and wrap around histone octamers (37, 124).

Notably, our restriction enzyme accessibility assay shows that H2A.Z nucleosomes are considerably more open and accessible compared to canonical H2A, regardless of methylation status. This difference is substantially larger than that induced by DNA methylation on H2A.Z, suggesting that the physical influence of DNA methylation on nucleosomes is overall subtle and noticeable only on an already destabilized nucleosome. Similar findings have been reported on canonical H2A 601 nucleosomes, where the effects of DNA methylation manifest greatest on an open nucleosome sub-population compared to their closed counterparts (38). This is likely why we saw no methylation-dependent structural differences with the 601L sequence (**Suppl Fig 5**),

as its innate nucleosome wrapping and positioning ability is even stronger than the classic Widom 601, highlighting the importance of careful DNA sequence consideration when assessing for more subtle physical influences. Overall, we suggest that the subtle nucleosome destabilization effect of DNA methylation is selectively pronounced on H2A.Z nucleosomes, which already exhibit an intrinsic instability compared to canonical H2A nucleosomes.

Beyond its interaction with DNA methylation, we show that H2A.Z promoter localization occurs immediately upon conditions mimicking fertilization in a transcription-independent manner. Unlike the almost complete loss of histones seen in mammalian sperm (125, 126), *Xenopus* sperm is packaged as a mixture of protamines and H3/H4 tetramers, which are then remodeled into full nucleosomes upon fusion with an egg (96, 127, 128). In our egg extract system, which lacks the maternal genome, the bulk of H2A.Z in sperm pronuclei is newly deposited yet still specifically targeted to promotors either via underlying sequence motifs (13, 101) or tetramer modifications maintained in sperm (96). This is similar to *Drosophila* where H2A.Z is recruited to TSSs prior to both Pol II binding and ZGA, marking genes for zygotic activation (101), indicating conservation of this initial transcription-independent H2A.Z positioning across both vertebrates and invertebrates.

Our work here provides a critical step in the pathways dictating genomic organization by uncovering the previously undescribed methylation sensitivity of SRCAP-C along with the destabilizing effects of DNA methylation on H2A.Z nucleosomes. Together, these findings provide molecular mechanisms driving the conserved antagonism between H2A.Z and DNA methylation in the genome. However, many questions remain to be answered, such as why the existence of both methylation sensitive and insensitive H2A.Z deposition pathways, the broader functional implications of the H2A.Z-DNA methylation antagonism as a whole, and mechanisms driving its maintenance in species that experience periods of global demethylation. These questions represent important areas of future study for our understanding of epigenome dynamics throughout development.

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AUTHOR CONTRIBUTIONS

R.M.S. and H.F. conceived and designed the study. R.M.S. and Y.A. designed and performed the Cryo-EM analysis. R.M.S. and H.A.K. designed physiological experiments. H.A.K. created the

custom α-SRCAP antibody, prepared XTC-2 nuclei, and purified pG-Tn5. R.M.S. conducted all experiments and analysis. R.M.S. and H.F. prepared the manuscript with input from H.A.K. and Y.A.

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CONFLICT OF INTEREST
H.F. is affiliated with the Graduate School of Medical Sciences, Weill Cornell Medicine and the Cell Biology Program at the Sloan Kettering Institute. The authors declare no competing interests.

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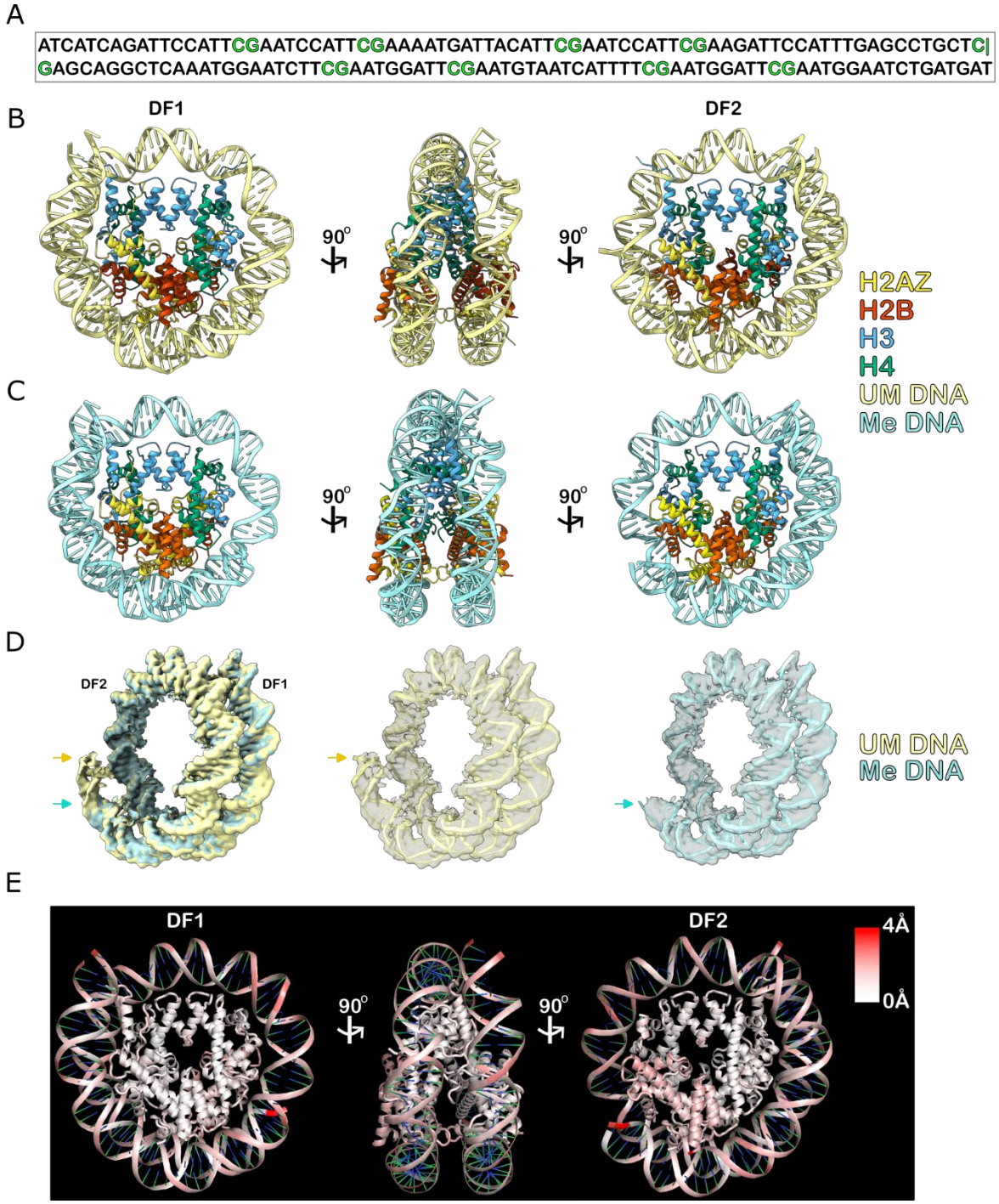


Figure 1. Cryo-EM structures of methylated and unmethylated Sat2R-P human H2A.Z nucleosomes. (A) Diagram of the palindromic Sat2R-P sequence. Methylated cytosines are labeled in green. Vertical bar denotes halfway point. **(B and C**) Atomic models of unmethylated (**B**) and methylated (**C**) Sat2R-P H2A.Z structures (the v1 model for both). Two face views (DF1 and DF2) and a side view (middle) are shown. **(D)** Left, overlay of unmethylated (UM) and methylated (Me) Sat2R-P DNA EM densities. Middle and right, overlays of DNA densities on top of the corresponding v1 DNA atomic models for either the unmethylated (middle) or methylated (right) structures. Arrows point to the visualizable end of the DF2 linker DNA for each structure. **(E)** RMSD analysis comparing differences between unmethylated and methylated v1 Sat2R-P H2A.Z atomic models.

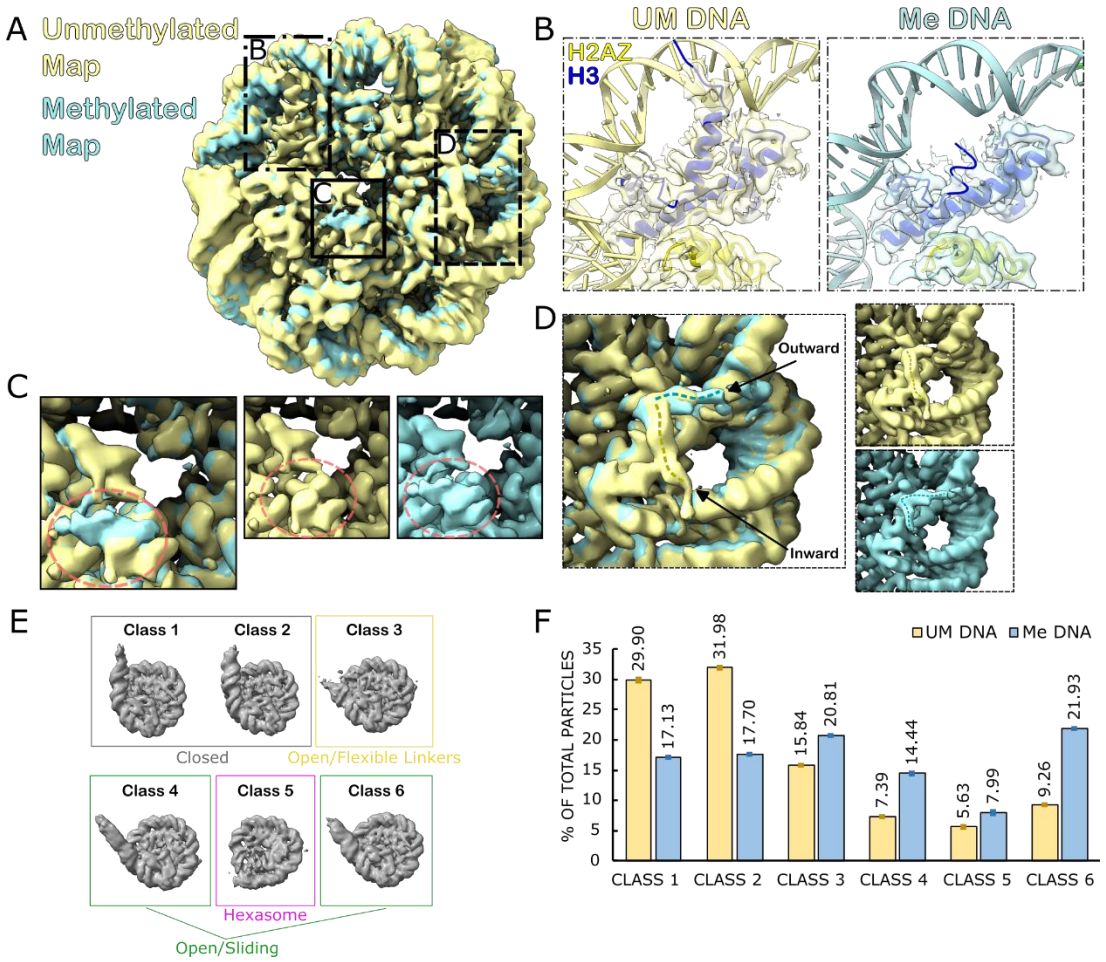


Figure 2. DNA methylation reduces H3-DNA contacts, alters H4 N-terminal tail orientations, and forms open nucleosome structures. (A) Density maps of unmethylated (UM) and methylated (Me) Sat2R-P H2A.Z nucleosomes filtered and resampled to ~3Å. The face shown, and all following zoom-ins, is of DF2. Maps are overlaid onto each other. Boxed regions highlight major areas of difference shown in **B-D**. **(B)** Zoom-in of the H3 N-terminus on DF2 of both structures. Density maps are shown as a transparent overlay on top of the corresponding v1 atomic models. **(C)** Zoom-in of the DF2 acidic patch on both structures. Area corresponding to the extra density found in the Me structure labeled in red. **(D)** Zoom-in of the H4 N-terminal tail. Dotted lines show each structure's preferred orientation **(E)** 3D Models used in the *in silico* mixing 3D classification analysis. Classes are as follows: (1 and 2) closed nucleosome structures with varying linker lengths; (3) open nucleosomes with highly flexible linkers; (4 and 6) open nucleosomes that have shifted position from center; (5) hexasomes. **(F)** Results of classification analysis. Y-axis is the proportion of particles sorted into each class as a percent of the total input particles from each sample (UM or Me). Error bars represent SEM, n = 3 technical replicates.

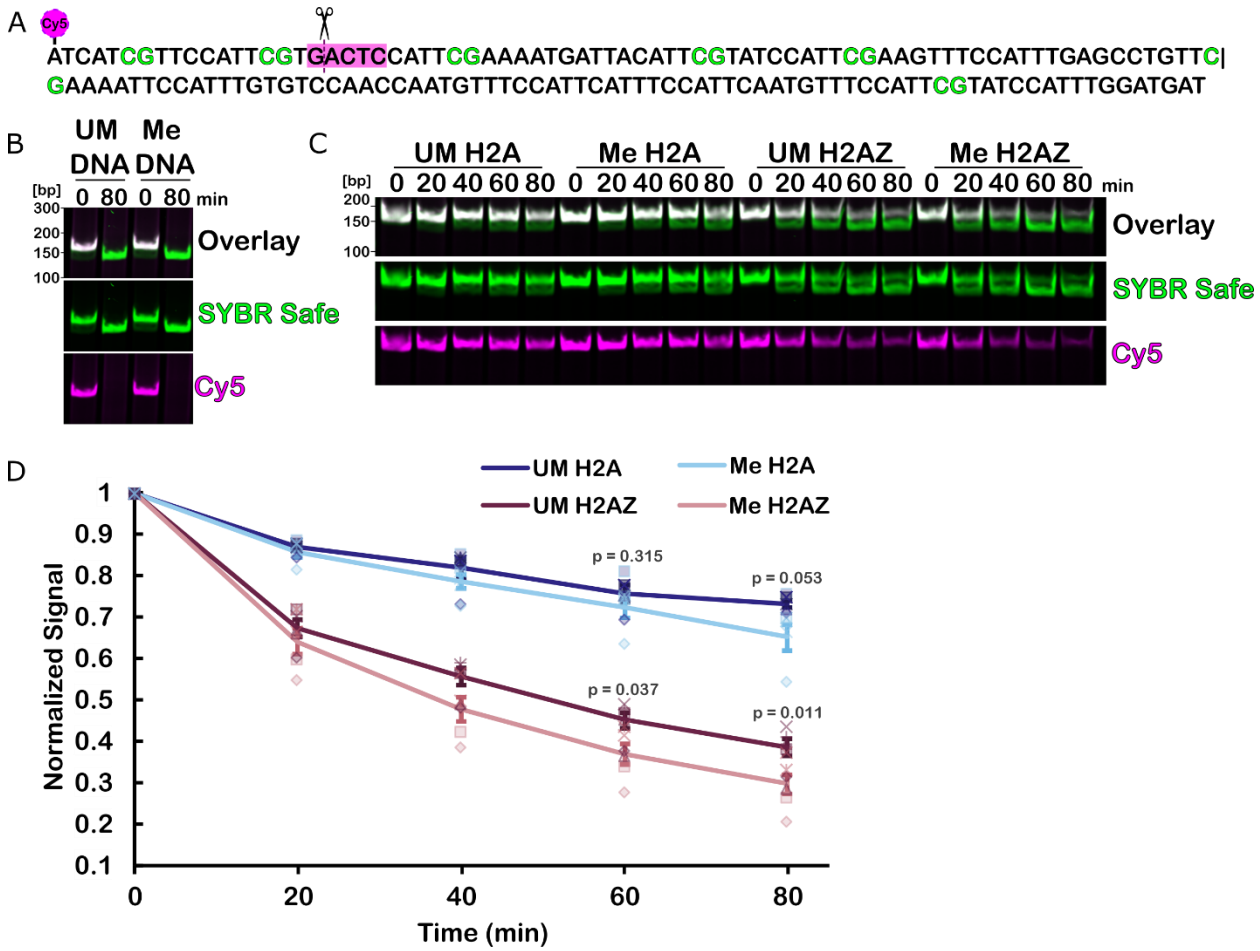


Figure 3. DNA methylation increases H2A.Z nucleosome accessibility. (A) Diagram of the 1HinfI_Sat2R DNA sequence used. CpG sites are shown in green. HinfI recognition site highlighted in magenta. A Cy5 fluorophore is attached to the 5′ end nearest the HinfI site. **(B)** Native gel showing HinfI digestion of bare control DNA over the time course of the assay. SYBR Safe was used to stain total DNA. Digestion efficiency was assessed via loss of Cy5 signal and a downward shift in the total DNA band. **(C)** Native gel showing HinfI digestion time course of UM and Me human H2A and H2A.Z nucleosomes. **(D)** Quantification of (**C**). Cy5 signals were normalized to total SYBR Safe signal for each sample. Error bars represent SEM. Each shape represents data from one independent experiment, n = 5 experiments. Statistics comparing unmethylated and methylated samples at indicated timepoints were completed with a two-tailed t-test assuming unequal variance with *p*-values listed above each comparison.

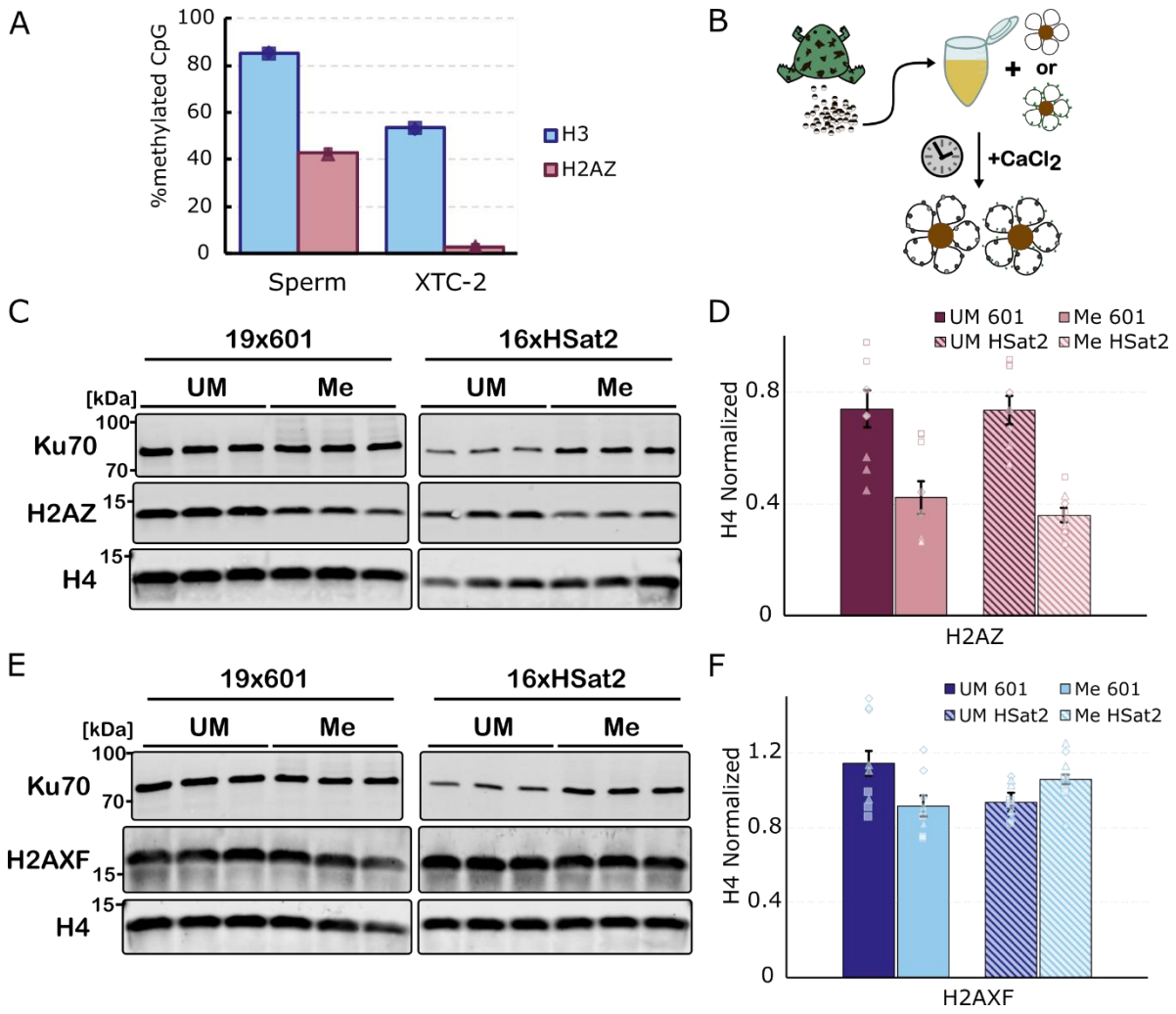


Figure 4. Presence or absence of DNA methylation influences H2A.Z deposition. (A) Percentage of methylated CpG sites (≥ 5x coverage) associated with H2A.Z peaks or H3 reads in sperm pronuclei incubated in interphase egg extract or XTC-2 nuclei following CnT-BS library preparation. H3 data is taken from regions outside of H2A.Z enriched peaks. Data points represent biological replicates (n = 2). **(B)** Schematic of chromatinization assay. Magnetic beads coated with DNA of interest were incubated in *Xenopus* egg extract with the addition of $CaCl_2$ to induce cycling into interphase. After 60 min, DNA beads were isolated to assess for histone composition. **(C and E)** Western blot results of chromatinization assay probing for either H2A.Z (**C**) or H2A.X-F (**E**). Ku70 and H4 signals are shown as loading controls. Beads coated with unmethylated (UM) or methylated (Me) 19-mer arrays of Widom 601 (19x601) or 16-mer arrays of HSat2 (16xHSat2) were used. A representative blot of technical triplicates for each condition is presented. **(D and F)** Quantification of (**C** and **E**). H2A.Z and H2A.X-F signals were normalized to H4 intensity. Error bars represent SEM. Data points represent technical triplicates across 3 biological replicates with each shape representing data from a single independent experiment (n = 9).

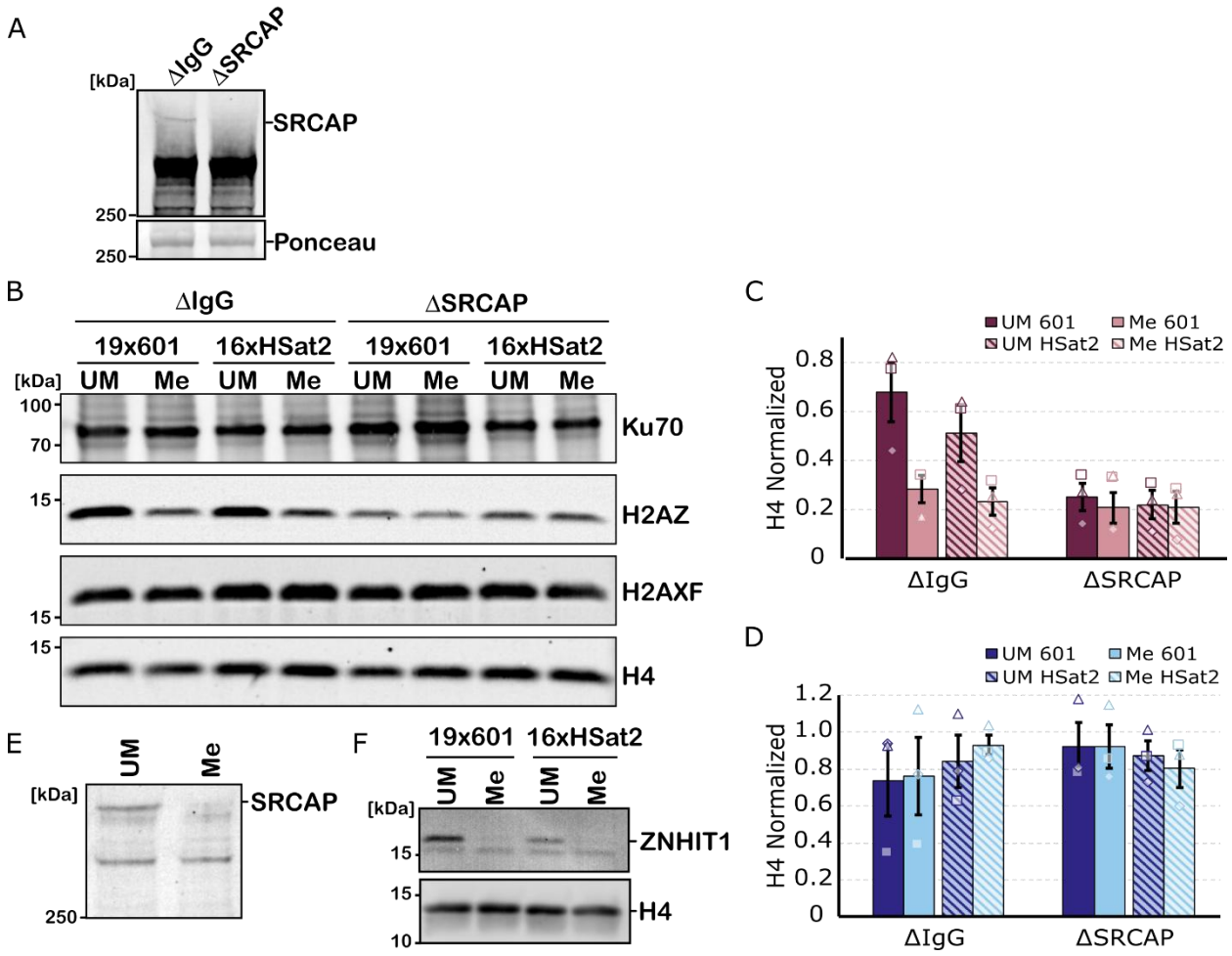


Figure 5. The SRCAP complex mediates H2A.Z's preferential association with unmethylated DNA. (A) Western blot to detect SRCAP in control IgG-depleted *Xenopus* egg extract (ΔIgG) or SRCAP-depleted extract (ΔSRCAP). Bottom panel shows ponceau staining as a loading control. **(B)** Western blots of chromatinization assay in IgG control vs SRCAP depleted egg extract. Magnetic beads coated with 19-mer arrays of Widom 601 (19x601) or 16-mer arrays of HSat2 (16xHSat2) were incubated with interphase egg extract for 60 min, before their isolation. **(C)** Quantification of H2A.Z signal normalized to H4 from **(B)**. **(D)** Quantification of H2A.X-F signal normalized to H4 from **(B)**. Error bars in (**C**) and (**D**) represent SEM from n = 3 biological replicates. **(E)** Western blot staining for SRCAP on 16xHSat2 DNA beads incubated in interphase egg extract. **(F)** Western blot staining for ZNHIT1 on specified DNA beads incubated in interphase egg extract. Representative image shown from two independent experiments.

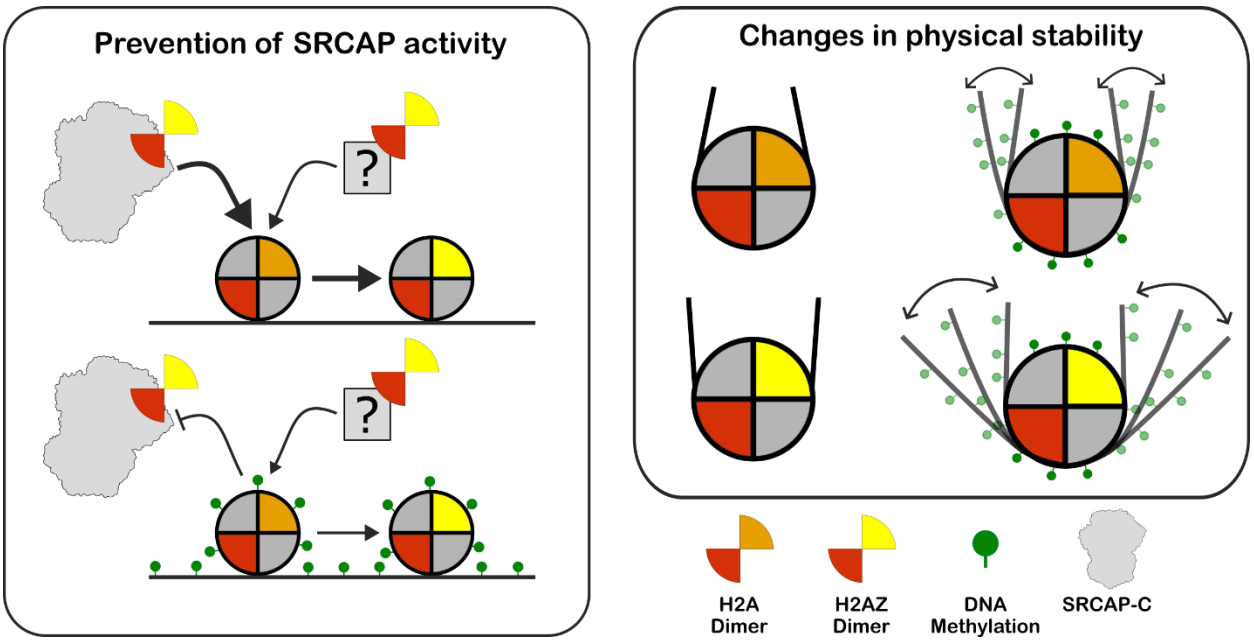
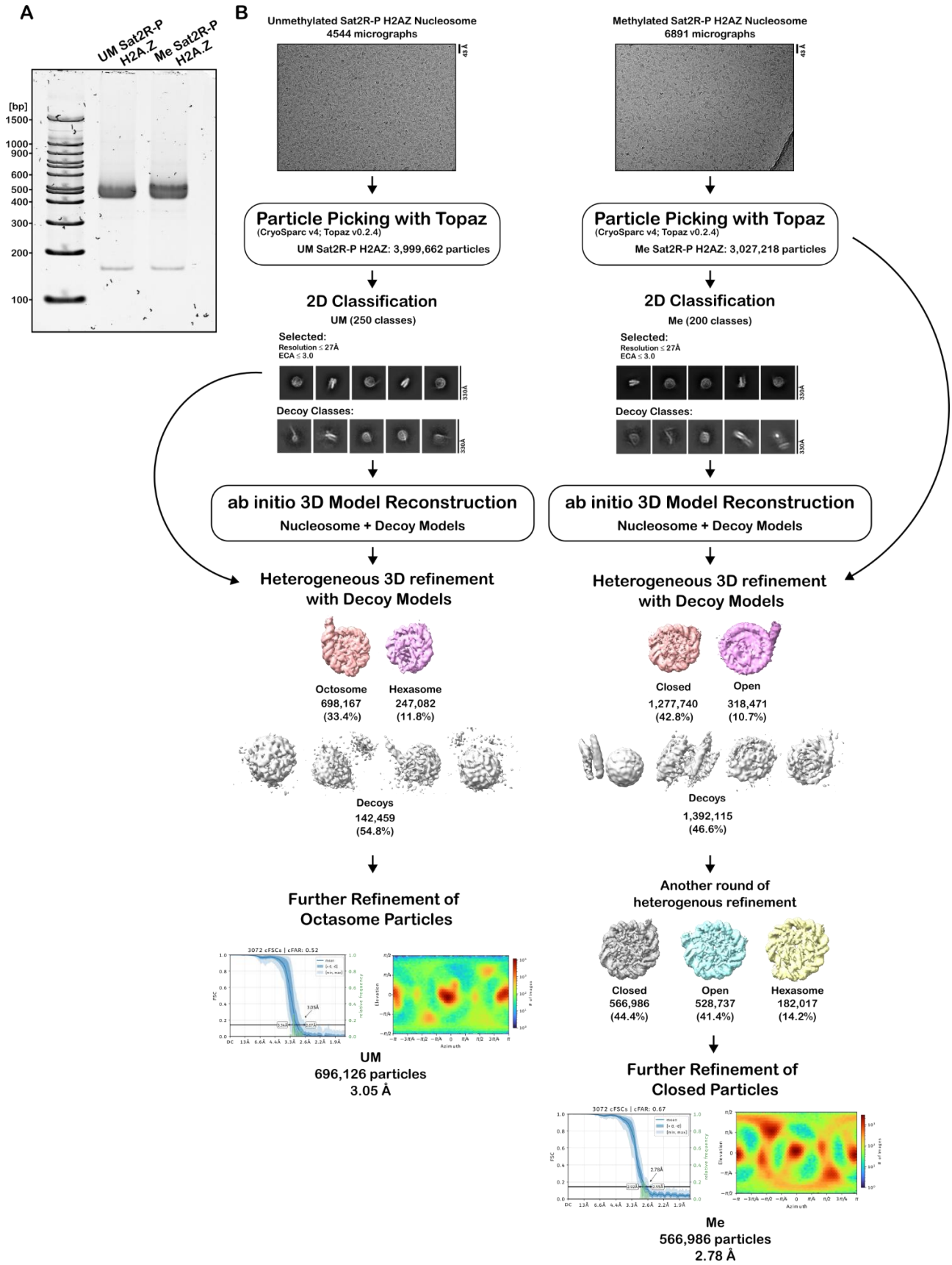


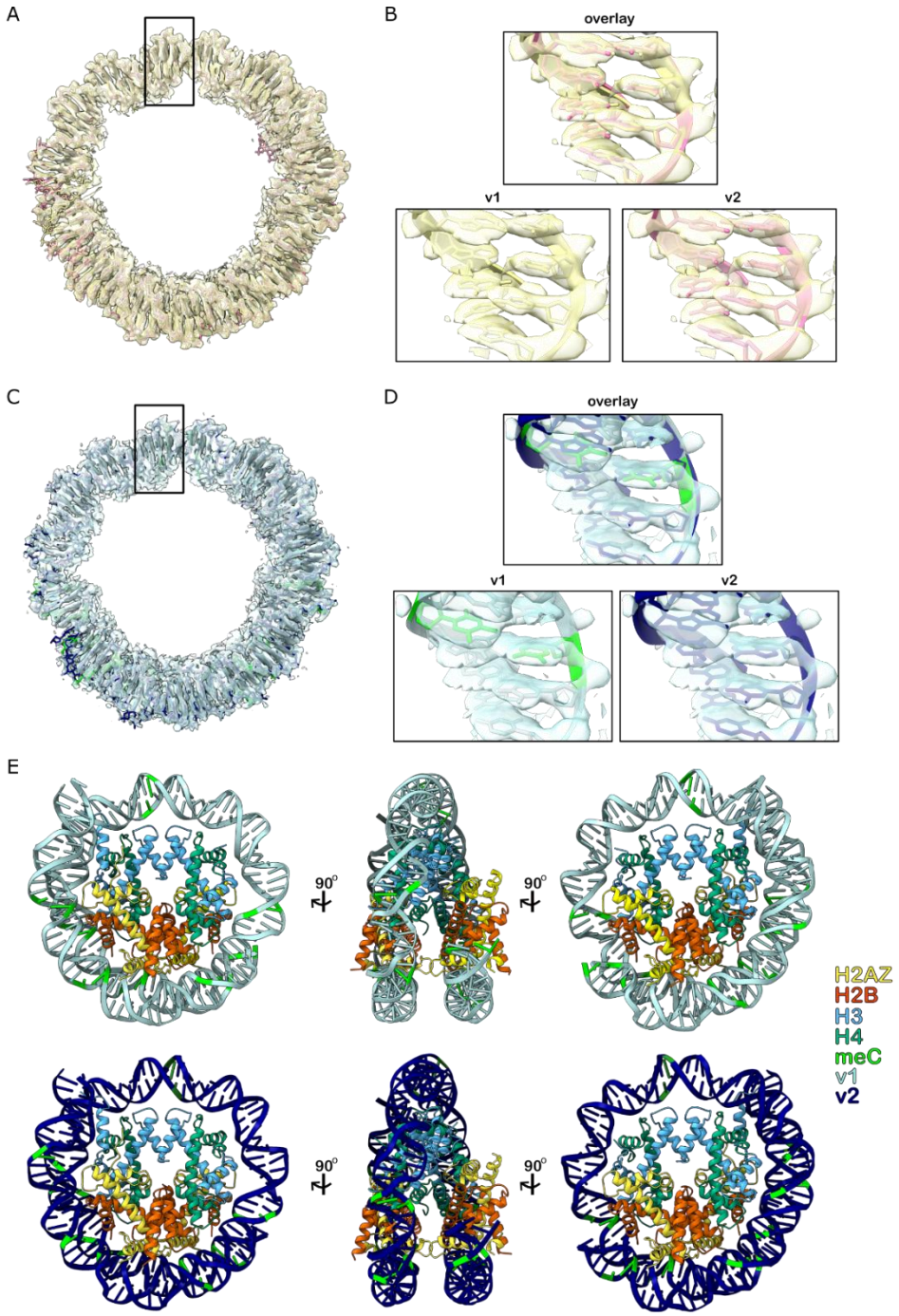
Figure 6. DNA methylation prevents SRCAP-C mediated H2A.Z deposition and creates more open and accessible H2A.Z nucleosome structures. Left, SRCAP-C is capable of depositing H2A.Z on unmethylated substrates, but presence of DNA methylation prevents binding and deposition activity. Another unidentified mechanism deposits H2A.Z in a DNA methylation insensitive manner. Right, DNA methylation decreases DNA wrapping stability, an effect felt greatest on the already open H2A.Z nucleosomes compared to the more tightly wrapped H2A nucleosomes, creating more open and accessible H2A.Z nucleosome structures.

Table 1. DNA sequences/oligos used in the study.							
Sequence Name	**Sequence**	**Vector**	**Plasmid Name**	**Insert Location**	**Source**	**Assay(s) Used In**	**Additional Notes**
Sat2R-P	ATCATCAGATTCCATTCGAATCCATTCGAAAATGATTACATTCGAATCCATTCGAAGATTCCATTTGAGCCTGCTCGAGCAGGCTCAAATGGAATCTTCGAATGGATTCGAATGTAATCATTTTCGAATGGATTCGAATGGAATCTGATGAT	pUC18	RMSp81	Between XbaI and BamHI sites	Generated and purchased from Integrated DNA Technologies (IDT™)	Cryo-EM	Modified from the Sat2R sequence in (Osakabe et al, 2015).
601L	ATCACAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGACAGCTCTAGCACCGCTTAAACGCACGTACGGAATCCGTACGTGCGTTTAAGCGGTGCTAGAGCTGTCTACGACCAATTGAGCGGCCTCGGCACCGGGATTGTGAT	pUC57	601L-145-8X	EcoRV	(601L-145-8X) was a gift from Curt Davey (Addgene plasmid # 158572 ; http://n2t.net/addgene:158572 ; RRID:Addgene_158572)	Cryo-EM	
1HinfI_Sat2R	ATCATCGTTCCATTCGTGACTCCATTCGAAAATGATTACATTCGTATCCATTCGAAGTTTCCATTTGAGCCTGTTCGAAAATTCCATTTGTGTCCAACCAATGTTTCCATTCATTTCCATTCAATGTTTCCATTCGTATCCATTTGGATGAT	pUC18	RMSp161	Between XbaI and BamHI sites	Generated and purchased from Integrated DNA Technologies (IDT™)	Restriction Digest Assay	DNA generated via PCR amplification using following primers: /5Cy5/ATCATCGTTCCATTCGTGAC; ATCATCCAAATGGATACGAATGG
HSat2 arrays (1 repeat shown)	CGTTTGATTCCATTTGATGTTGATTCCATTCGAGTCCATTCGATGATAATTCCATTCGATTCTTTGCGATGATTCCATTCCTTTCCATTTGAGATGATTCCATTCGAGACCATTCGATGATTGCATTCAATTCATTCGATGACGATTATTCAATTCCGTTCAATGATTCCATTCGATTCCAATTGATGATGATTCCATTCGATTCCATTTGATGATGATTCCATGCGATTCCATTCGATGATGACTCCGATATCGGATCTGATATC	pUC18	RMSp64	Between XbaI and BamHI sites	Generated and purchased from Integrated DNA Technologies (IDT™)	Chromatinization Assays	GenBank: X06199.1
601 arrays (1 repeat shown)	CTGGAGAATCCCGGTGCCGAGGCCGCTCAATTGGTCGTAGCAAGCTCTAGCACCGCTTAAACGCACGTACGCGCTGTCCCCCGCGTTTTAACCGCCAAGGGGATTACTCCCTAGTCTCCAGGCACGTGTCAGATATATACATCCTGTGCATGTATTGAACAGCGACTCGGGTTATGTGATGGACCCTATACGCGGCCGCC	pUC18	pAS696	EcoRI/XbaI	Gift from Aaron Straight	Chromatinization Assays	
Oligos for Sequencing Library Preparation							
Sequence Name	**Sequence**				**Source**	**Assay(s) Used In**	**Additional Notes**
Tn5mC-Apt1	T/iMe-dC/GT/iMe-dC/GG/iMe-dC/AG/iMe-dC/GT/iMe-dC/AGATGTGTATAAGAGA/iMe-dC/AG				(Li et al, 2021); Generated and purchased from Integrated DNA Technologies (IDT™)	CnT-BS adapter oligo	Annealed with Tn5mC1.1-A1block to form adapter oligo
Tn5mC1.1-A1block	/5Phos/CTGTCTCTTATACA/3ddC/				(Li et al, 2021); Generated and purchased from Integrated DNA Technologies (IDT™)	CnT-BS adapter oligo	Annealed with Tn5mC-Apt1 to form adapter oligo
Tn5mC-ReplO1	/5Phos//iMe-dC/TGT/iMe-dC/T/iMe-dC/TTATA/iMe-dC/A/iMe-dC/AT/iMe-dC/T/iMe-dC//iMe-dC/GAG/iMe-dC//iMe-dC//iMe-dC/A/iMe-dC/GAGA/iMe-dC//3InvdT/				(Li et al, 2021); Generated and purchased from Integrated DNA Technologies (IDT™)	CnT-BS	Used during oligonucleotide replacement step

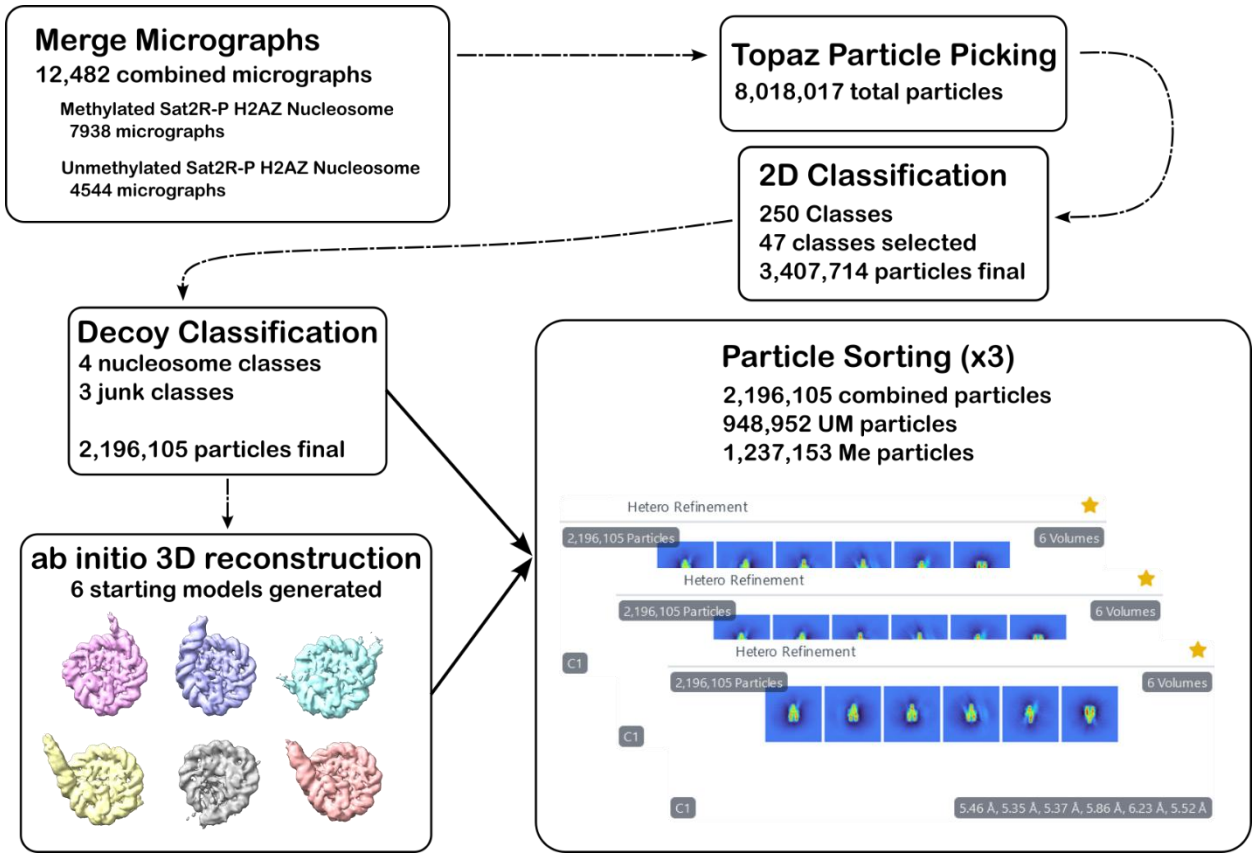
Table 2. Antibodies used in this study.						
Antigen	**Host**	**Company**	**Catalog No.**	**Peptide Used for Generation**	**Dilution**	**Assay(s) Used In**
H2A.Z	Rabbit	Custom		CIHKSLIGK KGQQKTV	2 µg/ml	Western Blots
H2A.Z	Rabbit	Active Motif	Cat#39013		1:10	CnT-BS
H3	Mouse	Cosmo Bio USA	MABI0001-100-EX		1:100	CnT-BS
H4	Mouse	Active Motif	Cat# 61521		1:1000	Western Blots
H2A.X-F	Rabbit	Custom, gift from David Shechter		Shechter et al. 2009	1:1000 (of serum)	Western Blots
Ku70	Rabbit	Custom		Postow et al. 2008	2 µg/ml	Western Blots
SRCAP	Rabbit	Custom		CPNSPSSH RVRKAKT	0.8 µg per 1 µl of beads (depletions); 12 µg/ml (WB)	Depletions; Western Blots
ZNHIT1	Rabbit	Proteintech	16595-1-AP		1:1000	Western Blots
INO80	Rabbit	Bethyl	A303-371A		1:1000	Western Blots
P400	Rabbit	Bethyl	A300-541A		1:1000	Western Blots
ALFA tag	Alpaca (single domain antibody purified from E. coli)	NanoTag Biotechnologies	N1502-Li800-L		1:1000	Western Blots
Secondaries						
IRDye® 800CW anti-Rabbit IgG	Goat	LICORbio	926-32211		1:10000	Western Blots
IRDye® 680RD anti-Mouse IgG	Goat	LICORbio	926-68070		1:10000	Western Blots
anti-Rabbit IgG	Guinea Pig	antibodies-online.com	ABIN101961		1:100	CnT-BS
anti-Mouse IgG	Rabbit	abcam	ab46540		1:100	CnT-BS



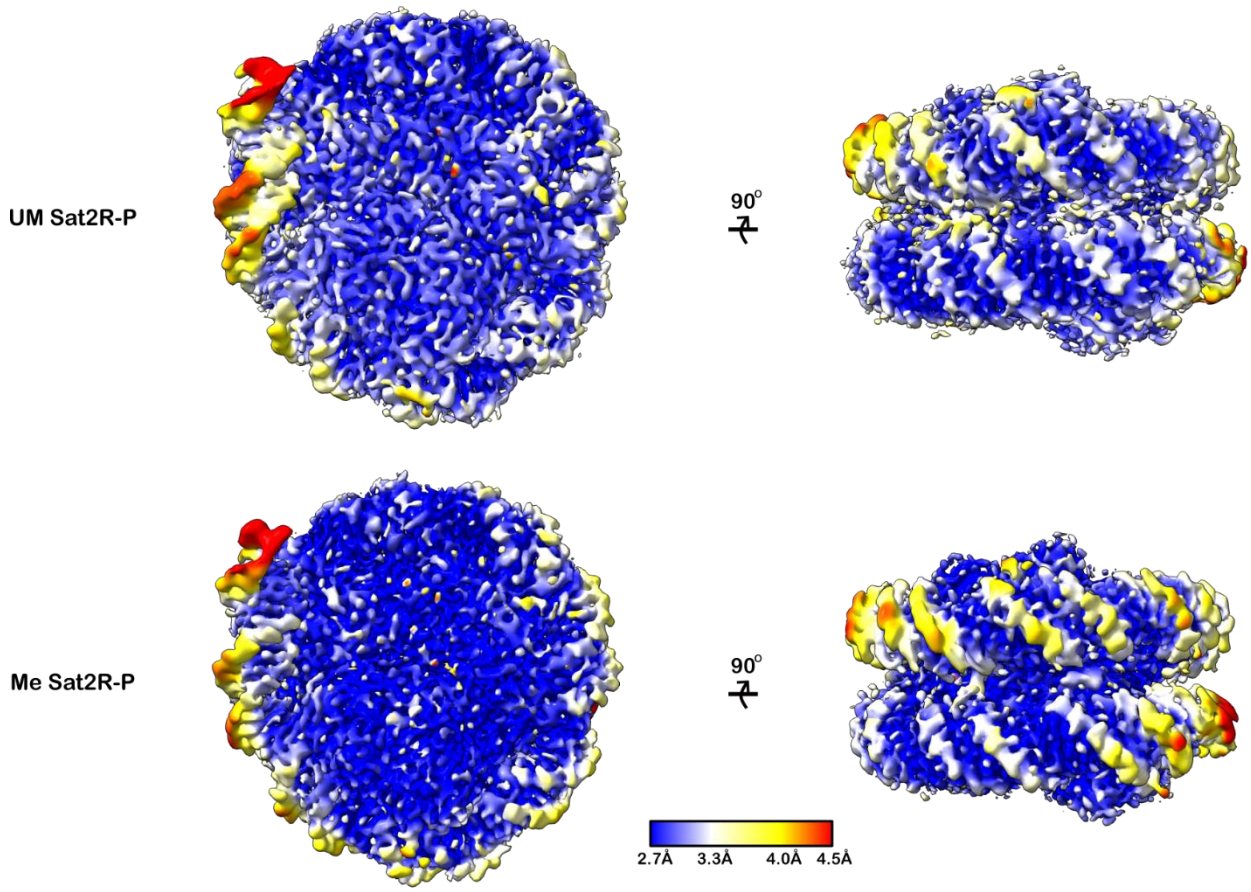
Supplementary Figure 1. Workflow of Sat2R-P cryo-EM analysis. (A) Native PAGE analysis of H2A.Z nucleosomes used for cryo-EM analysis. Bands visualized through SYBR Safe staining. **(B)** Diagram of structure analysis pipeline for either unmethylated (left) or methylated (right) H2A.Z SAT2R-P samples. Further details described in methods.



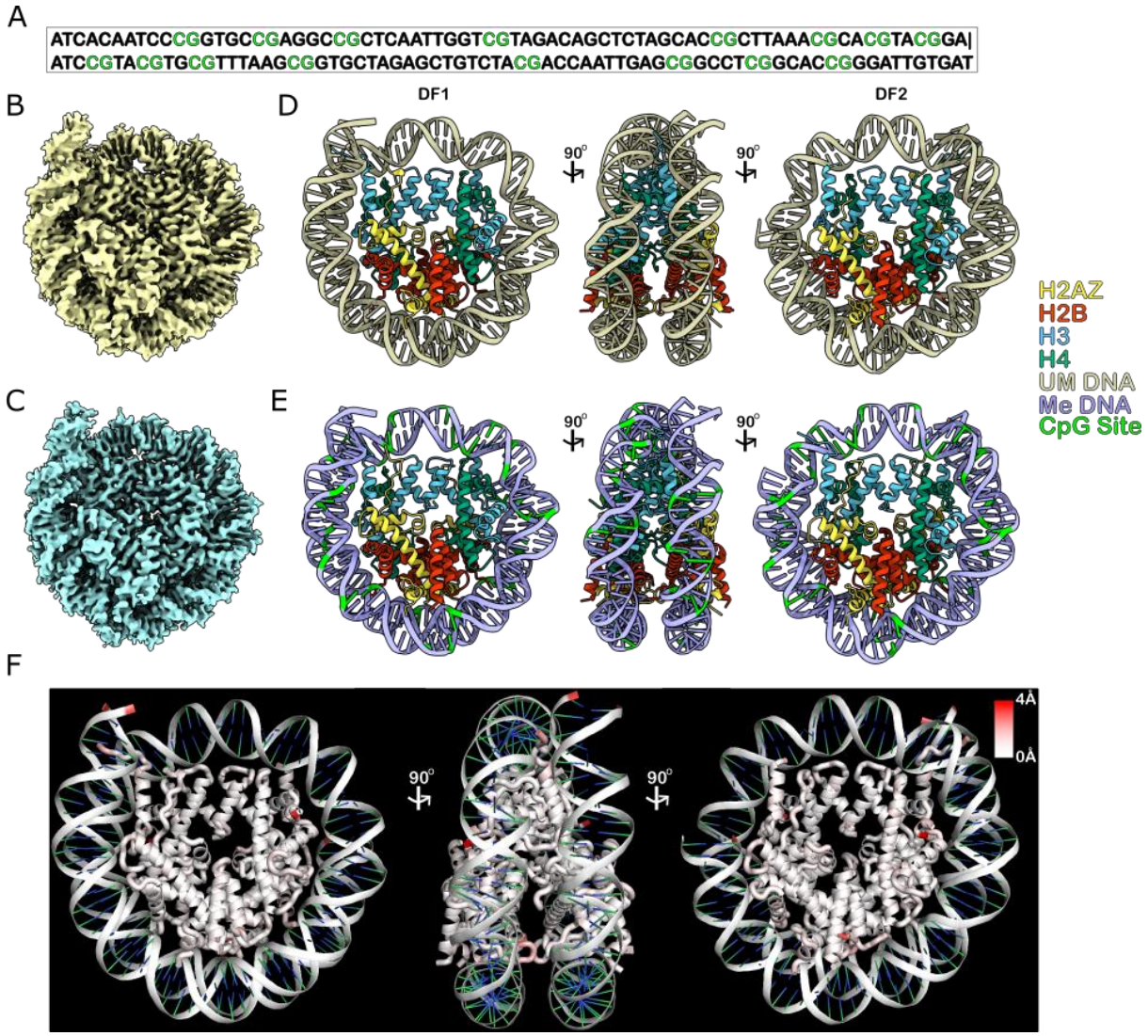
Supplementary Figure 2. DNA atomic model generation for Sat2R-P H2A.Z nucleosome structures. (A and C) Overlays of electron density maps for the DNA with both generated atomic models for either the unmethylated **(A)** or methylated **(C)** Sat2R-P H2A.Z nucleosome structure. **(B and D)** Zoom-in of dyad regions showing fitting of both DNA atomic models (v1 and v2) generated for either the unmethylated **(B)** or methylated **(D)** structure. Methylated cytosines are highlighted in green. Top panels are overlays of both models for the two structures. Positioning was determined by assessing densities around bases which retain purine/pyrimidine identity in both models vs those that switch. Bases that switched from purine to pyrimidine, and vice versa, showed more ambiguous densities than those that retained identity. Positioning was determined using the unmethylated structure and then used in modeling the methylated structure. (**E**) Comparison of predicted CpG positioning for the v1 (top) and v2 (bottom) atomic models generated for the methylated structure.



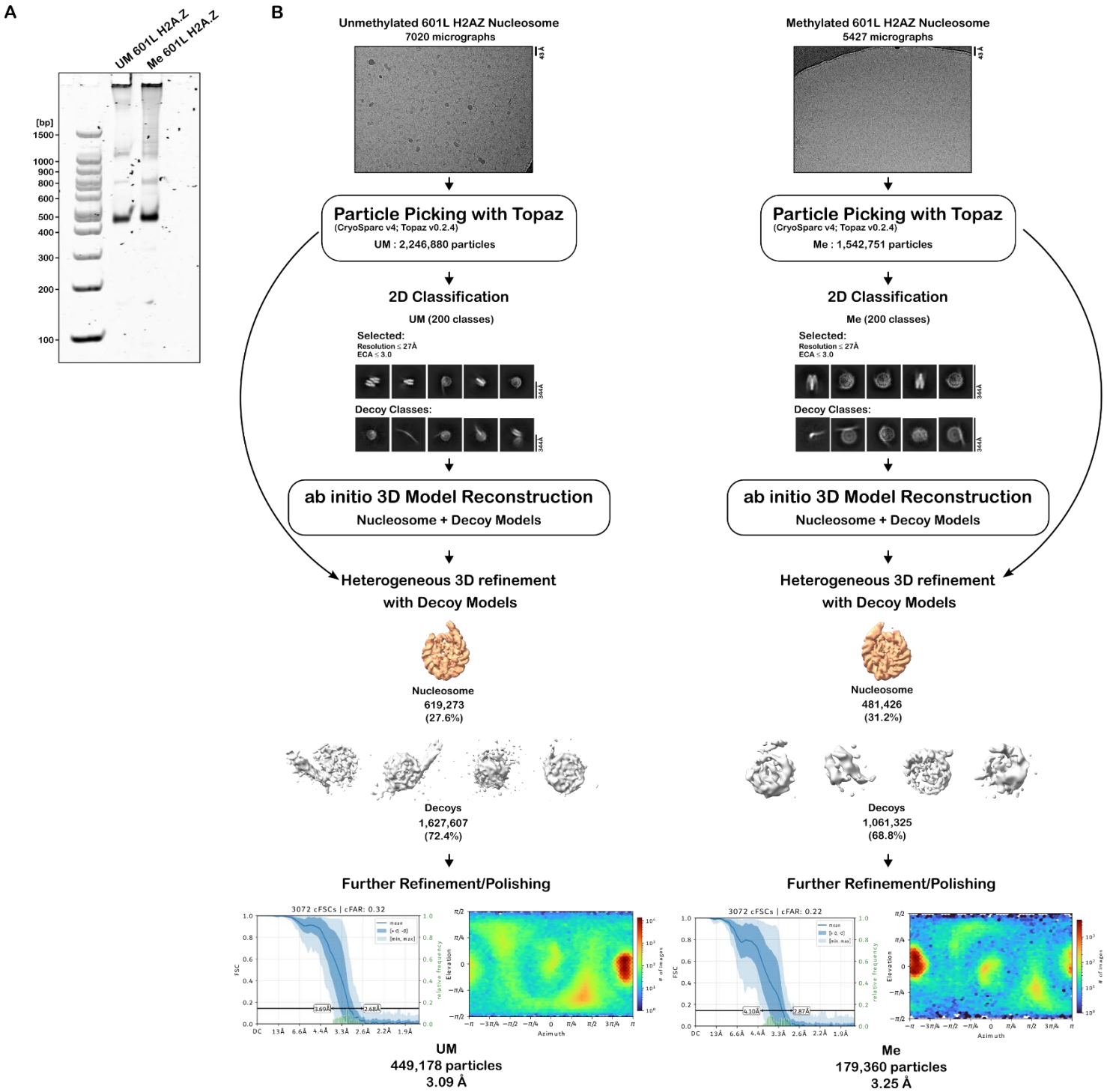
Supplementary Figure 3. Workflow of *in silico* mixing 3D classification analysis. Particles from a merged batch of unmethylated and methylated Sat2R-P H2A.Z nucleosome micrographs were picked and fed through the standard analysis pipeline. Six 3D models were then generated representing various open, closed, or hexasome-containing states of the nucleosome. Merged particles were then sorted to each of the classes through CryoSPARC's heterorefinement tool (n = 3 technical replicates).



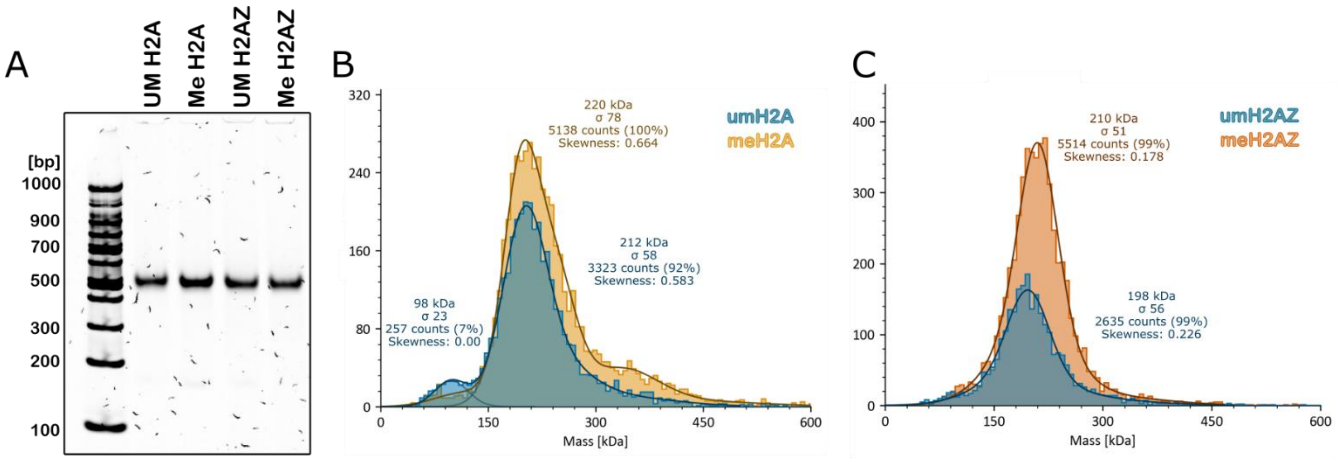
Supplementary Figure 4. Local resolution estimates for unmethylated and methylated Sat2R-P H2A.Z structures. Left, local resolution estimates of DF1 for both unmethylated (top) and methylated (bottom) Sat2R-P density maps. Right, side view showing local resolution of the DNA backbone for both methylated and unmethylated density maps. Local resolution maps were generated in CryoSPARC using the local resolution estimation tool and overlaid on density maps filtered using CryoSPARC's local filtering tool.



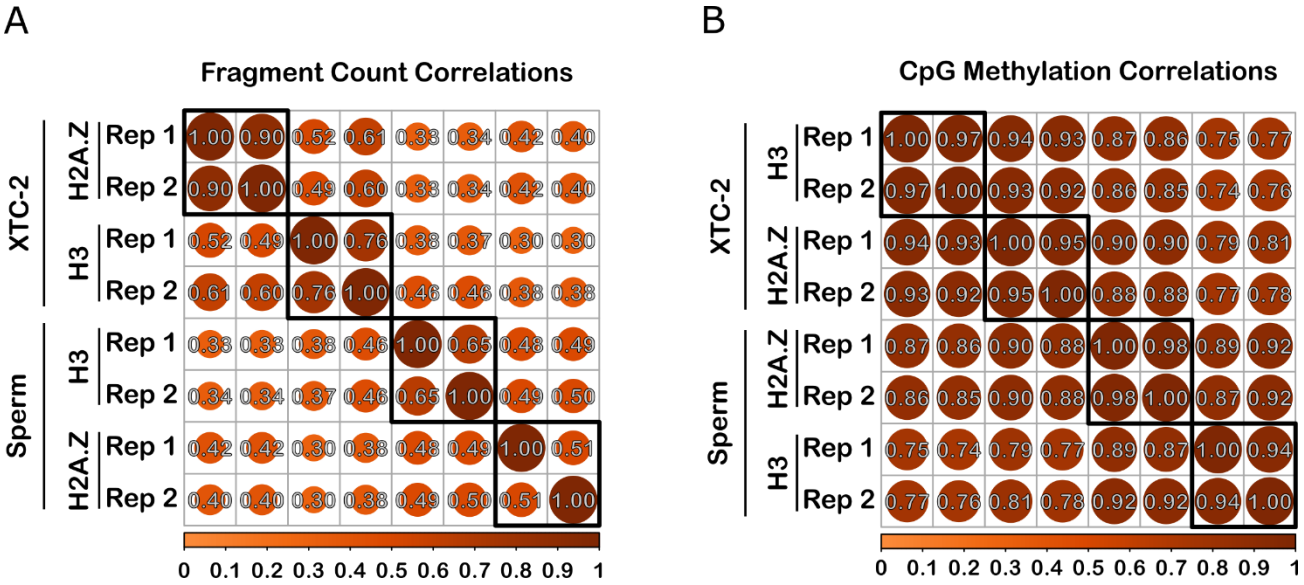
Supplementary Figure 5. Cryo-EM structures of 601L H2A.Z nucleosomes show no DNA methylation dependent differences. (A) Diagram of the palindromic 601L sequence. Bar indicates halfway point. Methylated cytosines are in green. **(B and C)** Final density maps of unmethylated **(B)** or methylated **(C)** H2A.Z nucleosomes. **(D and E)** Atomic models generated for **(B and C)**. Methylated cytosines shown in green **(F)** RMSD analysis comparing differences between methylated and unmethylated 601L H2A.Z atomic models.



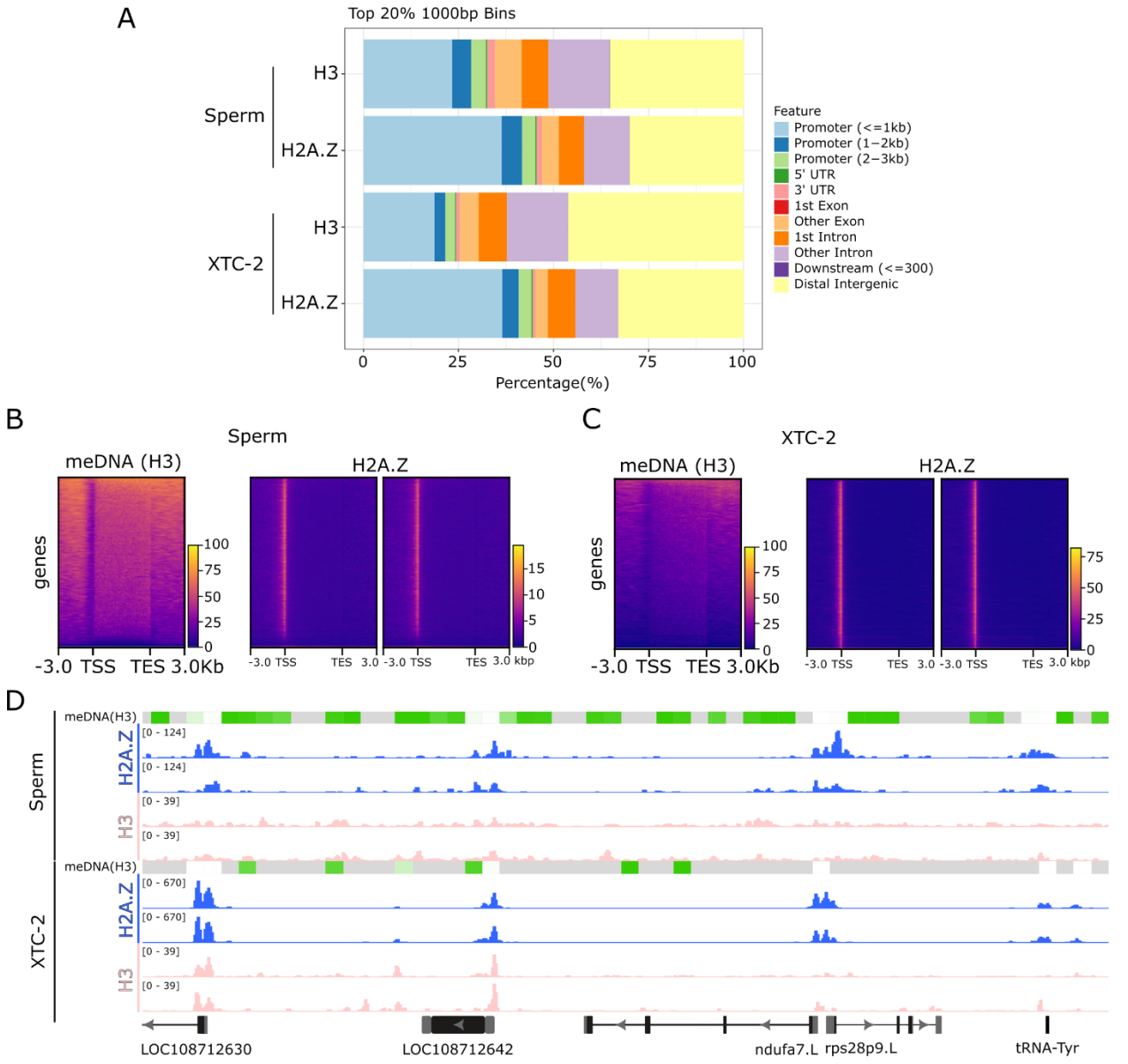
Supplementary Figure 6. Workflow of 601L H2A.Z nucleosome cryo-EM structures. (A) Native PAGE analysis of 601L H2A.Z nucleosome samples used for cryo-EM. Bands visualized through SYBR Safe staining. **(B)** Diagram showing analysis pipeline for solving 601L nucleosome structures. Further details described in methods.



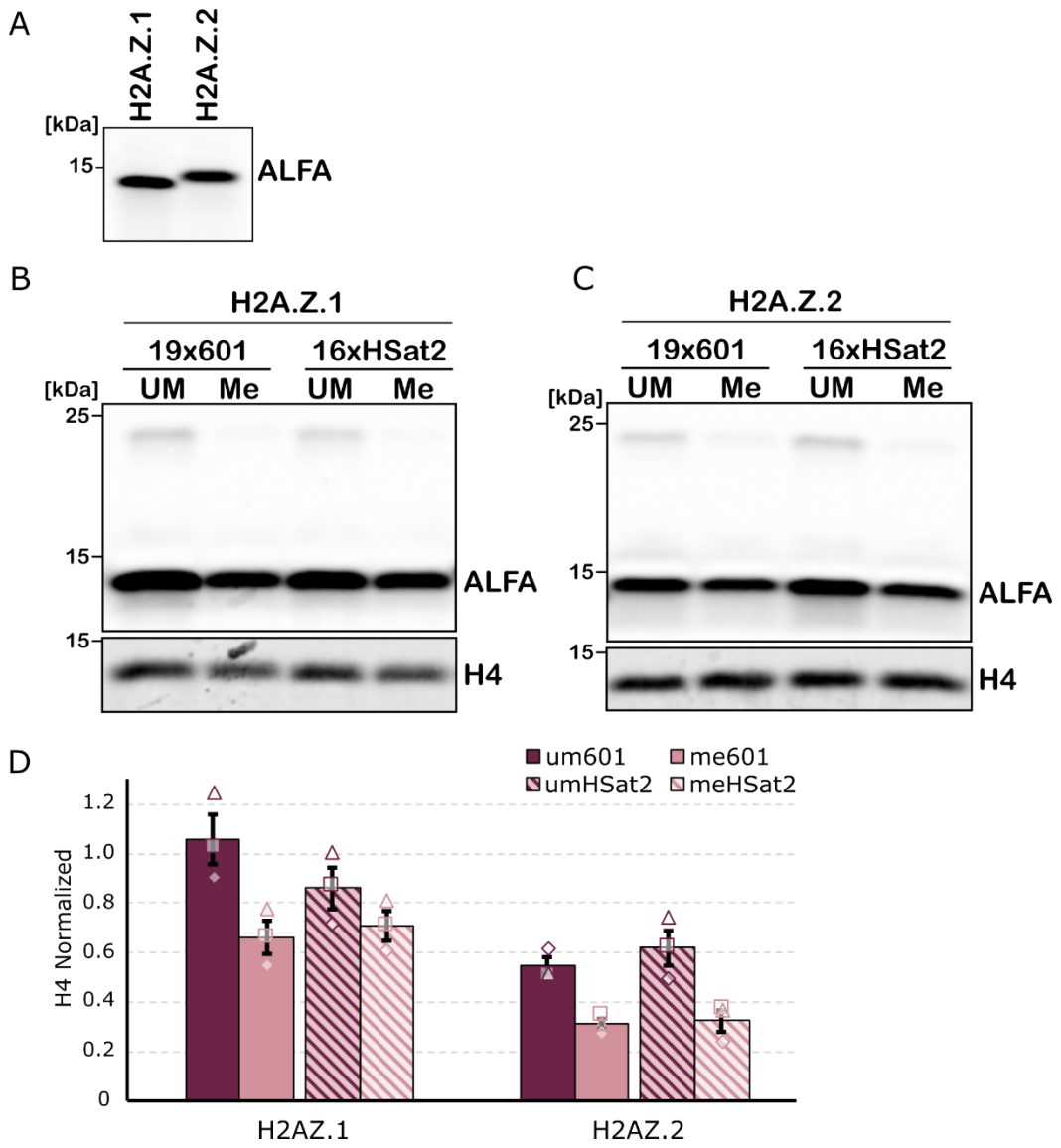
Supplementary Figure 7. Native PAGE and mass photometry analysis of nucleosomes used in HinfI digest assays. (A) Native PAGE analysis of nucleosome samples. Bands visualized through SYBR Safe staining. **(B and C)** Mass photometry analysis of methylated or unmethylated H2A **(B)** or H2A.Z **(C)** nucleosomes. Peaks around 200-220 kDa represent the fully formed nucleosome population



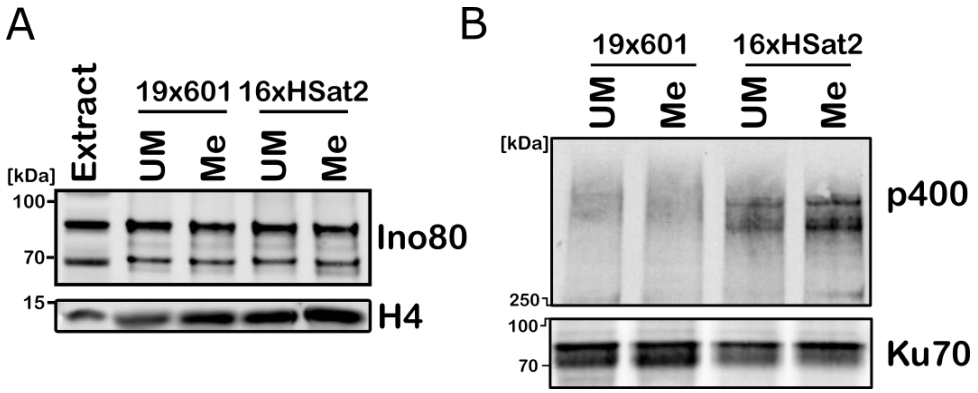
Supplementary Figure 8. Reproducibility comparison of CnT-BS samples (A) Pearson correlation plot comparing fragment counts mapped to 1000 bp genomic bins across samples. Bins containing zero fragments were first filtered out. Fragment counts were then normalized using DESeq2 to account for sequencing depth differences and underwent a variance stabilizing transformation (VST) before the correlation matrix was generated. **(C)** Pearson correlation plot comparing CpG methylation across samples. Percent methylation at individual CpG sites was calculated for high coverage CpGs (≥ 10 reads) and normalized using methylKit. The correlation matrix was then generated using pairwise observations across all eight samples. Both correlation matrices were ordered, and rectangles drawn (n = 4, user-specified), via hierarchical clustering.



Supplementary Figure 9. H2A.Z localizes to hypomethylated TSSs. (A) Genomic annotation plots for the top quantile of 1000 bp genomic bins containing the most mapped reads averaged between replicates for each specified condition. **(B and C)** Heatmaps of either H3 methylation calls averaged over 500 bp tiles across a representation of all annotated *Xenopus laevis* genes (left-most panel) or H2A.Z reads from two biological replicates across the same set of annotated genes (right panels) from either sperm (**B**) or XTC-2 (**C**) samples. **(D)** IGV snapshot of a region on chromosome 1L showcasing preferential H2A.Z deposition at hypomethylated TSSs. Average H3 methylation tracks were generated across 500 bp tiles for visualization purposes on a scale of 0 to 100% CpG methylation. Grey indicates regions with no methylation data availability. Two biological replicates of H2A.Z and H3 tracks are shown.



Supplementary Figure 10. Both H2A.Z variants preferentially associate with unmethylated DNA substrates. (A) Expression check of exogenously added mRNA expression for ALFA-tagged versions of both H2A.Z.1 and H2A.Z.2. **(B and C)** Blots of DNA beads incubated with interphase extract expressing either ALFA-tagged H2A.Z.1 **(B)** and H2A.Z.2 **(C).** Presence of the specified H2A.Z variant on DNA beads was determined by blotting against the ALFA-tag with H4 shown as a loading control. **(D)** Quantification of **(B and C)**. ALFA signals were normalized to H4 signals. Unique shapes denote results from three independent experiments (n = 3 biological replicates). Error bars represent SEM.



Supplementary Figure 11. Ino80-C and Tip60-C do not display DNA methylation sensitivity. Western blots of DNA bead pulldowns from interphase egg extract probing for endogenous **(A)** Ino80 or **(B)** p400 (ATPase for Tip60-C) along with respective loading controls.

Supplementary Table 1. Collection and model statistics for the Cryo-EM structures.

Sample	**UM Sat3R-P**	**Me Sat2R-P**	**UM 601L**	**Me 601L**
Data Collection				
Microscope	Titan Krios			
Magnification	81,000			
Voltage (kV)	300			
Camera	Gatan K3			
Electron exposure (e-/Å)	50.703		50	
Defocus range (μm)	-1.5 ~ -3.5			
Pixel size (Å)	0.86			
Micrographs Collected	4544	6891	7020	5427
Data Processing				
Particles (no.)	696,126	566,986	449,178	179,360
Symmetry imposed	C1	C1	C1	C1
Map resolution (Å, FSC = 0.143)	3.05	2.78	3.09	3.25
EMDR ID	***EMD-70474***	***EMD-70473***	***EMD-70480***	***EMD-70481***

	UM Sat2R-P		**Me Sat2R-P**		**601L**	
Atomic Models	**v1**	**v2**	**v1**	**v2**	**UM**	**Me**
Composition						
Chains	10	10	10	10	10	10
Atoms	11117 (Hydrogens: 0)	10905 (Hydrogens: 0)	10585 (Hydrogens: 0)	10558 (Hydrogens: 0)	11101 (Hydrogens: 0)	11477 (Hydrogens: 0)
Residues	Protein: 735 Nucleotide: 259	Protein: 721 Nucleotide: 254	Protein: 701 Nucleotide: 246	Protein: 698 Nucleotide: 246	Protein: 722 Nucleotide: 264	Protein: 738 Nucleotide: 274
Water	0	0	0	0	0	0
Ligands	0	0	0	0	0	0
Bonds (RMSD)						
Length (Å) (# > 4σ)	0.004 (0)	0.003 (0)	0.004 (0)	0.003 (0)	0.003 (0)	0.004 (0)
Angles (°) (# > 4σ)	0.580 (0)	0.525 (3)	0.617 (0)	0.619 (0)	0.578 (1)	0.650 (0)
MolProbity score	1.78	1.47	1.77	1.82	1.6	2.35
Clash score	5.17	4.66	5.86	7.13	8.64	9.79
Ramachandran plot (%)						
Outliers	0	0	0	0	0.14	0
Allowed	2.5	1.56	1.9	2.35	1.56	4.16
Favored	97.5	98.44	98.1	97.65	98.3	95.84
Rama-Z (Ramachandran plot Z-score RMSD)						
whole (N = 719)	1.58 (0.32)	1.80 (0.32)	1.67 (0.32)	1.84 (0.32)	1.99 (0.32)	0.95 (0.30)
helix (N = 518)	2.25 (0.23)	2.33 (0.23)	2.07 (0.23)	2.20 (0.23)	2.25 (0.22)	1.63 (0.22)
sheet (N = 0)	--- (---)	--- (---)	--- (---)	--- (---)	--- (---)	--- (---)
loop (N = 201)	-2.34 (0.36)	-2.21 (0.36)	-2.05 (0.39)	-1.87 (0.38)	-1.43 (0.42)	-2.12 (0.36)
Rotamer outliers (%)	3.44	1.99	3.96	2.94	1.5	5.37
Cβ outliers (%)	NA	NA	NA	NA	NA	NA
Peptide plane (%)						
Cis proline/general	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
Twisted proline/general	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0	0.0/0.0
CaBLAM outliers (%)	1.85	1.74	0.9	1.2	1.01	1.42
ADP (B-factors)						
Iso/Aniso (#)	11117/0	10905/0	10585/0	10557/0	11103/0	11477/0
min/max/mean						

Protein	0.00/41.12/5.00	0.28/93.40/15.80	0.23/30.35/4.02	0/89.15/13.38	1.75/82.28/22.40	2.38/89.91/24.49
Nucleotide	0.23/83.07/30.56	3.71/150.32/64.25	-0.00/88.15/40.33	0.06/161.03/73.80	4.85/140.55/59.47	6.08/156.41/67.10
Ligand	---	---	---	---	---	---
Water	---	---	---	---	---	---
Occupancy						
Mean	1	1	1	1	1	1
occ = 1 (%)	100	100	100	100	100	100
0 < occ < 1 (%)	0	0	0	0	0	0
occ > 1 (%)	0	0	0	0	0	0
Box						
Lengths (Å)	79.12, 119.54, 119.54	78.26, 118.68, 118.68	77.4, 116.96, 117.82	77.4, 116.96, 117.82	75.68, 116.1, 122.98	73.96, 116.1, 121.26
Angles (°)	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90	90, 90, 90
Supplied Resolution (Å)	3.3	3.3	3	3	3.2	3.3
Resolution Estimates (Å)	Masked | Unmasked	Masked | Unmasked	Masked | Unmasked	Masked | Unmasked	Masked | Unmasked	Masked | Unmasked
d FSC (half maps; 0.143)	--- | ---	--- | ---	--- | ---	--- | ---	--- | ---	--- | ---
d 99 (full/half1/half2)	3.5/---/--- | 3.5/---/---	3.5/---/--- | 3.5/---/---	3.2/---/--- | 3.2/---/---	3.2/---/--- | 3.2/---/---	3.3/---/--- | 3.3/---/---	3.4/---/--- | 3.3/---/---
d model	3.4 | 3.4	3.4 | 3.4	3.1 | 3.2	3.1 | 3.2	3.2 | 3.2	3.3 | 3.3
d FSC model (0/0.143/0.5)	2.9/3.1/3.3 | 3.1/3.2/3.3	2.9/3.1/3.3 | 3.0/3.2/3.3	2.6/2.9/3.0 | 2.8/3.0/3.1	2.6/2.9/3.0 | 2.8/3.0/3.1	2.9/3.0/3.2 | 2.9/3.1/3.3	3.1/3.1/3.3 | 3.1/3.2/3.4
Map min/max/mean	-39.16/62.30/0.79	-39.16/62.3/0.82	-41.24/58.89/0.40	-41.24/58.89/0.40	-34.81/61.05/0.68	-31.70/51.07/0.56
Model vs Data						
CC (mask)	0.86	0.88	0.83	0.83	0.82	0.8
CC (box)	0.78	0.78	0.7	0.68	0.74	0.7
CC (peaks)	0.75	0.75	0.68	0.66	0.7	0.65
CC (volume)	0.8	0.82	0.75	0.76	0.78	0.76
Mean CC for ligands	---	---	---	---	---	---
PDB ID	***9OGS***	***9OGZ***	***9OGR***	***9OH0***	***9OH1***	***9OH2***

Supplementary Table 2. Alignment statistics of CnT-BS sequencing libraries. XTC reads were downsampled to 30 million reads to match sperm samples, then all raw reads were trimmed and deduplicated using fastp v0.24.0 and aligned to the *Xenopus* laevis genome (Xenla 10.1) or lambda genome (to assess for bisulfite conversion efficiency) using Bismark v0.24.2. Methylated CpG percentages for H2A.Z samples were determined for the top 2.5 % of H2A.Z peaks by AUC (obtained using SEACR v1.3) while methylated percentages for H3 associated CpGs was conducted on regions outside of determined H2A.Z peaks. All CpGs met a cutoff of at least 5 reads to be counted. Methylated lambda CpG statistics were calculated from all mapped CpGs with no filtering.

		Alignment to Xenopus laevis			*Alignment to lambda*	
		Aligned %	**Total Aligned Reads (No.)**	**Methylated CpG %**	**Aligned %**	**Methylated CpG %**
Sperm Pronuclei						
H3	*Rep 1*	71.7	24141516	**85.6**	0.8	**0.6**
	Rep 2	71.0	24185147	**85**	1.0	**0.6**
H2AZ	*Rep 1*	64.7	25362485	**42.5**	4.0	**0.6**
	Rep 2	69.0	21963635	**43.5**	2.5	**0.6**
XTC-2						
H3	*Rep 1*	68.2	19539297	**53.2**	7.2	**0.4**
	Rep 2	62.4	17758471	**53.8**	15.9	**0.3**
H2AZ	*Rep 1*	69.1	19409396	**3.2**	12.4	**0.3**
	Rep 2	70.9	20109208	**2.8**	9.8	**0.3**

Supplementary Table 3. Fragment statistics of filtered genomic bins used for sequencing analysis. A count matrix was generated from processed bam files over 1,000 bp bins of the *Xenopus laevis* genome. Mitochondrial reads and bins containing fewer than 17 reads across less than 2 samples were removed to filter out low signal areas. Replicates for each sample were averaged and resulting count matrix used for genomic annotation analysis.

		Fragments in Bins (No.)	Fragments in Bins (%)
Sperm Pronuclei			
H3	*Rep 1*	9062125	34.04
	Rep 2	9265292	34.68
H2AZ	*Rep 1*	11482371	43.04
	Rep 2	9213698	39.58
XTC-2			
H3	*Rep 1*	7789646	36.43
	Rep 2	8145900	41.79
H2AZ	*Rep 1*	14276029	66.62
	Rep 2	15187573	69.13
Total Bins (No.)		506009	