Structure and regulation of ZCCHC4 in m^6^A-methylation of 28S rRNA

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N^6^-methyladenosine (m^6^A) modification provides an important epitranscriptomic mechanism that critically regulates RNA metabolism and function. However, how m^6^A writers attain substrate specificities remains unclear. We report the 3.1 Å-resolution crystal structure of human CCHC zinc finger-containing protein ZCCHC4, a 28S rRNA-specific m^6^A methyltransferase, bound to S-adenosyl-L-homocysteine. The methyltransferase (MTase) domain of ZCCHC4 is packed against N-terminal GRF-type and C2H2 zinc finger domains and a C-terminal CCHC domain, creating an integrated RNA-binding surface. Strikingly, the MTase domain adopts an autoinhibitory conformation, with a self-occluded catalytic site and a fully-closed cofactor pocket. Mutational and enzymatic analyses further substantiate the molecular basis for ZCCHC4-RNA recognition and a role of the stem-loop structure within substrate in governing the substrate specificity. Overall, this study unveils unique structural and enzymatic characteristics of ZCCHC4, distinctive from what was seen with the METTL family of m^6^A writers, providing the mechanistic basis for ZCCHC4 modulation of m^6^A RNA methylation.
Covariant modifications of RNA molecules represent an evolutionarily conserved epitranscriptomic mechanism that critically regulates diverse cellular activities. One of the most prevalent RNA modifications is N6-methyladenosine (m6A), which is widely present in mRNA and long noncoding RNA (lncRNA), as well as in the ribosomal RNA (rRNA) and small nuclear RNA (snRNA). Specific methylation of these RNA molecules functions to modulate RNA structure and protein–RNA interactions, which in turn influences RNA metabolism, cell signaling, cell survival, and differentiation. Dysregulation of m6A-based RNA modification has been associated with severe human diseases, such as cancer.

The dynamic profiling of m6A across the epitranscriptome is mediated by an array of distinct writer enzymes. For instance, mRNA and lncRNA are primarily methylated by the METTL3–METTL14 heterodimeric complex, which recognizes a DRACH (D: A, G, U; R: G, A; H: A, C, U) consensus sequence near 3′ UTR, whereas a subset of mRNA and snRNA sites is distinctly methylated by RNA methyltransferase METTL16, which recognizes a UACAGAGAA motif embedded in a stem-loop structure. In addition, m6A modification of cap adenosine at the transcription start nucleotide of mRNAs is achieved by cap-specific methyltransferase CAPAM, which then promotes translation of capped mRNAs. Recent studies have further demonstrated that the m6A modification on the site 4220 of 28S RNA and site 1832 of 18S RNA is, respectively, mediated by CCHC zinc finger-containing protein ZCCHC4 and the methyltransferase METTL5. Notably, these identified RNA methyltransferases bear no sequence homology, except for a remotely related methyltransferase (MTase) domain (Supplementary Fig. 1), raising a question of the molecular basis underlying their distinct substrate specificities.

ZCCHC4 is a newly identified m6A RNA methyltransferase and is highly conserved in multicellular organisms, ZCCHC4 knockout in human cells eliminates the m6A2220 methylation of 28S RNA, but not any other identified m6A sites, suggesting its exclusive role in methyllating 28S RNA. ZCCHC4-mediated m6A4220 methylation in 28S RNA promotes ribosome assembly and translation, which in turn impacts cell proliferation and tumor growth. In addition, a previous study on K-Ras-transformed cells has identified ZCCHC4 as a potential factor required for Ras-mediated gene silencing. However, due to the lack of structural information, the mechanism by which ZCCHC4 recognizes and methylates its RNA substrate remains unknown.

To provide mechanistic insights into the ZCCHC4-mediated m6A RNA methylation, we determined the crystal structure of human ZCCHC4 (residues 24–464; ZCCHC424–464) each into alanine (see the Methods section), which permitted crystallization and structure determination. The crystal structure of ZCCHC424–464, in complex with S-adenosyl-L-homocysteine (SAH), product of methyl donor S-adenosyl-L-methionine (SAM), was refined to 3.1 Å resolution (Fig. 1b; Supplementary Table 1). There are six ZCCHC424–464 complexes in one asymmetric unit, with one pair of m6A molecules in each complex (Supplementary Fig. 1), likely due to crystal packing effects. These two molecules also yield the highest-quality electron density map, with one of which permitting the modeling of the entire segment of residues M25-G440. We therefore chose the ZCCHC4 molecule for structural analysis. The ZCCHC4 molecules in the asymmetric unit of all the other ZCCHC4 molecules are not involved in domain swapping, in line with the fact that ZCCHC4 exists as a monomer in solution, as demonstrated by our size-exclusion chromatography analysis (Supplementary Fig. 2c).

The structure of ZCCHC424–464 reveals four closely packed domains: an N-terminal GRF zinc finger domain is followed by a C2H2 zinc finger domain, an MTase domain, and a C-terminal CCHC zinc finger domain, folded into an integrated structural unit (Supplementary Fig. 1b). The MTase domain is comprised of seven-stranded β-sheet, formed by five parallel strands and two antiparallel capping strands (Fig. 1b, c). This central β-sheet is further flanked by three intervening α-helices on each side (Fig. 1b). The SAH molecule snugly fits into a fully closed pocket, lined by a loop connecting the C2H2 domain and the MTase domain (cofactor loop: residues P163-F175), the terminal ends of α-helix and β-strand, and the DPPF (D276-P277-P278-F279) catalytic motif (Fig. 1d; Supplementary Fig. 3). The homocysteine moiety of SAH is surrounded by the side chains of L174-F175, T200-R202, and D276, with the amino group donating a hydrogen bond to the side-chain carboxylic of D276 and the carboxylate receiving a hydrogen bond from the backbone amide of F175 (Fig. 1d). On the other end, the adenosyl moiety of SAH is embraced by the side chains of I226, N243, M244, and F245 through van der Waals contacts, with the N6 atom further forming a hydrogen bond with the side-chain carbonyl group of N243 (Fig. 1d). In addition, the sugar moiety of SAH is recognized by Q172 and D225 through hydrogen-bonding interactions (Fig. 1d). It is worth noting that this closure of SAH-binding pocket is similar to that of CAPAM (Supplementary Fig. 4a, b), but distinct from those of METTL3, METTL5, and METTL6, in which the cofactor-binding pockets are rather exposed to the solvent (Supplementary Fig. 4c–e). In fact, the residues involved in the cofactor binding are poorly conserved among these m6A RNA methyltransferases (Supplementary Figs. 1, 4), highlighting their divergent cofactor-binding mechanisms.

The tandem GRF and C2H2 domains flank one end of the central β-sheet, with the GRF domain stacking on top of the SAH-binding pocket and the C2H2 domain docked in the groove formed by helices αG and αH of the MTase domain (Fig. 1b; Supplementary Fig. 5a). The structure of the GRF zinc finger domain is dominated by a three-stranded antiparallel β-sheet, with a CHCC zinc cluster embedded between the loop preceding β1 and the linker connecting β2 and β3 (Fig. 1b; Supplementary Fig. 5a, b). In addition, the three-stranded β-sheet of GRF is appended by a C-terminal α-helix that guides the packing of the
GRF domain toward the MTase domain (Fig. 1b; Supplementary Fig. 5a). The C2H2 zinc finger domain adopts an α/β-fold arranged in an α-β-α-β-α-β fashion, with a C2H2 zinc cluster formed between the loop connecting β4 and β5 and the loop connecting β6 and αD (Fig. 1b; Supplementary Fig. 5a, c). Association of the GRF and C2H2 domains with the MTase domain is both mediated by surface complementarity, which permits formation of extensive interdomain van der Waals and hydrogen-bonding contacts centered around the SAH-binding pocket (Supplementary Fig. 5a). The opposite end of the central β-sheet is sided by the CCHC domain, comprised of four consecutive two-stranded β-sheets and a C-terminal tail, stacking on top of each other to form four CCHC zinc clusters in a spacing of 10–11 Å (Fig. 1b; Supplementary Fig. 5a, d). Association of the CCHC domain with the MTase domain is mainly mediated by hydrogen bonds formed between residues from the start strands of the CCHC domain and the C-terminal end of the MTase domain (Supplementary Fig. 5a). As a result of these domain arrangements, the GRF and CCHC domains both position one end to flank the catalytic site of the MTase domain, while extending the other end away from the MTase domain, each adopting a claw-like conformation roofting the catalytic site (Fig. 1b).

Structural homology search using the Dali server reveals that the GRF domain of ZCCHC4 is well aligned with the corresponding domain of APE2 (apurinic/apyrimidinic endonuclease 2) nuclease (Supplementary Fig. 5e); among the most conserved sites are those involved in the interactions of APE2 with single-stranded DNAs for 3′–5′ exonuclease processing. The CCHC domain of ZCCHC4 bears a remote homology with the CCHC motif of the DHHC (Asp–His–His–Cys) palmitoyltransferases (Supplementary Fig. 5f), which harbors a catalytic center for protein palmitoylation. On the other hand, there is no structural homologue identified for the C2H2 domain.
The MTase-flanking domains are required for ZCCHC4 activity. The close association between MTase and the flanking domains potentially creates an extended RNA-binding surface, implying a possible contribution of the flanking domains to the RNA binding of ZCCHC4 (Fig. 1b). To test the role of the MTase-flanking domains in ZCCHC4-mediated RNA methylation, we have measured the methylation activity of ZCCHC4, either intact or domain-truncated, on a 30-mer RNA substrate, derived from the Ade4220-containing segment of 28S rRNA (Cyt4199-Gua4228) (Fig. 1e). As expected, the ZCCHC424-464 core derived from the Ade4220-containing segment of 28S rRNA either intact or domain-truncated, on a 30-mer RNA substrate, led to a dramatic reduction of enzymatic activity, while deletion of the N-terminal GRF domain, either alone (ΔGRF) or together with the C2H2 domain (ΔGRF-C2H2), completely abolished the activity of ZCCHC4, indicating the critical requirement of these domains for ZCCHC4-mediated RNA methylation. Consistently, electrophoretic mobility shift assays (EMSA) revealed that, in comparison with wild-type ZCCHC424-464 (ΔGRF, ΔGRF-C2H2, and ΔCCHC truncations) all substantially impair the RNA-binding affinity of ZCCHC4 (Supplementary Fig. 5 g). Analysis of 1D 1H NMR spectra of these domain-truncated ZCCHC4 fragments indicate that they remain folded in solution (Supplementary Fig. 6), supporting a direct role of the flanking domains in mediating substrate binding of ZCCHC4.

ZCCHC4 MTase domain adopts an autoinhibitory conformation. The MTase domain of ZCCHC4 merely shares ~6–17% sequence identity with the corresponding domains of all other m6A RNA methyltransferases identified so far (Supplementary Fig. 1). Nevertheless, their structures exhibit a similar Rossmann fold, with the catalytic motif aligned next to the SAH-binding pocket (Fig. 2a–c; Supplementary Fig. 7a–d), suggestive of a conserved catalytic mechanism. Astonishingly, structural comparison of ZCCHC4 with METTL16, as well as other RNA methyltransferases, also reveals a striking difference in the active-site conformation: the D/N-P-F/W catalytic motifs of METTL16, METTL3, METTL5, and CAPAM are all readily accessible for RNA contacts (Fig. 2b; Supplementary Fig. 7a–d); in contrast, the corresponding DPPF motif in ZCCHC4 is shielded from solvent exposure by the loop connecting αK and β13 (hereafter referred to as regulatory loop), which wedges into the catalytic center for interaction with the active-site residues (Fig. 2a, c). Of particular note, residue D276 forms a bifurcated side-chain hydrogen bond with regulatory loop residue Y340 (Fig. 2c), which potentially occludes D276 from nucleophilic attack during the methylation reaction. Furthermore, the aromatic ring of residue F279, which presumably aligns the target base during the methylation reaction (Fig. 2b), stacks against the aliphatic chain of regulatory loop residue L345 (Fig. 2c). In addition, regulatory loop residue D341 reaches over to form a salt bridge with R202 on the SAH-binding pocket, which further supports the positioning of the regulatory loop to the active site. These observations suggest that ZCCHC4 adopts an autoinhibitory conformation.

To test the effect of the intramolecular interactions observed above on the methylation activity of ZCCHC4, we have selected the active site-contacting residues, including Y340, D341, and L345, of the regulatory loop for mutagenesis and enzymatic assay. In comparison with wild-type ZCCHC4, mutation of residues D341 and L345 each into alanine led to a 1.3- and 2.2-fold increase in RNA methylation (Fig. 2d), supporting the idea that the intramolecular interaction between the regulatory loop and the catalytic motif results in enzymatic inhibition of ZCCHC4. On the other hand, introduction of the Y340A mutation severely impairs the methylation activity of ZCCHC4, suggesting that residue Y340 might also play an additional role in the enzymatic catalysis. Indeed, structural alignment of ZCCHC4 and the RNA-bound METTL16 reveals that in METTL16, the segment that corresponds to the regulatory loop engages extensive contacts with the RNA loop (Fig. 2a, b), implying a similar role of the regulatory loop of ZCCHC4 in substrate recognition, and a conformational readjustment of the regulatory loop accompanying this process. Together, these data suggest that ZCCHC4 adopts an autoinhibitory conformation that may regulate its RNA substrate specificity.

Regulation of ZCCHC4 on SAM binding and RNA methylation. Structural analysis of ZCCHC4 further reveals an interaction between the regulatory loop and the cofactor loop (Fig. 2e): residues N342 and H343 from the cofactor loop interact with residues L174 and Y173 on the cofactor loop through hydrogen-bonding and ring-stacking interactions, respectively (Fig. 2e). These interactions presumably stabilize the closed conformation of the SAM-binding pocket in the RNA-free state of ZCCHC4, therefore implying a role of the regulatory loop in controlling the cofactor binding of ZCCHC4. To test this possibility, we measured the SAM-binding affinities of wild-type and H343A-mutated ZCCHC4 by Isothermal Titration Calorimetry (ITC). Wild-type ZCCHC binds to SAM with a dissociation constant (Kd) of 6.7 μM (Fig. 2f; Supplementary Fig. 7e), consistent with the extensive interactions between ZCCHC4 and SAH (Fig. 1c). In contrast, titration of the H343A mutant with SAM gave a Kd of 1.6 μM (Fig. 2f; Supplementary Fig. 7f), ~four fold stronger than the binding between wild-type ZCCHC4 and SAM, indicating that disruption of the intramolecular interaction between the regulatory loop and cofactor loop significantly increases the SAM-binding affinity of ZCCHC4.

To explore the role of the cofactor loop in the ZCCHC4-mediated RNA methylation, we modeled the target adenosine into the active site of ZCCHC4 based on structural alignment of the ZCCHC4 MTase domain and HP1 RNA-bound METTL16 (PDB 6DU4) (Supplementary Fig. 7g). The structural model indicates that the cofactor loop is in close proximity to the RNA substrate, with loop residue Y173 flanking the adenosine ring in a fashion similar to that of residues L75 and I218 of METTL16 (Fig. 2b; Supplementary Fig. 7g). Consistently, mutation of cofactor loop residue Y173 into alanine largely abolishes the methylation activity of ZCCHC4 (Fig. 2g). Likewise, mutations of the regulatory loop residues N342 and H343 each into alanine also severely impair the activity of ZCCHC4 (Fig. 2g), reinforcing the notion that the regulatory loop is engaged in the ZCCHC4-mediated methylation. Note that EMSA assays indicated that the mutations on the regulatory loop do not significantly affect the RNA-binding activity of ZCCHC4 (Supplementary Fig. 8a), suggesting a role of these residues in enzymatic catalysis, rather than substrate binding of ZCCHC4. Together, these studies suggest that the intramolecular interaction between the regulatory loop and the cofactor loop might not only restrict the SAM-binding activity of ZCCHC4 but also strengthen the autoinhibitory regulation mediated by the regulatory loop, thereby establishing a link between the RNA recognition of ZCCHC4 and its SAM-binding activity.

Sequence- and structure-recognition of 28S rRNA by ZCCHC4. Previous studies have indicated that ZCCHC4, with substrate preference toward the AAC RNA motif in vitro, is highly specific toward Ade4220 of 28S rRNA9,22, located on the helix H8128. Structural analysis of the cryo-EM structure of human 80S ribosome30 reveals that the Ade4220-residing
segment adopts a stem-loop structure, with nucleotides Cyt4211-Cyt4221 extending as a twisted loop on top of a six base pair (bp)-long stem (Fig. 3a). To determine whether the RNA secondary structure contributes to the substrate specificity of ZCCHC4, we compared the methylation activity of ZCCHC4 on the 30-mer RNA (Cyt4199-Gua4228), encompassing the stem-loop segment as well as a 5 nucleotide (nt)-long 5′ overhang, and a 12-mer RNA (Cyt4215-Gua4226) that was used by a previous study9, which presumably lacks of any secondary structure (Fig. 3b). ZCCHC4 shows a 104-fold higher activity on the 30-mer RNA over the 12-mer RNA (Fig. 3b), suggesting that the structural feature of the 30-mer RNA may influence the methylation efficiency of ZCCHC4. Next, we aimed to disrupt the RNA stem structure by mutating four stem nucleotides, Ura4208, Gua4225, Gua4226, and Ura4227, into Ade4208, Cyt4225, Cyt4226, and Ade4227, respectively. The methylation activity of ZCCHC4 shows a 50-fold reduction on the "broken stem" RNA than on the native 28S RNA (Fig. 3b). In contrast, swapping of four complementary bases on the stem that are either 2- or 3-bp away from the RNA loop, only led to 1.2- and 1.5-fold reduction of the enzymatic activity, respectively (Fig. 3b). Similarly, removal of the 5′ overhang or retaining a same-length 3′ overhang instead only led to a modest decrease of the methylation activity of ZCCHC4, suggesting that the stem, rather than the overhang sequence, is essential for the substrate specificity of ZCCHC4. Together, these observations suggest that ZCCHC4 recognizes a combined sequence and structural feature of 28S rRNA.

Mapping of the RNA-binding surface of ZCCHC4. Structural analysis of ZCCHC4, along with the aforementioned RNA binding and enzymatic analyses, argues for an important role of
the MTase-associated domains in ZCCHC4-mediated RNA methylation. Analysis of the electrostatic surface of ZCCHC4 further reveals that the GRF domain forms a hydrophobic patch next to the active site (Fig. 4a, b), which might serve as an extended RNA-binding surface. In support of this notion, residues F61, R68, F76, and W78 on the GRF domain are evolutionarily conserved, with F61 representing one of the signature residues in the GRF domain-defining motif (Supplementary Figs. 1, 5e). In fact, a previous study, based on NMR chemical shift perturbation analysis, has demonstrated that the counterparts of these residues in the GRF domain of APE2 mediate the binding of APE2 to the single-stranded DNA. On the opposite side of the catalytic center, an interdomain cleft formed between the MTase and CCHC domains presents another surface patch containing basic and hydrophobic residues (Fig. 4a, b), which potentially also contributes to the substrate binding of ZCCHC4.

To further analyze the RNA-binding sites of ZCCHC4, we have selected a number of residues from these surface patches of the GRF and CCHC domains, along with the residues surrounding the catalytic site of the MTase domain, for mutagenesis and enzymatic assays. Our results showed that mutation of residues on the surface patches of the GRF and MTase domains severely compromised the activity of ZCCHC4 (Fig. 4c). Mutations of the residues on the surface patch in the interdomain cleft between the MTase and CCHC domains also significantly reduced the activity of ZCCHC4, albeit to a lesser extent (Fig. 4c). Note that none of these mutations led to a substantial decrease in RNA-binding activity of ZCCHC4 (Supplementary Fig. 8b), highlighting the delicacy of RNA substrate binding in ZCCHC4-mediated methylation. The mutational effects of these residues are well in line with their sequence conservation (Supplementary Fig. 8c), indicating their important roles in ZCCHC4-mediated RNA methylation. In contrast, mutation of residue K419 located further away from the MTase domain, residue Q153 on the C2H2 domain, or residue R87 on the GRF domain, did not appreciably affect the enzymatic activity of ZCCHC4 (Fig. 4c), which sets a boundary for the potential RNA-binding surface of ZCCHC4. It is worth noting that, out of the five surface mutations introduced for crystallization, sites K167 and K168 both fall into this RNA-binding surface of ZCCHC4, consistent with the observation for a large reduction of ZCCHC4 activity by these mutations (Supplementary Fig. 8d, e). Together, these mutational analyses provide a sketched view on the RNA-binding surface of ZCCHC4, spanning from the GRF domain to the CCHC domain.

**Discussion**

The m6A RNA methylation is increasingly appreciated as an important epitranscriptomic mechanism for regulation of the metabolism, trafficking and/or function of a diverse array of RNA molecules, which critically impacts on the behavior and phenotype of cells. To date, little is known about how different m6A writer enzymes target distinct RNA substrates for carrying out their respective roles in RNA modulation. Through structural, biochemical, and enzymatic characterization of ZCCHC4, a recently reported RNA m6A writer, this study reveals the molecular basis for the ZCCHC4-mediated m6A modification of 28S rRNA. Importantly, ZCCHC4 forms a multidomain RNA-binding platform, permitting its specific recognition of a stem-loop feature inherent in the 28S RNA substrate. Furthermore, it reveals a loop segment that controls both the enzymatic catalysis of ZCCHC4 and its SAM-binding activity, providing a potential mechanism by which ZCCHC4 achieves substrate specificity. This study therefore illustrates a previously unappreciated dynamic interplay between the RNA substrate and its m6A writer, with important implication in the functional regulation of ribosome assembly, translation, and cell proliferation.
Uniquely among all the m6A RNA methyltransferases identified so far, ZCCHC4 manifests an autoinhibitory conformation, with the regulatory loop occluding the catalytic site from RNA binding. Our structural modeling analysis, along with enzymatic assays, demonstrated that the regulatory loop may serve as a key element that couples RNA substrate recognition with the conformational state of ZCCHC4. Although the functional implication of this intramolecular regulation remains to be determined, it is conceivable that the interaction between the regulatory loop and the 28S rRNA leads to conformational rearrangement of ZCCHC4, thereby switching ZCCHC4 from an inactive state to an enzymatically active state (Fig. 4d). The other important observation is that the SAH-binding pocket adopts a fully closed conformation, which is in part strengthened by the intramolecular interaction between the regulatory loop and cofactor loop. It is therefore likely that the RNA substrate binding-triggered conformational change of the regulatory loop may also lead to destabilization of this intramolecular interaction, which consequently enhances the SAM-binding affinity of ZCCHC4. Together, these intramolecular interactions of the regulatory loop likely provide a gating mechanism for the RNA substrate and cofactor binding of ZCCHC4, which may contribute to its substrate specificity. This regulatory mechanism is reminiscent of mammalian DNA methyltransferase 1 (DNMT1)-mediated DNA methylation, in which an interdomain linker of DNMT1 mediates an autoinhibitory conformation to control its substrate specificity. It is worth mentioning that an autoinhibitory conformation was also previously observed for METTL16, but likely in a different functional context: the RNA binding of METTL16 triggers a conformational re-organization of the SAM-binding pocket (Supplementary Fig. 8f), which in turn reduces the activity of METTL16. Likewise, binding of SAM or SAH to METTL3 also induces a conformational change of a loop segment at the cofactor-binding site, namely gate loop, resulting in binding-induced folding of the SAM-binding pocket (Supplementary Fig. 8g). These observations suggest that cofactor binding-coupled conformational changes may represent a common mechanism for various m6A writers.

Previous studies have demonstrated that ZCCHC4 shows high specificity toward 28S rRNA: it specifically methylates Ade420 of 28S rRNA, but not Ade1832 of 18S rRNA, the other methylation site of rRNA, even though both sites are embedded in an AAC motif. Through nucleotide complementation analysis, this study demonstrates that the stem-loop structure critically influences the methylation activity of ZCCHC4. In this regard, structural analysis of 18S rRNA reveals a discrete structural environment for...
site 1832, which forms a base pair with Cyt1732 at the base of helix 44 (Supplementary Fig. 8h), providing an explanation for the stem-helix 44 (Supplementary Fig. 8h), suggesting aanking-module-directed regulation, as we herein show site 1832, which forms a base pair with Cyt1732 at the base of helix 44 (Supplementary Fig. 8h), providing an explanation for the stem-helix 44 (Supplementary Fig. 8h), suggesting aanking-module-directed regulation, as we herein show

Lastly, this study has demonstrated how various structural modules within ZCCHC4 act in synergy for mediating the binding and methylation of substrate. In particular, the GRF and CCHC modules, through direct association with the MTase domain, extend the substrate-binding surface, permitting a rather large RNA-binding platform to accommodate the substrate specificity. In fact, all the RNA m^A methyltransferases identified so far, except for METTL5, contain MTase-flanking domains, suggesting aflanking-module-directed regulation, as we herein show for ZCCHC4, as a widely adopted mechanism for m^A writers, enabling both binding affinity and substrate specificity. For instance, METTL3 harbors an N-terminal CCHC zinc finger domain that specifically recognizes the RNA substrate containing the GGAGCU motif, which is essential for the methylation activity of the METTL3–METTL14 complex. The detailed mechanism by which the MTase-associated domains of ZCCHC4 regulate its activity awaits further investigation.

Methods

Protein expression and purification. DNAs encoding human ZCCHC4, full-length or various fragments (ZCCHC4_24–513 residues 24–513; ZCCHC4_464–513 residues 24–464; ZCCHC4 residues 24–368; AGRF: residues 100–464; AGRF-C2H2: residues 159–464) were each inserted into a modified pRSF-Duet vector, preceded by an N-terminal hexahistidine (His<sub>6</sub>)-SUMO tag and a ubiquitin-like protease 1 (ULP1) cleavage site. To promote crystallization, five surface mutations (K55A, E56A, E57A, K167A, and K168A) were introduced onto the ZCCHC4_24–513 construct, which presumably would lead to surface entropy reduction. On the other hand, for biochemical and enzymatic characterizations, various point mutations were generated based on the wild-type ZCCHC4_24–513 construct. For protein expression, BL21(DE3) RIL cells harboring the expression plasmids were induced by addition of 0.4 mM isopropyl-β-D-thiogalactoside (IPTG) when the cell density reached optical density at 600 nm (A<sub>600</sub>) of 0.8, and continued to grow at 15 °C overnight. The cells were harvested and lysed in buffer containing 50 mM Tris–HCl (pH 8.0), 1 M NaCl, and 25 mM imidazole, 10% glycerol, 10 mM MgCl<sub>2</sub>, 1 mM TCEP (Tris (2-carboxyethyl) phosphine hydrochloride) (pH 7.0) using the sitting drop vapor-diffusion method. Subsequently, the fusion proteins were purified through a nickel column, followed by removal of His<sub>6</sub>-SUMO tag by ULP1 cleavage, ion-exchange chromatography and size-exclusion chromatography. The purified protein samples were concentrated in 20 mM Tris–HCl (pH 7.5), 10 mM NaCl, 5% glycerol, and 5 mM DTT, and stored at −80 °C freezer before use.

Crystallographic analysis. For crystallization of the ZCCHC4–SAH complex, 10 mg/mL ZCCHC4 was mixed with SAH in a molar ratio of 1:5, before incubation with 0.03 M citric acid, 0.07 M Bis-Tris, 12% PEG3350, 10% glycerol, and 5 mM TCEP (Tris (2-carboxyethyl) phosphine hydrochloride) (pH 6.7), 1–3 mM Tris–HCl, 150 mM NaCl, and 10% D2O. NMR spectra were collected on a Bruker Advance 700 MHz NMR spectrometer equipped with a TXI probe at 25 °C and processed using the Bruker TopSpin software.

Received: 26 August 2019; Accepted: 9 October 2019; Published online: 06 November 2019

Crystallographic data collection. For ZCCHC4 crystals, the crystals were soaked in the well solution containing 25 nM ZCCHC424–513, wild-type or mutants, 0.5 M sodium phosphate (pH 7.0), 7 mM MgCl<sub>2</sub>, 5% glycerol, 100 µg/mL BSA, and 10 µM zinc acetate. The reaction mixture was incubated at 37 °C for 15–20 min, before quenched by 2 mM cold SAM. Ten µL of the reaction mixture was applied onto DEAE filtermat (Perkin Elmer), sequentially washed with 0.2 M ammonium bicarbonate (pH 8.2) twice, water once, and ethanone. The filter paper was then air dried and soaked in ScintiVial cocktail (Thermo Fisher). The activity was measured by Beckman LS6500 scintillation counter. All the experiments were performed in biological replicates and repeated with consistent results.

ITC measurements. ITC measurements were performed using a MicroCal iTC200 instrument (GE Healthcare). To measure the bindings between the ZCCHC4_24–513, WT or H343A mutant, and SAM, 0.1 mM ZCCHC4_24–513 protein sample was dialyzed against buffer (20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 1 mM TCEP) overnight. 1 mM SAM compound (Sigma) was dissolved in the same buffer. Titration of SAM in the syringe over ZCCHC4_24–513 samples was carried out at 5 °C. The time between each injection was set at 180 s. All data were processed with the MicroCal Origin software and fitted with the single-site binding mode. The Kd values and error estimates were derived from the duplicated experiments.

NMR experiments. For 1H NMR experiments, 0.05 mM purified mutant ZCCHC4 proteins were dissolved in 250 µL buffer containing 50 mM sodium phosphate (pH 6.7), 1–3 mM Tris–HCl, 150 mM NaCl, and 10% D2O. NMR spectra were collected on a Bruker Advance 700 MHz NMR spectrometer equipped with a TXI probe at 25 °C and processed using the Bruker TopSpin software.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

Coordinates and structure factors for ZCCHC4–SAH complex have been deposited in the Protein Data Bank under accession code 6UCA. The PDB accession codes 6UG0, 6K7M, 6K7W, 5LAD, 5LH2, 6BYR, 6DU4, 6W92, 6BML, and 6H2U were used in this study. All other data are available from the corresponding authors upon reasonable request. The source data underlying Figs. 1e, 2d, 3b, and 4c, and Supplementary Figs. 5g and 8a, b, e are provided as a Source Data file.

References

1. Frye, M., Harada, B. T., Behm, M. & He, C. RNA modifications modulate gene expression during development. Science 361, 1336–1349 (2018).
2. Fu, Y., Dominissini, D., Rechavi, G. & He, C. Gene expression regulation mediated through reversible m(6)A RNA methylation. Nat. Rev. Genet. 15, 293–306 (2014).
3. Sergiev, P. V., Aleksashin, N. A., Chugunova, A. A., Polikanov, Y. S. & Dantsinov, O. A. Structural and evolutionary insights into ribosomal RNA methylation. Nat. Chem. Biol. 14, 226–235 (2018).
4. Podka-Przybylska, D., Donnar, V., A. & Pfeuffer, M. J. The 3D RNA modification maps database: with interactive tools for ribosome analysis. Nucleic Acids Res. 36, D178–D183 (2008).
5. Epstein, P., Reddy, R., Henning, D. & Busch, H. The nucleotide sequence of nuclear U6 (4.7 S) RNA. J. Biol. Chem. 255, 8901–8906 (1980).
6. Harada, F., Kato, N. & Nishimura, S. The nucleotide sequence of nuclear 4.8S RNA of mouse cells. Biochemical Biophysical Res. Commun. 95, 1332–1340 (1980).
7. Meyer, K. D. & Jeffery, S. R. Rethinking m(6)A readers, writers, and erasers. Annu. Rev. Cell Dev. Biol. 33, 319–342 (2017).
8. Roundtree, I. A., Evans, M. E., Pan, T. & He, C. Dynamic RNA modifications regulated with expression regulation. Cell 169, 1187–1200 (2017).
9. Ma, H. et al. N(6)-Methyladenosine methyltransferase ZCCHC4 mediates ribosomal RNA methylation. Nat. Chem. Biol. 15, 88–94 (2019).
10. Liu, J. et al. m(6)A mRNA methylation regulates AKT activity to promote the proliferation and tumorigenicity of endometrial cancer. Nat. Cell Biol. 20, 1074–1083 (2018).
11. Liu, J. et al. METTL3–METTL14 complex mediates mammalian nuclear RNA N6-adenosine methylation. Nat. Chem. Biol. 10, 93–95 (2014).
12. Bokar, J. A., Shambaugh, M. E., Polayes, D., Matera, A. G. & Rottman, F. M. Purification and cDNA cloning of the AdoMet-binding subunit of the human m(6)A-adenosine-methyltransferase. RNA 3, 1233–1247 (1997).
13. Wang, X. et al. Structural basis of N(6)-adenosine methylation by the METTL3-METTL14 complex. Nature 534, 575–578 (2016).
14. Wang, P., Doxtader, K. A. & Nam, Y. Structural basis for cooperative function of Mettl3 and Mettl14 methyltransferases. Mol. Cell 63, 306–317 (2016).
15. Sledz, P. & Jinek, M. Structural insights into the molecular mechanism of the m(6)A writer complex. eLife 5, e18434 (2016).
16. Dommisini, D. et al. Topology of the human and mouse m6A RNA methylomes revealed by m6A-seq. Nature 485, 201–206 (2012).
17. Meyer, K. D. et al. Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons. Cell 149, 1635–1646 (2012).
18. Linder, B. et al. Single-nucleotide-resolution mapping of m6A and m6Am throughout the transcriptome. Nat. Methods 12, 767–772 (2015).
19. Bendtzen, K. E. et al. The U6 snRNA m(6)A methyltransferase METTL16 regulates SAM synthetase intron retention. Cell 169, 824–835 (2017). e814.
20. Doxtader, K. A. et al. Structural basis for regulation of METTL16, an S-acyltransferase. Nature 503, 2132 (2004).
21. Mendel, M. et al. Methylation of structured RNA by the m(6)A writer METTL16 is essential for mouse embryonic development. Mol. Cell 71, 986–1000 (2018). e1011.
22. Akishika, S. et al. Cap-specific terminal N (6)-methylation of RNA by an RNA polymerase II-associated methyltransferase. Science 363, eaav0080 (2019).
23. van Tran, N. et al. The human 18S rRNA m6A methyltransferase METTL5 is stabilized by TRMT112. Nucleic Acids Res. 15, 7719–7733 (2019).
24. Gazin, C., Wajapeyee, N., Gobeil, S., Virbasius, C. M. & Green, M. R. An elaborate pathway required for Ras-mediated epigenetic silencing. Nature 449, 1073–1077 (2007).
25. Holm, L. & Rosenstrøm, P. Dalí server: conservation mapping in 3D. Nucleic Acids Res. 38, W545–W549 (2010).
26. Wallace, B. D. et al. APE2-ZF-GRF facilitates 3'-5' resection of DNA damage following oxidative stress. Proc. Natl Acad. Sci. USA 114, 304–309 (2017).
27. Rana, M. S. et al. Fatty acyl recognition and transfer by an integral membrane S-acyltransferase. Science 359, eaao6326 (2018).
28. Anger, A. M. et al. Structures of the human and Drosophila 80S ribosome. Nature 497, 80–85 (2013).
29. Natchiar, S. K., Myasnikov, A. G., Kratzat, H., Hazemann, I. & Klaholz, B. P. Visualization of chemical modifications in the human 80S ribosome structure. Nature 551, 472–477 (2017).
30. Khatter, H., Myasnikov, A. G., Natchiar, S. K. & Klaholz, B. P. Structure of the human 80S ribosome. Nature 520, 640–645 (2015).
31. Song, J., Rechkoibl, O., Bestor, T. H. & Patel, D. J. Structure of DNMT1-DNA complex reveals a role for autoinhibition in maintenance DNA methylation. Science 331, 1036–1040 (2011).
32. Huang, J. et al. Solution structure of the RNA recognition domain of METTL3-METTL14 N(6)-methyladenosine methyltransferase. Protein Cell 10, 272–284 (2019).
33. Minor, W., Cymborowski, M., Ołtyniak, Z. & Chruszcz, M. HKL3000: the integration of data reduction and structure solution from diffraction images to an initial model in minutes. Acta Crystallogr. Sect. D., Biol. Crystallogr. 62, 859–866 (2006).
34. Pannu, N. S. et al. Recent advances in the CRANK software suite for experimental phasing. Acta Crystallogr. Sect. D., Biol. Crystallogr. 67, 331–337 (2011).
35. Emsley, P. & Cowtan, K. Coot: model-building tools for molecular graphics. Acta Crystallogr. D. Biol. Crystallogr. 60, 2126–2132 (2004).
36. Adams, P. D. et al. PHENIX: building new software for automated crystallographic structure determination. Acta Crystallogr. D Biol. Crystallogr. 58, 1948–1954 (2002).

Acknowledgements

We would like to thank staff members at the Advanced Light Source (ALS), Lawrence Berkeley National Laboratory for access to X-ray beamlines. The Advanced Light Source is supported by the Director, Office of Science, Office of Basic Energy Sciences, of the U.S. Department of Energy under Contract No. DE-AC02-05CH11231. This work was supported by NIH grants (1R35GM119721 to J.S.; R01CA215284 and R01CA211336 to G.G.W.). G.G.W. is an American Cancer Society (ACS) Research Scholar and a Leukemia & Lymphoma Society (LLS) Scholar.

Author contributions

W.R., J.L. and J.S. designed and performed the enzymatic and structural studies. M.H. and L.G. assisted the protein purification and enzymatic assay. D.L. and G.G.W. provided construct support. J.S. conceived the project and prepared the manuscript with input from all authors.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at https://doi.org/10.1038/s41467-019-12923-x.

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Peer review information Nature Communications thanks the anonymous reviewers for their contribution to the peer review of this work.

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