Molecular basis for oncohistone H3 recognition by SETD2 methyltransferase

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Histone post-translational modifications [PTMs] are linked to tumorigenesis, mostly via dysfunction of their regulators [e.g., readers, writers, and erasers] that are frequently mutated in tumors [Dawson and Kouzarides 2012]. Recently, high-frequency mutations in genes encoding histones themselves, rather than the histone regulators, were identified in a number of cancer types. Exome sequencing studies have identified recurrent hot spot missense mutations in histone H3. Notably, these mutations are located at or adjacent to H3 lysine residues that undergo acetylation and/or methylation. For example, the H3K27M mutation was identified in the majority of pediatric diffuse intrinsic pontine gliomas [Schwartzentruber et al. 2012; Wu et al. 2012], and the H3K56M mutation was found to occur predominantly in chondroblastomas [Behjati et al. 2013] and rarely in other cancer types such as head and neck squamous cell carcinoma and colorectal cancer (Shah et al. 2014). In addition, the H3K36-neighboring G34 mutations, such as G34R/V and G34W/L, have been detected in pediatric non-brain stem gliomas [Schwartzentruber et al. 2012; Wu et al. 2012] and giant cell tumors of the bone [Behjati et al. 2013], respectively.

Biochemical and cellular studies showed that H3K27M reduced global H3K27 methylation in vitro and in vivo by inhibiting the methyltransferase activity of polycomb-repressive complex 2 [PRC2] [Chan et al. 2013; Lewis et al. 2013; Justin et al. 2016]. Recently, we and others identified a similar “poisoning” mechanism underlying H3K36M-driven tumorigenesis involving the inactivation of H3K36 methyltransferases [Fan et al. 2016; Lu et al. 2016]. H3K36 can be methylated by enzymes such as NSD1/2/3, ASH1L, and SETD2 [Wagner and Carpenter 2012]. Among them, SETD2 serves as the major H3K36 methyltransferase that is able to generate the trimethylated H3K36 from the unmethylated, monomethylated, or dimethylated states in vitro and in cells [Edmunds et al. 2008; Hu et al. 2010]. SETD2 plays important roles in cellular processes such as transcription elongation [Yoh et al. 2008], RNA splicing [de Almeida et al. 2011; Kim et al. 2011], and DNA damage repair [Li et al. 2013; Pai et al. 2014]. However, due to the lack of peptide-bound structures, the molecular basis underlying oncogenic histone H3 recognition by SETD2 remains unknown.

Here, we report the crystal structure of SETD2 catalytic domain bound to the H3K36M/I peptide and SAH [S-adenosylhomocysteine]. In the complex structure, the catalytic SET domain adopts an open conformation, with the K36M/I peptide snugly positioned in a newly formed substrate channel. Our structural and biochemical data reveal the molecular basis underlying oncohistone recognition by and inhibition of SETD2.

Results and Discussion

Overall structure of the SETD2–H3K36M–SAH complex

To decipher the molecular basis for H3K36M recognition by SETD2, we solved the ternary crystal structure of the human SETD2 catalytic domain [SETD2CD, amino acids 1434–1711] [Fig. 1A] bound to SAH and mutant histone H3.329Δ43K36M at 2.05 Å as well as a SAH-bound binary structure at 2.4 Å [Supplemental Table S1]. The SETD2CD is composed of an N-terminal AWS zinc finger motif, a center SET domain, and a C-terminal Post–SET motif [Fig. 1A]. The SET domain is characteristic of three discrete β sheets [I, β1–β2; II, β3–β8–β7; III, β4–β6–β5] that are arranged in a triangular shape [Fig. 1B,C]. The SAH is located at the pocket formed by loops Lαβ2, Lαβ7, and LPOST. Based on the electron densities, we could clearly trace histone H3.3 A29–R42 at the peptide-binding channel of the SET domain with the M36 long side chain pointing to SAH at the active center [Fig. 1B]. Upon complex formation, the C-terminal fragments of SETD2CD [loop LPOST and Post–SET] form a “knot”-like structure to cover the N-terminal part of the H3 peptide and stabilize its
dramatic structural changes of the LIN et al. (2012) as well as the one solved in this study revealed SAH-bound binary structures reported previously (Zheng et al. 2012). Conformational change trigged by histone peptide binding (Supplemental Movie S1). First, a short α helix (α8) is induced at the C terminus to interact with α6 of the SET domain through hydrophobic contacts, further fastening H3 binding [Fig. 2B]. In the presence of Pr-SNF [N-propyl sinefungin], a synthetic SAM [S-adenosyl methionine] analog, an open-state SETD2CD [PDB code: 4FMU] has been captured in the absence of bound peptide (Zheng et al. 2012). Structural superimposition revealed a number of conformational differences between the Pr-SNF-bound and peptide-bound “open” states of SETD2CD, which involves structural adjustments of the LIN loop and the ordering of the Post–SET C-terminal fragment in the latter case as an adaptation to peptide binding [Supplemental Fig. S1]. In addition, structural and sequence alignment of SETD2CD with other H3K36 methyltransferases [An et al. 2011; Qiao et al. 2011] revealed that C211 of NSD1 (PDB code: 3OOI) and S2259 of ASH1L (PDB code: 3OPE) are well superimposed with R1670 of SETD2 in its closed form [Fig. 2CD], which suggests similar conformational change-driven regulatory mechanisms shared among H3K36 methyltransferases.

**Conformational change trigged by histone peptide binding**

The structural alignment of the ternary complex with the SAH-bound binary structures reported previously [Zheng et al. 2012] as well as the one solved in this study revealed dramatic structural changes of the LIN–Post–SET fragment upon H3 binding [Supplemental Movie S1]. First, the LIN loop connecting the SET domain and the Post–SET motif undergoes stepwise conformational changes following H3 peptide binding [Fig. 2A in stereo view, closed in salmon, half-open in green, and open in magenta]. In the closed state [Protein Data Bank [PDB] code: 4H12], the LIN loop takes on a crouched conformation and inserts its residue, R1670, into the active center. Particularly, R1670 overlaps with H3K36M and occupies the K36 access pocket, therefore inhibiting the enzymatic activity of SETD2. Our binary structure captured a half-open state of SETD2 in which R1670 flips outwards from the active center by ~8.3 Å, thus priming the pocket for peptide entrance. In the open state, loopLIN adopts an extended conformation with R1670 further shifted ~4.5 Å away, which allows proper docking of the H3 peptide in the substrate channel. Interestingly, the autoinhibitory LIN loop in the closed state turned out to facilitate H3 peptide binding in the open state [Fig. 1D, detailed later], suggesting dualistic function of the LIN loop at different stages of the enzymatic cycle. A second conformational change trigged by H3 peptide binding is the ordering of an invisible C-terminal loop-α8 fragment (1692–1703) of Post–SET that stretches over the substrate channel [Fig. 2A]. Notably, a short α helix (α8) is induced at the C terminus to interact with α6 of the SET domain through hydrophobic contacts, further fastening H3 binding [Fig. 2B]. In the presence of Pr-SNF [N-propyl sinefungin], a synthetic SAM [S-adenosyl methionine] analog, an open-state SETD2CD [PDB code: 4FMU] has been captured in the absence of bound peptide (Zheng et al. 2012). Structural superimposition revealed a number of conformational differences between the Pr-SNF-bound and peptide-bound “open” states of SETD2CD, which involves structural adjustments of the LIN loop and the ordering of the Post–SET C-terminal fragment in the latter case as an adaptation to peptide binding [Supplemental Fig. S1]. In addition, structural and sequence alignment of SETD2CD with other H3K36 methyltransferases [An et al. 2011; Qiao et al. 2011] revealed that C211 of NSD1 (PDB code: 3OOI) and S2259 of ASH1L (PDB code: 3OPE) are well superimposed with R1670 of SETD2 in its closed form [Fig. 2CD], which suggests similar conformational change-driven regulatory mechanisms shared among H3K36 methyltransferases.

**Details of H3 peptide recognition by the SETD2 catalytic domain**

Interaction analysis by LigPlot [Laskowski and Swindells 2011] revealed 14 direct and eight water-mediated hydrogen-bonding pairs as well as 15 pairs of hydrophobic contacts between SETD2CD and the H3K36M peptide [Supplemental Fig. S2]. Notably, site-specific recognition of histone H3 by SETD2 is determined by extensive in-teractions involving residues A29–P30–S31–T32–G33–G34–V35 and K37–P38–H39–R40–Y41–R42 flanking position 36. The small, noncharged A29 residue and SAH-binding sites are shaded in lime green and slate blue, respectively. (C) The topology of SETD2CD (green) and the sequence of histone H3K36M (magenta) are represented by a cartoon. The H3K36M peptide is shown as yellow sticks. The rest of SETD2CD is represented as a gray surface.

**Figure 1.** Overall structure of the SETD2CD–H3K36M–SAH ternary complex. (A) The domain architecture of human SETD2 [hSETD2] and the sequence of histone H3K36M peptide used for crystallization. (B) Overall structure of SETD2CD bound to the H3K36M peptide and SAH. SETD2CD is colored in light gray for the AWS motif, green for the SET domain, and magenta for the Post–SET motif. The H3K36M peptide is shown as yellow sticks colored by the simulated annealing Fo–Fc omit map countered at the 2.5 σ level. The loops LPOST and LIN mentioned in the text are labeled. The H3K36M peptide and SAH-binding sites are shaded in lime green and slate blue, respectively. (D) Covering of the H3K36M peptide by the C-terminal segments of SETD2CD. The Post–SET motif [magenta] and loop LIN [green] are represented by a cartoon. The H3K36M peptide is shown as yellow sticks. The rest of SETD2CD is represented as a gray surface.
Histone H3G34 mutations (G34R/V/W/L) have been detected in brain (Schwartzentruber et al. 2012; Wu et al. 2012) and bone (Behjati et al. 2013) tumors. In the complex structure, the G33–G34 step is fully buried and threads through a narrow tunnel of the SETD2CD substrate channel [Fig. 4A], which is reminiscent of H3 G33–G34 recognition by the H3K36 demethylase KDM2A (Cheng et al. 2014). The inner wall of the H3 G33–G34 tunnel is formed by aromatic rings of Y1604, F1668, and Y1671 as well as main chains of Q1669–G1672 within loop L\text{IN} [Fig. 4B]. The dimension of the tunnel is highly restrictive, being perfect for accommodating side chain-free glycine residues. Conceivably, mutation of H3G34 into any other bulkier residues will severely block histone H3 binding [Supplemental Fig. S3A–C] and thereby abrogate subsequent H3K36 methylation by SETD2 as shown previously [Lewis et al. 2013]. This may serve as one fundamental molecular mechanism underlying the oncogenic activity of histone H3G34R/V/W/L mutants.

Sequence alignment showed that the tunnel-forming residues of SETD2 are essentially conserved in other H3K36 methyltransferases, including NSD1/2/3 and ASH1L [Fig. 4C]. In principle, an aromatic and planar feature is conserved at position Y1604 [Y, F, and H], which stacks against the T32–G33 amide plane, and a hydrophobic feature is conserved at positions F1668 [F, L, and F] and Y1671 [Y, L, and F], which encapsulate the side chain-free H3 G33–G34. These tunnel residues are well aligned in the peptide-free state [Fig. 4D], and structural modeling suggested that mutating Y1604/F1668/Y1671 to F/L/L of NSD1/2/3 can well restrict H3 G33–G34 in a narrow tunnel [Fig. 4E; Supplemental Fig. S3D,E]. Collectively, these results indicate that other H3K36 methyltransferase family members may be similarly inhibited by H3G34 oncogenic mutations.

**Structural basis for trans inhibition of SETD2 activity by H3K36M/I mutants**

Histone H3K36M is a high-frequency oncogenic mutation identified in chondroblastoma (Behjati et al. 2013). Our ternary structure in this study revealed registration and snug insertion of the K36M side chain into the active center of SETD2CD, where the S-methyl thioether group of K36M points to SAH—the product form of SAM after methyl transfer [Fig. 5A, panel I]. The K36M side chain is confined in a hydrophobic K36 access pocket formed by residues Y1579, M1607, F1664, and Y1666 with sound shape complementarity [Supplemental Fig. S4A, in stereo view]. These residues are well conserved in NSD1/2/3 and ASH1L [Supplemental Fig. S5], suggesting similar K36M preference by the lysine access pocket. Besides hydrophobic contacts, the K36M side chain stacks against the aromatic ring of Y1666 and is further stabilized by sulfur–aromatic [Valley et al. 2012]
as well as CH–π interactions (Fig. 5B, panel iii), Brandl et al. 2001). These features will conceivably promote SETD2_CD association and thus sequester and inhibit SETD2 activity in trans to block global H3K36 methylation.

Systematic amino acid substitution studies at H3K27 showed that only the K-to-I mutation displayed an inhibitory effect on PRC2 similar to that of K-to-M (Lewis et al. 2013), and we recently observed a similar oncoenic effect of H3K36l in cell culture models as well as the identification of the K36l mutation in pediatric undifferentiated sarcoma tumors (Lu et al. 2016). Interestingly, in an effort to obtain additional complex structures using wild-type, K36 methylated (me1/2/3), or other K36 mutated (K36I, K36R, K36L, and K36Q) H3.329 peptides, we were able to get only H3.329–42K36I-bound SETD2_CD crystal under essentially the same crystallization condition as that of H3.329–42K36M and K36I peptides; in contrast, addition of H3K36L, H3K36Q, H3K36R, or H3K36me3 peptide has a minimal effect on SETD2 activity. Taken together, our biochemical and structural studies suggest that H3K36M and H3K36l are preferred nonreactive ligands for SETD2 and likely its structurally conserved paralogs [Fig. 2C] to inhibit their enzymatic activities, therefore leading to global reduction of H3K36 methylation in cells in the process of tumorigenesis.

In summary, we report here the first peptide-bound structures of SETD2_CD in the open state, thus shedding new light on H3K36 methyltransferases with regard to substrate recognition and enzymatic regulation.

Our current work along with two recent structural studies on H3K27M recognition by the PRC2 complex (Justin et al. 2016) and H3K36M recognition by G9a (Jayaram et al. 2016) collectively emphasize the role of K to M among other oncohistone mutations in promoting loss of specific lysine methylation through recognition by and inhibition of SET domain methyltransferases. Given the importance of histone methylation in health and disease, the molecular mechanisms uncovered here not only help to elucidate the etiology associated with aberrant histone modification but also pave the way for new initiatives in the treatment of diseases linked to oncohistone mutation.

Materials and methods

Protein production and crystallographic studies

Wild-type and mutant human SETD2_CD [residues 1434–1711] were recombinantly produced in Escherichia coli and purified as His-SUMO-tagged proteins. Crystallization was performed via vapor diffusion method. Diffraction data were collected at Shanghai Synchrotron Radiation Facility beamline BL17U under cryo conditions and processed with the HKL3000 software packages. The structures were solved by molecular
replacement using the MolRep program (Vagin and Teplyakov 2010), with the free SETD2 SET domain structure (PDB code: 4H12) as the search model. All structures were refined using PHENIX (Adams et al. 2010) with iterative manual model building with COOT (Emsley and Cowtan 2004). Detailed structural refinement statistics are in Supplemental Table S1.

**In vitro methyltransferase assay**

Radiometric filter assay was used to measure the enzymatic activity of SETD2CD and its mutants. The mononucleosome samples were prepared from HeLa cells.

Detailed descriptions about the Materials and Methods are in the Supplemental Material.

**Accession codes**

The coordinates and structure factors for the SETD2–H3K36M–SAH, SETD2–H3K36–SAH, and SETD2–SAH structures have been deposited under accession codes 5JJY, 5JLB, and 5JLE, respectively.

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**Oncohistone recognition by SETD2**

Figure 4. The H3 G33–G34-accommodating tunnel of SETD2. (A) Burial of the G33–G34 fragment of H3 peptide in the SETD2CD substrate channel. SETD2CD is shown as half-transparent surface (gray). (B) Close-up view of CONO (blue), Sequence code: 3OOI) (cyan), and ASH1L (PDB code: 3OPE) (salmon). (C) Encapsulation of H3 G33–G34 (yellow) by a modeled NSD1/2/3 tunnel. Modelled tunnel residues are shown as cyan sticks.
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