Structural insight into HEMK2-TRMT112 mediated glutamine methylation

Jie Gao¹, Bin Wang², Huijuan Yu¹, Gao Wu¹, Cuihong Wan², Wenting Liu³, Shanhui Liao¹, Liansheng Cheng³, Zhongliang Zhu¹,#

¹ Hefei National Laboratory for Physical Sciences at Microscale and School of Life Sciences, University of Science and Technology of China, Hefei 230027, P. R. China.
² Hubei Key Lab of Genetic Regulation & Integrative Biology, School of Life Sciences, Central China Normal University, No. 152 Luoyu Road, Wuhan 430079, P. R. China.
³ Hanke Mab Biotechnologies Inc., Hefei 230088, P. R. China.

# Correspondence: charlson@ustc.edu.cn, zlzhu63@ustc.edu.cn

Abstract

Post-translational modifications (PTMs) play important roles in mediating protein functions in a wide variety of cellular events in vivo. HEMK2-TRMT112 heterodimer has been reported to be responsible for both histone lysine methylation and eukaryotic release factor 1 (eRF1) glutamine methylation. However, how HEMK2-TRMT112 complex recognizes and catalyzes eRF1 glutamine methylation is largely unknown. Here, we present two structures of HEMK2-TRMT112, with one bound to SAM and the other bound with SAH and methylglutamine (Qme). Structural analyses of the post-catalytic complex, complemented by mass spectrometry experiments, indicate that the HEMK2 utilizes a specific pocket to accommodate the substrate glutamine and catalyzes the subsequent methylation. Therefore, our work not only throws light on the protein glutamine methylation mechanism, but also reveals the dual activity of HEMK2 by catalyzing the methylation of both Lys and Gln residues.
Keywords
HEMK2, TRMT112, SAM/SAH, glutamine methylation, X-ray crystallography

Introduction

Post-translational modifications of proteins, including phosphorylation, acetylation, and methylation, are involved in numerous cross-regulatory cellular events. With extensive studies on histone modifications [1], lysine/arginine modifications have been found to be important for regulating gene transcription [2]. In addition, methylation of non-histone proteins was also found to be essential for their function or fate. In addition to methylation of lysine and arginine residues, methylation of other residues, such as aspartate [3], glutamate [3], histidine [4-6], and glutamine [7, 8], were also been reported.

Recently, SETD3, a SET-domain containing protein, has been found to affect the stability of cellular actin filaments by methylating β-actin His73 N$^3$ atom [4]. In contrast to well-studied lysine/arginine methyltransferases and recently identified histidine methyltransferase, how asparagine/glutamine (N/Q) methylation is catalyzed and erased, remains elusive.

Glutamine methylation was first reported 40 years ago in the ribosomal protein L3 of *Escherichia coli*, and is not as frequent as other modifications [9]. Prokaryotic and eukaryotic ribosomal polypeptide release factors (RFs) undergo this modification at a conserved GGQ motif aided by the HEMK protein family [10, 11]. The latter has been reported to be involved in heme biogenesis in *E. coli* [12]. Functional studies of glutamine methylation activity of prokaryotic HEMK suggest its essential role in ribosome assembly [11].

HEMK proteins contain a conserved [D/N]PPY motif, shared by DNA adenine methyltransferase [11]. There are two HEMK homolog genes (*HEMK1* and *HEMK2*) in human genome; HEMK2, also named as N6AMT1 ($N^6$-adenine-specific DNA methyltransferase). Although direct evidences show that HEMK2 serves as the
histone H4K12 methyltransferase and mediates the prostate tumor cell proliferation [7], the catalytic activity of HEMK2 towards 6mA DNA have been controversial [13-15]. tRNA methyltransferase 112 homolog (TRMT112) protein is an cofactor of HEMK2, which may boost the methyltransferase activity of HEMK2 [8]. The most recently structural and functional studies of histone H4 methylation mediated by HEMK2-TRMT112 not only unveiled the mechanism of lysine methylation activity of this enzyme, but also suggested that cellular effects induced by lysine methylation is independent of glutamine methylation [7]. However, how HEMK2-TRMT112 catalyzes glutamine methylation remains unknown.

In this work, we studied the methylation activity of human HEMK2-TRMT112 heterodimer towards eRF1 peptide containing GQ motif by mass spectrum. By solving the structures of human HEMK2-TRMT112 heterodimer bound with cofactor and eRF1 peptide, we revealed how HEMK2 specifically binds to TRMT112 and how HEMK2 recognizes the Gln185 of eRF1 and possible catalytic mechanism of glutamine methylation. Therefore, our work will not only complete the understanding of HEMK2-TRMT112 as a unique protein methyltransferase with dual activity, but also facilitate the design of potent inhibitors for HEMK2 in near future.

Materials and methods

Plasmid construction and protein expression

Genes encoding HEMK22214 and TRMT1122125 were synthesized by Sangon Biotech (Shanghai) and cloned into pETDuet-1 (Genbank accession number: 71146-3). Then, the plasmid was transformed into E. coli BL21 (DE3) that were cultured in Luria-Bertani (LB) at 37°C until OD600 to 0.8, and the recombinant protein was overexpressed at 16°C for 20 hrs in the presence of 0.5 mM isopropyl-D-1-thiogalactopyranoside (IPTG). Recombinant proteins were purified with a fast flow Ni-NTA column (GE Healthcare). N-terminal 6 x His-tag of recombinant proteins were removed with Tobacco Etch Virus (TEV) protease. Gel filtration and ion-exchange were employed for further purification. The final protein
was concentrated to about 10 mg/ml in buffer containing 20 mM Tris-HCl, pH 7.5, 50 mM NaCl.

**Crystallization, data collection and structure determination**

Crystals were grown using the sitting drop vapor diffusion method at 18°C. For crystallization of HEMK2-TRMT112, purified protein was pre-incubated with AdoMet at a molar ratio of 1:3, and mixed with the crystallization buffer containing 0.2 M ammonium acetate, 0.1 M sodium citrate tribasic dihydrate pH 5.6 and 30% PEG 4000 at the concentration of 10 mg/ml. For crystallization of HEMK2/SAH/Q(me), purified protein was pre-incubated with peptide corresponding to human eRF1 and mixed with buffer containing 1.26 M sodium phosphate monobasic monohydrate, 0.14 M potassium phosphate dibasic, pH 5.6. All crystals were soaked in a cryo-protectant consisting of 90% reservoir solution plus 10% glycerol. The diffraction data were collected on BL18U1 at the Shanghai Synchrotron Facility (SSRF). Data sets were collected at 0.979 Å and were processed using the HKL3000 program [16]. The initial structures of HEMK2-TRMT112 Complex with SAM was solved by molecular replacement using Phaser with a previously solved Mtq2 (PDB ID: 3Q87) [17, 18] and Trmt112(PDB ID: 4QTH) as a search model structure [19]. Then, the model was refined manually and built with Coot [20]. The final structure was further refined by PHENIX [21]. The statistics for data collection and refinement are summarized in table I.

**Mass spectrometry**

Reversed-phase microcapillary/tandem mass spectrometry (LC/MS/MS) was performed using an Easy-nLC nanoflow HPLC (Proxeon Biosciences) with a self-packed 75 mm x 15 cm C18 column connected to a QE-Plus (Thermo Scientific) in data-dependent acquisition and positive ion mode at 300 nL/min. Passing MS/MS spectra were manually inspected to ensure that all b- and y-fragment ions aligned with the assigned sequence and modification sites. A 30 ul reaction mixture contained 20
uM HEMK2-TRMT112 and 20 uM peptides (H2A99-110, eRF1178-194, CHD51384-1398, and NUTM11040-1054) in a buffer containing 10 mM Tris-HCl, (pH 7.5), 20 mM NaCl and 60 uM AdoMet. The reaction was incubated at 37°C for 4 hrs before being quenched (at 70°C for 15 mins), protein precipitates were removed by centrifuge at 16000g for 20 mins. Then, supernatant were analyzed by LC/MS/MS and Proteomics Browser software, with the relative abundances of substrate and product reflecting the methylation activities of protein.

Results and discussion

To elucidate the underlying mechanism, we solved two crystal structures of HEMK2/TRMT112 heterodimer, with one bound to SAM and the other bound with SAH and methylated glutamine (Qme), at resolutions of 2.2 Å and 1.9 Å, respectively (Supplementary Fig. S1, Fig. 1A and table I). Both HEMK2 and TRMT112 exhibit α, β-fold conformation, while HEMK2 adopting a Rossmann fold (Fig. 1A and Supplementary Fig. S2) [22]. The structure of human HEMK2 is similar to those previously reported homologs. Seven β-strands form a central β-sheet, with β1-β4 arranged in parallel and β5–β7 arranged in anti-parallel. α-helices were distributed on both sides of the central β-sheet (Supplementary Fig. S2C). TRMT112 consisted of four α-helices and three anti-parallel β-strands, all α-helices being on one side of β-sheet. TRMT112 interacted with HEMK2 via extensive hydrogen bonds, as indicated in Supplementary Fig. S3 A-B. Besides, TRMT112 also stabilized HEMK2 by masking its hydrophobic surface (Fig. 1B).

HEMK2 was reported to catalyze glutamine methylation of numerous substrates [23]. To study the enzymatic activity of HEMK2, we performed methylation assays on four synthetic peptides, H2A99-110, eRF1178-194, CHD51384-1398, and NUTM11040-1054, and analyzed the products by mass spectrometry. As expected, methylation of Q185 of previously reported substrate eRF1 was confirmed. In addition, peptide CHD51384-1398, and peptide NUTM11040-1054 were also found to be methylated. In contrast, no methylation was found on peptide H2A99-110 (Fig. 1C and Supplementary Fig. S3 C-E).
Histone H2A has been reported to be methylated by glutamine methyltransferase fibrillarin [23], indicating that HEMK2-mediated glutamine methylation depends on the flanking residues of glutamine residue in substrates.

To provide structure insight into the HEMK2-mediated glutamine methylation, we tried to co-crystallize of HEMK2-TRMT112 with above three substrate peptides, respectively. However, only eRF1\textsubscript{178-194} peptide was successfully co-crystallized with HEMK2/TRMT112. By solving the peptide-bound structure, we found that only the methylated glutamine (Qme) in eRF1 peptide is visible in the electron density map (Fig. 1A). In the same structure, the SAH was found to bind HEMK2 in a way similar to that in the SAM-bound one, which indicating it as a post-catalytic protein complex.

When two HEMK2-TRMT112 structures are superimposed, the root mean standard deviation (R.M.S.D) between two heterodimers are ~0.15 Å, indicating that no significant structural change were found upon peptide binding (Supplementary Fig. S1E). In both structures, the HEMK2 residues involved in SAH/SAM binding were almost identical (Supplementary Fig. S1). Three residues (Glu28, Tyr23, and Tyr 125) of HEMK2 are involved in the interaction with methylated Gln (Fig. 1D). The side chain nitrogen and carboxyl groups of Qme are hydrogen bonded to the side chain carboxyl group of Glu28 and the main chain nitrogen atom of Tyr125, respectively. Additionally, the side chain hydroxyl group of Tyr23 forms two hydrogen bond to mainchain N atom and side chain NE atom of Qme respectively ((Fig. 1E)). The Qme side chain further packs with the Tyr125 aromatic ring to enhance the intermolecular interaction. It’s difficult for us to crystallize the catalytically active complex, which may be due to a rapid methylation process during crystallization. The distance between side chain nitrogen atom of Qme and the Sulphur atom of SAH is ~ 3.9 Å, suggesting that above intermolecular interactions between HEMK2 and Qme would facilitate product release after catalysis.

Sequence alignment of eukaryotic HEMK proteins indicates that the SAH/SAM binding and Qme recognition residues are conserved among eukaryotes
Further DALI search results indicated that the HEMK2 adopts a fold similar to its prokaryotic homolog, as evidenced by the finding that R.M.S.D between core regions of human HEMK2 and *Escherichia coli* PrmC is ~4.8 Å [26] despite their low sequence identity (~18%) (Supplementary Fig. S4C-D). The overall structural similarity, as well as conserved residues involved in cofactor binding, implies the conserved HEMK-mediated glutamine methylation.

Comparing the structure of Qme-bound HEMK2 with that of the Kme-bound form, we found that HEMK2 utilizes the same pocket to methylate both lysine and glutamine, implying the catalytic plasticity of the pocket (Fig. 1D). However, only Asp28 of HEMK2 is conserved in recognizing Qme or Kme, which may imply Asp28 is the active residue for methyl transfer. Side chain of Asp28 and Asn122, which are involved in binding to Kme, forms two hydrogen bonds with Nε of Kme. While the main chain carbonyl group of Pro123 is h-bonded to Nε of Kme, the same group only forms one weak h-bond with the methyl moiety of Qme; the Glu204 side chain carbonyl group also provides a h-bond with the Kme main chain nitrogen atom, which enhanced the binding between Kme and HEMK2 (Fig. 1F).

In summary, recent studies have reported that HEMK2 displays multiple functions in the methylation of lysine, glutamine, and even nucleotides. Metzger *et al.* had revealed the lysine methylation mechanism of HEMK2 [7]. Here, we partially disclosure the mechanism of HEMK2-mediated glutamine methylation by solving a post-catalytic enzyme complex. Comparing the relative position of methylated residues (K/Q) and hydrogen bond formation with HEMK2, despite that some different residues involved in K/Q binding, we found HEMK2 to utilizes the same pocket to catalyze Lys methylation, suggesting the catalytic pocket catalyze both lysine and glutamine by employing different side chains to stabilize substrates. In addition, we found that both Kme and Qme are deeply buried in the catalytic pocket of HEMK2. Many residues with bulky hydrophobic sidechain are distributed around that pocket, which may prevent peptides with bulky sidechain residues neighbouring to Gln from glutamine methylation. That may explain why HEMK2 prefer GQ motif,
whether glutamine methylation or lysine methylation. Kusevic et al [27]. also reported GQ motif preference in substrates by massive substrate identification of HEMK2. However, further structural studies are warranted for verification.

Accession number
The coordinates and structural factors of structure of HEMK2-TRMT112 complex with SAM and with SAH and methylated glutamine have been deposited into the RCSB protein data bank under accession number 6K0X and 6KHS.

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Conflict of interest
The authors declare that they have no conflicts of interest with the contents of this article.

Author contributions
Z.Z., S.L., conceived the project; J.G. performed experiments and structure refinement under the guidance of S.L.; S.L. solved the initial structure; G.W., B.W., C.W. performed the mass spectrometry experiments; J.G., W.L., L.C., H.Y., wrote the manuscript and all authors contribute to editing.

Figure captions
Figure 1. A. Structure of human HEMK2/TRMT112, in complex with SAH, and
methylated glutamine residue. 2mFo-DFc electron density maps of SAH and methylated glutamine residue (Q(me)) are contoured at 1.0 σ, and displayed as red and blue mesh, respectively. **B.** The electrostatic surface of HEMK2, Q(me), and K(me) are colored in blue and yellow, respectively. Catalytic pocket is enclosed in a yellow dashed-line circle. **C.** Mass spectra of rRF1_{178-194} peptides; control (upper) and that reacted with HEMK2/TRMT112/SAM (lower) are shown. Black arrows and numbers indicate mass-to-charge ratio of unmodified peptides while red arrows and numbers indicate mass-to-charge ratio of methylated peptides. Numbers in parentheses mean the charge of peptides. **D.** Structural comparisons of HEMK2-Q(me) and HEMK2-K(me). Q(me) is colored in yellow, and K(me) is colored in red. the methyl group attached to the side chain amino group of Q and K are displayed in sphere. SAH is colored in green and blue respectively. Calculated R.M.S.D between heavy atoms (N, Ca, C, and O) of the two complex is 0.379. **E.** Hydrogen bonds between Q(me) (yellow) and HEMK2 are displayed. Hydrogen bonds are indicated in black dash lines, and bond distances are indicated besides the dash lines. The methyl group attached to the side chain amino group of glutamine residue is displayed in sphere. **F.** Hydrogen bonds between K(me) (red) and HEMK2 are displayed. Hydrogen bonds are indicated in black dash lines, and bond distances are indicated besides the dash lines. the methyl group attached to the side chain amino group of lysine residue is displayed in sphere.

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