An engineered variant of SETD3 methyltransferase alters target specificity from histidine to lysine methylation

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Edited by John M. Denu

Most characterized SET domain (SETD) proteins are protein lysine methyltransferases, but SETD3 was recently demonstrated to be a protein (i.e. actin) histidine-N3 methyltransferase. Human SETD3 shares a high structural homology with two known protein lysine methyltransferases—human SETD6 and the plant LSMT—but differs in the residues constituting the active site. In the SETD3 active site, Asn255 engages in a unique hydrogen-bonding interaction with the target histidine of actin that likely contributes to its >1300-fold greater catalytic efficiency ($K_{cat}/K_m$) on histidine than on lysine. Here, we engineered active-site variants to switch the SETD3 target specificity from histidine to lysine. Substitution of Asn255 with phenylalanine (N255F), together with substitution of Trp273 with alanine (W273A), generated an active site mimicking that of known lysine methyltransferases. The doubly substituted SETD3 variant exhibited a 13-fold preference for lysine over histidine. We show, by means of X-ray crystallography, that the two target nitrogen atoms—the N3 atom of histidine and the terminal ε-amino nitrogen of lysine—occupy the same position and point toward and are within a short distance of the incoming methyl group of SAM for a direct methyl transfer during catalysis. In contrast, SETD3 and its Asn255 substituted derivatives did not methylate glutamine (another potentially methylated amino acid). However, the glutamine-containing peptide competed with the substrate peptide, and glutamine bound in the active site, but too far away from SAM to be methylated. Our results provide insight into the structural parameters defining the target amino acid specificity of SET enzymes.

Enzymes that create posttranslational methylations on proteins, particularly histones, play a pivotal role in regulating gene expression and chromatin organization (1–3). For example, the histone lysine methyltransferases that catalyze site-specific lysine methylation have been extensively studied over the past two decades, ever since the discovery of the Suv39H1 as a histone H3 lysine 9–specific methyltransferase (MTase) (4). The vast majority of characterized histone lysine MTases contain a ~130-residue SET domain that possesses the methylation activity (5, 6), with the exception of DOT1L (on H3K79) (7–9) and KMT9 (on H4K12) (10). In humans, approximately half of the 55 SET domain family members methylate lysine residues on histone and/or nonhistone proteins (11, 12). Recently, SETD3 was identified as the first metazoan histidine MTase that works on an actin histidine residue, which promotes signal-induced smooth muscle contraction and in a catalytic-independent manner is important for virulence of enteroviruses (13–15). Structurally, SETD3 shares a high degree of global similarity with two characterized SET domain proteins (14, 16, 17), namely human SETD6 and rubisco LSMT, which act respectively on lysine residues of the ReLA subunit of nuclear factor NF-κB and the large subunit of rubisco (18–22). Here we investigate the structural and molecular determinant(s) of target specificity of histidine versus lysine versus glutamine in the active site of SETD3.

Most, if not all, enzymatic reactions of SAM-dependent MTases, including those of histidine methylation catalyzed by SETD3 and the lysine methylation catalyzed by SETD6 and LSMT, are thought to proceed with direct transfer of the methyl group to substrate from the methyl donor S-adenosyl-l-methionine (SAM) (23). This reaction also requires a deprotonation step, in which a proton is removed before, concurrent with, or after methyl transfer. Even within the structurally conserved family of SET domain MTases, a variety of mechanisms have evolved to activate the catalytic nucleophile, dependent on the polarizability of the target atom. For example, lysine methylation by DIM-5, LSMT, and SETD6 showed maximal in vitro activity at approximately pH 10, principally because of the pKₐ value of ~10 for a lysine substrate (19, 24, 25). Histidine methylation by SETD3 has an optimum pH of 7 and above, in agreement with the imidazole ring having a typical pKₐ value near 6 (17). Another aspect of these reactions is that the MTases have to position the target atom such that the lone-pair electrons on the (nitrogen) nucleophile point toward, and within a short distance of, the incoming methyl group. The methyl transfer is followed by an attack on the positively charged sulfonium of SAM with an inversion of symmetry in a S₈2-like mechanism.

This work was supported by National Institutes of Health Grants GM114306 (to X. C.) and GM133051 (to O. G.) and Cancer Prevention and Research Institute of Texas Grant RR160029 (to X. C.). X. C. is a CPRIT Scholar in Cancer Research. O. G. is a co-founder of EpiCypher, Inc. and Athelas Therapeutics. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. This article contains Table S1.

The atomic coordinates and structure factors (codes 6J62 and 6J63) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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The abbreviations used are: MTase, methyltransferase; ITC, isothermal titration calorimetry.

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Here we compare the active-site configurations of the histidine methyltransferase SETD3 with the lysine methyltransferases SETD6 and LSMT by mutation and assay its activities on histidine, lysine, and glutamine (as another physiologically methylated amino acid) in the context of actin peptide.

Results

Comparison of active sites of SETD3, SETD6, and LSMT

Our previous structural characterizations of SETD3 included the prereactive substrate complex and the postreactive product complex containing an actin peptide encompassing target His73 of actin (17). The histidine imidazole ring contains two nitrogen atoms (N1 and N3), both of which can be protonated. To ensure that the target atom N3 is deprotonated prior to the methyl transfer, Asn255 forms a hydrogen bond to the protonated N1 nitrogen (Fig. 1A). With substitution of Asn255 to alanine (N255A), valine (N255V), or phenylalanine (N255F), SETD3 loses the ability to form this hydrogen bond; this results in reduced activity for His73 methylation with slower \( k_{\text{cat}} \) values (N255A and N255V), and surprisingly for N255F, no measurable activity was observed for histidine methylation (Fig. 1B–D). We reasoned that the bulky side chain of phenylalanine at residue 255 could collide with neighboring Trp273 (Fig. 1B–D). Structure-based sequence alignment indicated that three of four residues (Asn255, Trp273, Ile310, and Tyr312) that form the active-site pocket in SETD3 are not conserved in LSMT and SETD6 (Fig. 1F). The corresponding SETD3 residues Asn255 and Trp273 are phenylalanine and alanine in both LSMT and SETD6 (Fig. 1G). Thus, we generated a variant of SETD3 with two alterations in the active site, N255F and W273A.

SETD3 and the N255F/W273A variant have opposite activity on His and Lys methylation

We first measured the activities of SETD3 (WT) and the double mutant on actin peptide residues 66–80 containing His73 (H73K) in the context of the same actin peptide. Interestingly, the activ-
SETD3 target specificity

(A) | H73 (66-80) | SETD3
--|------------|---
   | $k_{cat}$ (h$^{-1}$) | $K_m$ (µM) | $k_{cat}/K_m$ (h$^{-1}$ µM$^{-1}$)
--|-------------|---------|-----------------
WT | 43±1       | 24±2    | 1.8
N255F/W273A | 2.8±0.1 (15X1) | 54±5 (23X1) | 0.05 (36X1)

(B) | K73 (66-80) | SETD3
--|------------|---
   | $k_{cat}$ (h$^{-1}$) | $K_m$ (µM) | $k_{cat}/K_m$ (h$^{-1}$ µM$^{-1}$)
--|-------------|---------|-----------------
WT | 0.57±0.03 | 430±40  | 1.3×10$^{-3}$
N255F/W273A | 60±1 (105X1) | 91±5 (4.7X1) | 0.66 (500X1)

(C) | K73 (66-88) | SETD3
--|------------|---
   | $k_{cat}$ (h$^{-1}$) | $K_m$ (µM) | $k_{cat}/K_m$ (h$^{-1}$ µM$^{-1}$)
--|-------------|---------|-----------------
WT | 0.69±0.01 | 5.2±0.6 | 0.13
N255F/W273A | 62±2 (90X1) | 7.5±0.8 (1.4X1) | 8.3 (64X1)

Figure 2. Kinetics of SETD3 and the N255F/W273A variant on His$^{73}$ and Lys$^{73}$ peptides. A–C, comparison of WT and the mutant on peptides of (A) His$^{73}$ (residues 66–80), (B) Lys$^{73}$ (residues 66–80), and (C) Lys$^{73}$ (residues 66–88). The bottom three panels are enlarged for the lower activities. The assays were performed at 37 °C and pH 8.0 for His$^{73}$ peptide using WT = 0.18 µM (20 min) and N255F/W273A = 0.72 µM (1 h) (A) or pH 10.5 for Lys$^{73}$ peptides using WT = 15 µM (3 h) and N255F/W273A = 0.18 µM (1 h) (B). The assays were performed at 37 °C and pH 8.0 for His$^{73}$ peptide using WT = 0.18 µM (20 min) and N255F/W273A = 0.72 µM (1 h) (A) or pH 10.5 for Lys$^{73}$ peptides using WT = 15 µM (3 h) and N255F/W273A = 0.18 µM (1 h) (C). Data represent the mean ± S.D. of two independent determinations ($n=2$) performed in duplicate.

dities of WT and double mutant SETD3 on Lys$^{73}$ methylation flipped the order of preference (Fig. 2B). The double mutant demonstrated a ~500-fold gain in catalytic efficiency for Lys$^{73}$ methylation, compared to that of WT ($k_{cat}/K_m$ value of 0.66 h$^{-1}$ µM$^{-1}$ for the mutant and 1.3 × 10$^{-3}$ h$^{-1}$ µM$^{-1}$ for the WT). The gain in catalytic efficiency is driven by an improved reaction rate (~105× in $k_{cat}$) and stronger binding for the substrate (~5× in $K_m$). In essence, the double mutant variant of SETD3 has switched the identity of SETD3 from a histidine MTase into a lysine MTase. We note that the WT SETD3 demonstrated ~1385-fold preference of His$^{73}$ over Lys$^{73}$ methylation in catalytic efficiency, whereas the mutant showed a ~13-fold preference of Lys$^{73}$ over His$^{73}$ methylation. Thus, comparing the mutant to WT, the ratio of catalytic efficiency on Lys$^{73}$ to His$^{73}$ methylation is increased by ~18,000-fold ( = 1385 × 13).

Structure of the N255F/W273A variant in complex with Lys$^{73}$-containing peptide

To facilitate co-crystallization, we increased the peptide length to actin residues 66–88. As observed in the previous structural work, the additional C-terminal actin residues engage in additional intermolecular interactions with SETD3 (16, 17). The enhanced enzyme-peptide interactions increased binding affinity by decreasing the $K_m$ value but do not change the reaction rate by maintaining the same $k_{cat}$ value (Fig. 2C).

The double mutant was readily crystallized with the Lys$^{73}$ (66–88) peptide in the presence of SAH (Fig. 3A). The mutant-Lys$^{73}$ structure is highly similar to those of WT enzyme in complex with His$^{73}$ (PDB ID 6MBL), with pairwise comparison of ~0.4 Å across 458 pairs of Ca atoms. The target lysine residue is inserted into the active-site channel, where at the end of the channel the terminal ε-amino nitrogen atom meets the cofactor from the opposite end (Fig. 3B). The channel is bordered by the aromatic residues of Tyr$^{312}$ and N255F, which pack against the aliphatic portion of the target lysine. The side chains of the two mutated residues, N255F and W273A, are sufficiently separated (Fig. 3C). Superimposition of WT-His$^{73}$ and the mutant-Lys$^{73}$ complex structures indicated that the two target nitrogen atoms, i.e. the terminal ε-amino nitrogen of lysine and the N$_3$ atom of unmethylated histidine ring, reside at nearly the same position (Fig. 3D). The side chain aliphatic carbons of lysine trace along the edge of superimposed histidine ring in five-bond distances from the main chain Ca atom to the target nitrogen atom (Fig. 3D). The ε-amino group of the target lysine is 3.4 Å away from the sulfur atom of SAH, where a transferable methyl group would be attached (Fig. 3E). The distance between the sulfur of SAH and Ne of lysine is approximately the sum of the bond distance of donor–methyl (S$^–$–CH$_3$ = 1.82 Å) and the bond distance of acceptor–methyl (CH$_3$–N$^+$ = 1.47 Å). A water molecule (w1), coordinated by the main-chain carbonyl oxygen atoms of Cys$^{276}$ and W273A, could facilitate the deprotonation of the target ε-amino group of lysine during catalysis (Fig. 3, E and F).

Comparisons of the double mutant structure of SETD3 to that of LSMT and SETD6 show that the features of the active-
For negative, and the amino group (NH$_2$) of glutamine and lysine (Fig. 5B). The common feature of the two potential substrates is the glutamine) and the other in the nucleus (histone H4, lysine). Different target residues; one is located in the cytoplasm (eRF1, eRF2) and the other in the nucleus (eRF1, HemK2). The site specificity of this MTase by working on two different substrates with different target residues; one is located in the cytoplasm (eRF1, eRF2) and the other in the nucleus (eRF1, HemK2). The side chains of Tyr-Phe pair (Tyr$^{112}$–Phe$^{224}$ in SETD3, Tyr$^{287}$–Phe$^{224}$ in LSMT, and Tyr$^{285}$–Phe$^{225}$ in SETD6) guide the target lysine into a narrow channel. In addition, the hydroxyl oxygen atom of the Tyr interacts and stabilizes the positive charge on the SAM methylsulfonium group (CH$_3$–S$^+$). As a result, the deprotonated amino group (NH$_2$) of the target lysine is positioned at the right distance to be able to nucleophatically attack the positively charged SAM methylsulfonium without any general base.

SETD3 is not active on glutamine methylation

Human HemK2 has recently been documented to be a histone H4 lysine 12 MTase (renamed as KMT9) (10) in addition to its known activity of glutamine methylation of eukaryotic release factor eRF1 (27, 28). We confirmed that HemK2 is active on glutamine and lysine (29) and note the unique property of this MTase by working on two different substrates with different target residues; one is located in the cytoplasm (eRF1, glutamine) and the other in the nucleus (histone H4, lysine). The common feature of the two potential substrates is the amino group (NH$_2$) of glutamine and lysine (Fig. 5A). Unlike SET domain MTases, HemK2/KMT9 is a seven-$\beta$-stranded family MTase (23). As shown in Fig. 3D, the side chain of the lysine residue, with a length of 5-bond distance between the C$_\alpha$ atom and the terminal Ne nitrogen, adopts a bent conformation such that its aliphatic carbons could seemingly trace along one edge of the histidine imidazole ring. We asked whether the side chain of a glutamine residue, with a length of 4-bond distance, could trace along the shorter edge of the imidazole ring to reach the target position in the active site of SETD3 (Fig. 5A).

In the context of the same actin peptide residues 66–88, we replaced the His$^{73}$ by glutamine (Gln$^{73}$). Under the saturating conditions of an overnight reaction, in which SETD3 totally completes reaction on all given His$^{73}$ substrate, we observed no activity of SETD3 or its mutants on the Gln$^{73}$ peptide (Fig. 5B). However, we did observe direct binding between SETD3 and the Gln$^{73}$ peptide with a dissociation constant ($K_D$) of $\sim$0.7 $\mu$m by isothermal titration calorimetry (Fig. 5C). The Gln$^{73}$ peptide competes with the substrate peptide by inhibiting SETD3 activity on His$^{73}$ with a half-maximal inhibitory concentration (IC$_{50}$) of $\sim$1 $\mu$m (Fig. 5D).

To further understand the structural basis of the binding of SETD3 with Gln$^{73}$-containing peptide, we crystallized and determined the inactive complex structure at the resolution of 2.0 Å (Table S1). The Gln$^{73}$ structure is essentially the same to that of His$^{73}$-containing structure (root mean square deviation = 0.169 Å over 886 pairs of C$_\alpha$ atoms with two complexes per crystallographic asymmetric unit). The side chain of Gln$^{73}$ occupies the active site, but its amide group is >5 Å away from the sulfur of SAH (Fig. 5E). At the resolution of 2.0 Å, we were not able to determine the exact nature of oxygen versus nitrogen atom of Gln$^{73}$ side chain. However, the chemical nature of interacting functional groups, particularly the side chain of Asn$^{255}$ and its associated water molecule, allowed us to position the Gln$^{73}$ side chain as shown in Fig. 5E. The water molecule (w2) is saturated with tetrahedral coordination of four hydrogen bonds: two H-bond donors to the main-chain carbonyl oxygen atom of Arg$^{274}$ and one of the side chain carboxylate oxygen atoms of Asp$^{274}$ and two H-bond acceptors from the main chain amide nitrogen of Ile$^{270}$ and side chain amide nitrogen of Asn$^{255}$ (Fig. 5E). Asn$^{255}$ forms a weak hydrogen bond (via its side chain oxygen atom) with the amide group of Gln$^{73}$, which is positioned more than 5 Å away from the sulfur atom of SAH where a transferable methyl group would be attached. In com-
comparison, the corresponding distance is ~3.4 Å between the ε-amino group of the target lysine (or the N3 of histidine) and the sulfur atom of SAH (Fig. 3D).

Although we initially used SAH in the mixture for the crystallization, we were surprised to find that the difference electron density clearly shows one distinguishable location with
SETD3 target specificity

Discussion

A wide variety of macromolecules, including DNA, RNA, proteins, polysaccharides, lipids, and a range of small molecules are subject to methylation by highly specific SAM-dependent MTases acting on a particular target amino acid. Examples of methylation targets include nucleic acids (cytosine-C5, cytosine-N4, and adenine-N6), protein residues (arginine-N, lysine-N, glutamine-N, and histidine-N), and small molecules (catechol-O, histamine-N, glycine-N, and thiopurine-S). The SET domain–catalyzed protein methylation has demonstrated that these enzymes frequently have high substrate specificity because of recognition of the various sequences surrounding the target residue (30–34). SETD3 displays a greater substrate specificity than most by recognition of 17 ordered residues of a 23-residue long peptide used in this study (Fig. 3A), suggesting that SETD3 would inefficiently accommodate substrates divergent from actin.

Here, we investigated the determinants of target specificity of histidine versus lysine versus glutamine in the active site of SETD3 by replacing the His73 of actin with lysine (Lys73) or glutamine (Gln73). We engineered variants of SETD3 that have altered specificity. Once in the active site, the Nε atom of the imidazole ring hydrogen bonds to Asn255 of SETD3 that makes the histidine residue a preferred target (17). Substitution of Asn255 with phenylalanine (N255F) together with substitution of Trp273 with alanine (W273A) generates an active site mimicking known lysine MTases. The aromatic side chains of N255F and Tyr312 form the wall of a narrow channel that the aliphatic part of the target lysine side chain is well-accommodated. We have also shown that positioning the target nitrogen atom in the right position and distance is fundamental for its acceptance of the methyl group from the donor SAM. The N3 atom of histidine ring and the terminal Ne of lysine, both of which are substrates, can occupy the same position, whereas the amide nitrogen atom of glutamine is displaced because of the physical hindrance of an essential Tyr312 conformation. In summary, our studies delineate the governing principles of how SETD3 and its derivatives discriminate different target residues by organizing the architectures of the active sites.

Materials and methods

Purification of SETD3

Recombinant human SETD3 (pXC2003) was expressed in Escherichia coli BL21(DE3) CodonPlus™ cells (Stratagene) as a GST fusion. The SETD3 mutants, N255F (pXC2092), N255A (pXC2093), N255V (pXC2094), and N255F/W273A (pXC2159) were generated with the Q5 Site-Directed Mutagenesis Kit (New England Biolabs), confirmed by sequencing, and were expressed and purified using the same protocol as WT. Briefly, the purification was conducted in a Bio-Rad NGC™ system using three-column chromatography including GSH-Sepharose, HiTrap Q-HP and a Superdex 200 sizing column. The GST tag was removed by PreScission Protease (purified in house). The purified SETD3 proteins were concentrated to ~25–35 mg/ml in 20 mM Tris, pH 8.0, 200 mM NaCl, 5% glycerol, and 0.5 mM tris (2-carboxyethyl) phosphine (TCEP) and kept at ~80 °C for future use. The actin peptides (residues 66–80 or 66–88) containing His73, Lys73, or Gln73 were purchased from GenScript.

Crystallography

Ternary complex of the N255F/W273A mutant, SAH with Lys73 peptide (residues 66–88, GenScript) and SETD3, SAH with Gln73 peptide (residues 66–88, GenScript) were prepared as described (17). A molar ratio of 1:4.5 (protein:peptide:SAH) was used and incubated on ice for 1 h. An Art Robbins Gryphon Crystalization Robot was used to set up 0.4–μl sitting drops at ~20 °C of the ternary complexes (~14 mg/ml or ~0.2 mM) with a well solution of 0.2 M ammonium acetate, 0.1 M sodium citrate tris buffer pH 5.6, and 30% (v/v) PEG 4000. Under the same conditions, we observed two space groups, C2221 (one complex per crystallographic asymmetric unit) and P21 (two complexes per asymmetric unit) (Table S1).

Single crystals were flash frozen in liquid nitrogen by equilibrating in a cryoprotectant buffer containing the crystallization solution and 25% (v/v) ethylene glycol. X-ray diffraction data were collected at the SER-CAT beamline 22ID of the Advanced Photon Source at Argonne National Laboratory. Crystallographic datasets were first processed with HKL2000 (35). Molecular replacement was performed with PHENIX PHASER module (36) by using the known structure of human SETD3 (PDB ID 6OX3) as the search model. Structure refinement was performed with PHENIX Refine (37) with 5% randomly chosen reflections for the validation by the Rfree value. COOT (38) was used for the manual building of the structure model and coro...
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Restrictions between refinement rounds. Structure quality was analyzed during PHENIX refinements and finally validated by the PDB validation server. Molecular graphics were generated by using PyMol (Schrödinger, LLC).

Steady-state kinetics

Reaction mixtures contained 20 mM Tris/HCl, pH 8.0, for His73 peptide or 20 mM glycine/NaOH, pH 10.5, for Lys73 peptide, 50 mM NaCl, 0.1 mg/ml BSA, 1 mM DTT, 0.18–15 μM enzymes (SETD3 WT or mutants; details in legends of Figs. 1C and 2), 40 μM SAM, and varying concentration of peptides. The reactions were carried out at 37 °C for different times (20 min to 3 h; details in legends of reactions) were carried out at 37 °C for different times (20 min to 3 h; details in legends of Figs. 1C and 2) with a total reaction volume of 20 μl and terminated by the addition of TFA to 0.1% (v/v) for Tris/HCl, pH 8.0, or 0.4% (v/v) for glycine/NaOH (pH 10.5). Samples terminated with the addition of 0.4% (v/v) TFA were then further diluted 4× with buffer 20 mM Tris/HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml BSA, 1 mM DTT to reduce the TFA concentration. The methylation activity was measured using the Promega bioluminescence assay (MTase-Glo73) in which the reaction byproduct SAH is converted into ATP in a two-step reaction and ATP can be detected through a luciferase reaction (39). In general, 5 μl of reaction mixture was transferred to a low volume 384-well plate and the luminescence assay was performed according to the manufacturer’s protocol. A Synergy 4 Multi-Mode Microplate Reader (BioTek) was used to measure luminescence signal. The dependence of the velocity of product formation per enzyme on substrate concentration was analyzed according to the Michaelis-Menten equation.

Assay of SETD3 on Gln73 peptide

To test whether SETD3 could methylate Gln73 in the context of actin peptide residues 66–88, a reaction mixture containing 20 mM Tris/HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml BSA, 1 mM DTT, 3 μM SETD3, 40 μM SAM, and 10 μM peptide was performed overnight at room temperature (Fig. 5B). Then reactions were terminated by the addition of TFA to 0.1% (v/v).

Isothermal titration calorimetry

ITC experiments (Fig. 5C) were performed using a MicroCal PEAQ-ITC automated system (Malvern Instrument Ltd) at 25 °C. Purified SETD3 (20 μM in 20 mM Tris, pH 8.0, 50 mM NaCl, 0.1 mg/ml BSA, 1 mM DTT) was maintained in the sample cell. The actin peptide Gln73 (residues 66–88, 400 μM in the same buffer) were injected into the cell by a syringe under continuous stirring (750 rpm) with the reference power set as 8 μcal/s. The volume of each injection was 2 μl with a fixed duration time of 4 s and the spacing time between the injections was 250 s to achieve equilibrium. Binding constants were calculated by fitting the data using a single binding-site model by the ITC data analysis program supplied by the manufacturer.

Inhibition of SETD3 by Gln73 peptide

A mixture contained 20 mM Tris/HCl, pH 8.0, 50 mM NaCl, 0.1 mg/ml BSA, 1 mM DTT, 0.18 μM SETD3, 40 μM SAM, and was incubated with varied concentration of Gln73 peptide (residues 66–88, 0–400 μM) for 30 min. Reactions were started by addition of 20 μM His73 peptide (residues 66–80) (Fig. 5D). After 20 min at room temperature, the reactions were terminated by the addition of TFA to 0.1% (v/v).

Data availability

The X-ray structures (coordinates and structure factor files) have been submitted to the PDB under accession numbers 6V62 (N255F/W273A-Lys73) and 6V63 (SETD3-Gln73).

Author contributions—S. D. data curation; S. D. and J. R. H. investigation; J. R. H. formal analysis; A. W. W., O. G., and Z. X. writing-review and editing; X. Z. and X. C. conceptualization; X. Z. and X. C. supervision; X. C. funding acquisition.

Acknowledgments—We thank members of the Cheng laboratory for discussion.

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