Preliminary Results (PI: Jikui Song, University of California, Riverside)

Structure and regulation of DNMT1-mediated maintenance DNA methylation. To understand the molecular basis underlying the functional crosstalk between DNMT1-mediated DNA methylation and histone modifications (Fig. 1A), we plan to solve the cryo-EM structures of DNMT1 in complex with various nucleosome core particles (NCPs). Thanks to the support by NCCAT, in preliminary studies, we collected the cryo-EM data for the recruitment complex of DNMT1-NCP (Fig. 1B-C) and the productive complexes of DNMT1-NCP (Fig. 1D-I) at the mono-NCP level, at 3.7-6 Å resolution. Combined analysis of these DNMT1-NCP complexes reveals methylation dynamics of DNMT1 on nucleosome substrates. Importantly, these structural studies have uncovered two stages for DNMT1-medaited DNA methylation: the recruitment stage (Fig. 1B-C) and the production stage (Fig. 1D-I). This work is being summarized for publication in the near future. Building on the success of our structural characterization of DNMT1 with mono-NCPs, we plan to perform structural characterization of DNMT1 in complex with oligo-nucleosomes, which will provide deep insights into the DNMT1-mediated DNA methylation at the compact, heterochromatic regions. Toward this, we will generate the complex of DNMT1 with di- or tetra-nucleosome harboring various histone modifications (e.g. histone H3 ubiquitylation, H4K20me3, etc), following the same protocol as that used for the previous complexes. Structural study of the DNMT1-NCP complexes with various nucleosome context and histone modifications will permit us to capture the dynamic conformation and NCP contacts of DNMT1 in heterochromatin.

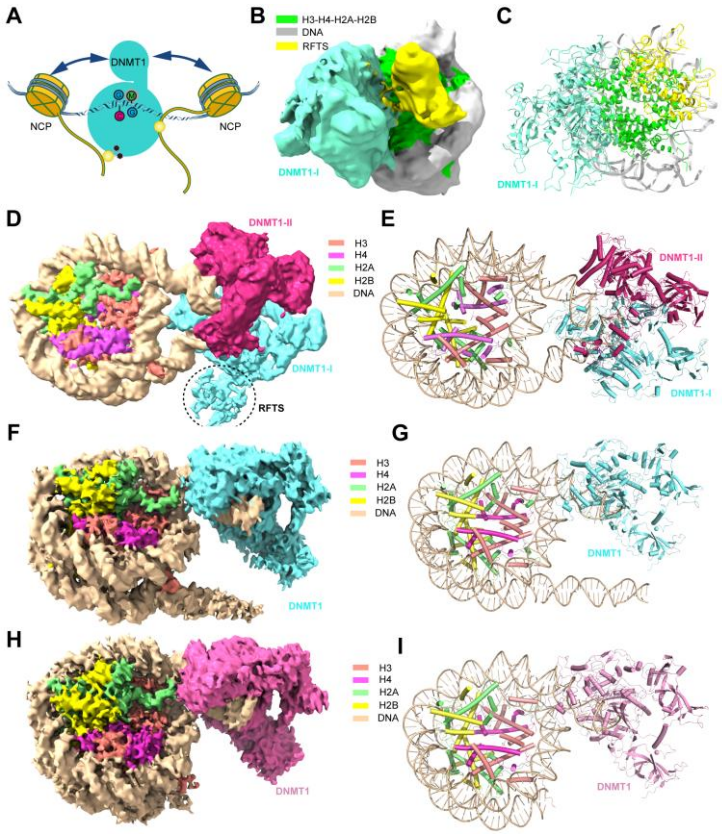

Figure 1. Structural study of the DNMT1 complexes. (**A**) Model for the crosstalk between DNMT1 and epigenetically modified NCPs in regulating maintenance DNA methylation. Unmodified and methylated cytosines are denoted as "C" and "M", respectively. (**B,C**) Cryo-EM density map (B) and atomic model for the recruitment complex of DNMT1 with NCP at ~7 Å-resolution . (**D,E**) Cryo-EM density map (D) and atomic model (E) of the productive complex of DNMT1 with the NCP containing linker sequence 1 at 3.7-Å resolution. (**F,G**) Cryo-EM density map (F) and atomic model (F) of the productive complex of DNMT1 with the NCP containing linker sequence 2 at 3.9-Å resolution. (**H,I**) Cryo-EM density map (F) and atomic model (F) of the alternative state the productive complex of DNMT1 with the NCP containing linker sequence 2 at 4.0-Å resolution.

DNMT1-mediated DNA methylation is further regulated by various protein factors, such as PCNA, PCNA-associated factor 15 ubiquitylated at lysine 15 and 24 (PAF15Ub2), deubiquitinate USP7, and DNMT1-associated protein 1 (DMAP1). To provide the molecular basis for the functional regulation of DNMT1, we will generate the DNMT1-NCP complexes in the presence of various chromatin factors. Our success in structural characterization of DNMT1 in complex with mono-NCP (Fig. 1) places us in an ideal position to investigate the regulatory complexes of DNMT1 in the chromatin environment.

Structure and mechanism of DNMT3A/3B-mediated *de novo* DNA methylation. DNMT3A and DNMT3B are both multi-modular proteins. To establish proper DNA methylation patterns in development, the activity of both proteins is subject to a multifaceted regulation. To understand how DNMT3A/DNMT3B-mediated *de novo* DNA methylation is regulated by their oligomerization and interactions with NCPs, we have investigated the structures of DNMT3A and DNMT3B under various functional states. For instance, we have recently determined the cryo-EM structures of DNMT3B at 3.1 Å resolution, providing molecular details for its intramolecular regulation (Lu et al. Nucleic Acids Res 2023). Thanks to the support by NCCAT, we have recently also determined the cryo-EM structure of DNMT3A ubiquitin-dependent recruitment (UDR) fragment complexed with H2AK119ub1-modified nucleosome (Fig. 2A-B) (Chen et al, Submitted). We

found that DNMT3A UDR occupies an extensive histone surface, involving the H2A-H2B acidic patch and a surface groove formed by H2A and H3 (Fig. 2A-B). In addition, the C-terminal α-helix of DNMT3A1 UDR bridges nucleosomal DNA with H2AK119ub1 (Fig. 2A-B). This study therefore provides a molecular basis for the functional regulation of DNMT3A1 by H2AK119ub1-modified nucleosome. Furthermore, we have performed preliminary structural characterization of DNMT3A in complex with functional regulator DNMT3L, free and in complex with NCPs. Our initially cryo-EM analysis of the DNMT3A-DNMT3L-NCP complex reveals that DNMT3A engages in a multi-layered interaction with the DNA and histone components of NCP (Fig. 2C-D). Currently, we have generated the cryo-EM grids for additional DNMT3A-DNMT3L-NCP complexes for further structural characterization. We expect that the cryo-EM data collection of the DNMT3A-DNMT3L complexes will ultimately unravel the structural basis of *de novo* DNA methylation.

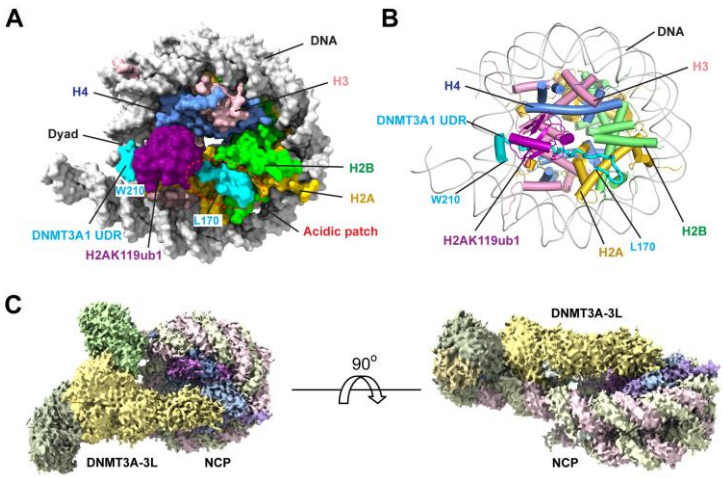

Figure 2. Structural study of the DNMT3A/3B-mediated de novo DNA methylation. (**A, B**) Cryo-EM density map (A) and atomic model (B) of the ubiquitin-binding-region of DNMT3A bound to H2K119ub-modified NCP. (**C**,**D**) Two orthogonal views of DNMT3A-DNTM3L complex bound to NCP substrate.

Structural study for the DNMT3A – inhibitor complex. Our recent studies have demonstrated that the AML-associated hot-spot mutations of DNMT3A, including R882H and R882C, shift the oligomerization state of DNMT3A toward high-order oligomer, leading to reduced chromatin association and DNA methylation activities of DNMT3A (Lu et al, *Nat Commun*. Accepted in principle). In cells, such aberrant protein oligomerization behavior of DNMT3A interferes with the proper functionalities of wild-type (WT) DNMT3A, causing a dominant-negative effect that contributes to the disease progression of AML. On the other hand, introducing oligomerization-attenuating mutations (R676K or R676K/M674T) helps revert the oligomerization of DNMT3A R882H or R882C, thereby inhibiting the dominant-negative effect of the disease mutations (Fig. 3A). Guided by this finding, we have further identified a small-molecule inhibitor that can effectively reduce the polymerization behavior of DNMT3A R882H (Fig. 3B), providing a basis for development of novel therapeutic strategies against AML. To characterize the structure-activity relationship of this lead compound, we plan to collect the cryo-EM data for DNMT3A, WT or R882H, in complex with the inhibitor for structure determination. Our preliminary analysis of the DNMT3A R882H sample indicates high homogeneity (Fig. 3C), providing a basis for the preparation of the cryo-EM sample of the DNMT3A R882H-inhibitor complex in the near future.

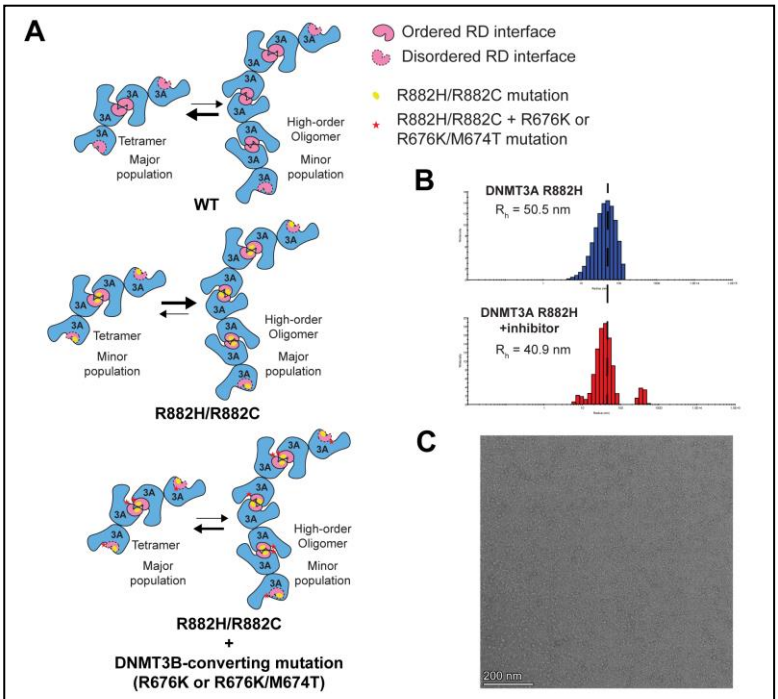

Figure 3. Inhibition of DNMT3A disease mutants. (**A**) A model for the dynamic assembly of DNMT3A, WT or mutant. (**B**) DLS analysis of R882H-mutated DNMT3A, free and in the presence of inhibitor. (**C**) Representative negative-stain image of DNMT3A R882H-inhibitor complex.

In summary, our proposed research on structural characterization of DNMT-NCP complexes promises to unravel the molecular basis for mammalian DNA methylation and its functional regulation, which is of enormous interest in the field of cancer epigenetics. Our preliminary studies have prepared us well to perform the proposed research. The BAG mechanism at NCCAT provides an excellent opportunity in collectively moving these projects forward.