Supplementary Data

Structural Insights into the Specific Recognition of Mononucleosome by the Catalytic Domain of Human KMT5A

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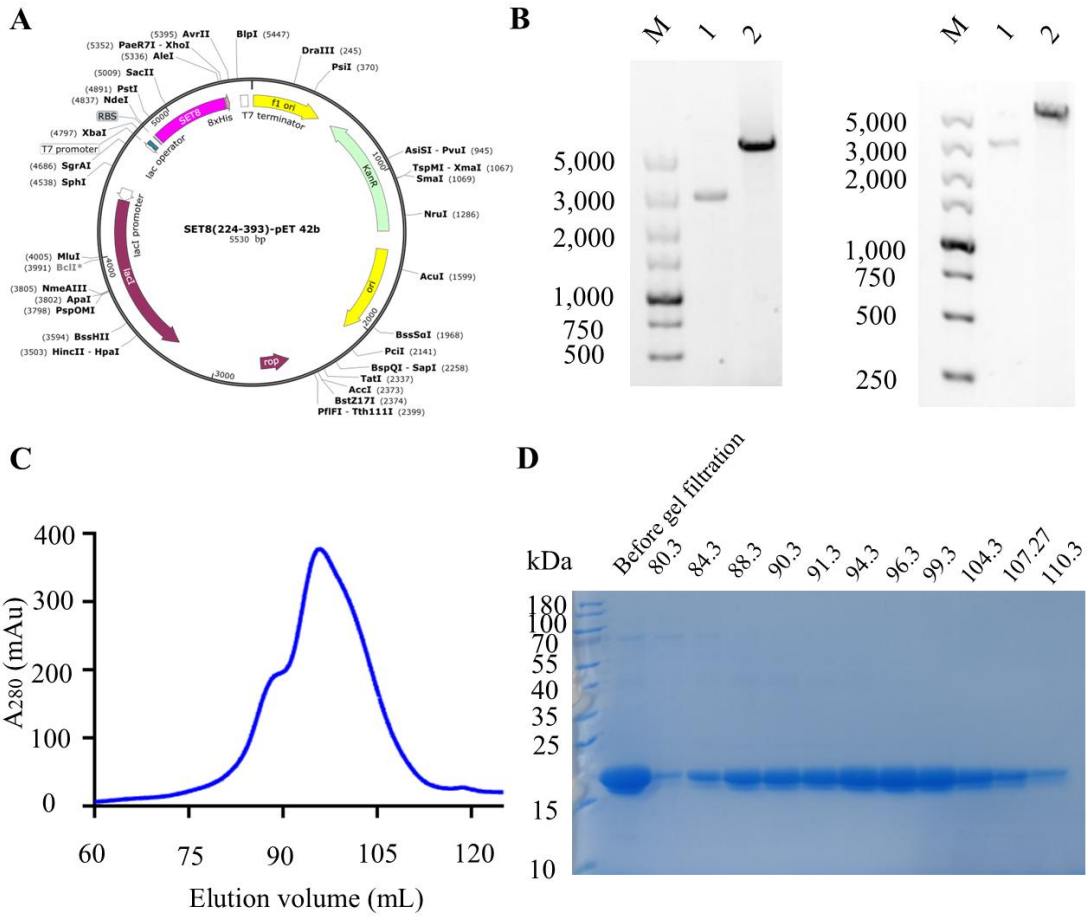


Figure S1. Preparation of SET 8 mutants and SET 8 WT protein

(A) Plasmid map of SET $8_{224\text{-}393}$-pET42b.

(B) Left panel: agarose gel electrophoresis of Y314A/F316A mutant PCR products. M: molecular weight marker; 1: SET 8 wild-type; 2: PCR amplification product. Right panel: agarose gel electrophoresis of deletion mutant PCR products. M: molecular

weight marker; 1: SET 8 wild-type; 2: PCR amplification product.

(C) Size-exclusion chromatography profile of SET $8_{224\text{-}393}$ wild-type protein.

(D) SDS-PAGE analysis of SET $8_{224\text{-}393}$ wild-type protein.

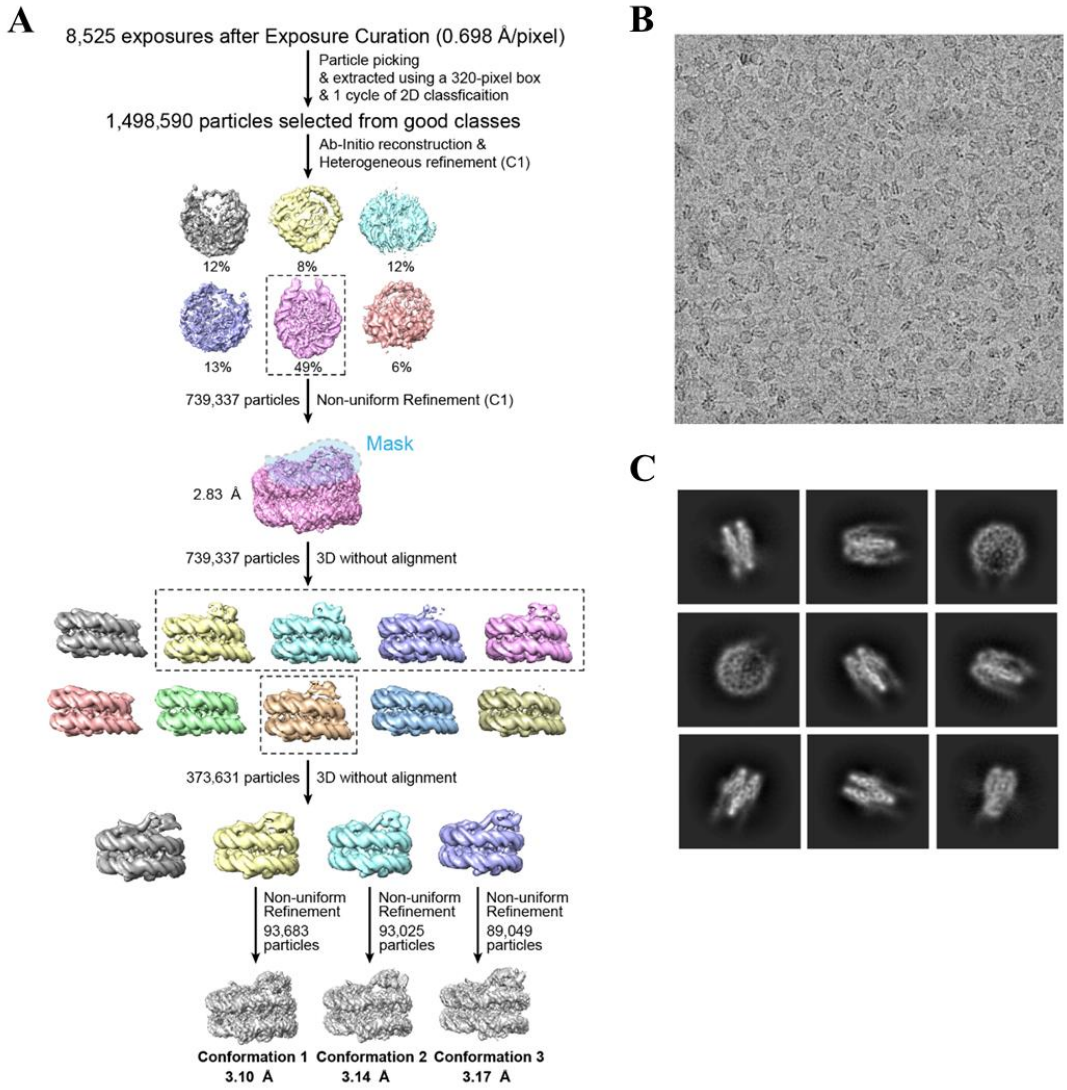


Figure S2. Cryo-EM data of SET $8_{224\text{-}393}$/nucleosome complex

(A) Flow chart of cryo-EM data processing for the complex. Three structural models with resolutions of 3.17 Å, 3.14 Å, and 3.10 Å were ultimately obtained.

(B, C) Representative cryo-EM micrographs of the complex and 2D class averages from different views.

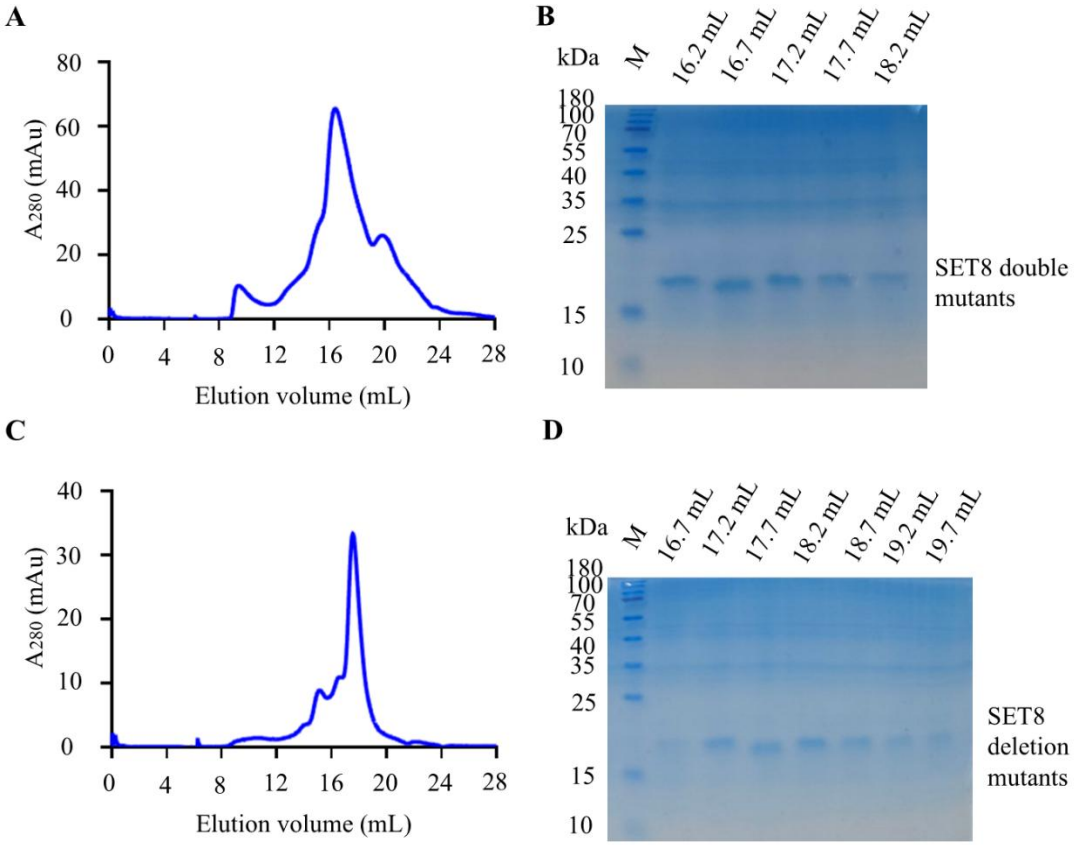


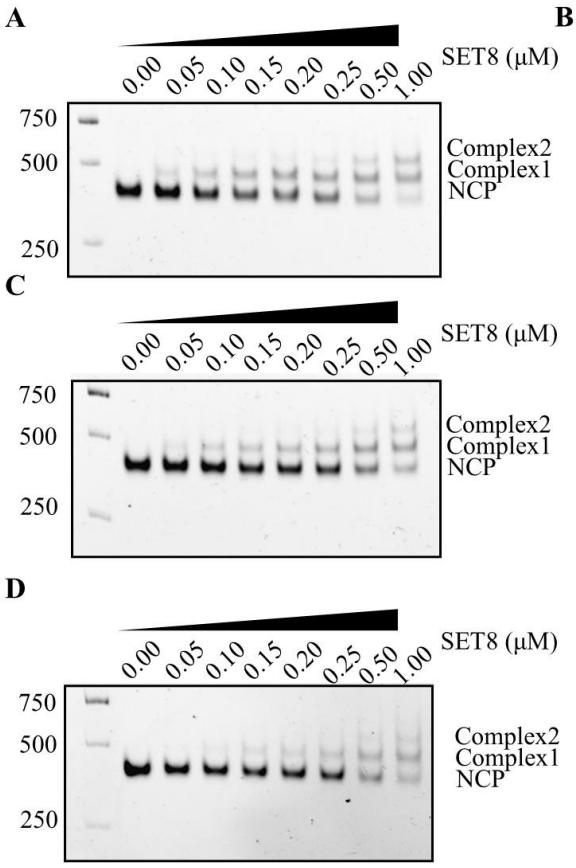
Figure S3. Preparation of SET 8 mutant proteins

(A) Size-exclusion chromatography profile of SET $8_{224\text{-}393}$Y314A/F316A mutant protein.

(B) SDS-PAGE analysis of SET $8_{224\text{-}393}$Y314A/F316A mutant protein.

(C) Size-exclusion chromatography profile of SET $8_{224\text{-}393}$ deletion mutant protein.

(D) SDS-PAGE analysis of SET $8_{224\text{-}393}$ deletion mutant protein.



B

	[SET8] (μM)	Measured area	Free NCP ratio	Fraction Bound
1	0.00	13027.96	1	0
	0.05	12551.67	0.963441	0.036559
	0.10	10165.08	0.780251	0.219749
	0.15	7909.255	0.607098	0.392902
	0.20	7222.376	0.554375	0.445625
	0.25	6730.669	0.516633	0.483367
	0.50	3620.134	0.277874	0.722126
	1.00	1824.598	0.140052	0.859948
2	0.00	14429.21	1	0
	0.05	13423.38	0.930292	0.069708
	0.10	12726.21	0.881975	0.118025
	0.15	10478.5	0.726201	0.273799
	0.20	10410.79	0.721508	0.278492
	0.25	9365.497	0.649065	0.350935
	0.50	5653.79	0.39183	0.60817
	1.00	2865.426	0.198585	0.801415
3	0.00	12579.96	1	0
	0.05	9945.426	0.790577	0.209423
	0.10	9438.719	0.750298	0.249702
	0.15	8934.841	0.710244	0.289756
	0.20	8467.012	0.673055	0.326945
	0.25	7886.719	0.626927	0.373073
	0.50	4701.426	0.373723	0.626277
	1.00	2811.477	0.223489	0.776511

Figure S4. In vitro analysis of SET 8 interaction with nucleosomes

(A) First EMSA analysis of SET $8_{224\text{-}393}$ binding to nucleosomes.

(B) EMSA area data were quantified using ImageJ (three times).

(C) Second EMSA analysis of SET $8_{224\text{-}393}$ binding to nucleosomes.

(D) Third EMSA analysis of SET $8_{224\text{-}393}$ binding to nucleosomes.

Experimental procedures

Construction of expression plasmids

The coden-optimized cDNA of SET 8 domain (224 to 393) of human KMT5A (Uniprot NO. Q9NQR1) was synthesized by General Biol. and cloned into the *E. coli* expression vector pET-42b (+) between *Nde* I (CATATG) and *Xho* I (CTCGAG).

SET 8 amino acid sequence:

MDFYPVRRSSRKSKAELQSEERKRIDELIESGKEEGMKIDLIDGKGRGVIATK
QFSRGDFVVEYHGDLIEITDAKKREALYAQDPSTGCYMYYFQYLSKTYCVDA
TRETNRLGRLINHSKCGNCQTKLHDIDGVPHLILIASRDIAAGEELLYDYGDRS
KASIEAHPWLKHLEHHHHHHHH*

3x Flag SET 8 amino acid sequence:

MDYKDHDGDYKDHDIDYKDDDDKLMARGRKMSKPRAVEAAAAAAAVAA
TAPGPEMVERRGPGRPRTDGENVFTGQSKIYSYMSPNKCSGMRFPLQEENSV
THHEVKCQGKPLAGIYRKREEKRNAGNAVRSAMKSEEQKIKDARKGPLVPFP
NQKSEAAEPPKTPPSSCDSTNAAIAKQALKKPIKGKQAPRKKAQGKTQQNRK
LTDFYPVRRSSRKSKAELQSEERKRIDELIESGKEEGMKIDLIDGKGRGVIATK
QFSRGDFVVEYHGDLIEITDAKKREALYAQDPSTGCYMYYFQYLSKTYCVDA
TRETNRLGRLINHSKCGNCQTKLHDIDGVPHLILIASRDIAAGEELLYDYGDRS
KASIEAHPWLKH*

Using the SET 8 wild-type plasmid as a template, primers for deletion mutation and double mutation (as shown in Table S1) were designed and synthesized by Sangon Biotech (Shanghai) Co., Ltd. SET 8 amino acids were mutated by PCR, with the mutation program shown in Table S2. The amplification products were analyzed by agarose gel electrophoresis. Upon successful amplification, *Dpn* I enzyme was added to digest the original template, followed by transformation into *E. coli* TOP 10 competent cells. The cells were plated onto LB plates containing 50 μg/mL kanamycin and incubated inverted at 37 °C overnight. Single colonies were picked for culture and plasmid extraction, and sent to Sangon Biotech (Shanghai) Co., Ltd. for sequencing.

Table S1. Mutation primers

Name	Primes 5'-3'
SET 8delVtoK_F	CATATGGATTTCTACCCGGCCGAACTGCAGAGC
SET 8delVtoK_R	GCTCTGCAGTTCGGCCGGGTAGAAATCCATATG
SET 8Y314A/F316A_F	AGCACCGGCTGCTATATGGCCTATGCACAGTATCTGAGC AAG
SET 8Y314A/F316A_R	CTTGCTCAGATACTGTGCATAGGCCATATAGCAGCCGGT GCT

Table S2. Mutation program

Programme No.	Temperature (°C)	Time	Step
1	95	5 min	Pre-denaturation
2	98	10 s	Denaturation
3	-	30 s	Annealing
4	72	1 min 30 s	Stretching
5	-	Goto 2，×15	Repeat steps 2-4
6	72	5 min	Final stretching
7	16	Forever	Storage

Expression and purification of mutant proteins

The SET 8 deletion mutant (deletion VRRSSRKSK) plasmid was transformed into *E. coli* BL21 (DE3) and incubated inverted at 37 °C overnight. The grown colonies were transferred to LB medium and cultured at 37 °C with shaking at 240 rpm. When OD_{600} reached 1.0, the culture was pre-chilled, and IPTG was added to a final concentration of 0.25 μM. Protein expression was induced at 18 °C with shaking at 220 rpm for 18 h. Cells were harvested by centrifugation at 3,500 rpm for 20 min, and

the medium was removed. The cell pellet was resuspended in 40 mL Ni binding buffer (0.5 M NaCl, 10 mM imidazole, 10 mM Tris-HCl pH 8.0, 0.2% glycerol, 1 mM β-ME, 0.1 M urea) and stored at -80 °C for at least 2 h. Subsequent steps were consistent with wild-type SET 8 purification to obtain purified SET 8 deletion mutant protein for lyophilization (500 μL/tube).

For the SET 8 double-point mutant (Y314A/F316A), when the culture reached OD_{600} of 1.0, IPTG was added to a final concentration of 0.25 μM, and protein expression was induced at 37 °C with shaking at 240 rpm for 4 h. The purification procedure for this mutant differed from the wild type. After sonication, the sample was centrifuged at 12,000 rpm for 1 h at 4 °C. The supernatant was discarded, and the pellet was resuspended in 30 mL of 6 M guanidine hydrochloride in 10 mM PBS pH 7.2 buffer, followed by shaking at 20 rpm on a rocking platform for 2 h. After centrifugation at 12,000 rpm for 1 h at 20 °C, the supernatant was loaded onto a Ni column pre-equilibrated with 7 M urea in 0.2 M PBS pH 7.2 buffer, and incubated at 4 °C for 3 h for binding. The column was washed with 40 mL of 10 mM imidazole in 7 M urea, 0.2 M PBS pH 7.2, and the target protein was eluted with 30 mL of 250 mM imidazole in 6 M guanidine hydrochloride, 10 mM PBS pH 7.2. The eluted protein was stored at -80 °C for refolding.

Protein refolding

Refolding buffer (0.3 M NaCl, 10 mM Tris-HCl pH 7.2, 0.1 mM $MgCl_2$) was prepared and pre-chilled to 4 L. Three tubes of lyophilized protein (500 μL liquid before lyophilization per tube) were taken, and 3 mL of 6 M guanidine hydrochloride in 10 mM PBS pH 7.2 buffer was added to dissolve the lyophilized protein. After complete dissolution, the solution was transferred to a 50 mL EP tube, followed by addition of 17 mL of 6 M guanidine hydrochloride in 10 mM PBS pH 7.2 buffer, and solid guanidine hydrochloride was supplemented to 0.2 M. The mixture was placed on a rocking platform at 20 rpm for 2 h at room temperature. After centrifugation at 12,000 rpm for 1 h at 20 °C, the supernatant was collected and placed into a 3 kDa dialysis bag, which was then immersed in the refolding buffer for overnight refolding

at 4 °C. After refolding, the sample was centrifuged at 12,000 rpm for 1 h at 4 °C. The supernatant was concentrated using a 3 kDa ultrafiltration concentrator and further purified by size-exclusion chromatography (using Superdex^{TM} 200 Increase 10/300 GL column, with buffer containing 0.3 M NaCl, 10 mM Tris-HCl pH 7.2, 0.1 mM $MgCl_2$). Each fraction was analyzed by SDS-PAGE, and the target protein was collected.

Cell culture

Frozen Hela cells were rapidly thawed in a 37 °C water bath, followed by centrifugation at 1,000 rpm for 5 min at 20 °C. The supernatant (cell freezing medium) was discarded, and the cells were gently resuspended in 1 mL of pre-warmed complete medium (10% FBS + DMEM + 1% penicillin/streptomycin) at 37 °C. The cell suspension was transferred to a culture dish containing 5 mL of complete medium, gently swirled to spread evenly, and placed in a 37 °C, 5% CO_2 incubator. When cells reached approximately 80% confluence, the old medium was discarded, and the cells were washed twice with 1× PBS buffer. Trypsin was added to digest the cells (incubated at 37 °C, 5% CO_2 for 1-2 min), and digestion was terminated by adding 1 mL of complete medium. After centrifugation at 1,000 rpm for 5 min at 20 °C, the supernatant was discarded, and the cells were resuspended in 1 mL of complete medium. The cells were passaged at a 1:20 ratio into fresh medium, and cell transfection was performed when cell viability was good.

Western blot (WB) analysis

The immunoprecipitation samples were subjected to SDS-PAGE and transferred to membrane at 100 V for 2 h. The membrane was blocked with 5% (w/v) non-fat milk at room temperature for 2 h. After washing with 1× TBST buffer, the primary antibody was added and incubated at 4 °C overnight. After washing 5 times with 1× TBST buffer, the secondary antibody was added and incubated at room temperature for 2 h. After washing 5 times with 1× TBST buffer, ECL chemiluminescent substrate was applied to the PVDF membrane surface, and imaging was performed using the

Tanon automated chemiluminescence imaging analysis system. Primary and secondary antibodies were diluted with 1× TBST buffer at appropriate ratios. Flag-Tag (DYKDDDDK), Mouse mAb, 30505ES20; Anti-Histone H3 Antibody, JJ090-07; HRP-conjugated Goat Anti-Mouse IgG, D110097; HRP-conjugated Goat Anti-Rabbit IgG, D110058.

Table S3. EMSA reaction system

Ratio	[SET 8] (μM)	SET 8 (μL)	[NCP] (μM)	NCP (μL)	Buffer (μL)
0.00	0.00	0.00	0.10	2.80	17.00
0.50	0.05	2.00	0.10	2.80	15.00
1.00	0.10	2.00	0.10	2.80	15.00
1.50	0.15	2.00	0.10	2.80	15.00
2.00	0.20	2.00	0.10	2.80	15.00
2.50	0.25	2.00	0.10	2.80	15.00
5.00	0.50	2.00	0.10	2.80	15.00
10.00	1.00	2.00	0.10	2.80	15.00