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A rapid mass spectrometric method for the measurement of catalytic activity of ten-eleven translocation enzymes

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ABSTRACT

Enzymatic methylation at carbon five on cytosine (5mC) in DNA is a hallmark of mammalian epigenetic programming and is critical to gene regulation during early embryonic development. It has recently been shown that dynamic erasure of 5mC by three members of the ten-eleven translocation (TET) family plays a key role in cellular differentiation. TET enzymes belong to Fe (II)- and 2-ketoglutarate (2KG) dependent dioxygenases that successively oxidize 5mC to 5-hydroxymethyl cytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxycytosine (5CaC), thus providing a chemical basis for the removal of 5mC which once was thought to be a permanent mark in mammalian genome. Since then a wide range of biochemical assays have been developed to characterize TET activity. Majority of these methods require multi-step processing to detect and quantify the TET-mediated oxidized products. In this study, we have developed a MALDI mass spectrometry based method that directly measures the TET activity with high sensitivity while eliminating the need for any intermediate processing steps. We applied this method to the measurement of enzymatic activity of TET2 and 3, Michaleis-Menten parameters (K_M and k_{cat}) of TET-2KG pairs and inhibitory concentration (IC_{50}) of known small-molecule inhibitors of TETs. We further demonstrated the suitability of the assay to analyze chemoenzymatic labeling of 5hmC by β-glucosyltransferase, highlighting the potential for broad application of our method in deconvoluting the functions of novel DNA demethylases.

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Introduction

Chemical modifications on DNA and histones are primary epigenetic mechanisms that play critical role in gene regulation [1]. Notable example includes 5-methylcytosine (5mC) in DNA, and acetylation and methylation of lysine ε-amine in the unstructured tail of nucleosomal histones [2]. Ten-Eleven Translocation (TET)-mediated successive oxidation of 5mC to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC) and 5-carboxylcytosine (5CaC) is considered a landmark finding as it provides a biochemical basis for active DNA demethylation and is essential to early mammalian development (Fig. 1A) [3–5]. Humans have three TET isoforms (TET 1, 2 and 3), all of which are members of the Fe (II) and 2-ketoglutaric acid **1** (2KG)-dependent dioxygenases, a superfamily that is present in all forms of life (Fig. 1B) [6]. TET-mediated oxidation of 5mC is critical to cellular differentiation [7]. Growing evidence also suggests that each of the TET oxidized products (5hmC, 5fC and 5CaC) can independently regulate gene expression, aside from playing a role as intermediates in the demethylation pathway [8–10]. Overexpression and catalytically inactive mutations of TET enzymes are involved in the development of certain cancers as well [11].

Efforts to understand the functions of TET proteins in eukaryotic gene regulation have fueled the development of novel analytical methods to detect and quantify the enzymatic activity of TETs as well as their oxidized products both *in vitro* and *in vivo* [12,13]. Several chemical approaches coupled with next-generation sequencing techniques have been developed for base-resolution mapping of TET products in cell- and tissue-specific manners [14,15]. Such methods have led to a detailed understanding of TET mediated processes and their relevance in gene regulation. In parallel, efforts are underway for the biochemical and structural characterization of TET proteins *in vitro* [16,17]. Representative methods for the analysis of modified nucleotides are (1) one- and two-dimensional thin-layer chromatography (1D- and 2D-TLC), (2) tandem liquid chromatography and mass spectrometry (LC-MS/MS), (3) Western blotting with cytosine modification-specific

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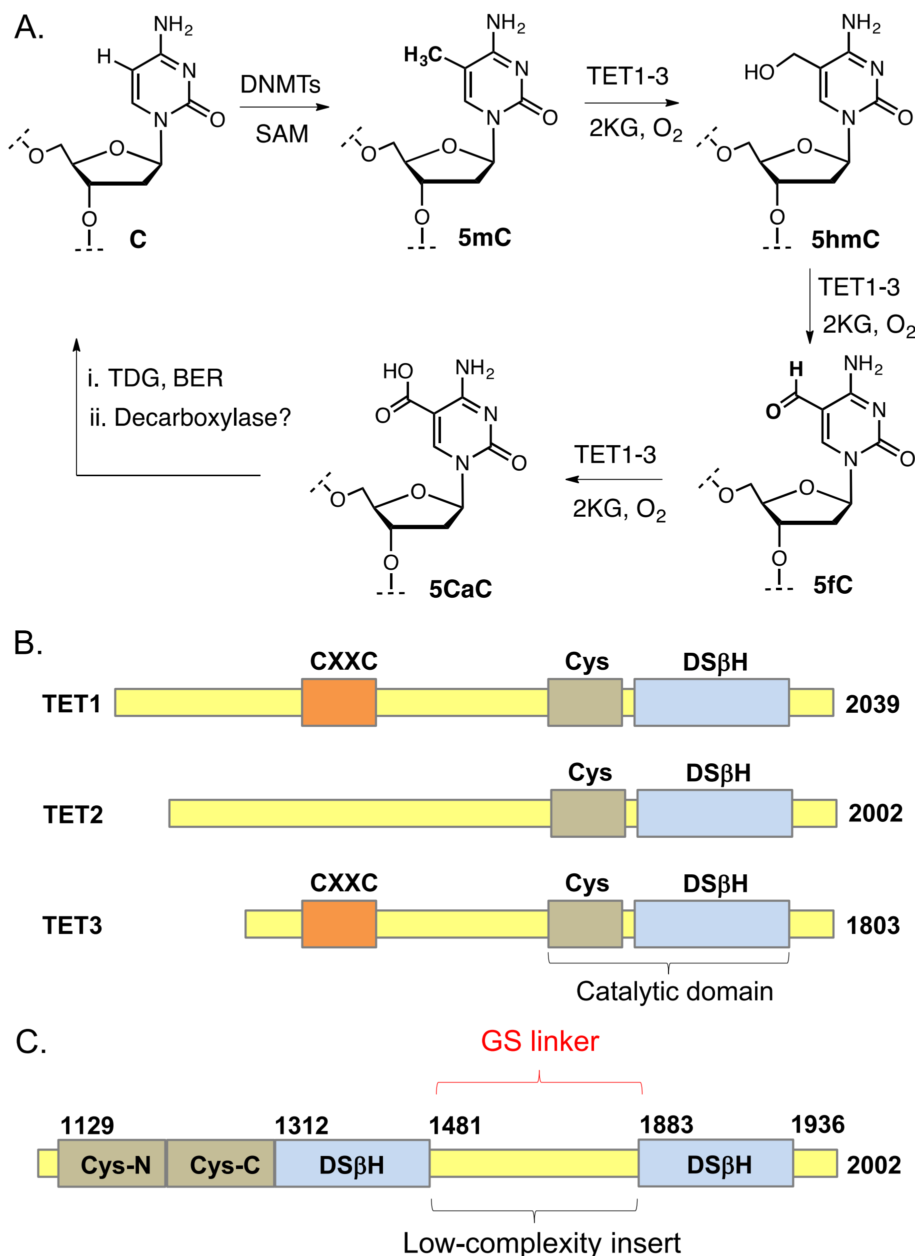

Fig. 1. Ten-eleven translocation (TET) enzymes and their biochemistry. (A) Successive oxidation of 5mC (generated by DNMTs and SAM) to 5hmC, 5fC and 5CaC by TET enzymes and 2KG leads to active DNA demethylation. Decarboxylation of 5CaC to C is achieved primarily by Thymine DNA glycosylase (TDG) followed by base excision repair (BER) mechanism. Putative decarboxylase may also be involved in direct removal of the carboxylic group. (B) Domain structures of TET1-3 showing double stranded β helix (DSβH) fold, preceding cysteine (Cys)-rich domain and a CXXC domain in TET1 and 3. (C) Colored-coded domain structure of catalytic portion of the human TET2 enzyme. A low-complexity insert (aa 1481-1843) was replaced with a GS linker of 15 amino acids.

antibodies, and (4) chemoenzymatic labeling of the modified cytosines particularly 5hmC [13]. Novel methods are also being developed for strand-specific quantitative analysis of TET activity [13].

A common assay to characterize TET activity involves LC-MS/MS analysis of the oxidized oligonucleotide products. Following enzymatic oxidation, oligonucleotide product(s) are purified using nucleotide purification kits and subjected to enzymatic degradation to individual nucleotides prior to mass spectrometric assignment. Most of the current approaches involve multiple enzymatic reactions and purification steps that invariably lead to the loss of oxidized products 5hmC, 5fC and 5CaC) and errors in quantification. Herein we report the development and optimization of a Matrix-assisted laser desorption/ionization (MALDI) based mass spectrometric assay that facilitates direct and efficient measurement of enzymatic activity of TET and eliminates the need for subsequent enzymatic digestion and purification of the oxidized oligonucleotide products. Employing the optimized conditions, we further determine the kinetic constants of TET enzymes and IC_{50} of the selected small-molecule inhibitors of TETs. Finally, we demonstrate the utility of the assay to directly examine the subsequent chemoenzymatic functionalization of TET enzymatic products particularly 5hmC.

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