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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 made a library of putative 2-oxoglutarate (2OG) analogs. This library is used to enhance the activity of TET2 enzymes from MDS patient. Our results shows that the activity of TET2 can be dramatically modified using 2OG analogs.

15. SUBJECT TERMS
Nothing 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). One of the fundamental causes of these diseases is the presence 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. We have developed large scale expression of TET2 dioxygenase and a convenient assay for TET2 dioxygenase, which will be utilized to determine the kinetics of wild-type and various clinical mutants of TET2 dioxygenase. Further, we made a library of putative 2-oxoglutarate analogs which will be used to rescue the activity of TET2 mutants from MDS patient. Initial results shows that the activity of TET2 can be modified using 2-oxoglutarate analogs.

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.

100% completed. Along with expression in the insect cell lines, we have developed a convenience expression of active TET2 enzymes in bacterial system, which is described later.

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.

50% completed. Delays in progress is due to development of a reliable assay. As describe below we have tried several assays and found mass spectrometry-based assay to be most reliable.

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

50% completed.

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.

Ongoing. ***Our initial 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 continuing on this extremely exciting results and hope to publish some very significant papers from this grant.

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.

2-5 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).

100% 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.

Ongoing with selected compounds.

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).

Not started yet.

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).

Not started yet.

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

In Year 3 (June, 2015 – May, 2016) we continued with our focus on enhancing the activity of TET2 enzymes found in MDS patients. Please find below a brief description of our results:

Cloning, expression and purification of TET2: TET proteins play critical role in DNA demethylation by performing iterative oxidation of 5mC, 5hmC, and 5fC. TET2 is one of the

most frequently mutated genes in MDS, MDS-MPN, and sAML. 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. 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 (Figure 1).

Using the optimized expression conditions in E. coli BL21 trxB (DE3) cells, the untagged TET2 demethylase was produced at ~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 (Figure 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 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).

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. We optimized LC retention time (tr), 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). The LOD is defined as the lowest concentration of samples required to give reliable and reproducible results with at least 300 counts/sec in intensity. 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 (Figure 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. Moreover, a comparison of untagged TET2 with GST-tagged TET2 demonstrated insignificant differences in activity. These results establish that untagged TET2 described here is equally active to tagged TET2 proteins described earlier. Further, in agreement with a recent report, we found that TET2 prefers 5mC as a substrate to 5fC and 5caC (Figure 3F). These results further establish that untagged TET2 oxygenase is very similar to the previously characterized TET2 enzyme.

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 (12% and 34%, respectively) (Figure 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 by 41%, 20%, and 60%, respectively. 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. 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 μM in the reaction buffer absence of ATP and DTT. However, results from these studies demonstrated that TET2 activity did not reach to its normal levels in the absence of ATP and DTT (Figure 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 (Figure 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 (Figure 4D). This behavior is typical of 2OG-dependent oxygenases such as phytanoyl-CoA 2-hydroxylase.

Analyses of clinical TET2 mutants: Numerous TET2 clinical mutations have been reported in the literature mainly from MDS, MDS-MPN, and sAML patients. These mutations include frame-shifts, nonsense, and missense mutations. To date, over a hundred different missense mutations have been identified with most being clustered in the oxygenase domain. 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 (Figure 5). Since the presence of the TET2 protein inhibited the detection of 5hmC (Figure 5A), 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 the commonly used dot-blot assays (Figure 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 (Figure 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 can be cured by commonly used dietary 2OG supplements.

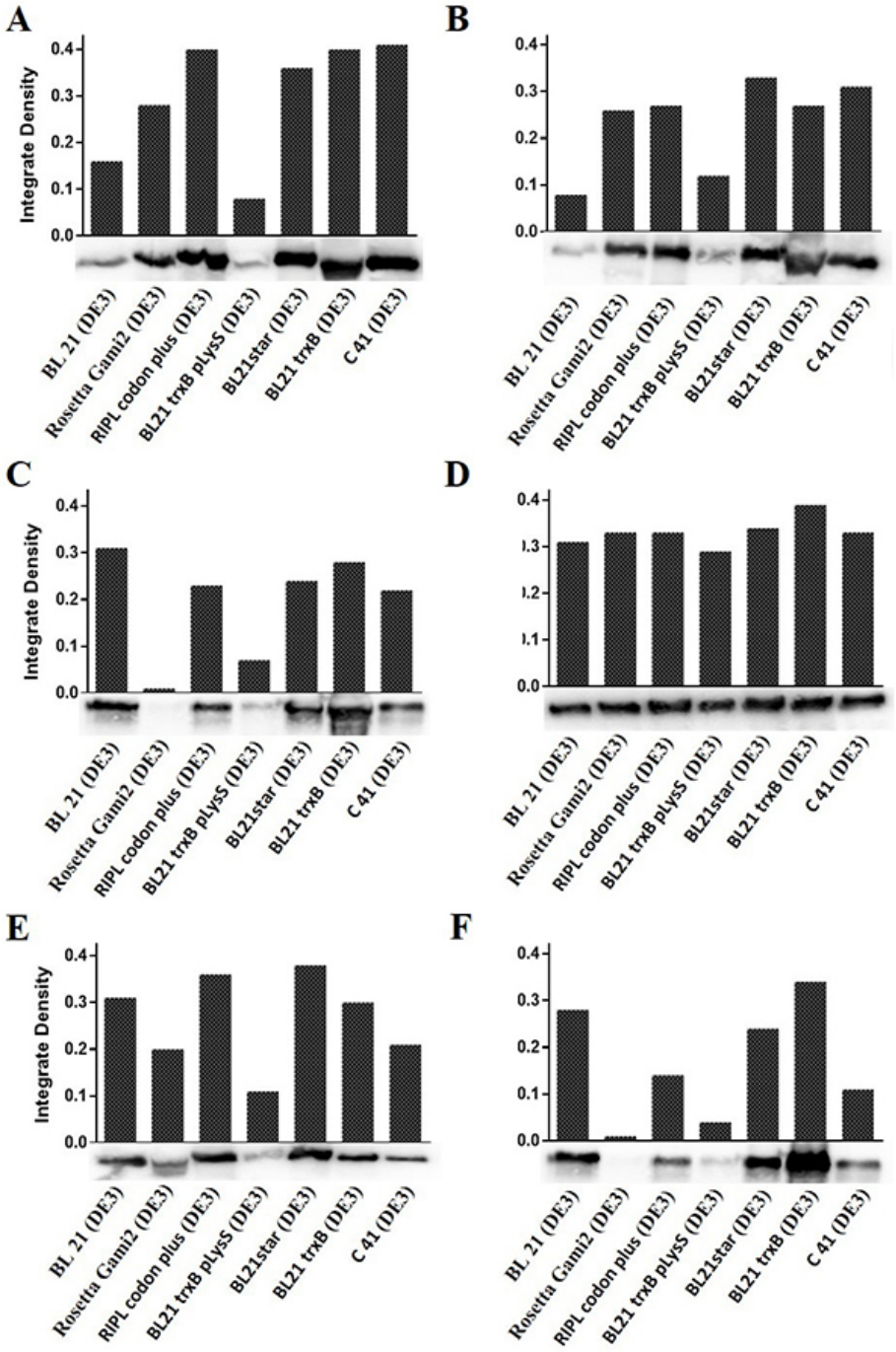

Figure 1: Western blot analysis of TET2 oxygenase expression in seven different strains of *E. coli* when induced at the indicated temperature and IPTG concentration: 16°C for 16 h with 0.5 mM IPTG (A), 27°C for 10 h with 0.5 mM IPTG (B), 37°C for 4 h with 0.5 mM IPTG (C), 16°C for 16 h with 1 mM IPTG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

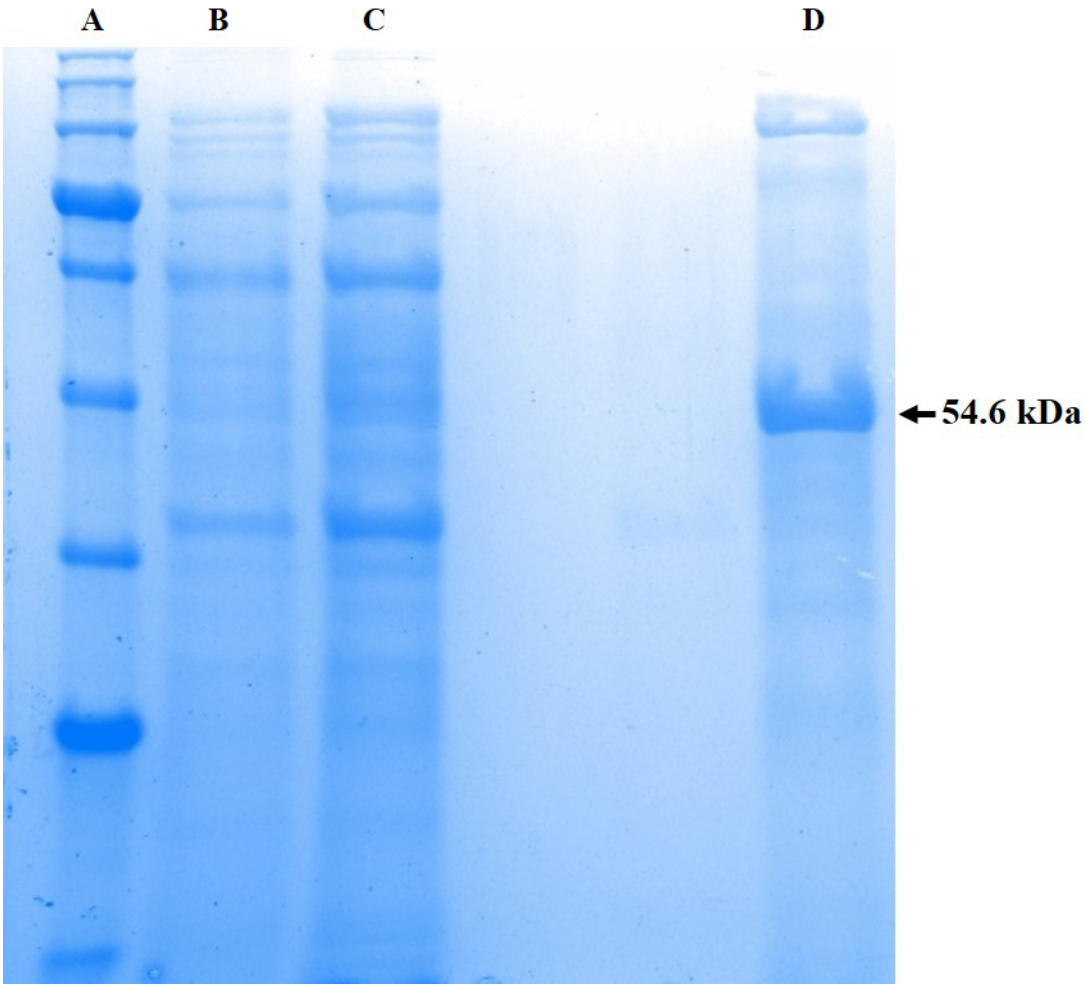

Figure 2: SDS-PAGE analysis of purified untagged TET2 demethylase from *E. coli* BL21 trxB (DE3) cells. Lane indicates: marker (A), unintroduced TET2 (B), induced TET2 protein (C), and TET2 protein purified using SP sepharose high-performance strong cation exchange resin (D). The total size of the untagged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

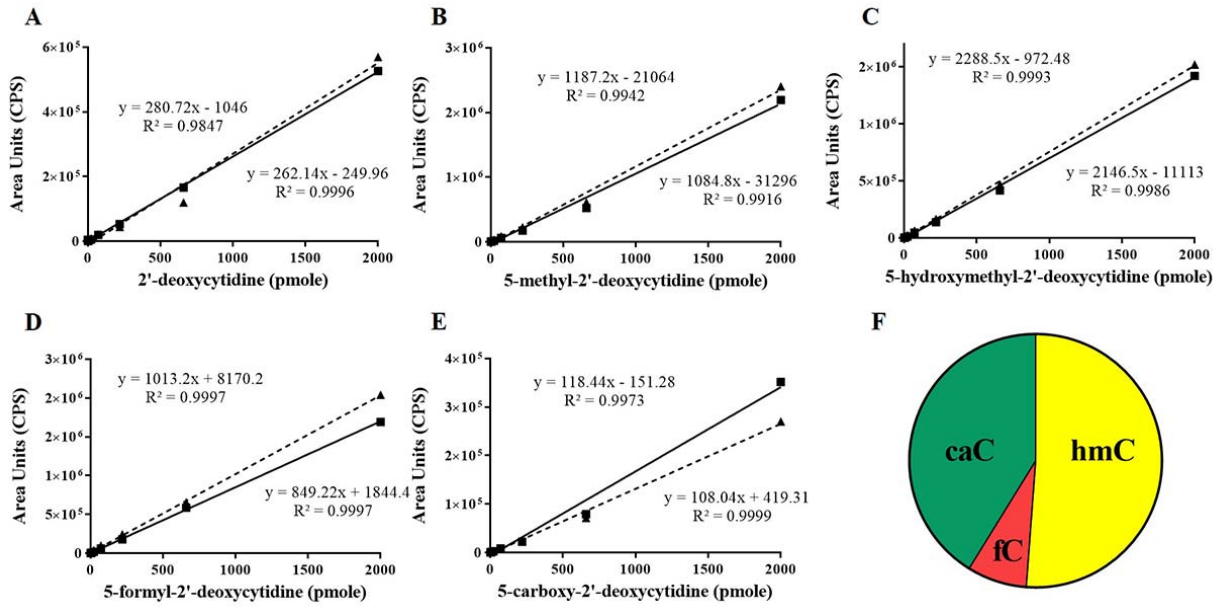

Figure 3: Standard curves of modified cytosine nucleosides dissolved in TET2 reaction conditions (straight line) or HPLC-grade water (doted line) showing insignificant effect on the quantification (A-E). A pie chart showing LC-MS/MS detection of 5hmC, 5fC, and 5caC formed after TET2 reaction (F).

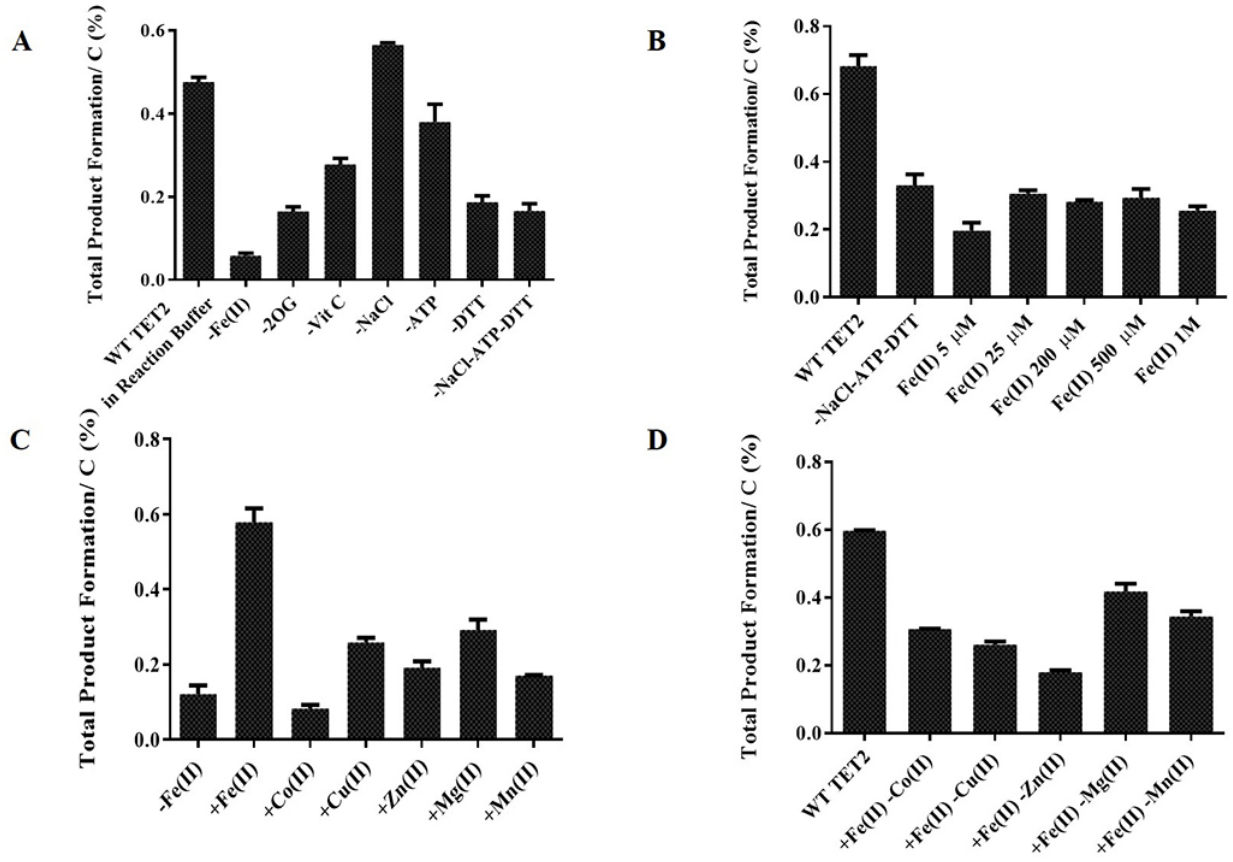

Figure 4: Characterization of the WT-TET2 demethylase. Effect of co-factors on the TET2-mediated demethylation (A). An increase in Fe(II) concentration from 75-1000 µM in the reaction buffer in the absence of ATP and DTT did not increase TET2 activity (B). Fe(II) could not be replaced by any of the alternative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

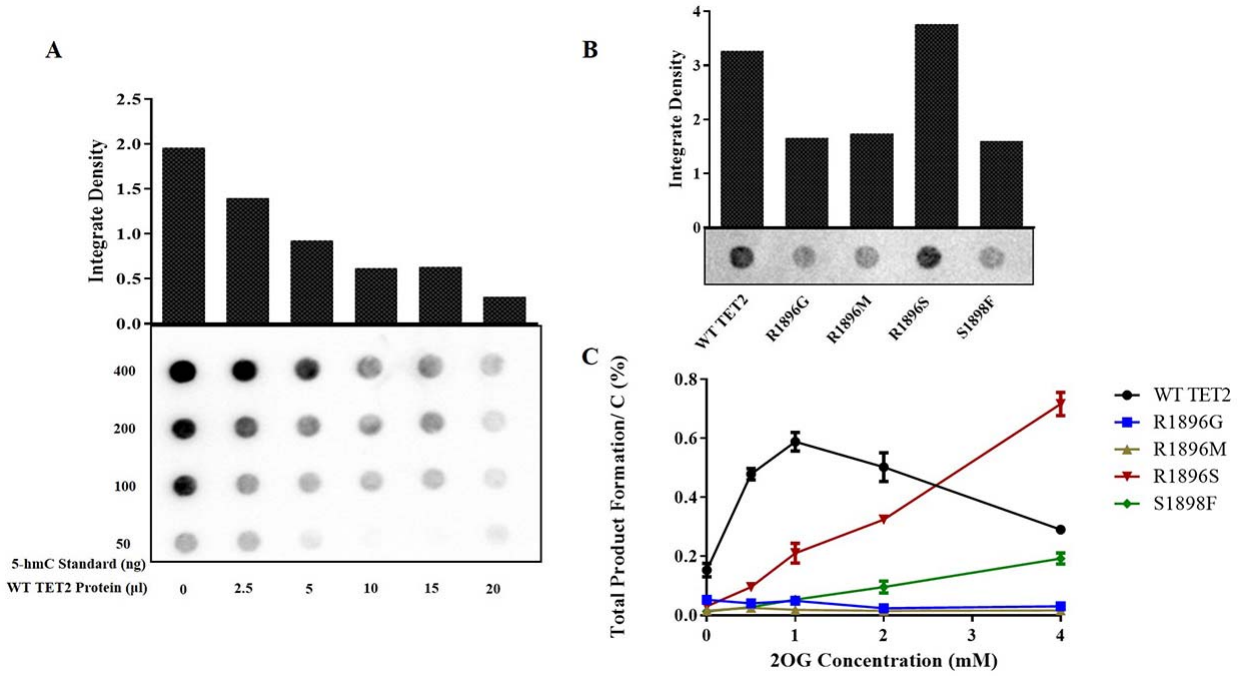

Figure 5: A comparison of the activities of wild type and some TET2 clinical mutants by dot-blot and our quantitative LC-MS/MS-based assay. The presence of the TET2 protein inhibited the detection of 5hmC (A), DNA substrate was purified using a Zymo Oligo purification columns before the blotting and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

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

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.

- **What opportunities for training and professional development has the project provided?**
  - This project has provided important opportunities to Dr Mridul Mukherji to attain conferences.
- **How were the results disseminated to communities of interest?**
  - Preliminary results were disseminated in a Protein Engineering conference-2015 in Chicago, IL. We are also writing 3 manuscripts, which would be submitted for publication soon in peer reviewed national/ international journals by the end of 2016.
- **What do you plan to do during the next reporting period to accomplish the goals?**
  - After some delays with the assay development, the project is moving forward. We are very excited with our initial findings showing that the activity of TET2 dioxygenase can be modified using 2-oxoglutarate analogs.

4. **IMPACT:**

- **What was the impact on the development of the principal discipline(s) of the project?**
  - For the very first time we have found that the activity of TET2 dioxygenase can be modulated using 2-oxoglutarate analogs.
- **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**
  - 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). However, now we are able to use ammonium salt in the LCMS methods.

    While we were waiting for the permission to use LCMS system with ammonium salt, which took over one year, we spent significant time on developing alternative TET2 assays using (i) colorimetric TET Activity Assay from Epigentek, and (ii) Dot blot. However, both these assays detect 5mhC, using an antibody, and therefore these two assays were not very reliable because they don't detect 2nd (5fmC) and 3rd (5cmC) TET2 products. Only the LCMS assay detects all three products, namely 5hmC, 5fmC, and 5cmC, of TET2 enzyme.

    Due to delays in the progress of project a 12 month no cost extension was requested which was granted by DOD.
- **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.
- **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:	Mohit Jaiswal

Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	161-29-343 (EMPLID #)
Nearest person month worked:	3
Contribution to Project:	He is working on purification and assay of TET2.
Funding Support:	

Name:	Subhradeep Bhar
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	162-02-782 (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:** Nothing to Report.