Supplementary Information

# Mechanistic basis for maintenance of CHG DNA methylation in plants

Jian Fang[1,4], Jianjun Jiang[2,3,4], Sarah M. Leichter[2,3,4], Jie Liu[3], Mahamaya Biswal[1], Nelli Khudaverdyan[1], Xuehua Zhong[2,3,*], Jikui Song[1,*]

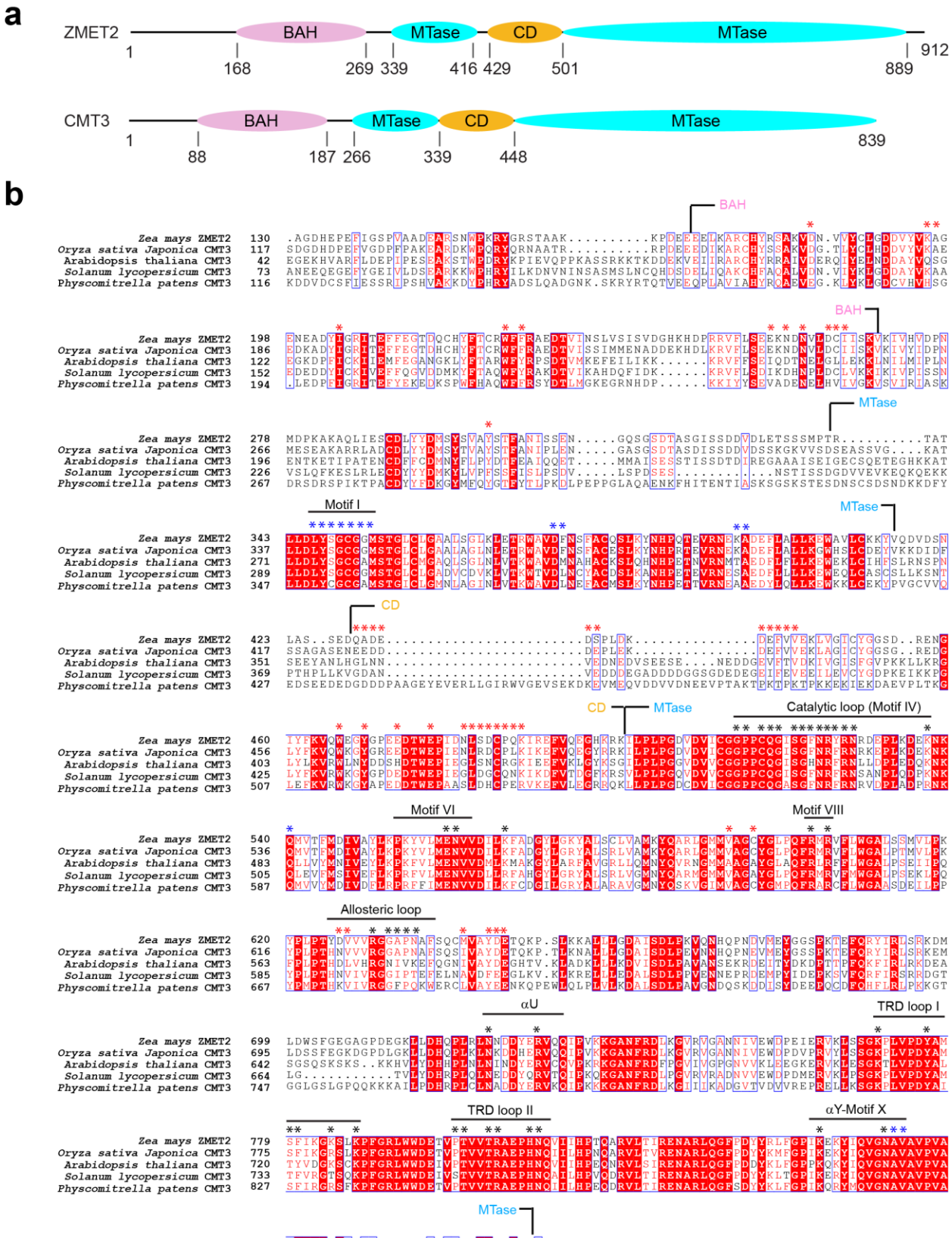
[1]Department of Biochemistry, University of California, Riverside, CA 92521, USA

[2]Laboratory of Genetics, University of Wisconsin-Madison, Madison, WI 53706, USA

[3]Wisconsin Institute for Discovery, University of Wisconsin-Madison, Madison, WI 53715, USA.

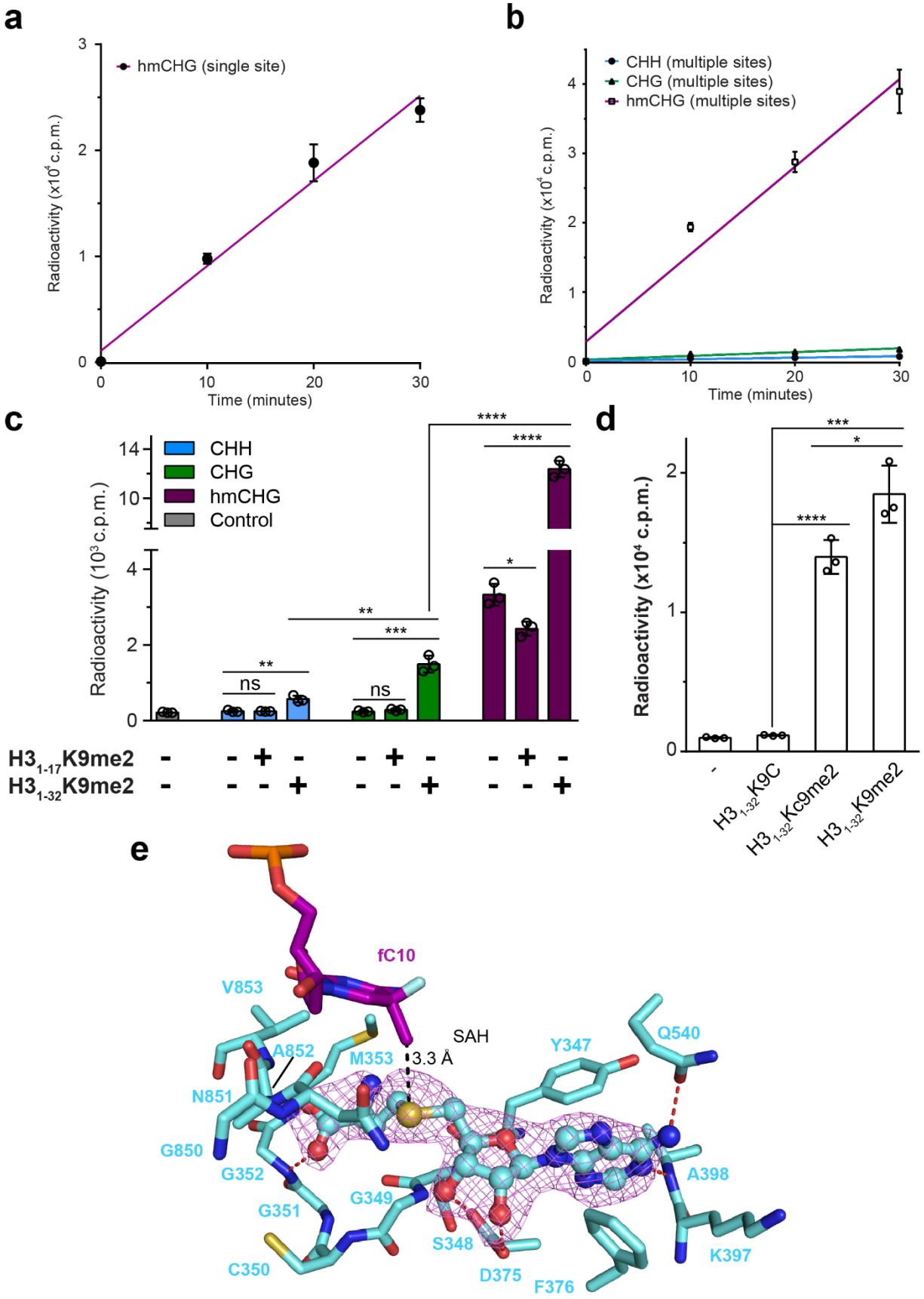
[4]These authors contributed equally to this work

*Correspondence: xuehua.zhong@wisc.edu or jikui.song@ucr.edu


a
ZMET2
BAH
MTase
CD
MTase
1
168
269 339
416 429
501
889
912
CMT3
BAH
MTase
CD
MTase
1
88
187 266
339
448
839
b
BAH
MTase
Motif I
MTase
CD
MTase
Catalytic loop (Motif IV)
Motif VI
Motif VIII
Allosteric loop
αU
TRD loop I
TRD loop II
αY-Motif X
MTase
Zea mays ZMET2
Oryza sativa Japonica CMT3
Arabidopsis thaliana CMT3
Solanum lycopersicum CMT3
Physcomitrella patens CMT3

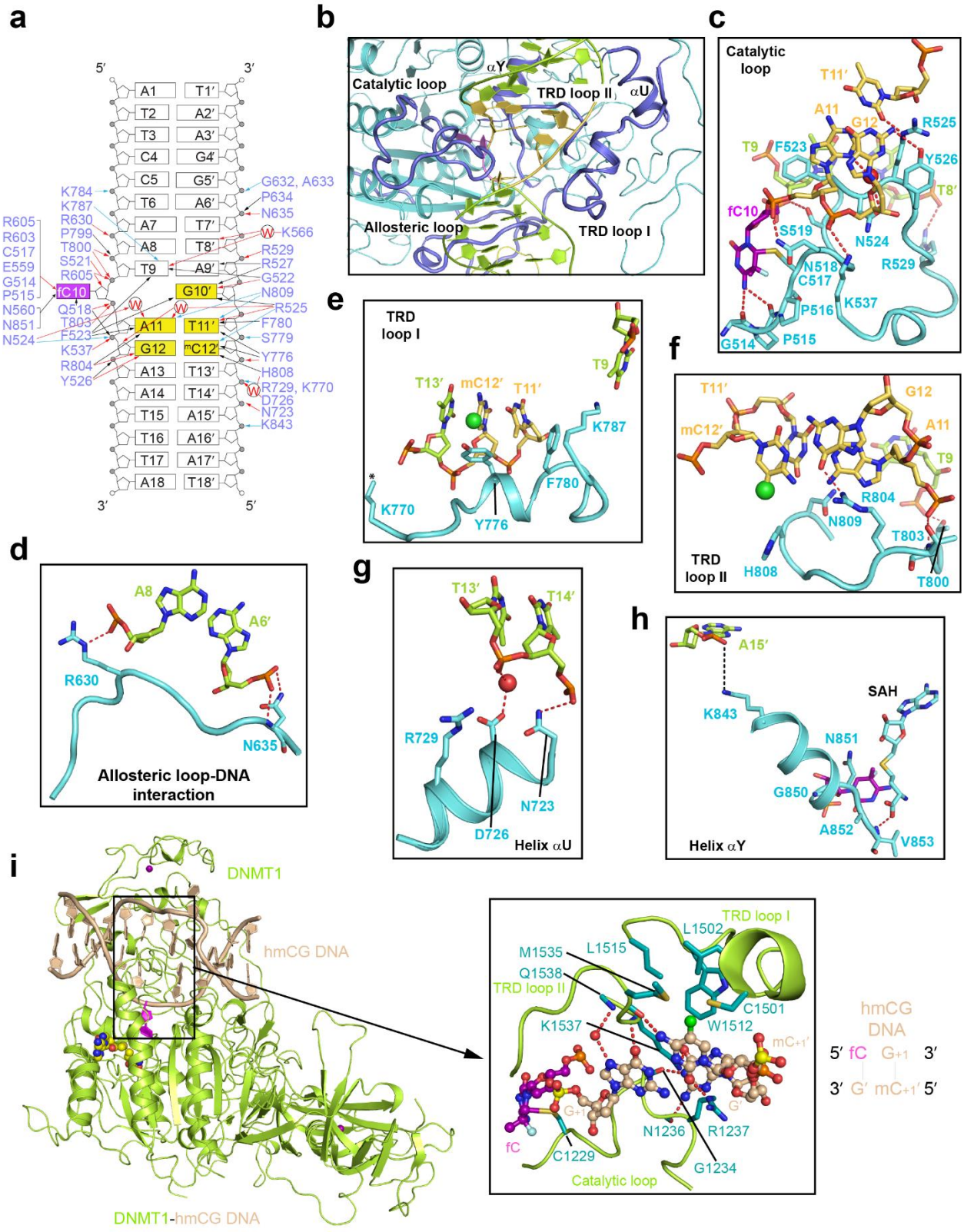
**Supplementary Fig. 1 | Sequence analysis of CMT3 proteins from various species.**

(**a**) Domain architecture of ZMET2 and *Arabidopsis* CMT3, with individual domains color-coded and delimited by residue numbers. (**b**) Sequence alignment of ZMET2 with CMT3 proteins from various plant species. Strictly conserved residues are colored white in red background. Partially conserved residues are colored red. The DNA-, H3K9me2 peptide-, and SAH-binding sites are indicated by red, black and blue asterisks, respectively, on top. Individual domains and enzymatic motifs are also labeled on top.


a
hmCHG (single site)
Radioactivity (x10^4 c.p.m.)
Time (minutes)
b
CHH (multiple sites)
CHG (multiple sites)
hmCHG (multiple sites)
Radioactivity (x10^4 c.p.m.)
Time (minutes)
c
CHH
CHG
hmCHG
Control
Radioactivity (10^3 c.p.m.)
ns
ns
H3 1-17 K9me2
H3 1-32 K9me2
d
Radioactivity (x10^4 c.p.m.)
H3 1-32 K9C
H3 1-32 Kc9me2
H3 1-32 K9me2
e
fC10
V853
A852
M353
SAH
3.3 Å
Y347
Q540
N851
G850
G352
G351
G349
S348
C350
D375
F376
A398
K397

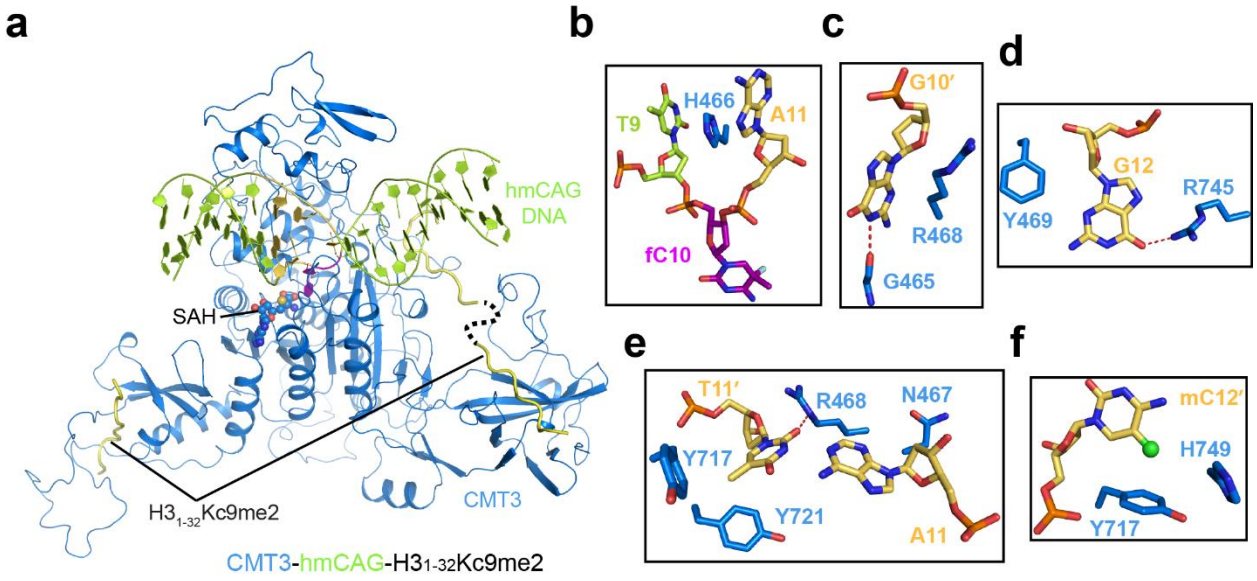
**Supplementary Fig. 2 | Enzymatic and structural analysis of CMTs.**

(**a,b**) *In vitro* enzymatic assays of ZMET2 on DNA containing single hmCHG site (a) or multiple CHH, CHG or hmCHG sites (b). (**c**) *In vitro* DNA methylation assay of CMT3 on DNA duplex containing multiple CHH, CHG, or hmCHG sites, in the presence or absence of H3K9me2 peptides. Control, no substrate present. Data are mean ± s.d. (n = 3 biological replicates). Statistical analysis used two-tailed Student's t-test. No adjustments were made for multiple comparisons. ns, not significant; *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$; ****, $p < 0.0001$. (**d**) *In vitro* DNA methylation assays of ZMET2 on DNA containing single hmCHG target site in the presence or absence of K9C-mutated $H3_{1-32}$, $H3_{1-32}$Kc9me2 or $H3_{1-32}$K9me2 peptide. Data are mean ± s.d. (n = 3 biological replicates). Statistical analysis used two-tailed Student's t-test. No adjustments were made for multiple comparisons. ns, not significant; *, $p < 0.05$; ***, $p < 0.001$; ****, $p < 0.0001$. (**e**) Close-up view of the SAH-binding site of ZMET2, with the Fo-Fc omit map (violet) for the SAH molecule contoured at 2.0$\sigma$ level. The distance between the 5-methyl group of fC10 and sulfur atom of the SAH molecule is marked. Source data are provided as a Source Data file.


a
b
c
d
e
f
g
h
i
5′
3′
A1
T1′
T2
A2′
T3
A3′
C4
G4′
C5
G5′
T6
A6′
A7
T7′
A8
T8′
T9
A9′
fC10
G10′
A11
T11′
G12
mC12′
A13
T13′
A14
T14′
T15
A15′
T16
A16′
T17
A17′
A18
T18′
K784
K787
R630
P799
T800
S521
R605
R605
R603
C517
E559
G514
P515
N560
N851
N524
Q518
T803
F523
K537
R804
Y526
G632, A633
P634
N635
K566
R529
R527
G522
N809
R525
F780
S779
Y776
H808
R729, K770
D726
N723
K843
Catalytic loop
αY
TRD loop II
αU
Allosteric loop
TRD loop I
Catalytic loop
T11′
A11
G12
R525
T9
F523
Y526
T8′
fC10
S519
N524
R529
N518
C517
P516
K537
G514
P515
TRD loop I
T9
T13′
mC12′
T11′
K787
F780
K770
Y776
T11′
G12
mC12′
A11
T9
R804
N809
T803
TRD loop II
H808
T800
A8
A6′
R630
N635
Allosteric loop-DNA interaction
T13′
T14′
R729
N723
D726
Helix αU
A15′
SAH
K843
N851
G850
A852
V853
Helix αY
DNMT1
hmCG DNA
DNMT1-hmCG DNA
TRD loop I
L1502
M1535
L1515
Q1538
TRD loop II
C1501
K1537
W1512
mC+1′
G+1
G′
fC
N1236
R1237
C1229
G1234
Catalytic loop
hmCG DNA
5′ fC G+1 3′
3′ G′ mC+1′ 5′

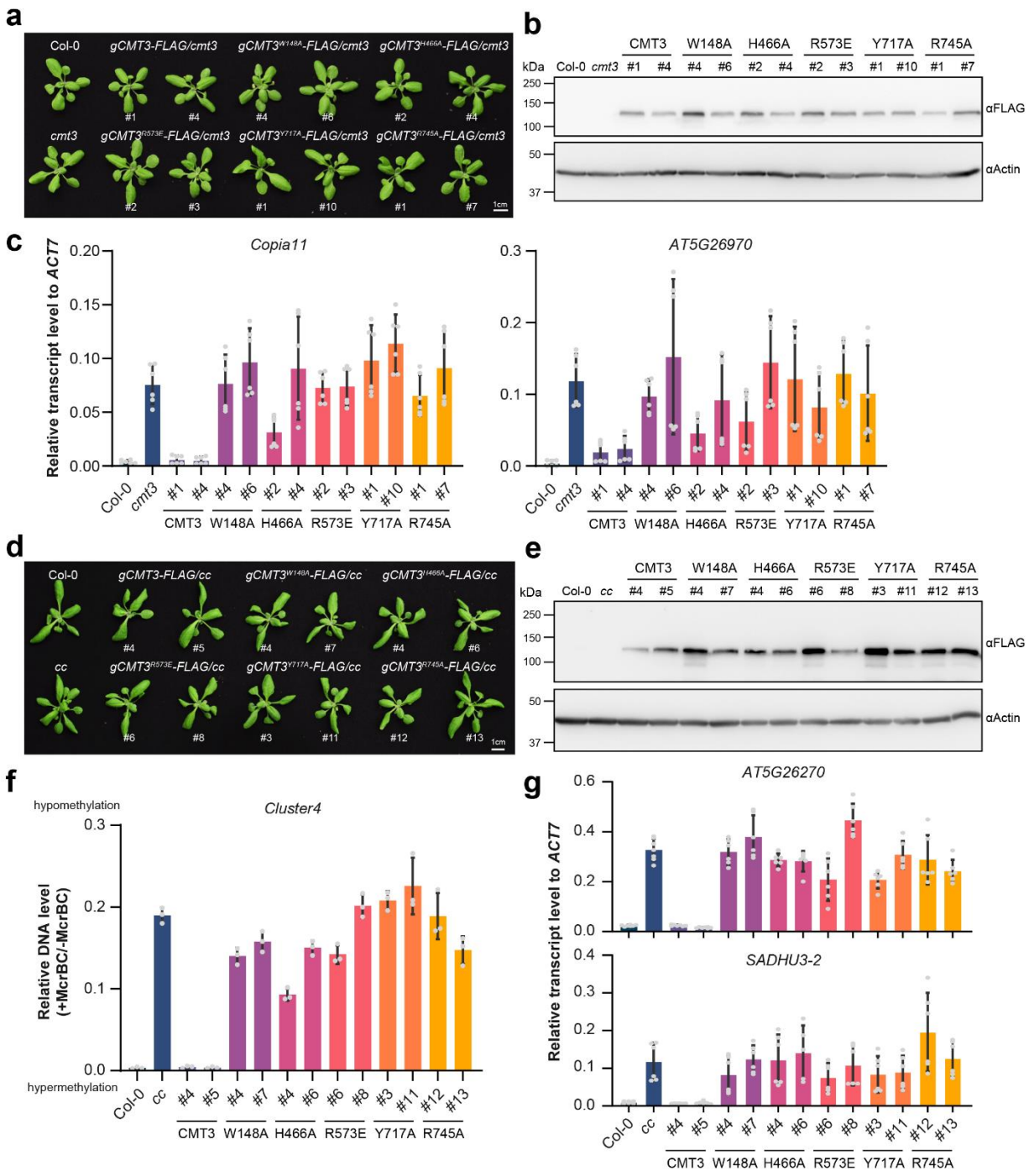
**Supplementary Fig. 3 | Structural details for the ZMET2-hmCAG interaction.**

(**a**) Schematic view of the ZMET2-hmCAG interactions. Hydrogen-bonding, electrostatic, and van der Waals contacts are indicated by red, cyan, and black arrows, respectively. Water-mediated hydrogen bonds are labeled by letter 'W'. (**b**) Structural overview of the structural elements of ZMET2 responsible for DNA binding, colored in slate. (**c-h**) Close-up view of the DNA interaction by the catalytic loop (c), allosteric loop (d), TRD loop I (e), TRD loop II (f), Helix αU (g) and Helix αY (h). The hydrogen-bonding and van der Waals contacts are depicted as red and black dashed lines, respectively. The water molecule is shown as red sphere. (**i**) Ribbon diagram of mouse DNMT1 (limon) in complex with hemimethylated CG DNA (wheat) (PDB 4DA4), with the interactions for base-specific recognition of the fCG/mCG motif shown in expanded view. The hydrogen bonds are shown as dashed lines. The DNA-interacting residues of DNMT1 is labeled. The 5-methyl group of 5mC on the target strand is shown as a green sphere.




**Supplementary Fig. 4 | Structural model of the CMT3-hmCAG-H3Kc9me2 complex.**

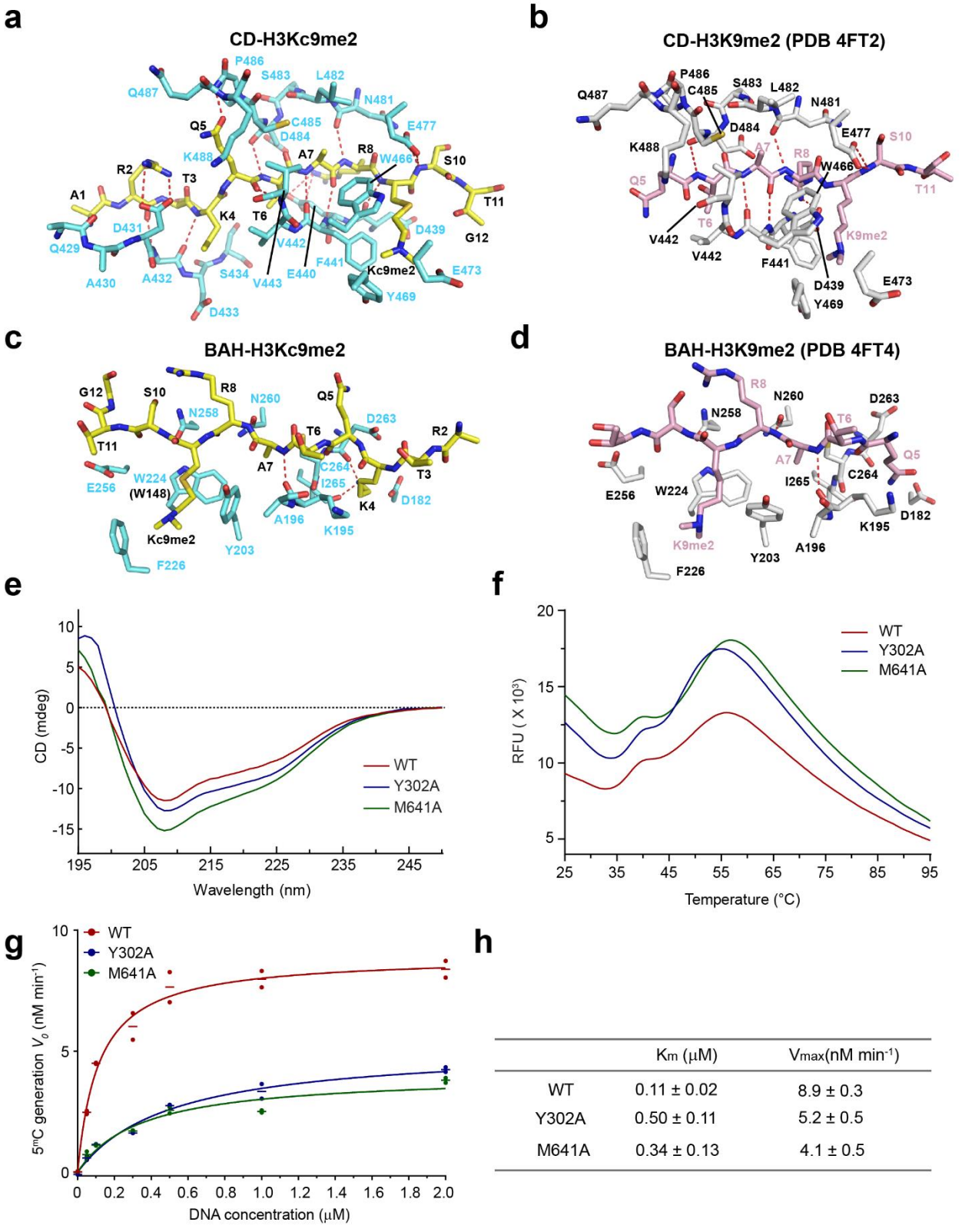
(**a**) Structural overview of the CMT3-hmCAG-H3$_{1-32}$Kc9me2 complex. The disordered residues T11-G13 of the H3$_{1-32}$Kc9me2 peptide is shown as a dashed line. (**b-f**) Close-up view of the CMT3-DNA interactions at the DNA cavity after fC10 flipping (b), orphan G10′ site (c), CHG guanine G12 (d), +1-flanking A11·T11′ pair (e), and mC12′ (f). The hydrogen bonds are shown as dashed lines.




**Supplementary Fig. 5 | Effect of CMT3 mutations on genomic DNA methylation.**

(**a**) Phenotypes of 3-week-old Arabidopsis expressing wild-type and mutant CMT3-FLAG in *cmt3* mutant background. Col-0 and *cmt3* served as controls. (**b**) Immunoblots showing the CMT3 protein level of the same lines in panel (a). Actin served as a loading control. (c) The relative transcript level of TE *Copia11* and gene *AT5G26970* was measured with reverse-transcription quantitative PCR in the transgenic lines. The RNA

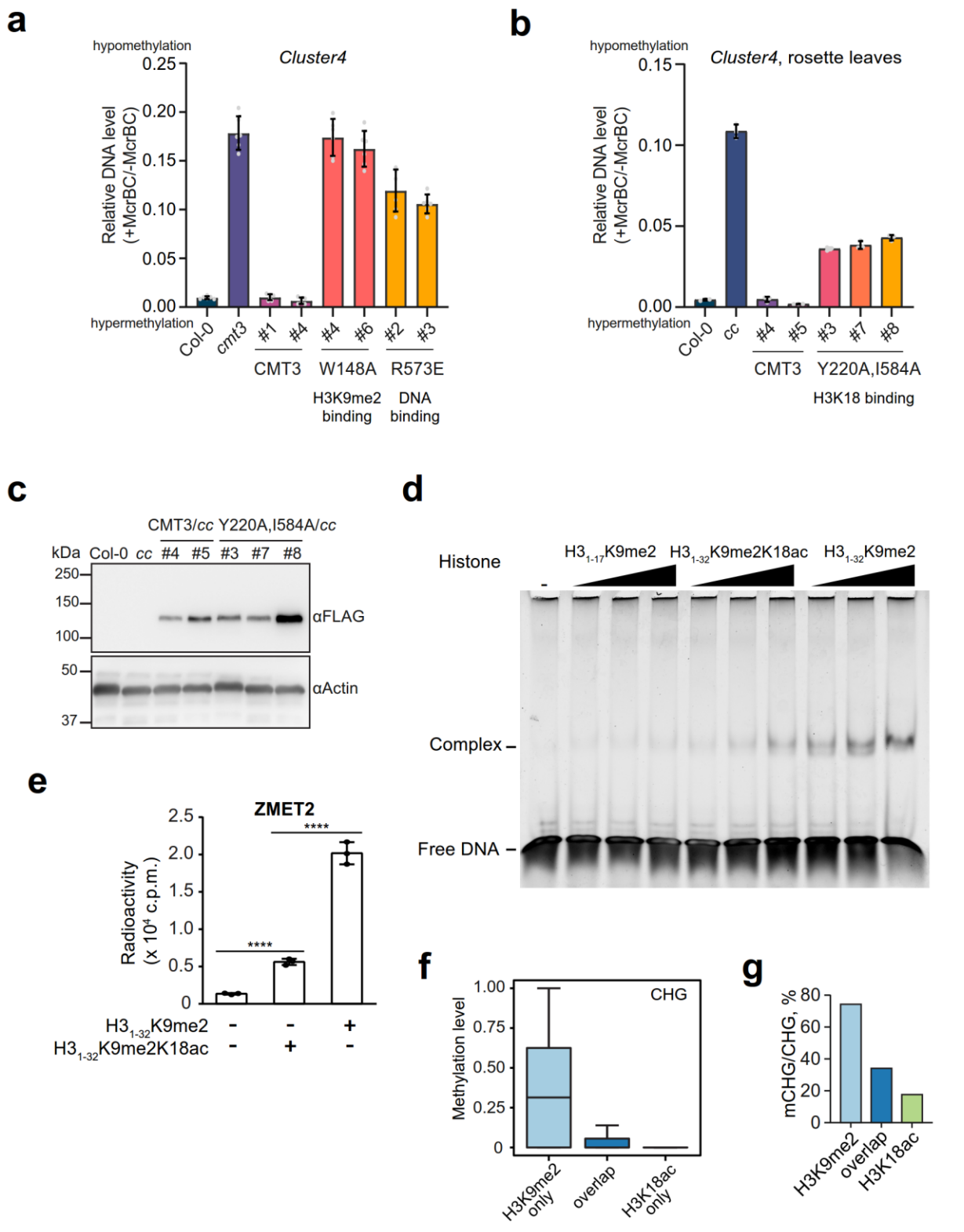
transcript was normalized to *ACT7*. Data are mean ± s.d. (n=6 technical replicates from n=2 independent experiments). (d) Phenotypes of 3-week-old Arabidopsis expressing wild-type and mutant CMT3-FLAG in *cmt2 cmt3* (*cc)* mutant background. (e) Immunoblots showing the CMT3 protein level of the same lines in panel (d). Actin served as a loading control. (f) DNA methylation of *Cluster4* locus in Arabidopsis transgenic lines in *cc* background measured by McrBC-qPCR assay. Data are mean ± SD from three technical replicates. (g) The relative transcript level of TE *SADHU3-2* and gene *AT5G26270* in the transgenic lines in *cc* background. Data are mean ± s.d. (n=6 technical replicates from n=2 independent experiments). Source data are provided as a Source Data file.




| | Km (μM) | Vmax(nM min⁻¹) |
|---|---|---|
| WT | 0.11 ± 0.02 | 8.9 ± 0.3 |
| Y302A | 0.50 ± 0.11 | 5.2 ± 0.5 |
| M641A | 0.34 ± 0.13 | 4.1 ± 0.5 |

**Supplementary Fig. 6 | Structural and biochemical analysis of the H3 peptide readout by CMTs**

(**a, b**) Close-up view of the interaction between ZMET2 CD and $H3_{1-32}K9me2$ peptide in the ZMET2-hmCAG-$H3_{1-32}Kc9me2$ (a) or ZMET2-$H3_{1-15}K9me2$ (4FT2) (b) complex. The hydrogen bonds are shown as dashed lines. Note that residues Q429-S434 of ZMET2 are disordered in the ZMET2-$H3_{1-15}K9me2$ complex (PDB 4FT2), presumably due to the crystal packing effect. (**c,d**) Close-up view of the interaction between ZMET2 BAH and the N-terminal half (residues 1-12) of the $H3_{1-32}Kc9me2$ peptide in the ZMET2-hmCAG-$H3_{1-32}Kc9me2$ (c) or ZMET2-$H3_{1-32}K9me2$ (4FT4) (d) complex. (**e**) Overlaid CD spectra of wild type (WT), Y302A, and M641A ZMET2 protein. (**f**) Thermal shift assay for the WT, Y302A, and M641A ZMET2 protein, with the raw fluorescence data shown. (**g**) DNA methylation rate of WT, Y302A, and M641A ZMET2 protein as a function of substrate concentration. Data points and the corresponding mean (bar) are derived from n=2 independent measurements. (**h**) The steady-state Michaelis–Menten parameters of WT, Y302A and M641A ZMET2 derived from (g). The mean and error estimate were derived from curve fitting. Source data are provided as a Source Data file.




**Supplementary Fig. 7 | Functional analysis of the allosteric activation of CMT3/ZMET2 by the H3K9me2/H3K18 readout**

(**a**) DNA methylation of *Cluster4* locus in *Arabidopsis* transgenic lines in *cmt3* background measured by McrBC-qPCR assay. 10-d-old seedlings were used. Data are mean ± s.d. (n=6 technical replicates from n=2 independent experiments). (**b**) DNA methylation of *Cluster4* locus in *Arabidopsis* transgenic lines in *cc* background measured by McrBC-qPCR assay. The rosette leaves from 3-week-old plants were used. The Y220A,I584A/*cc* transgenic lines were in $T_1$ generation. Data are mean ± s.d. (n=3 technical replicates). (**c**) Immunoblots showing the wild type and Y220A/I584A mutant CMT3 protein level in *cc* background. Actin served as a loading control. (**d**) EMSA analysis of the DNA-binding activity of ZMET2, in presence of different H3 peptides. The experiment was repeated twice with consistent results. (**e**) *In vitro* DNA methylation assays of ZMET2 on DNA containing single hmCHG site. Data are mean ± s.d. (n = 3 biological replicates). Statistical analysis used two-tailed Student's t-test. ****, $p < 0.0001$. (**f, g**) CHG methylation level (f) and the ratio of methylated CHG over all CHG sites (mCHG/CHG) (g) of Col-0 in the peaks in Fig. 3j. The numbers of ChIP peaks are 1852 (H3K9me2 only), 206 (overlap), and 12036 (H3K18ac only). The lower and upper box edges correspond to the first and third quartiles, the horizontal lines indicate the median, and the lower and upper whiskers denote the smallest and largest values at most 1.5 × IQR, respectively. Source data are provided as a Source Data file.

## Supplementary Table 1. Data collection and refinement statistics.

| | ZMET2–hmCAG-H3Kc9me2 (PDB: 7UBU) |
|---|---|
| **Data collection** | |
| Space group | P $2_1$ $2_1$ $2_1$ |
| Cell dimensions | |
| *a*, *b*, *c* (Å) | 64.6, 121.4, 160.4 |
| *a*, *b*, *g* (°) | 90.00, 90.00, 90.00 |
| Resolution (Å) | 160.45-2.39(2.47-2.39)[a] |
| $R_{merge}$ | 0.129(1.873) |
| *I*/*s*(*I*) | 10.1(0.8) |
| $CC_{1/2}$ | 0.992(0.380) |
| Completeness (%) | 99.53(99.9) |
| Redundancy | 5.7(6.0) |
| | |
| **Refinement** | |
| No. reflections | 50,221 |
| $R_{work}$ / $R_{free}$ | 0.211/0.256 |
| No. atoms | |
| Protein | 5571 |
| Peptide | 240 |
| DNA | 733 |
| SAH | 26 |
| Water | 120 |
| *B* factors (Å$^2$) | |
| Protein | 74.0 |
| Peptide | 101.1 |
| DNA | 100.0 |
| SAH | 64.9 |
| Water | 68.0 |
| r.m.s deviations | |
| Bond lengths (Å) | 0.006 |
| Bond angles (°) | 0.855 |

[a]Values in parentheses are for highest-resolution shell, which was determined based on $CC_{1/2}$ > 0.3. Each structure was determined using the dataset collected from a single crystal.

## Supplementary Table 2. Sequencing Statistics

| Sample | Total reads | Aligned reads | % aligned | unique reads | % unique | *Fold Coverage | Bisulfite conversion |
|---|---|---|---|---|---|---|---|
| Col-0 | 59444216 | 56484853 | 95 | 44932612 | 75.6 | 18.9 | 99.6 |
| *cmt3* | 58331559 | 55602517 | 95.3 | 44187132 | 75.8 | 18.6 | 99.6 |
| R745A/*cmt3* | 63566301 | 62199752 | 97.9 | 49485354 | 77.8 | 20.8 | 99.6 |
| CMT3/*cmt3* | 52995070 | 51707665 | 97.6 | 41898929 | 79.1 | 17.60 | 99.5 |
| H466A/*cmt3*, 2 | 58822265 | 55626051 | 94.6 | 43660615 | 74.2 | 18.3 | 99.8 |
| H466A/*cmt3*, 4 | 41510535 | 36800534 | 88.7 | 29581372 | 71.3 | 12.4 | 99.8 |

*Fold coverage was calculated by multiplying the unique reads by the read length (50bp) and dividing it by the size of the Arabidopsis genome (119Mb).

## Supplementary Table 3. List of primers used in this study

| *Primers for Cloning and Site Directed Mutagenesis of CMT2/CMT3 in plants* | | | |
|---|---|---|---|
| Gene/TE | Gene/TE ID | Name | Primer Sequence (5'→3') |
| CMT3 | AT1G69770 | gCMT3-F | TTGTTCCGCGACATTGAATTCgatggtgatacagagacactact |
| CMT3 | AT1G69770 | gCMT3-R | CCAAGGGCGAATTGGTCGACTGCAAGCTCGGAAGGAAGAG |
| CMT3 | AT1G69770 | W148A-F | CACGGCTCGGgcaTTTTATAGACCT |
| CMT3 | AT1G69770 | W148A-R | AGGTCTATAAAAtgcCCGAGCCGTG |
| CMT3 | AT1G69770 | H466A-F | TCAGTGGTgcCAACCGCTTC |
| CMT3 | AT1G69770 | H466A-R | GAAGCGGTTGgcACCACTGATT |
| CMT3 | AT1G69770 | R573E-F | GATCTAGTTCATgaAGGAAATATTGT |
| CMT3 | AT1G69770 | R573E-R | ACAATATTTCCTtcATGAACTAGATC |
| CMT3 | AT1G69770 | Y717A-F | cagGTTCCCGATgcTGCCTTAACAT |
| CMT3 | AT1G69770 | Y717A-R | ATGTTAAGGCAgcATCGGGAACctg |
| CMT3 | AT1G69770 | R745A-F | CTGTTGTCACAgcGGCAGAACCCC |
| CMT3 | AT1G69770 | R745A-R | GGGGTTCTGCCgcTGTGACAACAG |
| CMT3 | AT1G69770 | Y220A-F | CTATTTCTTGCCTgcCGATACATTTGAAG |
| CMT3 | AT1G69770 | Y220A-R | TTCAAATGTATCGgcAGGCAAGAAATAG |
| CMT3 | AT1G69770 | I584A-F | atgcagGGAAACgcAGTAGCCTATGATG |
| CMT3 | AT1G69770 | I584A-R | CATCATAGGCTACTgcGTTTCCctgcat |
| *Primers for qPCR* | | | |
| Cluster4 | AT4G27930 | Cluster4-F | CGTCCTCAAAGTTCCAGA |
| | | Cluster4-R | GGTATTCTCCATCCCAAAG |
| Copia11 | AT3G44215/ | Copia11-F | TTGGAGACTAATCATACTTGGGAC |

| | | | |
|---|---|---|---|
| | AT3TE64435 | Copia11-R | TAACCTTTGGCAACGAGACG |
| AT5G26970 | AT5G26970 | AT5G26970-F | TTGTCTCTATCCAAAGCCAC |
| | | AT5G26970-R | GTCGGGAAAACTTTGGATTC |
| TM protein | AT5G26270 | AT5G26270-F | CAAAATTTCGTAGTGGTGCG |
| | | AT5G26270-R | TTTCAAACTGCCACCAAGAA |
| SADHU3-2 | AT3G42658/ AT3TE60310 | SADHU3-2-F | TTTTCTAGGTTTCGGTTCATAC |
| | | SADHU3-2-R | TCCAATAGTACCAGCGATTACA |
| ACT7 | AT5G09810 | ACT7-F | CCTTCCAACAGATGTGGATTTC |
| | | ACT7-R | GTTCAATTCCCATCTCAACTAGG |
| *Primers for Cloning and Site Directed Mutagenesis of CMT2, CMT3, ZMET2 and histone H3 tail for protein expression* | | | |
| CMT3 | AT1G69770 | CMT3_42F | A CAG ATT GGT GGATCC GAAGGGG AAAAGCATGT TG |
| CMT3 | AT1G69770 | CMT3_839R | C TTT ACC AGA CTCGAG TTATGCAAGCTCGGAAGGAA |
| H3 | | H3K9C_1WF | ACAGATTGGTGGATCCTGGGCCCGTACCAAGCAGACCGCCCGTTGTTCCA CCGGAG |
| H3 | | H3K9C_32R | CTTTACCAGACTCGAGTTAGGTAGCAGGAGCGGACTTC |
| ZMET2 | | Y302A_F | CTCTGTTGCAGCTTCTACATTTGCTA |
| ZMET2 | | Y302A_R | AATGTAGAAGCTGCAACAGAGTAAGAC |
| ZMET2 | | F523A_F | TCAGTGGGGCTAATCGGTACAGAAAC |
| ZMET2 | | F523A_R | GTACCGATTAGCCCCACTGATACCTTGGCA |
| ZMET2 | | R630A_F | GTTGTAGTAGCTGGAGGAGCCCCTAATGC |
| ZMET2 | | R630A_R | CTCCTCCAGCTACTACAACATCATAGGTG |
| ZMET2 | | M641A_F | TCGCAATGTGCTGTTGCATATGACGAGAC |
| ZMET2 | | M641A_R | ATGCAACAGCACATTGCGAAAAGGCATTAG |
| ZMET2 | | D645AE646A _F | GGTTGCATATGCCGCTACACAAAAACCATCCCTG |
| ZMET2 | | D645AE646A_ R | GTGTAGCGGCATATGCAACCATACATTGCG |
| ZMET2 | | Y776A_F | GGTTCCTGACGCTGCAATGTCATTCATCAAG |
| ZMET2 | | Y776A_R | ACATTGCAGCGTCAGGAACCAGTGGTTTCCC |
| ZMET2 | | R804A_F | AGTTGTAACCGCAGCAGAGCCTCACAACCAG |
| ZMET2 | | R804A_R | GCTCTGCTGCGGTTACAACTGTAGGAACTG |
| ZMET2 | | H808A_F | AGCAGAGCCTGCCAACCAGGTTATAATTCATCCG |
| ZMET2 | | H808A_R | CCTGGTTGGCAGGCTCTGCTCTGGTTACAAC |
| ZMET2 | | R630E_F | GTTGTAGTAGAAGGAGGAGCCCCTAATGCC |
| ZMET2 | | R630E_R | CTCCTCCTTCTACTACAACATCATAGGTGGGC |
| ZMET2 | | N635P_F | AGGAGCCCCTCCTGCCTTTTCGCAATGTATGG |
| ZMET2 | | N635P_R | GAAAAGGCAGGAGGGGCTCCTCCACGTACTAC |
| ZMET2 | | D645R_F | GGTTGCATATCGCGAGACACAAAAACCATCCCTG |
| ZMET2 | | D645R_R | TTGTGTCTCGCGATATGCAACCATACATTGCGAA |
| ZMET2 | | E646P_F | TGCATATGACCCTACACAAAAACCATCCCTGAAG |
| ZMET2 | | E646P_R | TTTTGTGTAGGGTCATATGCAACCATACATTGC |

| | | | |
|---|---|---|---|
| ZMET2 | | N524A_F | AGTGGGTTTGCTCGGTACAGAAACCGTGAT |
| ZMET2 | | N524A_R | TCTGTACCGAGCAAACCCACTGATACCTTG |
| ZMET2 | | R525A_F | GGGTTTAATGCGTACAGAAACCGTGATGAG |
| ZMET2 | | R525A_R | GTTTCTGTACGCATTAAACCCACTGATACC |
| ZMET2 | | Y526A_F | TTTAATCGGGCCAGAAACCGTGATGAGCC |
| ZMET2 | | Y526A_R | ACGGTTTCTGGCCCGATTAAACCCACTGATAC |
| CMT3 | AT1G69770 | Y220A_F | TTCTTGCCTGCGGATACATTTGAAGCTATACAAC |
| CMT3 | AT1G69770 | Y220A_R | AAATGTATCCGCAGGCAAGAAATAGTTCATGTC |
| CMT3 | AT1G69770 | I584A_F | CAGGGAAACGCAGTAGCCTATGATGAAGGAC |
| CMT3 | AT1G69770 | I584A_R | ATAGGCTACTGCGTTTCCCTGAAACTCCTTG |