Enzymatic Characterization of In Vitro Activity of RNA Methyltransferase PCIF1 on DNA

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ABSTRACT: PCIF1 and FTO are a pair of human mRNA cap-specific modification enzymes that have opposing activities. PCIF1 adds a methyl group to the N6-position of 2′O-methyladenosine (Am), generating N6, 2′O-dimethyladenosine (m6Am), when Am is the cap-proximal nucleotide. FTO removes the N6-methyl group from m6Am. In addition, FTO has a demethylase activity on a broad spectrum of various RNA substrates, as well as on DNA N6-methyldeoxyadenosine (m6dA). While the existence of m6dA in mammalian DNA remains controversial, we show here that PCIF1 has significant methylation activity on single stranded DNA deoxyadenosine, double stranded RNA/DNA hybrids, and double stranded DNA, though with lower catalytic efficiency than that on its preferred RNA substrate. PCIF1 has activities in the order ssRNA > RNA/DNA hybrid > ssDNA > dsDNA. We discuss the implications of PCIF1 generation, and FTO removal, of DNA adenine methylation.

INTRODUCTION

While detected immunochemically as early as 1983,1 the existence of N6-methyl deoxyadenosine (m6dA) in mammalian DNA remains a controversial topic2−7 ever since it was reported again in 2016.8 One key issue is uncertainty regarding the methyltransferases (MTases) responsible for generating m6dA in mammalian DNA, suggesting the inclusion of N6AMT1/HemK2/KMT9 (henceforth HemK2), MettL4, and MettL3-MettL14 heterodimers, as well as regarding the enzymes removing the methyl group from m6dA (demethylases). We note that many nucleic acid-modifying enzymes modify both DNA and RNA (ref 9 and references therein), including members of the AlkB family (see below) involved in the direct reversal of alkylation damage to DNA and RNA10 and members of the Apcobec family of cytidine deaminases.11 Tet2, one of the ten-eleven translocation proteins initially discovered as DNA 5-methylcytosine (5mC) dioxygenases,11 also mediates oxidation of 5mC in mRNA.13,14 These considerations prompted us to investigate whether known human RNA m6A MTases also possess methyl transfer activity on DNA adenine.

Human HemK2 forms a heterodimer with Trm112 and was thought to be a DNA m6A MTase (N6AMT1 activity) based on sequence comparisons15 but is actually a protein MTase active on glutamine (HemK2 activity) and lysine (KMT9 activity)16−19 and has no N6AMT1 activity. Murine MettL4 is responsible for DNA m6A deposition in gene elements associated with transcriptional silencing.20 Intriguingly, recombinant human MettL4 purified from Escherichia coli has RNA MTase activity on U2 snRNA that adds a methyl group to the N6-position of 2′O-methylxyloxyadenosine (Am), generating N6, 2′O-dimethylxyloxyadenosine (m6Am).22,23 [We note that, in searching of the Protein Data Bank (PDB), a homolog of Arabidopsis thaliana MettL4 has been crystallized in complex with the mononucleotide Am (which could occur in the context of RNA) (PDB 7CV6) and in complex with dsDNA (PDB 7DPE).] While MettL3-MettL14 had been characterized extensively as generating internal m6A in mRNA (ref 24 and references therein), human MettL3-MettL14 is also active in vitro on single-stranded DNA, as well as on double-stranded DNA containing short lesion-associated bubbles.25−27 From the opposing demethylation side, nine mammalian AlkB homologs exist (ALKBH1−8, and FTO), but only a subset functions as DNA/RNA (de)modification enzymes.10 The AlkB family members are Fe(II)- and α-ketoglutarate-dependent dioxygenases that direct reversal of DNA/RNA damage, removing alkyl adducts from nucleobases via oxidative dealkylation, including in some cases direct demethylation.28−31 Focusing on the m6A substrates (Table 1), ALKBH1 is active on DNA containing unpaired lesions.32−34

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In a study using HEK293T cells, DNA m6dA deposition and removal are catalyzed by the METTL4 MTase and ALKBH4 demethylase, respectively, corresponding with transcriptional silencing. An in vitro study found that ALKBH5 and FTO do not discriminate between RNA and DNA substrates.

FTO, a fat mass and obesity-associated protein, was initially characterized as a repair enzyme active on 3-methyldeoxythymidine (m3dT) in single-stranded (ss) DNA, or on 3-methyloxuracil (m3U) in ssRNA. Later, five additional FTO activities were identified: (a) a demethylase that removes the methyl group from internal m6A residues in mRNA, (b) a cap-dependent demethylase, which preferentially removes the N6 methyl group of m6Am, (c) a demethylase active on internal m6A and m6Am in small nuclear RNA (snRNA) and a demethylase of (d) 1-methyladenosine (m1A) in tRNA and of (e) m6dA in ssDNA and dsDNA lesions. Among all the known FTO substrates, including multiple RNA species, structures have only been determined for human FTO and P2. FTO modulates mRNA splicing, is required for adipogenesis, and mediates stem-like properties in colorectal cancer cells. Among all the known FTO substrates, including multiple RNA species, structures have only been determined for human FTO and P2. FTO modulates mRNA splicing, is required for adipogenesis, and mediates stem-like properties in colorectal cancer cells.43

Although the demethylase activity of FTO on RNA cap-specific m6Am modification was characterized first, the opposing activity of the MTase PCIF1 was discovered soon after. PCIF1 adds a methyl group to the N6-position of 2′-O-methylated A, generating dimethylated m6Am, when Am is the cap-proximal nucleotide. FTO removes the N6-methyl group from m6Am. Since FTO has a broad spectrum of substrates, including the demethylase activity on DNA m6dA, we asked whether PCIF1 is active on DNA substrates in vitro.

### RESULTS

Reanalysis of Existing Structures for FTO and PCIF1.

Human FTO and PCIF1 have both been structurally characterized. FTO contains an N-terminal double-strand β-helix fold, conserved among almost all Fe(II) and α-ketoglutarate (αKG) dependent dioxygenases, and a C-terminal helical domain (Figure 1A). PCIF1 contains a small N-terminal WW domain for binding the polymerase II C-terminal domain (CTD) and a C-terminal region consisting of a helical domain and MTase domain, the latter having a canonical Rossmann fold containing a conserved catalytic motif for binding of SAM (Figure 1B). Because the WW

| Table 1. Examples of Mammalian MTases and Demethylases Tested for DNA m6dA Activity |
|---------------------------------|---------------------------------|---------------------------------|
| **MTases** | **adenine in DNA** | **references** |
| MetTL3−14 | GGGCT | Woodcock et al.25 |
| MetTL4 | NNAGNN (nuclear) | Kweon et al.20 |
| CTHATC (mitochondrial) | | Hao et al.21 |
| PCIF1 | A | this study |
| demethylases | FTO | m6dA | Jia et al.38 |
| | | | Zou et al.35 |
| | | | Zhang et al.31 |
| ALKBH1 | m6dA in dsDNA lesions | Li et al.32 |
| | | | Zhang et al.33 |
| | | | Tian et al.34 |
| ALKBH4 | m6dA | Kweon et al.20 |
| ALKBH5 | m6dA | Zou et al.35 |

The oligonucleotides were synthesized by Integrated DNA Technologies (IDT). N6-methyladenosine (D9D9W) Rabitz mAb #56593 was purchased from Cell Signaling Technology. MetTL3 inhibitors, two racemates of UZH1(R) and UZH1-(S), and STM2457 were purchased from MCE (MedChemExpress).
The overall topological arrangement of FTO and PCIF1 are remarkably similar: a catalytic domain (with opposite activities) associated with a helical domain comprising six helices. Potential functions of the helical domains have been suggested, including protein stability and mediating interactions with the catalytic domain in FTO, or binding the RNA substrate in PCIF1. Both helical domains have additional basic- and acidic-rich surfaces (Figure 1C,D), which could mediate interactions with factors common to both proteins including chromatin remodeling and DNA repair (see Discussion).

The active site of FTO could accommodate both m^6dA of ssDNA and mononucleotide m^3dT (which could occur in the context of DNA) (Figure 1E,F). The (removable) methyl groups of m^6dA and m^3dT occupy similar positions relative to the metal ion and αKG, implying that the same oxidative reaction would occur to both methyl groups upon the binding of dioxygen. However, the conformations of the deoxyribose differ markedly between the two deoxynucleotides (Figure 1G). Thus, the current structural information is insufficient to explain the preference of FTO for either mRNA caps or for RNA 2'O-methylation.

**PCIF1 Activity on DNA Substrates.** Inspired by the dual activity of FTO (on RNA and DNA), as well as by the overall similarity of FTO and PCIF1 architecture, we asked whether PCIF1 is also active on DNA. Using the recombinant human PCIF1 (Figure 1H), we first tested PCIF1 activity on an ssDNA sequence (oligo D2 in Figure 2A). The sequence of the D2 DNA oligo corresponds to that of the 18-nt uncapped RNA oligo R2 that contains a S'-adenosine and additional internal Ade residues, which was used in our recent study of
PCIF1 activity on uncapped RNAs. For the RNA R2 oligo, PCIF1 showed high activity, resulting in fully N6-methylated 5′A, with a second methylation site at the internal adenosine, three or four residues away from the 5′ end (AGCGAA). In the case of the DNA D2 oligo, PCIF1 exhibited substantial activity, at about half the level, after a saturated overnight reaction, which was observed on the corresponding RNA (Figure 2A). The 5′ adenine is not required for PCIF1 activity on ssDNA, as the complimentary strand D3 yields similar activity to that of D2 (Figure 2B).

However, the double-stranded substrates, either dsDNA (D2/D3) or in a RNA/DNA hybrid (R2/D3) or DNA/DNA hybrid (D2/D3), exhibit significantly reduced activity compared to the corresponding single-stranded substrates (Figure 2B). We estimated that the rate of PCIF1 activity on R2/D3 is reduced by more than 13-fold comparing to that on R2 alone: the similar activities are reached by 2 h on R2/D3 and in merely 10 min on R2, under the same reaction conditions (Figure 2C). To ensure that the remaining activity is not due to unpaired R2 (in which PCIF1 has much higher activity), we added excess D3 to assure that R2 is fully annealed. Thus, the activity observed on R2/D3, which is in between R2 and D3 (R2 < R2/D3 < D3), is not from the single-stranded D3.

To further evaluate the weak activity of PCIF1 on dsDNA, we included three controls. First, we prepared dsDNA with the adenines contained only in one strand and paired it with no-A complimentary strand excessively (Figure 2D). Second, because the E. coli strain used here expresses Dam MTase, which modifies GATC sites, we confirmed that there was no observable activity of Dam on non-GATC dsDNA (Figure 2E). Third, a catalytically dead mutant of PCIF1 (N553APF to A553APF) is inactive (Figure 2F).

Figure 2. PCIF1 activity on DNA. (A) Comparison of PCIF1 activity on oligo 2 of corresponding RNA and DNA sequences under the saturated overnight reactions ([E] = 5 μM, [S] = 10 μM and [SAM] = 100 μM) (N = 6). The underlined As in R2 oligo indicates the RNA methylation sites confirmed by mass spectrometry. The CATT site in D3 oligo is used in Figure 3, and the GGACT site in D4 oligo is used in Figure 4. (B) Comparison of PCIF1 activities on single-stranded oligos (D2 and D3) and double-stranded duplexes (R2/D3 and D2/D3) (N = 4). (C) A 15% native gel (top) showing substrates used in the assays (bottom) (N = 2). (D) PCIF1 activity on dsDNA (D2-1/D3-1) with an increased complementary strand (D3-1) that contains no As (N = 3). (E) EcoDam activity (top) and PCIF1 activity (bottom) on double-stranded GATC (N = 3). (F) The PCIF1 N553A mutant is inactive (N = 3). For panels (B–F), assay conditions were [E] = 1 μM, [S] = 10 μM, [SAM] = 40 μM, pH 8.0, T = 37 °C.
though eventually, the same level of methylation was reached in the saturated overnight reaction (Figure 3C). As a control, no activity was observed on a DNA oligo without any adenines (Figure 3C). Thus, PCIF1 has significant in vitro methylation activity on adenine in ssDNA.

Finally, we tested the effects of varying the nucleotides immediately preceding and following the target adenine. By varying the 5′ or 3′ nucleotides with all four possibilities (X = G, A, T, and C), we found that PCIF1 has roughly equal activity on all substitutions of CAXT or XATT (Figure 3C). This observation agrees with FTO showing no specificity for the flanking sequence around the target methyladenine.41 We note that PCIF1 had slightly higher activity on GATT at an early time point (4 h), but methylation reached similar levels after saturated overnight reactions (Figure 3C).

Comparison of PCIF1 and MettL3−14 Activities on DNA. To compare the PCIF1 DNA methylating activities with those of other MTases (HemK2-Trm112 and MettL3-MettL14 heterodimers), using the same DNA substrates, we prepared DNA oligo D4, containing GGACT, the consensus sequence for MettL3-MettL14. [At the time of this experiment, we did not have recombinant MettL4 for comparison.] First, we observed high activity of MettL3-MettL14 on the ssDNA (K_{app} = 130 h^−1; Figure 4A), which is the same as or even better than the corresponding RNA containing GGACU (K_{app} = 120 h^−1).56 Second, as expected, we observed no activity of HemK2-Trm112 on DNA (Figure 4B). Observations on both the positive control (MettL3-MettL14)25−27 and the negative control (HemK2-Trm112)17−19 agree with previous publications. Third, we observed weak activity of PCIF1 on the DNA, which is marginal in comparison to MettL3-MettL14 on the same DNA molecule. In summary, MettL3-MettL14 has high DNA adenine methylation activity, at least in vitro. As a control, the DNA methylation activity of MettL3-MettL14 was inhibited by known selective MettL3−MettL14 inhibitors51,52 (Figure 4C), which had no effect on PCIF1 activity (Figure 4D).

Methylation Activity Detection by Antibody. Antimethyladenosine antibodies have been used to detect adenine modifications in mammalian DNA, and the results are controversial, partly because the antibodies cross-react with both m^6dA and m^6A.3 Here, we characterized one commercially available antibody (D9D9W from Cell Signaling Technology), using our in vitro enzyme reaction products (Figure 5A). First, the antibody detects but does not distinguish quantitatively an internal m^6dA in synthesized DNA from m^6A in RNA (lanes 1 and 9 of Figure 5A). However, it does not recognize 2′O-methylation in RNA, as 5′Am (lane 2 of Figure 5A), at an internal position (lane 11 of Figure 5B), or in a capped structure (lane 13 of Figure 5B). Thus, the antibody-based dot blots agree with our enzymatic assays.

Third, we asked whether the antibody can distinguish monomethylated m^6A and dimethylated m^6Am, located either

![Figure 3.](https://example.com/figure3.jpg) PCIF1 is a sequence-independent MTase on internal A. (A) PCIF1 activities on RNA (CAUU) and DNA (CATT) as a function of substrate concentration (N = 4). (B) PCIF1 activity on CATT as a function of temperature (N = 4). (C) PCIF1 activities on CAXT and XATT (X = G, A, T, C). (E] = 1 μM, [S] = 10 μM and [SAM] = 40 μM) (N = 4).

![Figure 4.](https://example.com/figure4.jpg) Comparison of PCIF1 and MettL3-MettL14 activities on the same DNA substrate. (A, B) Comparison of activities of three enzymes (MettL3-MettL14, PCIF1, and HemK2-Trm112). Panel (B) is an enlarged portion of panel A in order to visualize the low activity of PCIF1. (C) Inhibition of MettL3-MettL14. The compounds tested were known inhibitors of MettL3, and included sinefungin, 5′-deoxy-5′-methylthioadenosine (MTA), the two racemates UZH1(R) and UZH1(S),51 and STM2457.52 (D) The MettL3-selective inhibitors had no effect on PCIF1. Compounds were used at a concentration of [I] = 50 μM. (Figure 5A). First, the antibody detects but does not distinguish quantitatively an internal m^6dA in synthesized DNA from m^6A in RNA (lanes 1 and 9 of Figure 5A). However, it does not recognize 2′O-methylation in RNA, as 5′Am (lane 2 of Figure 5A), at an internal position (lane 11 of Figure 5B), or in a capped structure (lane 13 of Figure 5B). Second, PCIF1-catalyzed RNA modification is more intensive than its DNA modification (comparing lanes 3 and 4 to lane 5 in Figure 5A). Among the three enzymes tested for DNA methylation, MettL3-MettL14 has higher activity than PCIF1 (comparing lanes 6 and 7), while HemK2-Trm112 has no detectable activity (lane 8). Thus, the antibody-based dot blots agree with our enzymatic assays.
cap-proximally or internally. The m₆Am can be cap-proximal (driven by PCIF1) or internal (in U2 snRNA added by MettL4). However, the transcriptome-wide distribution of m₆Am and m₆A sites do not overlap (initially observed in 2019 and reaffirmed in 2021), suggesting distinct functions between the two modifications. In any case, the tested antibody does not distinguish between these two RNA modifications. Shown in lanes 14 and 12 of Figure 5B, the antibody recognizes PCIF1-driven m₆Am as the first nucleotide after the m₇G cap as well as at an internal position.

### DISCUSSION

Here, we analyzed existing structures for human FTO and PCIF1, which illustrated a general arrangement of a well-characterized catalytic domain (methylase or dioxygenase) in association with a less-characterized helical domain. In sum, FTO and PCIF1 have opposite enzymatic activities on cap-specific m₆Am, on internal adenosines (m₆Ao rm m₆Am) in the context of RNA, and on DNA adenosine and DNA double-strand break (DSB) repair enzyme XRCC4, the E3 ubiquitin-protein ligase RNF8 coordinating the repair of DNA lesions in specific chromatin topologies, the tyrosyl DNA phosphodiesterase 2 (TDP2) potentially involved in topoisomerase-mediated DNA damage, or PHRF1, a protein that contains a plant homeodomain (PHD) and a RING domain, possibly promoting genome integrity by modulating non-homologous end-joining. Covelo-Molares et al. noted that the identification of proteins involved in DNA replication and repair was the most striking result, with the most enriched and significant FTO interactor being the ssDNA-binding protein RADX.

Indeed, we have recently proposed a role for DNA m₆dA in DNA damage repair, which involves m₆dA in ssDNA generated transiently, and in preventing or reducing incorporation of 8-oxo-2′-deoxyguanosine during the gap-filling DNA synthesis. We note that the initial observation in embryonic stem cells identified m₆dA enrichment in H2A.X deposition regions, where H2A.X is a histone variant typically associated with DSBs. m₆dA modification is also elevated in glioblastoma (a disease partially associated with the cumulative effects of high-dose exposure to ionizing radiation, or to chemical carcinogens), but not in normal adult tissues or mammalian cells. Recently, the same laboratory that made the initial observation has reported that m₆dA increases during the development of mouse trophoblast stem cells (which eventually give rise to the placenta), specifically at regions of stress-induced DNA double helix destabilization (SIDD). ssDNA that persists in that state is one of the possible SIDD aftereffects.

Although there is no direct evidence yet for PCIF1 involvement in DNA repair, there is growing evidence for a functional link between m₆A-mediated activities of writers (MTases), readers (YTH domain proteins) and erasers (demethylases), and DNA damage repair after UV irradiation. The biochemically characterized candidates for the m₆dA writer (MettL3-MettL14), reader (YTHDC1), and eraser (ALKBH1) all prefer locally unpaired DNA substrates in vitro. Of these three proteins, the activities of YTHDC1 and ALKBH1 are independent of sequence context aside from the methylated adenine itself. In contrast, MettL3-MettL14 is an adenine methylase complex, long studied for its activity on RNA, where it targets the sequence RRACH (R is purine and H is not G). Thus, while PCIF1 is the mRNA cap-proximal adenine methylase, this enzyme might also be a versatile methyltransferase for modifying internal adenine in RNA and DNA.

### ASSOCIATED CONTENT

**Accession Codes**

PCIF1 (NCBI: NP_071387.1; PDB: 2JX8 and 6IRW); MettL3 (NCBI: NP_062826.2); MettL14 (NCBI: NP_071389.1).
NP_066012.1); HemK2 (NCBI: NP_037372.4); Trm112 (NCBI: NP_057488.1); FTO (PDB: 5ZMD and 3LFM).

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D.Y. and J.Z. contribute equally to this work.

Author Contributions
D.Y. and J.Z. performed protein purification and enzymatic assays. D.Y. and Q.C. performed the antibody dot blot assay. T.W. participated in discussion and provided the antibody. R.M.B. participated in discussions and assisted in preparing the manuscript. X.Z and X.C organized and designed the scope of the study.

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■ ABBREVIATIONS

m2dA = N6-methyl deoxyadenosine in DNA; m6A = N6-methyladenosine in RNA; m6Am = N6, 2′O-dimethyladenosine in RNA; A6m = 2′O-methyladenosine in RNA

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