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Catalytic Space Engineering as a Strategy to Activate C−H Oxidation on 5-Methylcytosine in Mammalian Genome

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ABSTRACT: Conditional remodeling of enzyme catalysis is a formidable challenge in protein engineering. Herein, we have undertaken a unique active site engineering tactic to command catalytic outcomes. With ten−eleven translocation (TET) enzyme as a paradigm, we show that variants with an expanded active site significantly enhance multistep C−H oxidation in 5-methylcytosine (5mC), whereas a crowded cavity leads to a single-step catalytic apparatus. We further identify an evolutionarily conserved residue in the TET family with a remarkable catalysis-directing ability. The activating variant demonstrated its prowess to oxidize 5mC in chromosomal DNA for potentiating expression of genes including tumor suppressors.

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Enzymes that modify postreplicative genetic material are crucial for safeguarding and accessing the information encoded therein. DNA methyltransferase, which methylates carbon 5 on cytosine to form 5-methylcytosine (5mC) to drive mammalian gene expression, is a prime example.[1] 5mC has been shown to undergo iterative C−H oxidation by the ten−eleven translocation (TET) enzymes to 5-hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) in the presence of 2-ketoglutarate (2KG). Subsequently, 5fC and 5caC are both excised by thymine DNA glycosylase (TDG) for repair, constituting an intriguing mechanism for DNA demethylation (Figure 1A).[2−6] Due to their distinct stereoelectronic features, the 5mC oxidative intermediates ($5mC_{ox}$) interact with a range of chromatin-associated proteins and regulate gene expression.[7−9]

Much is unknown about the context-dependent functions of the intermediates as demethylation mediators and transcriptional regulators. Assigning a distinct and compensatory role to $5mC_{ox}$ species, particularly 5fC and 5caC, in mammalian physiology has remained a challenge due to their intrinsic low abundance and fleeting existence. Limited efforts to modulate $5mC_{ox}$ distribution in DNA include ascorbic acid mediated TET activation.[10] Ascorbic acid, however, activates several 2KG-dependent dioxygenases, obscuring its exclusive role in controlling TET activity. Other approaches focus on conditional reconstitution of wild-type TET2, which are less likely to regulate the $5mC_{ox}$ distribution.[11,12]

We envisioned an active site remodeling tactic to stimulate C−H oxidation for enriching the human genome with the low-abundant intermediates for functional analysis. In TET-mediated C−H oxidation, hydrogen abstraction by oxo-ferryl species is the rate-limiting step with a relative activation barrier following the order 5fC > 5hmC > 5mC (Figure 1B).[13] Computational analyses indicate that restrained conformations of 5hmC and 5fC within the active site likely prevent the abstractable hydrogens from adopting a favorable orientation for C−H oxidation; in contrast, all the rotational conformers of 5mC lead to productive hydrogen abstraction.[14−16] We

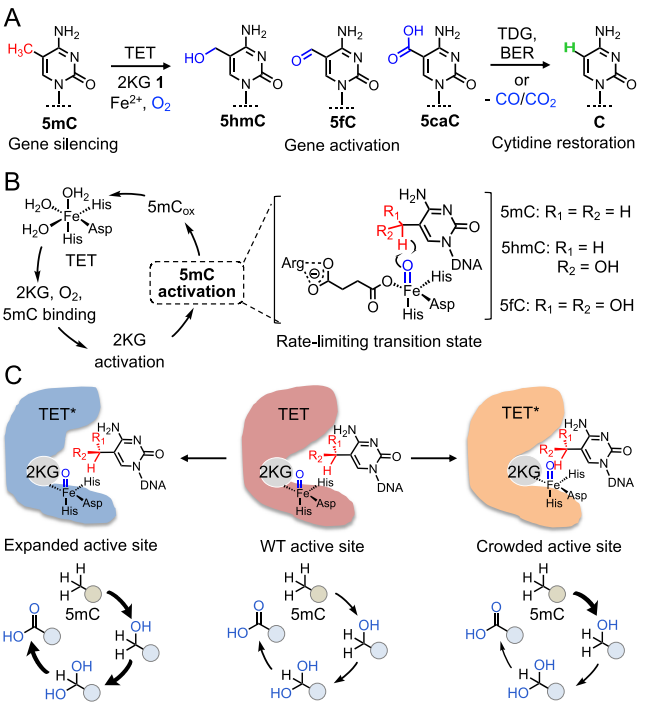

Figure 1. Catalytic cycle of TET-mediated 5mC oxidation. (A) Cytidine demethylation by TET and TDG. (B) In a catalytic cycle, proton abstraction by $Fe^{IV}(O)$ constitutes the rate-determining transition state. (C) Engineering active site space of TET enzymes to control the $5mC_{ox}$ distribution in DNA.

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reasoned that systematic alteration of the stereoelectronic component at the TET−2KG−5mC interface could modulate the transition state barrier toward a more controlled product distribution (Figure 1C).

To test our hypothesis, we generated a panel of TET2 mutants by replacing several hydrophobic and polar residues in the active site with alanine, one at a time (Figure 2A).[17]

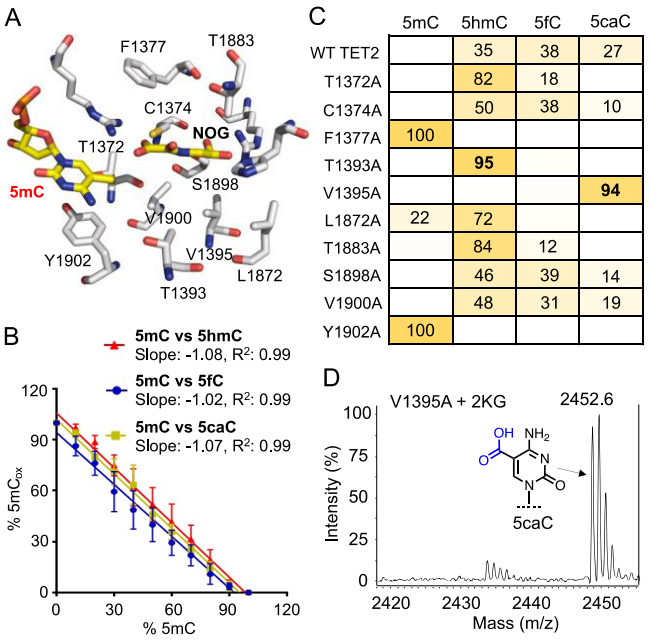

Figure 2. Active site engineering toward novel TET2 variants. (A) Structure of TET2 bound with 5mC DNA (PDB: 4NM6).[17] (B) Percent intensities of MALDI-MS signals for a range of mixtures of 5mC DNA with either 5hmC, 5fC, or 5caC. (C) Heat-map representation of % $5mC_{ox}$ furnished by TET2 mutants. (D) MALDI-MS spectrum showing activating effect of V1395A to generate 5caC.

Employing a MALDI-MS-based assay, we examined the oxygenase activity of these mutants toward a short dsDNA substrate, CAC5mCGGTG.[18,19] To analyze the product distribution in a quantitative manner, we first examined if the DNA segments carrying various $5mC_{ox}$ intermediates displayed comparable ionization potentials. For this, we synthesized three additional DNAs (CACXGGTG; X = 5hmC, 5fC, and 5caC) (Figure S1, Schemes S1−3). 5mC DNA was mixed with each synthesized $5mC_{ox}$ DNA separately in different ratios and subjected to MALDI-MS. Peak intensity of the individual DNA reflected their known ratio in the mixture, indicating equivalent degrees of ionization in MALDI analyses (Figures 2B, S2−S4).

In an *in vitro* assay, wild-type TET2 converted 5mC DNA to 5hmC, 5fC, and 5caC. In contrast, the mutants oxidized substrate DNA with varied degrees of specificity (Figures 2C, S5). T1372A, T1393A, and T1883A mutants showed remarkable specificity for generating primarily 5hmC. It has been shown that a H-bonding network involving T1372 is critical for rotating 5hmC to switch from product to substrate orientation for further oxidation; lack of such interaction in T1372A locks 5hmC in the product conformation.[20] We suspect a similar mechanism is operating for T1393A and T1883A mutants as well to impart the degree specificity in C−H oxidation. Among the hydrophobic residues screened, the most notable difference was observed for the V1395A mutant, as it generated 5caC as the dominant product (>90%) (Figure 2C,D). To the best of our knowledge, such an activating TET mutant is yet to be reported. Collectively, our systematic screening experiment identified TET2 variants that could significantly modulate $5mC_{ox}$ distribution by either stalling the oxidation pathway at the first step to exclusively generate 5hmC or accelerating to the fully oxidized product. The V1395A mutant is particularly important given that 5caC is rare in the mammalian genome.

We further analyzed 5caC formation using base-resolution sequencing. It has been shown that 5caC is deaminated upon treatment with bisulfite and read as T during sequencing (Figure 3A).[21] In contrast, 5mC and 5hmC remain as C due to

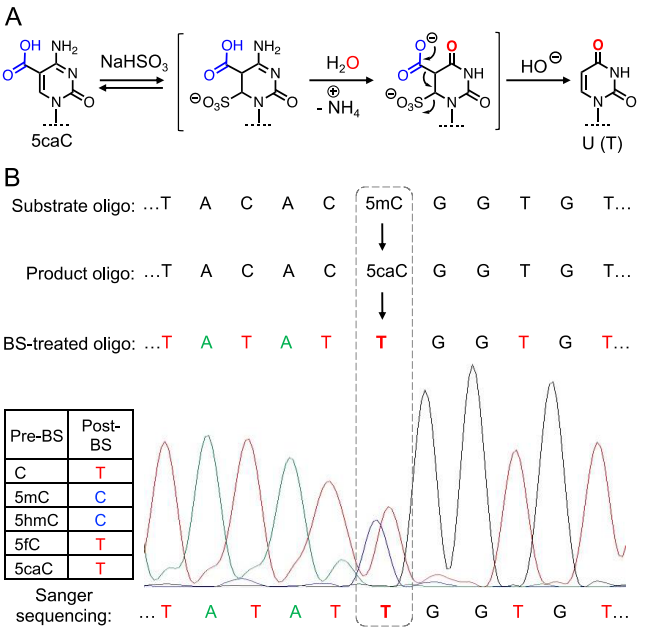

Figure 3. Analysis of $5mC_{ox}$ using Sanger sequencing. (A) Scheme showing bisulfite-mediated 5caC deamination and decarboxylation. (B) V1395A-catalyzed 5mC oxidation followed by bisulfite treatment and PCR, confirming 5caC as the major product based on Sanger sequencing data. BS = bisulfite.

their inertness toward the reagent. We subjected a duplex 76-mer DNA carrying a central 5mCpG unit to TET-mediated oxidation followed by bisulfite treatment, PCR amplification, and Sanger sequencing. While 5mC in samples exposed to either no protein or wild-type TET2 read as C, the equivalent site in the V1395A-treated sample emerged as T (Figures 3B, S6). These results demonstrate the ability of the mutant to predominantly generate 5caC.

We next compared the activity of V1395A with that of wild-type TET2 on three individual substrates (Figures 4A−C, S7−S12). With respect to the wild-type enzyme, V1395A led to a significantly higher production of 5hmC from 5mC, while both enzymes showed comparable activity on 5hmC DNA. The mutant displayed further improved activity on 5fC leading to higher 5caC formation, demonstrating that it is indeed acting as a superior dioxygenase in two discrete cycles involving conversions of 5mC to 5hmC and 5fC to 5caC in a three-step catalytic process. To further gauge the relative activity, we measured catalytic efficiencies of V1395A and wild-type TET2 on 5mC with varying concentrations of 2KG (Figure S13).

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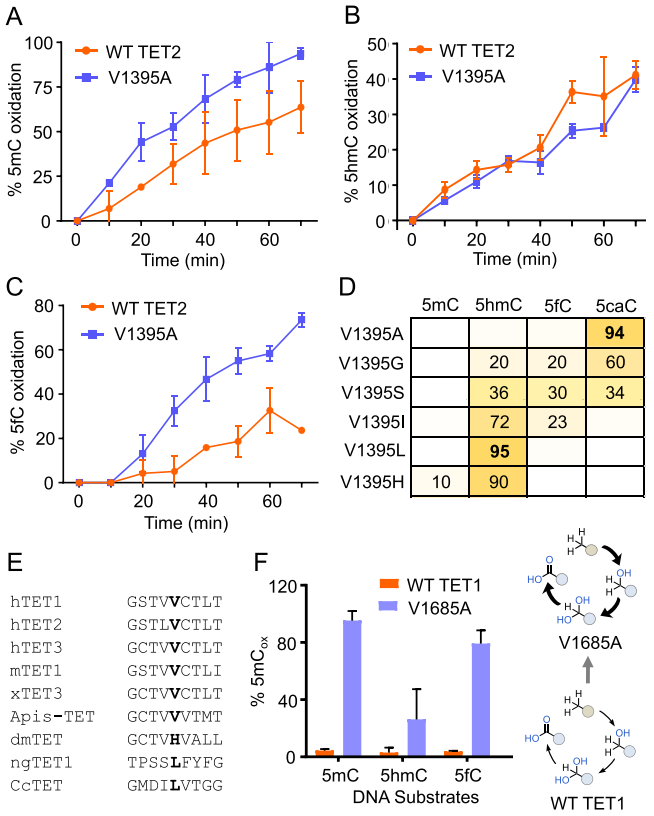

Figure 4. Characterization of the TET variants. (A–C) Time-dependent activity of wild-type TET2 and V1395A toward 5mC (A), 5hmC (B), and 5fC (C) substrate DNAs. (D) Heat-map representation of % $5mC_{ox}$ generated by V1395A congeners. (E) Sequence alignments of eukaryotic TETs highlighting the gatekeeper residue. (F) Activity of wild-type TET1 and the V1685A mutant on substrate DNAs.

While the mutant had improved turnover (k_{cat}), both enzymes showed comparable K_M values, ruling out the possibility that enhanced activity of V1395A could be from improved cofactor binding.

To further examine the influence of active site space on product distribution, we generated a panel of V1395 mutants differing in amino acid side chains (Figures 4D, S14). As expected, the glycine variant generated 5caC as the major product. Remarkably, the mutants with a crowded active site furnished primarily 5hmC, demonstrating a clear relationship between catalytic pocket size and $5mC_{ox}$ distribution. This switch from three-step catalysis (5mC → 5hmC → 5fC → 5caC) by V1395A to just one (5mC → 5hmC) by its congeners thus must be a result of crowding the active site.

Intrigued by the unique catalysis-directing feature of V1395 in TET2, we analyzed the sequences of representative TET homologues and found that a bulky hydrophobic residue at equivalent sites is strictly conserved among eukaryotes, indicating its gatekeeper-like role in controlling $5mC_{ox}$ derivatives (Figure 4E).[22−26] We further noted that the space-creating mutation is absent in human genome variants (Figure S15, Table S1). To access a similar activating variant of human TET1, we generated a V1685A mutation that indeed showed remarkably higher enzymatic activity compared to wild-type TET1 (Figures 4F, S16–S18). Our work thus identifies a conserved gatekeeper residue in eukaryotic TETs that can be engineered to regulate $5mC_{ox}$ content in DNA.

Such mutants are valuable for probing member-specific activity of TET1–3 with identical catalytic pockets but distinct expression patterns and domain organizations.[27]

We next examined the ability of the V1395A mutant to oxidize 5mC on genomic DNA isolated from HEK293T cells. The product distribution was analyzed in a dot-blot assay using $5mC_{ox}$-specific antibodies. The mutant furnished 5fC and 5caC more than 3-fold in excess compared to wild type, a result consistent with the variant having a higher enzymatic activity (Figures 5A, S19). It is notable that the variant with an

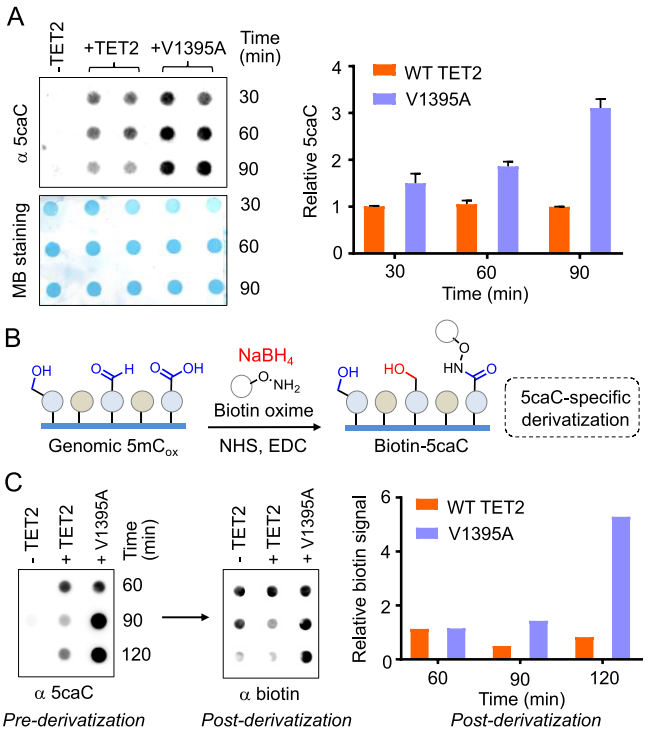

Figure 5. Activity of TET2 and V1395A on genomic DNA. (A) Dot-blot assay showing time-dependent increase in 5caC by V1395A. The bar diagram provides relative fold changes in 5caC. (B) Chemical strategy to selectively derivatize 5caC. (C) Dot-blot assay with 5aC and biotin antibodies before and after derivatization, respectively. Bar representation of biotinylated 5caC on genomic DNA. Nonlinearity in biotin signal is likely due to multistep functionalization of 5caC.

expanded active site is capable of generating an excess of 5caC on the entire human DNA carrying millions of 5mC moieties. Remarkably, V1685A also enhanced $5mC_{ox}$ content on genomic DNA in comparison to wild-type TET1, consistent with the activating nature of the expanded catalytic site (Figure S20).

This result prompted us to apply the TET2 variant in combination with a chemo-functionalization strategy to enrich 5caC-containing DNA (Figure 5B).[21,28] To selectively derivatize 5caC, the 5fC group in TET-treated DNA was reduced using $NaBH_4$ followed by coupling with biotinylated hydroxylamine on an activated carboxylate substrate. Subsequently, the samples were analyzed in a dot-blot assay using 5caC- and biotin-specific antibodies to gauge pre- and postfunctionalized 5caC (Figure 5C). Consistent with the mutant's enhanced prowess to generate 5caC, we observed a higher signal of biotinylated 5caC for the mutant over wild-type TET2, implying that the activating variant can be

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exploited for improved isolation of the low-abundant intermediates.

To examine if the TET2 variant could activate 5mC oxidation on the DNA within human cells, we expressed the catalytic domain of wild-type TET2 or the mutant in HEK293T cells (Figure 6A). Dot-blot analysis of the isolated

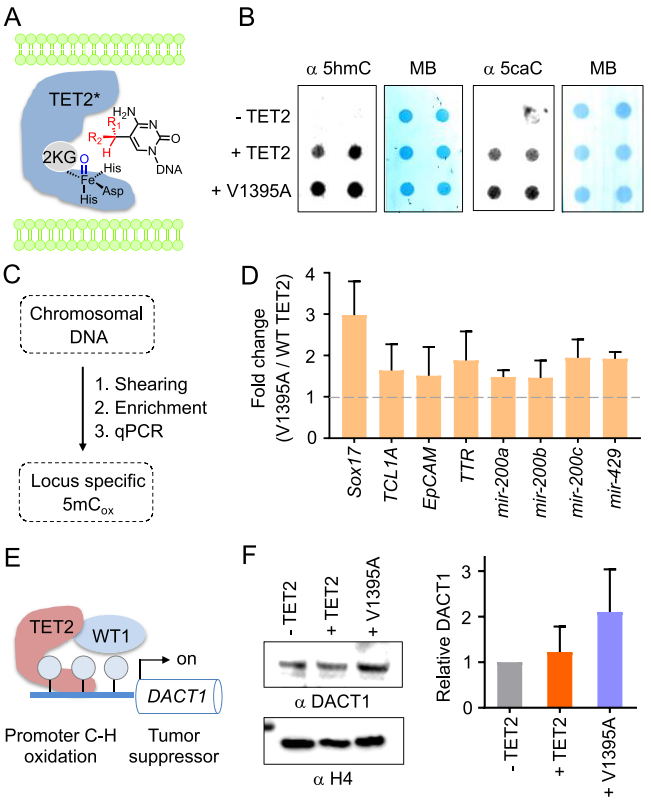

Figure 6. *In cellulo* activity of TET2 variant. (A) Schematic displaying activity of V1395A on chromosomal DNA. (B) Dot-blots confirming higher level of 5hmC and 5caC generated by the mutant. (C) Steps involved in DIP-qPCR. (D) Fold change (mutant/wild-type) in enrichment of selected genes using 5caC antibody. (E) TET2 interacts with WT1 to regulate DACT1. (F) Expression of DACT1 is enhanced by V1395A. MB = methylene blue.

DNA revealed the presence of ~2-fold higher 5hmC and 5caC DNA in V1395A-expressing cells compared to those expressing the wild-type enzyme (Figures 6B, S21), demonstrating that the activating effect of V1395A is well preserved in the complex environment of mammalian cells.

We then assessed the ability of V1395A to oxidize 5mC at individual promoters. We selected a panel of eight genes including *microRNA-200* and *EPCAM*, as TET-mediated oxidation of promoter 5mC leads to reactivation of the corresponding transcripts.[29] We performed DNA immunoprecipitation (DIP) from HEK293T cells expressing wild-type or the variant enzymes with $5mC_{ox}$-specific antibodies followed by quantitative polymerase chain reaction (qPCR) using gene-specific primers (Figure 6C).[30] Results from independent replicates suggested that the mutant indeed boosted 5mC oxidation at these loci compared to wild-type TET2 as revealed by ~2-fold enrichment of the genes in the DIP-qPCR assay (Figures 6D, S21).

Finally, we examined if V1395A is functionally relevant to potentiate the expression of tumor suppressors. This is particularly important given that inactivating TET2 mutations are known to downregulate tumor suppressors in hematological malignancies.[31] We focused on DACT1, a tumor suppressor that is frequently silenced in cancers via promoter methylation.[32] It has been shown that the transcription factor WT1 interacts with TET2 to regulate expression of DACT1 in normal hematopoietic stem cells via 5mC oxidation (Figure 6E).[33] In Western blot, we noted that wild-type TET2 marginally increased the DACT1 level in cells compared to a control vector. Remarkably, the mutant catalyzed DACT1 expression up to 2-fold increase over TET2, consistent with its higher dioxygenase activity toward 5mC (Figures 6F, S22). Such results imply that the activating mutant is capable of enhancing the expression of a tumor suppressor that is aberrantly silenced in cancers.

In this work, we explored active site space engineering as a novel tactic to direct catalytic outcome. Mutations at an evolutionarily conserved residue led to TET variants with tunable oxygenase activity: an enlarged cavity augmented multistep oxidation of 5mC to 5caC as the dominant product, whereas reduced catalytic space led to primarily 5hmC via a single-step oxidation. Remarkably, the spacious variant exhibited superior activity to potentiate expression of antimetastatic genes in human cells. We anticipate the engineered TET in combination with genome editing strategies would constitute a powerful tool for establishing a locus-specific $5mC_{ox}$ pattern to reprogram downstream processes. The V1395A mutant will find further application in the enrichment and analysis of 5caC content in the genome using TET-assisted bisulfite[34] and related sequencing approaches. Furthermore, our work provides a blueprint for remodeling the catalytic apparatus of 2KG-dependent dioxygenases, which are emerging as critical regulators of mammalian gene expression.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/jacs.1c03815.

Methods for phosphoramidite synthesis, protein expression, biochemical assays, and supplementary figures and tables (PDF)

Table S1: TET1−3 exome summary analysis (XLS)

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Notes

The authors declare no competing financial interest.

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