Convenient expression, purification and quantitative liquid chromatography-tandem mass spectrometry-based analysis of TET2 5-methylcytosine demethylase

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Abstract

5-Methylcytosine within CpG islands in DNA plays a crucial role in epigenetic transcriptional regulation during metazoan development. Recently, it has been established that the Ten-Eleven Translocation (TET) family, Fe(II)- and 2-oxoglutarate (2OG/ α KG)-dependent oxygenases initiate 5-methylcytosine demethylation by iterative oxidation reactions. Mutations in the TET2 gene are frequently detected in patients with myeloid malignancies. Here, we describe the cloning of untagged human TET2 demethylase using Gateway technology and its efficient expression in *E. coli*. The untagged TET2 enzyme was purified using cation exchange and heparin sepharose chromatography. In addition, a reliable quantitative liquid chromatography-tandem mass spectrometry-based assay was utilized to analyze the activity of TET2 oxygenase. This assay was further used to analyze the activity of a number of clinical TET2 variants with mutations in the 2OG binding sites. Our results demonstrate that the activity of one TET2 mutant, TET2-R1896S, can be restored using an excess of 2OG in the reaction mixture. These studies suggest that dietary 2OG supplements, which are commonly used for several other conditions, may be used to treat some patients with myeloid malignancies harboring TET2-R1896S mutation. Results described in this paper serve as a foundation for better characterization of wild type as well as mutant TET2 demethylases.

Keywords

Epigenetics, TET2, DNA demethylase, oxygenase, LC-MS/MS, leukemia.

1. Introduction

5-Methylcytosine (5mC) within the CpG dinucleotides in DNA is one of the most widely studied epigenetic mark. This modification is carried out by DNA methyltransferases, which play an important role in normal development and disease states by regulating gene expression [1]. Three putative DNA demethylating enzymes, TET1-3, were later identified in mouse and human genomes using bioinformatics [2]. Initial biochemical characterization of TET1 oxygenase showed that it converts 5mC into 5-hydroxymethylcytosine (5hmC) in mammalian cells [3]. Recently, it has been established that the TET-family of oxygenases sequentially converts 5mC into 5hmC, 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) by iterative oxidation steps [4,5,6]. Demethylation takes place by replacing 5caC with an unmodified cytosine by thymine-DNA glycosylase, a base excision repair enzyme [4].

The *TET1* gene was first identified as a fusion partner of the mixed-lineage-leukemia gene in acute myeloid leukemia (AML) [7], while the *TET2* gene was identified as one of the most frequently mutated genes in myelodysplastic syndromes (MDS) [8,9,10]. TET2 mutations are also observed in a number of myeloid malignancies such as MDS-myeloproliferative neoplasms (MDS-MPN) and acute myeloid leukemia derived from MDS and MDS-MPN (sAML) [10]. Patients with TET2 mutations show low levels of genomic 5hmC in the bone marrow compared to those with wild type (WT)-TET2 [8]. In order to elucidate the role of TET2 in normal hematopoiesis and myeloid transformation, TET2-knockout mouse models were developed [11,12,13,14]. These studies demonstrated that diverse myeloid malignancies develop in TET2-knockout mice. Interestingly, TET2-knockout mice were initially viable and grossly normal, but as they aged they started manifesting different hematopoietic malignancies that

caused their death. These studies established that the WT-TET2 plays critical roles during normal hematopoietic differentiation. The loss of TET2 causes an expansion of hematopoietic stem cells (HSCs) and other myeloid progenitor cells. TET2-deficient HSC and progenitor cells also demonstrated progressively reduced hematopoiesis. HSCs with TET2^{-/-} and TET2^{+/-} genotype had a greater ability to self-renew, providing these cells a competitive advantage over WT-TET2 HSCs for repopulating hematopoietic lineages. Further, heterozygous HSCs with TET2^{+/-} behaved similarly to TET2^{-/-} cells and developed diverse myeloid malignancies, consistent with a large number of patients with heterozygous TET2 mutations. These results suggest that haploinsufficiency of TET2 enzymatic activity is sufficient to change the properties of HSCs, leading to the induction of myeloid malignancies.

Recently, the crystal structure of TET2 oxygenase/demethylase domain bound to methylated double-stranded DNA (dsDNA) was reported [15]. This structure demonstrated that the two zinc fingers bring the Cys-rich domain and catalytic core formed by anti-parallel β -sheets, also known as the ‘jelly-roll’ motif, together to form a compact oxygenase domain. The Cys-rich domain stabilizes the DNA above the ‘jelly-roll’, a characteristic domain of 2OG-dependent oxygenases. Interactions between TET2 and CpG dinucleotide in dsDNA flips the 5mC into a cavity with the methyl group projected towards the catalytic Fe(II) for oxidation. The TET2 active site also accommodates 5mC derivatives (*i.e.* 5hmC and 5fC) for their further oxidation [5,6].

Clinically described TET2 mutations include frame-shift, nonsense and missense mutations [16]. Since the dioxygenase domain is present toward the TET2 C-terminus with the

critical 2OG binding motif located toward the very end, most frame-shift and nonsense mutations upstream to it will result in an inactive enzyme. In addition to the frame-shift and nonsense mutations, most of the identified TET2 missense mutations are clustered in the C-terminal oxygenase domain. Little is known about the characteristics of the WT-TET2 protein and its clinical mutants partly due to complications associated with the production of recombinant TET2 protein and its assay. In this study, we developed an efficient two-step purification method using cation exchange and heparin sepharose chromatography to yield the native TET2 oxygenase domain without the use of an affinity tag. In addition, a reliable, sensitive, and quantitative liquid chromatography-tandem mass spectrometry (LC-MS/MS)-based assay was used to characterize the activity of purified WT-TET2 oxygenase and selected TET2 clinical mutations in the 2OG binding sites. The outcomes from the study suggest that the activity of one of the TET2 mutants, TET2-R1896S, can be restored with an excess of 2OG.

2. Materials and Methods

2.1 Chemicals and Reagents

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise stated. *Escherichia coli* (*E. coli*) TOP10 and DH5 α competent cells were purchased from Invitrogen (Carlsbad, CA). *E. coli* expression strains BL21 (DE3), BL21 trxB (DE3), BL21 trxB pLysS (DE3), and Rosetta Gami2 (DE3) pLysS were purchased from Novagen (now EMD Millipore, Billerica, MA). *E. coli* BL21-CodonPlus (DE3)-RIPL cells were purchased from Agilent Technologies (Santa Clara, CA). *E. coli* C41 (DE3) was purchased from Lucigen (Middleton, WI). *E. coli* BL21star (DE3), pDONR221 and pDEST14 plasmid vectors, as well as BP and LR clonase enzymes, were purchased from Invitrogen. All growth media were from

Difco Laboratories (Detroit, MI). Ni-nitrilotriacetic acid (Ni-NTA)-agarose was supplied by Gold Biotechnology (Olivette, MO). The anti-6×His antibody (catalog number MA1-21315) and anti-5hmC antibody (catalog number 39769) were obtained from Thermo Fisher Scientific (Waltham, MA) and Active Motif (Carlsbad, CA), respectively. The anti-rabbit HRP conjugated IgG (catalog number 111/035/144) was purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

2.2 *Cloning of human TET2 DNA demethylase*

A human TET2 (1219-1936) clone with residues 1481-1843 replaced by a 15-residue GS linker (designated as TET2 1219-1936 Δ1481-1843) was obtained as a gift from Fudan University. This TET2 clone was missing 90 nucleotides from the N-terminal of the crystallized TET2 clone, (TET2 1129-1936 Δ1481-1843) [15]. Therefore, the missing nucleotides were added by PCR (Thermal Cycler DNA Engine®, Bio-Rad, Hercules, CA) using the following primers: 5'-*GAATTCCATATGTCTGTTCTCAATAATTTTATAGAGTCACCTTCCAAATTACTAGATACTCCTATAAAAAATTTATTGGATACACCTGTCAAGACTCAATATGATTTCCCATCTTGCAGATGTG-3'* (forward Primer) and 5'-*CTCGAGGCGGCCGCGTCGACTCAGCCATACTTTTCACAC-3'* (5' Reverse Primer). After purification on the agarose gel, the TET2 1129-1936 Δ1481-1843 DNA fragment was cloned into the pJET1.2 vector and verified by DNA sequencing. To produce the GST-tagged human TET2 protein, the catalytic domain of human TET2 (TET2 1129-1936 Δ1481-1843 DNA) was inserted into pGEX4T-1 vector using EcoR1 and Xho1 restriction enzymes. The final recombinant clone was sequence verified. A previous study has demonstrated that the C-terminal TET2 fragment (from 1129-1936 amino acids) is catalytically active [15]. This C-terminal fragment carries a low complexity insert (approximately from 1481-1843 amino

acids), which is less conserved in the Tet subfamily [2]. Deletion of this insert resulted in a minimum catalytically active TET2 fragment (TET2 1129-1936 Δ 1481-1843 DNA) [15]. Therefore, we have used this minimum catalytically active TET2 fragment for our studies in this manuscript.

To produce the untagged human TET2 enzyme, the TET2 1129-1936 Δ 1481–1843 catalytic domain fragment was amplified by following primers: 5'-*GGGGACAAGTTTGTACAAAAAGCAGGCTTCGAAGGAGATAGAACCATGTCTGTTCTCAATAATTTTATAG*-3' (forward primer) and 5'-*GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGCCATACTTTTCACAC*-3' (5' Reverse Primer). While the 6×His tagged human TET2 was amplified using the following primers: 5'-*GGGGACAAGTTTGTACAAAAAGCAGGCTTCGAAGGAGATAGAACCATGCATCATCATCATCATCATCTGTTCTCAATAATTTTATAG*-3' (forward primer) and 5'-*GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAATGATGATGATGATGATGGCCATACTTTTCACAC*-3' (reverse primer). In order to clone the untagged or 6×His tagged TET2 fragment in the pDONR221 donor vector using Gateway technology, Shine Dalgarno and Kozak sequences were also incorporated in the forward primer. The amplified linear *attB*-TET2-*attB* fragment was gel eluted using EasyPrep™ Gel Extraction kit (Bio-land Scientific, Paramount, CA) and the TET2 fragment was recombined into the pDONR221 entry vector using BP clonase II enzyme mix kit. The BP reaction mixture (2 μ l) was transformed into chemically competent *E. coli* DH5 α cells using kanamycin (50 μ g/mL) as a selection antibiotic. Following transformation, all entry clones were confirmed by DNA sequencing. The entry clone, *i.e.* pDONR221 with the

TET2 fragment, was used to transfer the TET2 fragment into pDEST14 destination vector using LR recombination reaction as per manufacturer's protocol. The LR recombination reaction (2 µl) was used to transform *E. coli* DH5α cells by the heat shock method using ampicillin (100 µg/mL) as a selection antibiotic.

2.3 *Site-directed mutagenesis of human TET2*

Point mutations were made in the pGEX4T-1 vector containing TET2 1129-1936 Δ1481–1843 catalytic domain fragment by PCR using the QuickChange site-directed mutagenesis kit (Stratagene) with modifications [17], using the following primers: (1) TET2-R1896M mutant: 5'-CACCCCACCATCATCTCCCTCGTCTTTTACCAGCAT-3' (forward primer), 5'-GAGGGAGATCATGGTGGGGTGATTCCTATTGGGATTC-3' (reverse primer); (2) TET2-R1896S mutant: 5'-CACCCCACCAGCATCTCCCTCGTCTTTTACCAGCAT-3' (forward primer), 5'-GAGGGAGATGCTGGTGGGGTGATTCCTATTGGGATTC-3' (reverse primer); (3) TET2-R1896G mutant: 5'-CACCCCACCGGGATCTCCCTCGTCTTTTACCAGCAT-3' (forward primer), 5'-GAGGGAGATCCCGGTGGGGTGATTCCTATTGGGATTC-3' (reverse primer); (4) TET2-S1898F mutant: 5'-ACCAGGATCTTCCTCGTCTTTTACCAGCATAAGAGCATG-3' (forward primer), 5'-AAAGACGAGGAAGATCCTGGTGGGGTGATTCCTATTGGG-3' (reverse primer). All the mutant constructs were confirmed by DNA sequencing. The GST-tagged human WT-TET2 and mutant proteins were purified using a standard GST affinity purification procedure using standard procedure.

2.4 *Expression of 6×His tagged human TET2 protein*

The recombinant pDEST14 vector with 6×His tagged human TET2 fragment was transformed in to seven different types of *E. coli* expression hosts: BL21 (DE3), BL21star (DE3), BL21 trxB (DE3), BL21 trxB pLysS (DE3), BL21 (DE3) RIPL codon plus, C41 (DE3), and Rosetta Gami2 (DE3) pLysS to screen for the best expression system. Multiple colonies from every transformed host cell were grown in 100 mL LB media at 37°C. After the cultures reached a density of 1.0 at OD₆₀₀, they were maintained at three different temperatures (16°C for 16 h, 27°C for 10 h, and 37°C for 4 h) with two different isopropyl β-D-1-thiogalactopyranoside (IPTG) concentrations (0.5 mM and 1 mM). An equal amount of cells from each condition were analyzed using SDS-PAGE gel and transferred onto PVDF membrane using western blot. The TET2 expression levels were quantitated using anti-6×His antibody. The best *E. coli* strain expressing the protein was selected for transformation with pDEST14-TET2 plasmid using the standard heat shock method.

2.5 Purification of untagged TET2 protein

The pDEST14-TET2 plasmid was transformed into *E. coli* BL21 trxB (DE3) cells. The cells were grown in 5 L of LB media at 37°C. After the cultures reached a density of 1.0 at OD₆₀₀, they were induced by 1 mM IPTG and then further grown at 16°C for 16 h. Bacterial cells were centrifuged at 4,500 rpm for 15 min using a Beckman J2-HS centrifuge (Beckman Coulter, Indianapolis, IN) and either stored at –80°C or lysed immediately for protein purification. All the remaining protein purification steps were performed on ice or at 4°C. The cells were resuspended in 50 mM MES (2-(N-morpholino)ethanesulfonic acid) buffer at pH 6 and lysed by sonication using Sonic Dismembrator 550 (Fisher Scientific, Pittsburgh, PA). A protease inhibitor cocktail of 1 mM PMSF, 0.5 mM benzamidine-HCl, 0.2 mM 1,10 *o*-phenanthroline, and 1 mM EDTA was added to the cell lysate. The lysate was spun at 18,000 rpm for 45 min. The supernatant was

collected and filtered through a 0.45-micron filter. SP sepharose fast flow (GE Healthcare, Piscataway, NJ) strong cation exchange resin (5 mL) was packed into a XK16/20 FPLC column (Pharmacia now GE Healthcare) and was employed as the first step to purify untagged TET2 protein. The column was equilibrated with 10-bed volumes of wash buffer (50 mM MES buffer, pH 6) at constant flow rate of 0.25 mL/min using an ÄKTA FPLC system (Pharmacia now GE Healthcare). Clarified cell lysate was loaded onto the column and washed with the wash buffer (~10-bed volumes) until the flow through was clear. Elution of the bound protein was achieved using a 0-100% gradient from the wash buffer to the elution buffer (50 mM MES buffer, pH 6, 1 M NaCl) in 10-bed volumes followed by holding at 100% elution buffer for two-bed volumes. Samples of cell lysate before and after column loading along with all the elution fractions (3 mL each) were collected and analyzed by SDS-PAGE. The fractions with desired protein were pooled and dialyzed for 10 h in 50 mM MES buffer, pH 6.5. After dialysis, the fractions were passed through a 5 mL prepacked heparin Hi-trap column (GE Healthcare) with a flow rate of 0.5 mL/min. Un-bound proteins were removed by washing with 10-bed volumes of MES buffer, pH 6.5. The gradient elution was performed by up to 1 M NaCl in 50 mM MES buffer (pH 6.5) and 2 mL fractions were collected. All the eluted fractions were run on SDS-PAGE gels and desired fractions were again dialyzed in 50 mM MES buffer, pH 6.5 at 4°C overnight and stored in 15% glycerol at -80°C.

2.6 *5mC Demethylation reactions*

The TET2-mediated in vitro oxidation reactions were performed using a 25-mer dsDNA (sense strand: 5'-GTGTTCTTTCAGCTC5-mCGGTCACGCT-3') as a substrate. All demethylation reactions were performed in triplicate with 5 µM of substrate and 2.5 µM of purified TET2

enzyme in 50 μ l reaction buffer containing 50 mM HEPES (pH 8.0), 75 μ M FeSO₄, 1 mM 2OG, 2 mM ascorbate, 100 mM of NaCl, 1 mM ATP, and 1 mM DTT at 37°C for 1 h [15]. Reactions were quenched with 10 mM EDTA.

2.7 *In vitro dot-blot analysis of TET2 activity*

The TET2 reaction was quenched and used for an *in vitro* dot-blot analysis by denaturing the DNA with 0.4 M NaOH at 98°C for 10 min following its neutralization by an equal volume of cold 2 M ammonium acetate. The DNA was spotted on pre-activated nylon membranes (Bio-Rad) under vacuum. The DNA spotted membranes were UV-crosslinked for 10 min and blocked by 5% non-fat milk. The membranes were washed twice with 1 $\times$ TBST (Tris-buffered saline with 0.1% Tween 20) and incubated with anti-5hmC antibodies (1:10000) for 1 h at room temperature. The membranes were then washed three times with 1 $\times$ TBST and incubated for 45 min with HRP conjugated secondary antibody (Jackson ImmunoResearch Laboratories, Inc.). Finally, the membranes were washed again for three times with TBST and visualized using BM chemiluminescence (Sigma) blotting substrate.

2.8 *Quantitative LC-MS/MS-based assay development*

An LC-MS/MS method was developed for the separation and quantification for different cytosine nucleotides using a Phenomenex Gemini 5 μ C18 column (Phenomenex, Torrance, CA). A gradient elution method was employed with solvent A (10 mM ammonium acetate, pH 4.0) and solvent C (100% acetonitrile with 0.1% acetic acid) as mobile phase solvents. The chromatographic elution was performed with a gradient of 0-11% solvent C in 14 min followed by a post equilibration with solvent A for 4 min using a flow rate of 0.3 mL/min. The UV

detector was set at 280 nm. MS detection was done in the positive ESI mode on an AB Sciex API 3200 Q-Trap tandem mass spectrometer (Concord, Ontario, Canada) equipped with an ESI source. The mass-spectrometry parameters were optimized by infusing different cytosine nucleosides and using the automated quantitative optimization routine in the Analyst software. The optimized source parameters were as follows: heater temperature 550°C, ion source voltage 5500 V, curtain gas 20, nebulizer gas (GS1) 50, and sheath gas (GS 2) 40. Tandem mass-spectrometric analyses were performed using nitrogen as the collision gas (CAD Medium). For each parent ion nucleoside (Q1), the most intense product ion (Q3) was selected and used in an LC-MS/MS analysis.

A 10 mM stock solution of all modified cytosine nucleosides [2'-deoxycytidine (dC), 5-methyl-2'-deoxycytidine (5mdC), 5-hydroxymethyl-2'-deoxycytidine (5hmdC), 5-formyl-2'-deoxycytidine (5fdC), and 5-carboxy-2'-deoxycytidine (5cadC)] were prepared in the HPLC-grade water for the development of the LC-MS/MS method. Method development included determination of response linearity, limit of detection (LOD), lower limit of quantification (LLOQ), and possible matrix/reaction buffer effect in sample extract and carryover. Serial dilutions (steps of 3-fold) of a mixture of all modified cytosine nucleosides were prepared in pure HPLC grade water and matrix simultaneously. The matrix was prepared in 50 µl reaction buffer (50 mM HEPES at pH 8.0, 75 µM FeSO₄, 1 mM 2OG, 2 mM ascorbate, 100 mM of NaCl, 1 mM ATP, and 1 mM DTT) described above without the TET2 enzyme and substrate. The matrix was passed through a Zymo Oligo purification columns and treated with DNase, S1 nuclease, and CIAP and analyzed by LC-MS/MS. Carryover between analytical runs was

assessed by alternately analyzing blank samples and samples containing mid to high levels of cytosine nucleotides. Calibration curves were plotted for all modified cytosine nucleosides.

2.9 *Quantitative LC-MS/MS-based analysis of TET2 activity*

The TET2 reaction was quenched and prepared for LC-MS/MS analysis by separating the DNA from the reaction mixture using Zymo Oligo purification columns (Zymo research, Irvine, CA) according to the manufacturer's protocol. The separated DNA was denatured by heating at 95°C for 10 min and digested with 2 units of DNase I and 60 units of S1 nuclease at 37°C for 12 h to produce individual nucleoside-monophosphates. Following the digestion, 2 units of calf intestinal alkaline phosphatase (CIAP) was added at 37°C for 12 h to remove the terminal phosphate groups from nucleoside-monophosphates to obtain nucleosides. All modified cytosine nucleosides were quantified using the LC-MS/MS method described above.

3. **Results and Discussions**

3.1 *Cloning, expression and purification of TET2*

TET2 oxygenase plays a critical role in demethylation of 5mC in DNA and is one of the most frequently mutated genes in MDS, MDS-MPN, and sAML [16]. To better characterize the TET2 enzyme and its clinical mutants, we cloned the wild-type demethylase domain of TET2 (TET2 1129-1936 Δ 1481-1843) into pDEST14 vector using the recombination-based Gateway technology without any affinity tag (described in Materials and Methods section). We evaluated the expression of recombinant TET2 in seven different strains of *E. coli* at different IPTG concentrations and temperatures. To determine the expression levels of TET2 by western blot, we also produced a TET2 clone with poly-histidine tag (since no commercial antibody is

available for the TET2 demethylase domain) in the pDEST14 vector. Although these experiments identified a number of *E. coli* strains as suitable expression hosts, *E. coli* BL21 trxB (DE3) was selected as the most robust host based on the minimum time needed by this host to reach the log phase (Fig. 1).

Using the optimized expression conditions in *E. coli* BL21 trxB (DE3) cells, the untagged TET2 demethylase was produced at ~2-5% of the total soluble protein by SDS-PAGE analysis. The wild-type demethylase domain of TET2 has a relatively high pI (7.49 calculated) compared with other *E. coli* proteins. Therefore, a convenient purification by an SP sepharose high-performance strong cation exchange resin was developed. This purification yielded TET2 protein of >90% purity in a single step (Fig. 2). Since the TET2 demethylase contain cysteine-rich DNA binding domains and the structure and negative charge of heparin enable it to mimic DNA in its overall binding properties, the heparin sepharose was used, if needed, as the polishing step (data not shown). This purification yielded ~1 mg/ L of TET2 protein of >95% purity. However, using the heparin sepharose as the first purification step did not yield highly pure TET2 enzyme (data not shown).

3.2 *Development of quantitative LC-MS/MS-based assay for TET2 oxygenase*

In order to separate and quantify individual nucleosides from the TET2 reaction, a quantitative LC-MS/MS-based assay was developed as described in the Materials and Methods section. We optimized LC retention time (t_r), declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), and collision energy (CE) for all cytosine derivatives (Table 1). To further validate our LC-MS/MS method, LOD and LLOQ of all modified cytosine nucleosides

were also determined (Table 1). In order to determine the potential effect of the TET2 reaction conditions (e.g. Fe(II), 2OG, etc.) on the quantification of each nucleoside, the standard modified cytosine nucleosides were dissolved in matrix/reaction buffer and their peak areas were compared with standards dissolved in water. The results from these studies demonstrated that TET2 reaction buffer had insignificant effect on the quantification of cytosine nucleosides (Fig. 3A-E).

In order to characterize the activity of untagged TET2 demethylase, we used a 25-mer dsDNA containing one 5mC in a CpG island in each DNA strand as a substrate. Following incubation with or without TET2 enzyme, oligonucleotides were processed and quantitative LC-MS/MS analyses were performed as described above. In the negative control reaction, i.e. without the TET2 enzyme, peaks were observed for only dC and 5mdC. However, in the positive control reaction which contained the TET2 enzyme, three additional peaks corresponding to 5hmdC, 5fdC, and 5cadC were observed. Further, in agreement with a recent report, we found that untagged TET2 prefers 5mC as a substrate to 5fC and 5caC (Fig. 3F) [5].

3.3 Characterization of WT-TET2 5-methylcytosine demethylase

Interestingly, the WT-TET2 oxygenase demonstrated small but significant activities in the absence of Fe(II) or 2OG (Fig. 4A), presumably due to pre-bound cofactors from bacteria during expression. In addition, sequential removal of other reported cofactors (i.e. ascorbate, NaCl, DTT, and ATP) from the TET2 reaction mixture had varied effect on the 5mC demethylation. Specifically, removal of ascorbate, ATP, and DTT in the reaction buffer significantly decreased the activity of TET2, while removal of NaCl had little to no effect on the TET2-mediated

demethylation. The role of ascorbate in the activity of 2OG-dependent oxygenases is well documented [18]. To investigate whether ATP and DTT regulate the oxidation state or chelation of Fe(II) in TET2-mediated demethylation, we gradually increased Fe(II) concentration from 75-1000 μ M in the reaction buffer in the absence of ATP and DTT. However, results from these studies demonstrated that TET2 activity did not reach peak levels in the absence of ATP and DTT (Fig. 4B). Further, Fe(II) could not be effectively replaced for TET2 activity by any of the alternative metal ions [Co(II), Cu(II), Mg(II), Mn(II), Zn (II), and Mn(II)] at 75 μ M final concentration (Fig. 4C). Several metal ions [Co(II), Cu(II), and Zn(II)] at 75 μ M (almost) completely inhibited TET2 activity in the presence of Fe(II) at 75 μ M (Fig. 4D) similar to other 2OG-dependent oxygenases [19].

3.4 Analyses of clinical TET2 mutants

Numerous TET2 clinical mutations have been reported in the literature mainly from MDS, MDS-MPN, and sAML patients [16]. However, limited characterizations of these mutations have been reported in the literature partly due to lack of a quantitative assay. Interestingly, some of the most frequently mutated residues, i.e. TET2-R1896M, -R1896G, -R1896S, and -S1898F (-R1896M/S/G, -S1898F), bind the 5-carboxylate group of 2OG in the TET2 oxygenase active site. It is tempting to speculate that the impaired binding of 2OG in these mutants possibly account for the loss of TET2 activity in patients with myeloid malignancies. Therefore, the activities of wild type and some TET2 clinical mutants were compared by the dot-blot and our quantitative LC-MS/MS-based assay (Fig. 5). Since the presence of the TET2 protein inhibited the detection of 5hmC (Fig. 5A), the DNA substrate was purified using a Zymo Oligo purification columns before the blotting. These experiments demonstrated that the quantitative

LC-MS/MS-based assay described here is far more accurate, sensitive, and reproducible than commonly used dot-blot assays (Fig. 5B and 5C). We further characterized the catalytic activities of the wild type and clinical TET2 mutants (-R1896M/S/G, -S1898F) in the presence of different 2OG concentrations using the quantitative LC-MS/MS-based assay (Fig. 5C). All of the studied TET2 mutants showed significantly less activity compared to the WT-TET2 at 1 mM of 2OG. Further, the activities of TET2-R1896M/G and -S1898F mutants did not significantly increased with increasing the concentration of 2OG. However, the activity of TET2-R1896S mutant can be enhanced by increasing the concentration of 2OG in the assay. Our results suggest that patients with TET2-R1896S mutation may be treated using dietary 2OG supplements [20].

During the course of our studies, *Laukka et al.* produced the mouse TET proteins (mTET1-3) in insect cells. They studied the $V_{\max}$ and K_m values for 2OG in the case of mTET2-R1817M and -R1817S mutants, which are analogous to AML-associated human TET2-R1896M and -R1896S mutants [21]. Results from these studies demonstrate that the activity of mTET2-R1817M and -R1817S mutants can be rescued by using an excess of 2OG in the reaction mixture. In fact, the mTET2 demethylase activity did not reach saturation, even when increasing 2OG concentration to 8mM, making it unfeasible to determine the $V_{\max}$ and K_m values for 2OG for these mutants. In contrast, our analyses of these two mutants demonstrated that the activity of only TET2-R1896S but not TET2-R1896M mutant can be restored by using an excess of 2OG in the reaction mixture. It is interesting to note that *Laukka et al.* used a C-terminal mTET2 fragment (from 916–1921 amino acids) without the deletion of the low complexity insert [21]. Although, it has been suggested that the low complexity insert of human TET2 (from 1481-1843 amino acids) may play a role in protein-protein interaction [2], deletion of this insert didn't

diminish the activity of TET2 demethylase [15]. Thus, our contrasting results with TET2-R1896M mutant suggest (i) differences between human and mouse TET2 demethylases produced from different heterologous sources or (ii) a role of the low complexity insert in the regulation of TET2 activity. It is interesting to note that *Iyer et al* have suggested that the TET demethylases may be regulated through sumoylation of this low complexity insert [2]. Further studies need to be carried to further define the role of this region in TET2 activity.

The observation that the activity of some TET2 mutants can be enhanced by excess of 2OG has far-reaching implications. For example, the normal function of isocitrate dehydrogenases (IDH1/2) is to convert isocitrate into 2OG. In some AML and glioblastoma patients mutations in IDH1/2 create neomorphic variants of the enzymes, which produce 2-hydroxyglutarate (2HG) instead of 2OG [22]. Due to the structural similarity with 2OG, 2HG binds in the 2OG binding pocket in the active site of oxygenases, which impairs their function. Thus, 2HG acts as a competitive inhibitor for a number of 2OG-dependent oxygenases, like TET2 [23,24]. The clinical features of some IDH1/2 gain-of-function mutations are similar to TET2 mutations in AML patients. Thus, in addition to TET2-R1896S mutation, our unpublished results suggest that 2OG supplements may also benefit a large number of patients with IDH1/2 gain-of-function mutations.

4. **Conclusion**

In conclusion, in this paper we described the cloning of untagged human TET2 demethylase using Gateway technology and its efficient expression in *E. coli*. The untagged TET2 enzyme was purified using cation exchange and heparin sepharose chromatography. In addition, a

reliable quantitative liquid chromatography-tandem mass spectrometry-based assay was used to analyze the activity of TET2 oxygenase. This assay was then used to analyze the activity of a number of clinical TET2 variants with mutations in the 2OG binding sites. Our results demonstrate that the activity of one TET2 mutant, TET2-R1896S, can be restored using an excess of 2OG in the reaction mixture. These studies suggest that the dietary 2OG supplements, which are commonly used for several other conditions, may be used to treat cancer patients with the TET2-R1896S mutation.

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Figure Legends:

Fig. 1. Western blot analysis of TET2 oxygenase expression in seven different strains of *E. coli* when induced at the indicated temperature and IPTG concentration: 16°C for 16 h with 0.5 mM IPTG (A), 27°C for 10 h with 0.5 mM IPTG (B), 37°C for 4 h with 0.5 mM IPTG (C), 16°C for 16 h with 1 mM IPTG (D), 27°C for 10 h with 1 mM IPTG (E), and 37°C for 4 h with 1 mM IPTG (F).

Fig. 2: SDS-PAGE analysis of purified untagged TET2 demethylase from *E. coli* BL21 *trx*B (DE3) cells. Lane indicates: marker (A), uninduced TET2 (B), induced TET2 protein (C), and TET2 protein purified using SP sepharose high-performance strong cation exchange resin (D). The total size of the untagged TET2 demethylase is ~54.6 kDa as indicated by the arrow.

Fig. 3. Standard curves of modified cytosine nucleosides dissolved in TET2 reaction conditions (straight line) or HPLC-grade water (dotted line) showing insignificant effect on the quantification (A-E). A pie chart showing LC-MS/MS detection of 5hmC, 5fC, and 5caC formed after TET2 reaction (F).

Fig. 4: Characterization of the WT-TET2 demethylase. Effect of co-factors on the TET2-mediated demethylation (A). An increase in Fe(II) concentration from 75-1000 μ M in the reaction buffer in the absence of ATP and DTT did not increase TET2 activity (B). Fe(II) could not be replaced by any of the alternative metal ions for TET2 activity (C). Several metal ions inhibited TET2 activity in the presence of Fe(II) (D).

Fig. 5: A comparison of the activities of wild type and some TET2 clinical mutants by dot-blot and our quantitative LC-MS/MS-based assay. The presence of the TET2 protein inhibited the detection of 5hmC (A), DNA substrate was purified using a Zymo Oligo purification columns before the blotting and detection by dot-blot (B), Catalytic activities of the wild type and clinical TET2 mutants (-R1896M/S/G, -S1898F) were characterized in the presence of different 2OG concentrations using the quantitative LC-MS/MS-based assay (C).

Table Legends:

Table 1: Summary of optimized positive mode IP-LC-MS/MS parameters and characteristics of different cytosine derivatives. For each parent ion nucleoside (Q1), the most intense product ion (Q3) was detected.

Cytosine modification	Q1	Q3	t _r (min)	DP (V)	EP (V)	CEP (V)	CE (V)	LOD (pmol)	LLOQ (pmol)
2'-deoxycytidine	228.1	112.1	11.50	11	6	16	21	1.00	3.30
5-methyl-2'-deoxycytidine	242.2	126.2	12.57	30	6	14	23	0.10	0.33
5-hydroxymethyl-2'-deoxycytidine	258.2	142.2	11.68	36	3.5	14	27	0.30	1.00
5-formyl-2'-deoxycytidine	256.2	140.2	14.49	26	4	14	17	0.30	1.00
5-carboxy-2'-deoxycytidine	272.2	156.2	11.84	56	6	24	15	3.00	9.90