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TITLE: Rescue of TET2 Haploinsufficiency in Myelodysplastic Syndrome Patients Using Turbo Cosubstrate

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14. ABSTRACT
During this period, we have demonstrated that 2-oxoglutarate (2OG) analogs can dramatically enhance the activity of TET2 enzymes from MDS patient. Further, so far we have published three manuscripts describing the results generating using this funding. Finally, we have identified novel substrate specificity of TET2 oxygenase. I hope these results will further generate 3-5 more publications in future.

15. SUBJECT TERMS
None listed

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TABLE OF CONTENTS

1. **INTRODUCTION:** TET2 is one of the most frequently mutated genes in myelodysplastic syndromes (MDS). The TET2 mutations are also prevalent in a number of myeloid malignancies such as MDS-myeloproliferative neoplasms (MDS-MPN) and acute myeloid leukemia derived from MDS and MDS-MPN (sAML). TET2 mutations results in presence of higher levels of 5-methylcytosine (5mC) marks, particularly in the CpG islands of promoters, leading to gene silencing. The wild-type (wt)-TET2 protein, a putative tumor suppressor, is a non-heme iron(II), 2-oxoglutarate (2OG)-dependent dioxygenase which initiates 5mC demethylation by hydroxylating it into 5-hydroxymethylcytosine (5hmC). TET2-knockout mice, which are viable and grossly normal initially, with age, develop diverse myeloid malignancies similar to humans. The objective in this project is to develop effective strategies using 2OG analogs to enhance the activity of the wt-TET2 enzyme both in vitro and in vivo in order to overcome TET2 haploinsufficiency.

2. **KEYWORDS:** Myelodysplastic syndromes (MDS), MDS-myeloproliferative neoplasms (MDS-MPN), Acute myeloid leukemia (AML), 5-methylcytosine (5mC), Mutation, Haploinsufficiency, Small molecule activators, TET2, Dioxygenase, 2-oxoglutarate (2OG).

3. **ACCOMPLISHMENTS:**

- **What were the major goals of the project?**

  Task 1: Screen a library of 2OG analogs and identify cosubstrates with better K_M values towards wt-TET2 under *in vitro* assay conditions (months 1-26).

  1a. Scale-up the purification of wt-TET2 from the insect cells (SF9) using an N-terminal his-tag by affinity chromatography (months 1-6). We already have a stock of P1 virus, which will be used to produce ≈10 mg of pure wt-TET2 dioxygenase.

  Completed. Along with expression in the insect cell lines, we have developed a convenient expression of active TET2 enzymes in bacterial cells.

1b. Determination of kinetic properties (V_{max}, K_M, and k_{cat}) of wt-TET2 with respect to 2OG using the standardized *in vitro* HPLC assay (months 4-6). Using our reported methods we will determine the kinetic properties of wt-TET2 dioxygenase.

Completed. Using LC-MS/MS-based assay we have determined the specific activity of TET2.

1c. Synthesize, purify and characterize a library of 2OG analogs using the scheme reported in the application (months 1-18).

Partly completed. We have had several problems with the synthesis of 2OG analogs. However, we were able to synthesize some analogs based on our results and tested those compounds using wt-TET2 and mutant-TET2 from MDS patients.

1d. Determination of kinetic properties (V_{max}, K_M, and k_{cat}) of wt-TET2 with respect to 2OG analogs using the *in vitro* HPLC assay (months 6-24). This will be followed by selection of ≈10 best 2OG analogs with an improved K_M value.

Completed. However, due to issues associate with kinetic characterization (V_{max}, K_M, and k_{cat}) of TET2, we focused on specific activity of TET2. Our results shows for the first time that the activity of TET2 can be modulated, even enhanced in case of some MDS mutants, using 2-oxoglutarate analogs. We are making attempts to crystalize some 2OG analogs with mutant TET2 enzymes from MDS patients.

1e. The shortlisted 2OG analogs (≈10) will be assayed with histone lysine demethylases, HIF prolyl hydroxylases and AlkB2 dioxygenases to identify 2OG analogs that show specificity towards wt-TET2 activation compared to other dioxygenases (months 12-24). From these experiments two 2OG analogs will be selected for the cell-based studies.

3-4 2OG analogs are found to enhance the activity of TET2 mutants found in MDS patients. No compounds are found that enhanced the activity of wt-TET2.

Task 2: Develop strategies to improve wt-TET2 activity in haploinsufficient lymphoid cells from MDS patients (months 9-36).

2a. Selection at least two TET2 mutated haploinsufficient patient cell line and one normal cell line with wt-TET2, used as a control (months 12-18).

Completed.

2b. Chemical modification (esterification) of the two 2OG analog selected from task 1e (months 24-26) and 2OG, which will be used as a control in every experiment.

Partly completed. Ongoing studies.

2c. Co-culture of the two TET2 mutated haploinsufficient patient cell line from task 2a in the presence or absence of the two modified 2OG analogs at five times K_M concentration. As a control, a normal cell line from healthy donor with wt-TET2 will be grown without any 2OG analogs (months 26-28).

Will be performed in future.

2d. Quantitation and analysis of 5mC/5hmC and gene expression levels in the three cell lines cultured in the presence or absence of modified 2OG analogs (months 28-36).

Will be performed in future.

- **What was accomplished under these goals?**

  Please find below a brief description of our accomplishments:

1. *Expression and LC-MS/MS-based TET2 assay:* In this project, 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 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.

2. *Rescue of TET2 activity using 2OG analogs*: This was the main focus of this project and as stated above the activity of TET2 can be modulated, even enhanced in case of some MDS mutants, using 2-oxoglutarate analogs. We are in process of understanding these dramatic results based on crystallography (detailed results will be published).

3. *Substrate specificity of Tet2 oxygenase*: As a side project we have for the first time demonstrated the detailed substrate specificity of Tet2 oxygenase. These remarkable results will be published within next few months (detailed results will be published soon).

4. *Rescue of TET2 haploinsufficiency using vitamin-c*: Our results also suggest that TET2 haploinsufficiency can be overcome using vitamin-c.

Resulting Publications:

1. Convenient expression, purification and quantitative liquid chromatography-tandem mass spectrometry-based analysis of TET2 5-methylcytosine demethylase. Protein Expr Purif. 132, 143-151, 2017.

2. Efficient Purification and LC-MS/MS-based Assay Development for Ten-Eleven Translocation-2 5-Methylcytosine Dioxygenase. J. Vis. Exp. (140), e57798, doi:10.3791/57798, 2018.

3. The mechanism and context of ascorbic acid mediated TET2 activation. Journal of Clinical Investigation, under review (2018).

- **What opportunities for training and professional development has the project provided?**

  This project has provided important opportunities to Dr Mridul Mukherji and his students to attain conferences.

- **How were the results disseminated to communities of interest?**

  Preliminary results were disseminated in the 34th Midwest Enzyme Chemistry Conference (MECC), University of Illinois at Chicago, Chicago, IL, 2016 and 35th Midwest Enzyme Chemistry Conference (MECC), Loyola University, Chicago, IL, 2017. We have published/ submitted 3 manuscript and planning to publish at least 3-4 more manuscripts in peer reviewed national/ international journals.

- **What do you plan to do during the next reporting period to accomplish the goals?**

  Although the project period has ended but we would be publishing at least 3-4 more manuscripts in peer reviewed national/ international journals.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**

  For the very first time we have demonstrated that the activity of TET2 dioxygenase can be modulated using 2-oxoglutarate analogs. In addition, our results demonstrate that the activity of one TET2 mutant, TET2-R1896S, can be restored using an excess of 2OG. These studies suggest that dietary 2OG supplements, which are commonly used for several other conditions, may be used to treat patients with myeloid malignancies

harboring TET2-R1896S mutation. Our results also suggest that TET2 haploinsufficiency can be overcome using vitamin-c supplement by MDS patients. In addition, we have established novel substrate specificity of TET2 oxygenase.

- **What was the impact on other disciplines?**

  There are many 2-oxoglutarate-depent dioxygenases like histone demethylases that regulate critical biological processes like HIF signaling, epigenetics etc. Our methods would make it possible to regulate the activity of these dioxygenases using 2-oxoglutarate analogs.

- **What was the impact on technology transfer?**

  A patent application will be filed soon.

- **What was the impact on society beyond science and technology?**

  Nothing to Report.

5. **CHANGES/PROBLEMS:**

- **Changes in approach and reasons for change**

  1. We have had issues with development of a reliable TET2 dioxygenase assay. Progress of the project has been hampered by delays in permission to use the mass spectrometry instrument with ammonium salt (please note that our LCMS method requires use of ammonium salt in the buffer for proper separation of nucleosides).

  2. We also had delays due to three graduate students from my lab transferring to other pharmacy programs/ schools.

  Due to above mentioned delays, some of the main objectives of the project were not accomplished with in the duration of the project.

- **Actual or anticipated problems or delays and actions or plans to resolve them**

After one year we were able to use our LCMS system with ammonium salt. This has allowed us to progress with the project as proposed in the application. Further, I have admitted two new graduate students and they are making good progress.

- **Changes that had a significant impact on expenditures**
  Since the alternative colorimetric assay from Epigentek requires a plate reader, we received a written permission from DOD to buy a demo molecular devices plate reader costing over $19,000. Along with the purchase of the plate reader, which was not allocated in the original approved budget by DOD, I had to pay students and buy reagents in order to develop alternative assays.

- **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents**: Nothing to Report.

- **Significant changes in use or care of human subjects:** Nothing to Report.

- **Significant changes in use or care of vertebrate animals:** Nothing to Report.

- **Significant changes in use of biohazards and/or select agents:** Nothing to Report.

6. **PRODUCTS:** Nothing to Report.

7. **PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**

- **What individuals have worked on the project?**
  Provide the following information for: (1) PDs/PIs; and (2) each person who has worked at least one person month per year on the project during the reporting period, regardless of the source of compensation (a person month equals approximately 160 hours of effort). If information is unchanged from a previous submission, provide the name only and indicate "no change."

Name:	Mridul Mukherji
Project Role:	PI
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	0.36
Contribution to Project:	*Dr Mukherji supervises the project on day-to-day basis and ensure that the participating graduate students learn appropriate skills to conduct their daily research independently and timely manner. He participate in writing progress reports and research publications.*
Funding Support:	

Name:	Chayan Bhattacharya
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	16236402 (EMPLID #)
Nearest person month worked:	3
Contribution to Project:	He is working on purification and assay of TET2.
Funding Support:	

Name:	Aninda Sundar Dey
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	16245682 (EMPLID #)
Nearest person month worked:	3

Contribution to Project:	He is responsible for LCMS-based TET2 assays.
Funding Support:	

- **Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?**
  Nothing to Report.

- **What other organizations were involved as partners?**
  Nothing to Report.
- **Organization Name:**
- **Location of Organization:** *(if foreign location list country)*
- **Partner's contribution to the projec**t *(identify one or more)*

8. **SPECIAL REPORTING REQUIREMENTS**: Nothing to Report.

9. **APPENDICES:** Two published articles are attached. Another one manuscript, which is under revision in JCI, is not attached. I expect 3-4 additional publications in near future, which would result from this grant.

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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/αKG)-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.
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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 [4–6]. 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) [8–10]. 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 [11–14]. 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

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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 DH5α 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 39,769) 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 to 1936 amino acids) is catalytically active [15]. This C-terminal fragment carries a low complexity insert (approximately from 1481 to 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 Δ1481–1843 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 Δ1481–1843 catalytic domain fragment was amplified by following primers: 5′-*GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGTCTGTTCTCAATAATTTTATAG*-3′ (forward primer) and 5′-*GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGCCATACTTTTCACAC*-3′ (5′ Reverse Primer). While the 6 × His tagged human TET2 was amplified using the following primers: 5′-GGG*GACAAGTTTGTACAAAAAAGCAGGCTTC*GAAGGAGATAGAACCATG*CATCATCATCATCATCATTCTGTTCTCAATAATTTTATAG*-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 *att*B-TET2-*att*B fragment was gel eluted using EasyPrep™ Gel Extraction kit (Bioland 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 μl) was transformed into chemically competent *E. coli* DH5α cells using kanamycin (50 μg/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′-*GAGGGA-GATCATGGTGGGGTGATTCCTATTGGGATTC*-3′ (reverse primer); (2) TET2-R1896S mutant: 5′-*CACCCCACCAGCATCTCCCTCGTCTTTTAC-CAGCAT*-3′ (forward primer), 5′-*GAGGGA-GATGCTGGTGGGGTGATTCCTATTGGGATTC*-3′ (reverse primer); (3) TET2-R1896G mutant: 5′-*CACCCCACCGGGATCTCCCTCGTCTTTTAC-CAGCAT*-3′ (forward primer), 5′-*GAGGGA-GATCCCGGTGGGGTGATTCCTATTGGGATTC*-3′ (reverse primer); (4) TET2-S1898F mutant: 5′-*ACCAGGATCTTCCTCGTCTTTTACCAGCATAA-GAGCATG*-3′ (forward primer), 5′-*AAAGACGAGGAA-GATCCTGGTGGGGTGATTCCTATTGGG*-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 OD_{600}, 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 OD_{600}, they were induced by 1 mM IPTG and then further grown at 16 °C for 16 h. Bacterial cells were centrifuged at 4500 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 pre-packed 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. 5 mC 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 μl reaction buffer containing 50 mM HEPES (pH 8.0), 75 μM $FeSO_4$, 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 × TBST (Tris-buffered saline with 0.1% Tween 20) and incubated with anti-5hmC antibodies (1:10,000) for 1 h at room temperature. The membranes were then washed three times with 1 × 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 5μ 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 $FeSO_4$, 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 5 mC 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 Δ1481–1843) 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 to 1000 μM 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 μM final concentration (Fig. 4C). Several metal ions [Co(II), Cu(II), and Zn(II)] at 75 μM (almost) completely inhibited TET2 activity in the presence of Fe(II) at 75 μM (Fig. 4D) similar to other 2OG-dependent oxygenases [19].

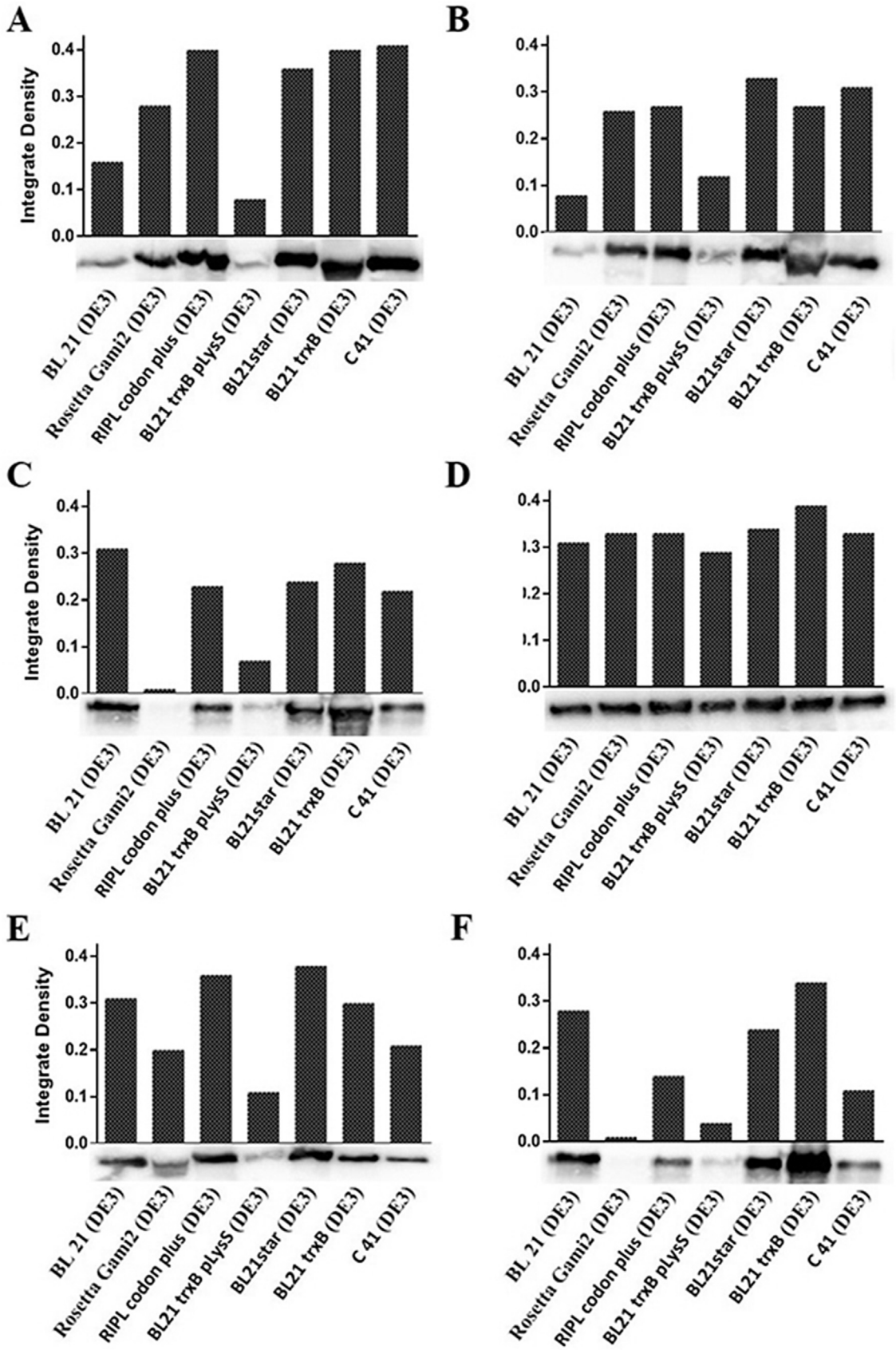

Fig. 1. Western blot analysis of TET2 oxygenase expression in seven different strains of *E. coli* when induced at the indicated temperature and IPTG concentration: 16 °C for 16 h with 0.5 mM IPTG (A), 27 °C for 10 h with 0.5 mM IPTG (B), 37 °C for 4 h with 0.5 mM IPTG (C), 16 °C for 16 h with 1 mM IPTG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

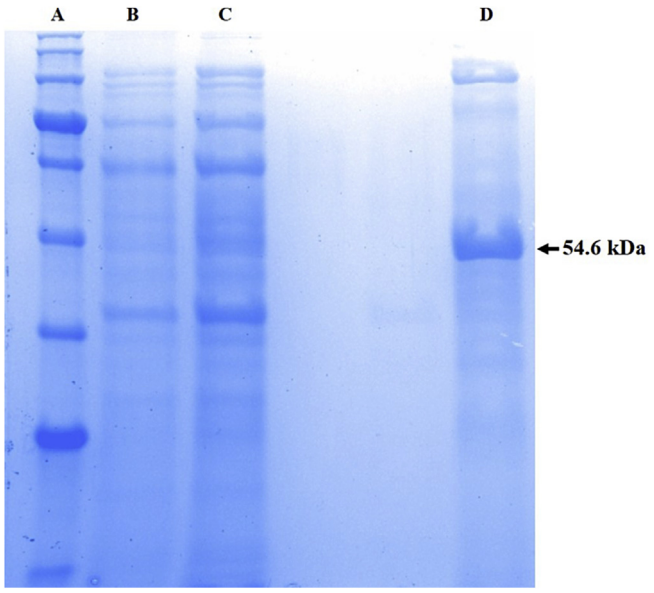

Fig. 2. SDS-PAGE analysis of purified untagged TET2 demethylase from *E. coli* BL21 trxB (DE3) cells. Lane indicates: marker (A), uninduced TET2 (B), induced TET2 protein (C), and TET2 protein purified using SP sepharose high-performance strong cation exchange resin (D). The total size of the untagged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

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 C). 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.

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

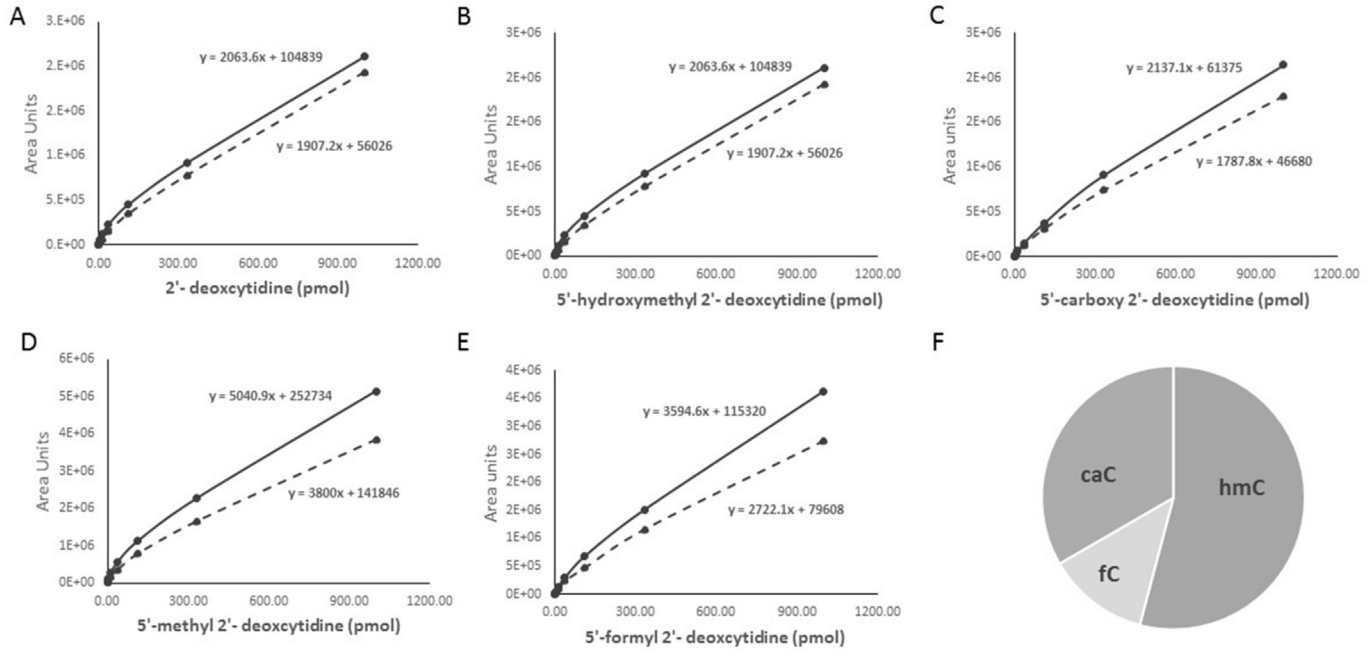

Fig. 3. Standard curves of modified cytosine nucleosides dissolved in TET2 reaction conditions (straight line) or HPLC-grade water (doted line) showing insignificant effect on the quantification (A–E). A pie chart showing LC-MS/MS detection of 5hmC, 5 fC, and 5caC formed after TET2 reaction (F).

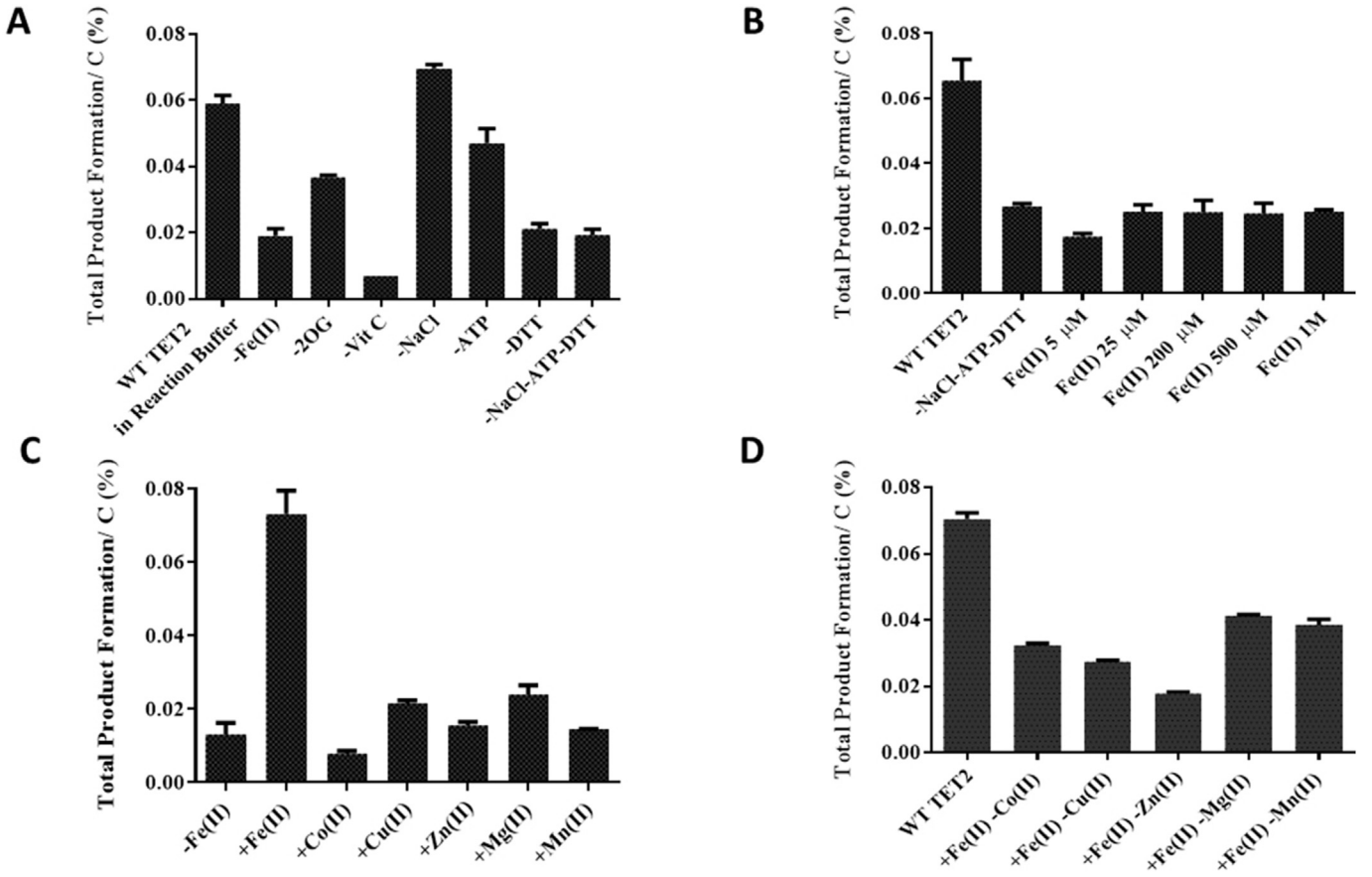

Fig. 4. Characterization of the WT-TET2 demethylase. Effect of co-factors on the TET2-mediated demethylation (A). An increase in Fe(II) concentration from 75 to 1000 μM in the reaction buffer in the absence of ATP and DTT did not increase TET2 activity (B). Fe(II) could not be replaced by any of the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

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 8 mM, 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 to 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 to 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-

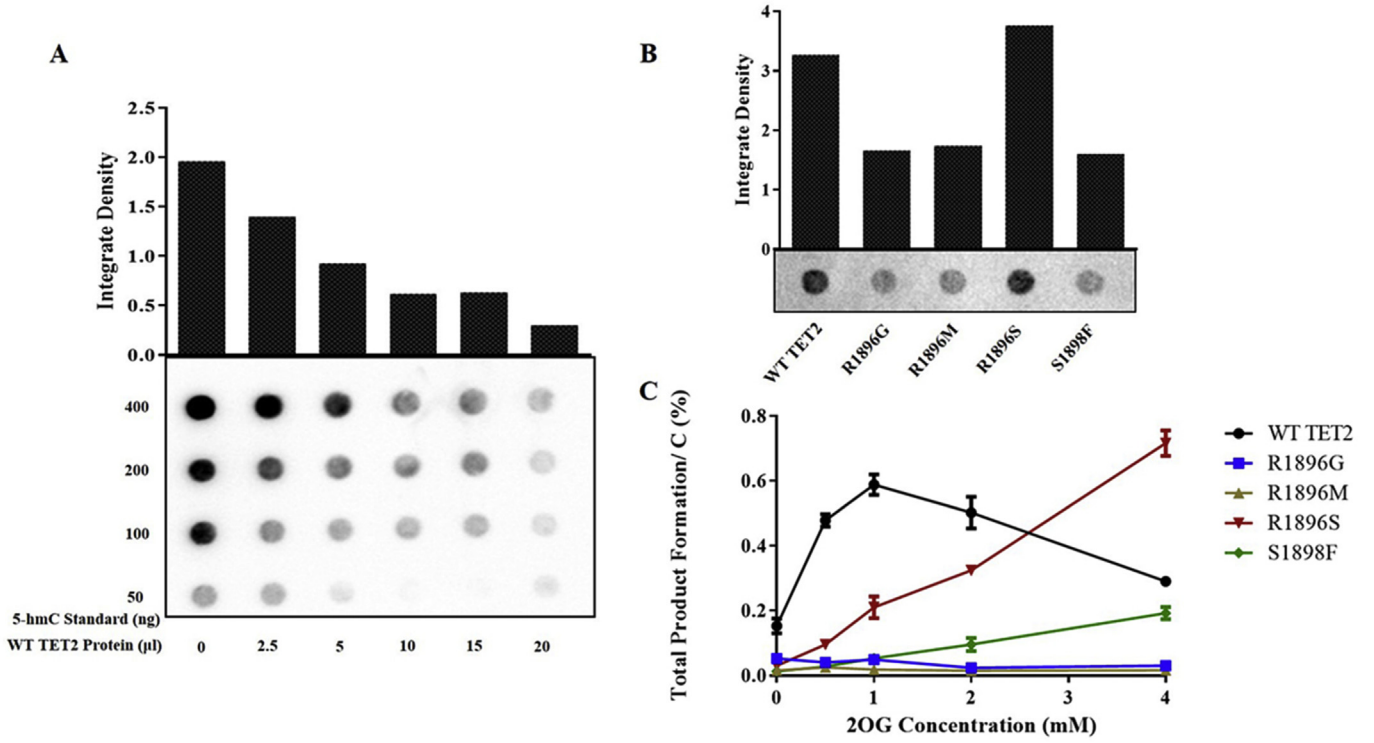

Fig. 5. A comparison of the activities of wild type and some TET2 clinical mutants by dot-blot and our quantitative LC-MS/MS-based assay. The presence of the TET2 protein inhibited the detection of 5hmC (A), DNA substrate was purified using a Zymo Oligo purification columns before the blotting and detection 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).

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.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Video Article

Efficient Purification and LC-MS/MS-based Assay Development for Ten-Eleven Translocation-2 5-Methylcytosine Dioxygenase

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Abstract

The epigenetic transcription regulation mediated by 5-methylcytosine (5mC) has played a critical role in eukaryotic development. Demethylation of these epigenetic marks is accomplished by sequential oxidation by ten-eleven translocation dioxygenases (TET1-3), followed by the thymine-DNA glycosylase-dependent base excision repair. Inactivation of the TET2 gene due to genetic mutations or by other epigenetic mechanisms is associated with a poor prognosis in patients with diverse cancers, especially hematopoietic malignancies. Here, we describe an efficient single step purification of enzymatically active untagged human TET2 dioxygenase using cation exchange chromatography. We further provide a liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach that can separate and quantify the four normal DNA bases (A, T, G, and C), as well as the four modified cytosine bases (5-methyl, 5-hydroxymethyl, 5-formyl, and 5-carboxyl). This assay can be used to evaluate the activity of wild type and mutant TET2 dioxygenases.

Video Link

The video component of this article can be found at https://www.jove.com/video/57798/

Introduction

The C5 position of cytosine bases within CpG dinucleotides is the predominant methylation site (5mCpG) in mammalian genomes[1]. In addition, a number of recent studies have uncovered extensive C5 cytosine methylation (5mC) in non-CpG sites (5mCpH, where H = A, T, or C)[2,3]. 5mC modification serves as a transcriptional silencer at endogenous retrotransposons and gene promoters[3,4,5]. DNA methylation at 5mC also plays important roles in X chromosome inactivation, gene imprinting, nuclear reprogramming and tissue-specific gene expression[5,6,7]. Methylation of cytosine at the C5 position is carried out by DNA methyltransferases, and mutations in these enzymes cause significant developmental defects[8]. The removal of 5mC marks are initiated by TET1-3 5mC oxidases[9,10]. These TET-family dioxygenases convert 5mC into 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) by sequential oxidation steps[11,12,13]. Finally, thymine-DNA glycosylase replaces 5fC or 5caC to unmodified cytosine using the base excision repair pathway[11].

The human *TET2* gene was identified as a frequently mutated gene in diverse hematopoietic malignancies including myelodysplastic syndromes (MDS)[14,15,16], MDS-myeloproliferative neoplasms (MDS-MPN), and acute myeloid leukemia (AML) originating from MDS and MDS-MPN[16]. The levels of 5hmC modification in the bone marrow DNA are lower in patients with TET2 mutations compared to those with wild type (wt)-TET2[14]. A number of groups have developed TET2-knockout mouse models to elucidate its role in normal hematopoiesis and myeloid transformation[17,18,19,20]. These mice with mutations in the TET2 gene were initially normal and viable, but manifested diverse hematopoietic malignancies as they aged causing their early death. These studies showed the important roles played by the wt-TET2 in normal hematopoietic differentiation. In these mouse models, the heterozygous hematopoietic stem cells ($TET2^{+/-}$ HSCs) and homozygous $TET2^{-/-}$ HSCs had a competitive advantage over homozygous wt-TET2 HSCs in repopulating hematopoietic lineages as both $TET2^{+/-}$ and $TET2^{-/-}$ HSCs developed diverse hematopoietic malignancies[17,18]. These studies demonstrate that haploinsufficiency of TET2 dioxygenase alters the development of HSCs and results in hematopoietic malignancies.

Similar to mice with mutations in the TET2 gene, most leukemia patients manifest haploinsufficiency of TET2 dioxygenase activity. These mostly heterozygous somatic mutations include frame-shift and nonsense mutations dispersed throughout the TET2 gene body while missense mutations that are most clustered in the dioxygenase domain[12]. To date, little characterization of wt- and mutant-TET2 is reported in the literature mainly due to difficulties with the production of TET2 dioxygenase and its assay[21]. Here, we report a simple single-step purification of native TET2 dioxygenase using ion exchange chromatography. Further, a quantitative LC-MS/MS assay was optimized and used to measure the enzymatic activity of native TET2 dioxygenase.

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Protocol

1. Cloning and Purification of Untagged Human TET2 Dioxygenase

1. Clone human TET2 dioxygenase (TET2 1129-1936, Δ1481-1843) into the pDEST14 destination vector using the site-specific recombination technique as previously described[22].
   NOTE: Previous studies have demonstrated that the C-terminal TET2 dioxygenase (TET2 1129-1936, Δ1481-1843) domain is the minimal catalytically active domain[21,23]. In order to express the untagged TET2 dioxygenase domain using the pDONR221 vector, Shine Dalgarno and Kozak sequences were incorporated in the forward primer during PCR (**Table 1**).
2. For bacterial transformation, add 1 µL of recombinant pDEST14 expression vector containing untagged human TET2 dioxygenase (TET2 1129-1936, Δ1481-1843) to 100 µL of chemically competent *E. coli* BL21 (DE3) cells in a 1.7 mL tube. Keep the mixture on ice for at least 15 minutes followed by heat shock at 42 °C for 30 s in a water bath.
   1. Immediately after heat shock, keep the cells back on ice for minimum of 2 min. Following this, add 250 µL of super optimal broth with catabolite repression (S.O.C media) to cells. Incubate the bacterial cells for 1 h at 37 °C in a shaker.
   2. After incubation, spin down the cells by centrifuging the tube at 9,000 x g for 1 min. Discard 70% supernatant by pipetting and dissolve the pellet in left over media.
   3. Spread the cell suspension on a Luria broth (LB) agar plate containing 100 µg/mL ampicillin. Incubate the plate for 16 h at 37 °C.
3. Select one isolated colony and inoculate it into 10 mL of LB-ampicillin media in a 50 mL tube. Incubate the tube at 37 °C in a shaker for overnight. Following this, use 100 µL of culture from the 50 mL tube to inoculate 100 mL of LB-ampicillin media. Incubate the flask at 37 °C on a shaker at 180 rpm and use it as primary culture. Next day, use 6 mL each of primary culture to inoculate 15 flasks, each containing 600 mL LB-ampicillin media. Now, incubate 15 flasks at 37 °C on a shaker at 180 rpm.
   NOTE: For verifying transformed clones, perform DNA sequencing or restriction digestion with the isolated plasmid DNA.
4. To check the density of bacterial culture, measure its OD_{600} using a spectrophotometer. After the culture reaches a density of 0.8 at OD_{600}, induce the expression of TET2 protein with 300 µL of 1 M (final concentration of 0.5 mM in 600 mL) IPTG in each flask and further grow the culture for 16 h at 17 °C.
5. After 16 h, transfer the bacterial culture to centrifuge bottles. Centrifuge the bacterial culture expressing the TET2 enzyme at 5,250 x g for 45 min. Use the bacterial pellet for TET2 purification.
   NOTE: Perform all remaining protein purification steps either on the ice or at 4 °C.
6. Resuspend the bacterial pellet in 100 mL of 50 mM MES (2-(N-morpholino)ethanesulfonic acid) buffer, pH 6 and sonicate for 5 × 30 s at power 20 with 60 s cooling intervals.
7. Spin the lysate at 5,250 x g for 45 min. Collect the supernatant containing the soluble TET2 enzyme and pass through 0.45-µm filters before loading on an FPLC system.
   NOTE: In these experiments, the TET2 enzyme was very stable, but if needed a protease inhibitor cocktail containing 1 mM benzamidine-HCl, 1 mM phenylmethylsulphonyl fluoride (PMSF), and 0.5 mM 1,10-*o*-phenanthroline can be added to the cell lysate to prevent degradation of TET2 enzyme. Avoid using EDTA or EGTA in the inhibitor cocktail as these may interfere with the following cation-exchange chromatography.
8. Pack 30 mL of a strong cation exchange resin into a FPLC column. Equilibrate the column with 10 bed volumes of wash buffer (50 mM MES buffer, pH 6) at the constant flow rate of 0.3 mL/min using a FPLC system.
9. Load the clarified lysate onto the pre-equilibrated column and wash with #10 bed volumes of wash buffer until the flow through becomes clear.
10. Elute TET2 using a 0-100% gradient from the wash buffer to the elution buffer (50 mM MES buffer, pH 6, 1 M NaCl) in 15 bed volumes followed by holding at 100% elution buffer for two-bed volumes.
    NOTE: Collect samples (100 µL each) of cell lysate before and after column loading along with all the elution fractions and analyze on 10% resolving SDS-PAGE.
11. Pool the fractions containing TET2 protein, freeze dry, dissolve enzyme pallet in 10 mL water and store at -80 °C.

2. 5mC Oxidation by TET2 Dioxygenase

1. Perform all demethylation reactions in triplicate with 3 µg of substrate (25-mer double stranded DNA, **Table 2**).
   1. Add 100 µg of purified TET2 enzyme in 50 µL of total reaction buffer containing 50 mM HEPES (pH 8.0), 200 µM $FeSO_4$, 2 mM 2OG (2-oxoglutarate/α-ketoglutarate), and 2 mM ascorbate and incubate at 37 °C for 1 h[21].
   2. Quench TET2 catalyzed oxidation reactions with 5 µL of 500 mM EDTA.
2. After quenching TET2 reactions, prepare samples for LC-MS/MS analysis by separating the DNA from TET2 reaction mixture using oligo purification columns.
   1. Add 100 µL of oligo binding buffer to 55 µL of quenched reaction.
   2. Following this, add 400 µL of 100% ethanol to the mixture. Pass this mixture through an oligo binding column.
   3. Wash bound DNA with 750 µL wash buffer and elute in 20 µL water.
3. Digest the isolated DNA with 2 units of DNase I and 60 units of S1 nuclease at 37 °C for 12 h to produce individual nucleoside-monophosphates.
4. Following digestion, add 2 units of calf intestinal alkaline phosphatase (CIAP) in the samples and incubate for addition 12 h at 37 °C to remove the terminal phosphate groups from nucleoside-monophosphates to obtain nucleosides.
5. Quantify all nucleosides, especially modified cytosines, using the LC-MS/MS method described below.

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3. Quantitative LC-MS/MS-based Assay Development

1. Prepare 100 µM stock solution of all modified cytosine nucleosides [5-methyl-2′-deoxycytidine (5mdC), 5-hydroxymethyl-2′-deoxycytidine (5hmdC), 5-formyl-2′-deoxycytidine (5fdC), and 5-carboxy-2′-deoxycytidine (5cadC)] and normal DNA bases (adenine, thymine, cytosine, and guanine) in HPLC-grade water for the development of the LC-MS/MS method.
2. Optimize nucleoside-dependent MS/MS parameters by infusing stock solutions, one at a time, in mass spectrometer at a flow rate of 10 µL/min in EMS scan mode. Optimize following parameters: Declustering Potential (DP), Entrance Potential (EP), Collision Cell Entrance Potential (CEP), Collision Energy (CE), and Collision Cell Exit Potential (CXP) for each DNA nucleoside using automated quantitative optimization feature of the software.
3. Optimize source-dependent MS/MS parameters by injecting 10 µL of stock solution using a gradient with 25% solvent B at a flow rate of 0.3 mL/min where solvent A is 10 mM ammonium acetate (pH 4.0) and solvent B is 20% acetonitrile with 10 mM ammonium acetate (pH 4.0). Optimize following parameters: Curtain Gas (CUR): 10-50, Temperature: 0-600 °C, Gas Flow 1 (GS1): 0-50, Gas Flow 2 (GS2): 0-50, Collisionally Activated Dissociation (CAD): Low-Medium-High, Ion spray Voltage (IS): 4000-5500 for each DNA nucleoside using manual quantitative optimization feature of the software in FIA (Flow Injection Analysis) mode.
4. To separate all eight DNA nucleosides, perform liquid chromatography using the following gradient: 0% solvent B (0-2 min), 0-20% solvent B (2-5 min), 20-60% solvent B (5-9 min), 60-0% solvent B (9-10 min) and then equilibrate with solvent A for 5 min at a flow rate of 0.3 mL/min on a C18 column (Particle Size: 5 µm, Pore Size: 120 Å).
5. Using the optimum MS/MS parameters (**step 3.2 and 3.3**) coupled with the above-mentioned liquid chromatography gradient (**step 3.4**), determine the response linearity, limit of detection (LOD), and lower limit of quantification (LLOQ) using a two-fold serial dilution of a 100 µM standard mixture containing all eight nucleosides. Draw standard curves for all eight DNA nucleosides.
6. Detect and quantify all nucleosides, especially modified cytosines, produced in **step 2.4** using the LC-MS/MS method and standard curves.

Representative Results

Dynamic modification of 5mC in DNA by TET-family dioxygenases plays important roles in epigenetic transcriptional regulations. TET2 dioxygenase is frequently mutated in diverse hematopoietic malignancies[12]. To investigate the role of the TET2 enzyme in normal development and disease, we have cloned its minimal catalytically active domain without any affinity tag into the pDEST14 vector[22]. The untagged TET2 dioxygenase was produced at #5% of the total soluble protein by SDS-PAGE analysis in bacterial *E. coli* BL21 (DE3) cells. Since the catalytic domain of TET2 has a relatively high isoelectric point (#7.49), compared with most indigenous *E. coli* proteins[24,25,26], an efficient purification process utilizing a cation exchange chromatography was developed. This purification yielded >90% pure TET2 enzyme in a single step (**Figure 1**).

In order to separate and quantify different deoxycytidines derivatives and other four natural DNA bases following the TET2 enzymatic reaction, a sensitive LC-MS/MS-based assay was optimized. The liquid chromatography used a reversed-phase C18 columns. Standard curves were drawn using serial dilutions of a mixture containing all nucleosides (**Figure 2**). The gradient used for liquid chromatography, described in the experimental procedure, was able to resolve all eight nucleosides (**Figure 3**). The LC retention times (t_r) for all eight nucleosides are described in **Table 3**. We further optimized the MS detection of each parent ion nucleoside (Q1), the most intense product ion (Q3) by determining their declustering potential (DP), entrance potential (EP), collision cell entrance potential (CEP), collision energy (CE), limit of detection (LOD), and lower limit of quantification (LLOQ) (**Table 3**). Finally, an LC-MS/MS method was developed that can separate and quantify the four normal DNA bases (A, T, G, and C), as well as the four modified cytosine bases (5-methyl, 5-hydroxymethyl, 5-formyl, and 5-carboxyl) (**Figure 3**).

The activity of untagged TET2 dioxygenase was determined using a 25-mer dsDNA containing one 5mC in a CpG island in each DNA strand (**Table 2**). After TET2 enzymatic reactions, DNA oligonucleotides were purified and converted into nucleosides. Then these nucleosides were subjected to LC-MS/MS assay. In the reactions without the TET2 enzyme (negative control), only dA, dT, dG, dC, and 5mdC peaks were observed. However, in the positive control reaction, which contained the TET2 dioxygenase, two new peaks corresponding to d5hmC and d5fC were observed. We were not able to detect the formation of d5caC nucleoside possibly due to its poor detection levels (**Figure 2**). These results demonstrate that the untagged TET2 dioxygenase purified in this procedure is catalytically active and can be used to characterize the wt-TET2 enzyme and its clinical mutants.

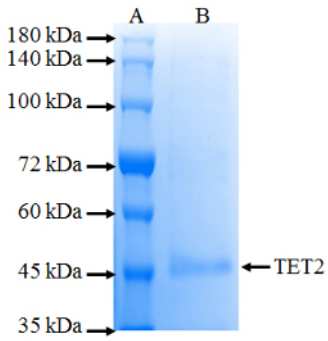

Figure 1. SDS-PAGE analysis of purified TET2 dioxygenase from *E. coli* BL21 (DE3) cells. Lane A indicates marker while lane B indicates TET2 protein purified using SP sepharose ion exchange resin. The total size of the untagged TET2 dioxygenase is #54 kDa as indicated by the arrow. Please click here to view a larger version of this figure.

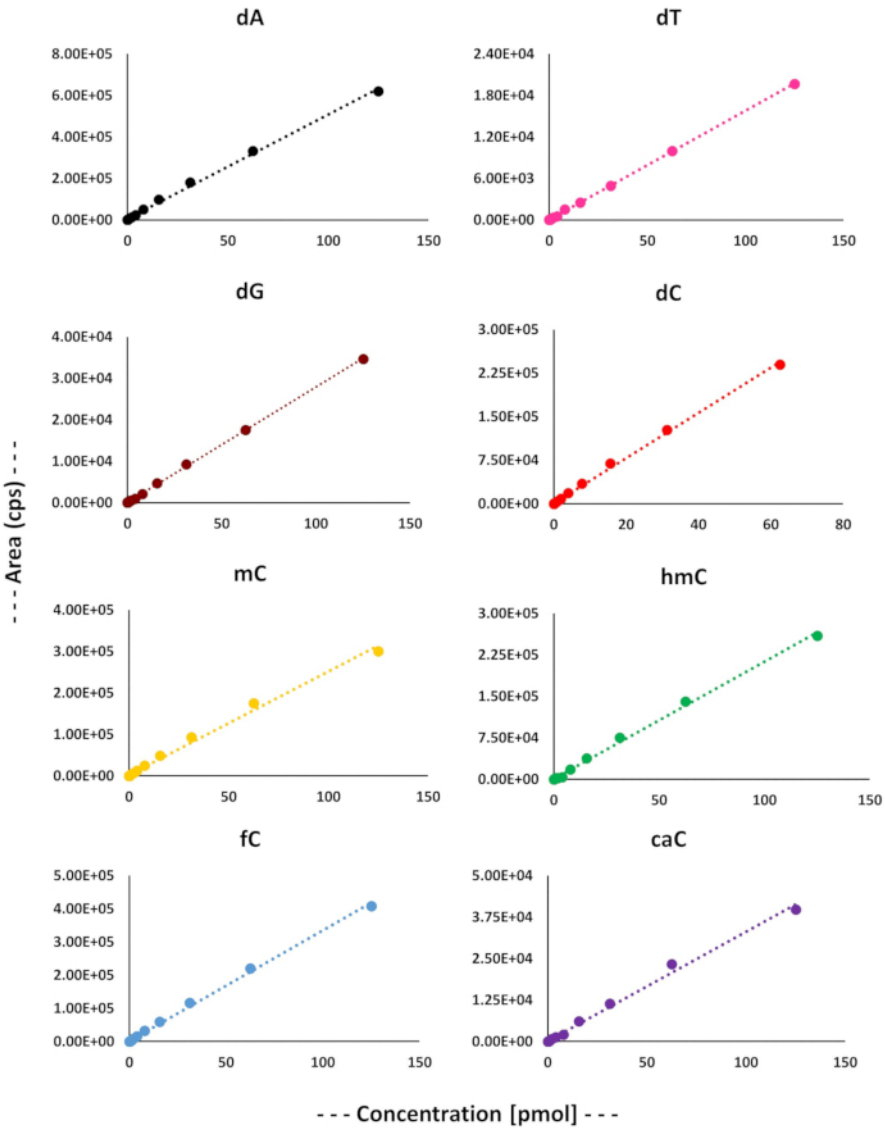

Figure 2. Standard curves was draw for four natural DNA nucleosides and different cytosine derivatives, which were then used for their quantification. Please click here to view a larger version of this figure.

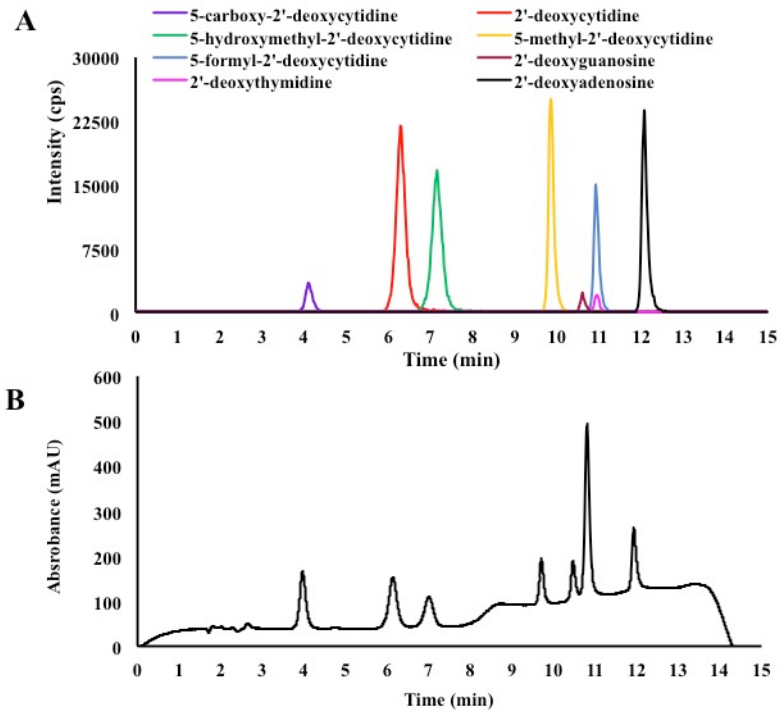

Figure 3. Liquid chromatography (bottom) and MS/MS (above) method used to separate and characterize four natural DNA nucleosides and different cytosine derivatives.

Primer Name	Primer Sequence
TET2 forward primer	5'-GGGGACAAGTTTGTACAAAAAAGCAGGCTTCGAAGGAGATAGAACCATGTCTGTTCTCAATAATTTTATAG-3'
TET2 Reverse Primer	5'-GGGGACCACTTTGTACAAGAAAGCTGGGTCTCAGCCATACTTTTCACAC-3'

Table 1: Sequence of DNA oligonucleotide primers used for PCR amplification of the catalytic domain of untagged human TET2 dioxygenase.

Primer Name	Primer Sequence
Sense Strand	5'-AGCCCGCGCCG/iMe-dC/GCCGGTCGAGCGG-3'
Antisense Strand	5'-CCGCTCGACCGGCG/iMe-dC/GGCGCGGGCT-3'

Table 2: Sequence of the sense and anti-sense 25-mer dsDNA oligonucleotide used as a TET2 substrate for *in vitro* oxidation reactions.

Nucleosides	Q1	Q3	t_r (min)	DP (V)	EP (V)	CEP (V)	CE (V)	LOD (pmol)	LLOQ	R^2
2'-deoxyadenosine	252.2	136.1	12.07	41	9	14	17	0.06	0.198	0.997
2'-deoxythymidine	243.2	117.1	10.95	16	8	14	15	1.8	5.94	0.999
2'-deoxyguanosine	268.2	152.1	10.6	21	7	14	37	7.8	25.74	0.999
2'-deoxycytidine	228.1	112.1	6.29	21	7	14	15	0.1	0.33	0.998
5-methyl-2'-deoxycytidine	242.2	126.1	9.85	31	6.5	24	13	0.03	0.1	0.998
5-hydroxymethyl-2'-deoxycytidine	258.2	142.1	7.15	16	6	14	13	0.6	1.98	0.993
5-formyl-2'-deoxycytidine	256.2	140.1	10.92	11	6	14	15	0.2	0.66	0.998
5-carboxy-2'-deoxycytidine	272.2	156.1	4.1	6	7	94	23	3.9	12.87	0.993

Table 3: Optimized LC-MS/MS parameters of four natural DNA nucleosides and different cytosine derivatives under positive ion mode. For each parent ion nucleoside (Q1), the most intense product ion (Q3) was detected.

Discussion

Mutations in TET2 gene are some of the most frequently detected genetic changes in patients with diverse hematopoietic malignancies. To date hundreds of different TET2 mutations, which include nonsense, frame-shift, and missense mutations, have been identified in patients[12]. Patients with *TET2* mutations show low levels of genomic 5hmC in the bone marrow compared to those with wt-TET2[14]. Mutant *TET2* knock-in experiments have recapitulated the effects of these mutations on 5hmC levels in transfected cells[14]. Results from TET2-knockout mouse models demonstrated that level of TET2 enzyme inversely correlated with the progression of hematopoietic malignancies[17,18,19,20]. Consistently, Zhang *et al.* recently demonstrated that down-regulation of TET2 expression levels is a potential prognostic and predictive biomarker in cytogenetically normal acute myeloid leukemia[27].

Despite growing evidence that TET2 plays a fundamental role in normal hematopoiesis and myeloid transformation, biochemical characterization of wt- and mutant TET2 remain at rudimentary stages due to difficulties associated with the production of active TET2 and its assay. Most studies have produced recombinant TET2 either using time-consuming baculovirus system in insect cells[14], or as a glutathione S-transferase affinity tag in bacterial cells which requires removal of affinity tag[21].

In this experimental procedure, we described the cloning of untagged human TET2 dioxygenase catalytic domain using a site-specific recombination technique and its efficient expression using destination vector (pDEST14) in *E. coli*. Because the isoelectric point of untagged TET2 is relatively high (#7.49) compared with most indigenous *E. coli* proteins, we developed an efficient purification process utilizing a cation exchange chromatography yielding >90% pure untagged TET2 enzyme in a single step.

Further, additional challenges exist in the quantification of wt- and mutant TET2 dioxygenase activity. For these experiments, most studies have relied on antibody-based assays such as dot-blots[14,28], enzyme-linked immunosorbent assays (ELISA)[29], *etc.* Because these assays generally use only one antibody, *e.g.,* 5hmC or 5fC or5caC, for detection of 5mC modification in substrate DNA, they do not provide a full picture of the catalytic reaction carried by TET isoforms. For these reasons, the LC-MS/MS-based assay has emerged as the only assay to quantify different cytosine modifications. In this regard, we have developed a novel liquid chromatography method that can separate the four normal DNA bases (A, T, G, and C), as well as the four modified cytosine bases (5mC, 5hmC, 5fC, and 5caC).

To quantify the eight nucleosides from TET2 catalyzed reactions, we have coupled our improved liquid chromatography method with tandem mass spectrometry. This sensitive LC-MS/MS assay was then utilized to determine the activity of recombinant untagged human TET2 enzyme. The approach described here will greatly enhance the evaluation of wt- and mutant TET2 dioxygenase activities.

Disclosures

The authors have no financial interests to declare.

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