Unusual Activity of a *Chlamydomonas* TET/JBP Family Enzyme

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The Great Oxygenation Event, which occurred on earth around 2.45 billion years ago, opened up the niche for enzymes that could directly oxidize substrates using molecular oxygen (O_2), including the 2-oxoglutarate (2OG) and Fe(II)-dependent dioxygenases. 2OGFe-dioxygenases possess a double-stranded b-helix fold, with an iron center chelated by two histidines and an aspartate.[1] 2OGFe-dioxygenases catalyze the incorporation of one atom of O_2 into 2-oxoglutarate, oxidizing it to succinate, and the second atom into an organic substrate. The AlkB family and related clades catalyze this reaction on alkyl adducts on nitrogens of bases, whereas the TET/JBP family catalyzes the oxidation of methyl groups attached to carbons of bases.[1] As enzymes that generate epigenetic marks by modifying DNA or RNA, members of both the AlkB and TET/JBP families have been repeatedly recruited to different eukaryotic lineages.

In eukaryotes, enzymes of the TET/JBP family were first recognized in kinetoplastids (e.g., the human parasite *Trypanosoma*), where they act on thymine in a pathway that results in the synthesis of the hypermodified base J in DNA (Figure 1A). In metazoans and fungi, however, TET/JBP enzymes catalyze the serial oxidation of 5mC to 5-hydroxy-, 5-formyl-, and 5-carboxylcytosine (5hmC, 5fC, and 5caC, respectively) (Figure 1B, top). All three oxidized methylcytosines are intermediates in DNA demethylation; in mammalian cells, 5hmC marks highly expressed genes and active enhancers.[2]

The chlorophyte alga *Chlamydomonas reinhardtii* possesses a lineage-specific expansion of at least 12 genes predicted to encode active TET/JBP enzymes (in genome assembly Genbank GCA_000002595.3). A recent study of one of these enzymes,[3] termed CMD1, revealed an unexpected feature. Unlike other characterized 2OGFe-dioxygenases, *Escherichia coli*-expressed CMD1 uses ascorbate instead of 2OG as its essential co-substrate *in vitro* (Figure 1B, bottom). Enzymes typically do not generate a mixture of stereoisomeric products, but CMD1 produces a skewed (3:1) mixture of stereoisomeric glyceryl modifications of 5mC as its major products, with the glyceryl moiety directly linked to the $-CH_2$ group at position 5 of cytosine [5-glyceryl-methylcytosines (5gmC)]. This has functional implications, because protein readers of major groove modifications would typically show a preference for a defined stereochemistry. On the basis of biosynthetic isotope tracer experiments in *E. coli*, the authors propose that the two stereoisomeric 5gmCs are produced directly from the ascorbate co-substrate via its oxidation to CO_2 and glyoxylate, along with transfer of the glyceryl moiety to 5mC. 5hmC and 5caC are produced in minor amounts, through a mechanism that apparently does not require 2OG but needs ascorbate.

Ascorbate (>1 mM) is believed to help reduce Fe(III) at the active site of 2OGFe-dioxygenases back to Fe(II), thereby "resetting" the active site for catalysis. Addition of ascorbate results in a dose-dependent improvement in catalysis by mammalian TET1 and an increased amount of 5hmC in cells.[4] Thus, instead of the usual 2OG substrate, *C. reinhardtii* CMD1 appears to have evolved to use a cofactor normally involved in restoration of enzyme activity as its essential co-substrate, to produce both the glyceryl adducts and the minor oxidized 5mC products.

From the known structures of TET/JBP proteins, a total of 10 residues other than the three (H, D/E, and H) that form the iron-chelating center can be inferred to be proximal to the 2OG. Of these, the most important is the nearly absolutely conserved arginine, which forms a salt bridge with the distal carboxylate of 2OG. This residue is also conserved in all *C. reinhardtii* TET/JBP proteins and might form a salt bridge with ionized ascorbic acid in CMD1. This basic mechanism of ascorbate binding is likely to be conserved across the TET/JBP superfamily. The remaining less conserved positions might play a role in the increased selectivity of CMD1 for ascorbate over 2OG. In principle, certain family members could utilize ascorbate analogues to catalyze distinct modifications.

The 5gmC base constitutes a very minor fraction (~0.25%) of 5mC in genomic DNA of *C. reinhardtii*, ~1000 5gmC in the entire *C. reinhardtii* genome. Nevertheless, like oxidized 5mC generated by mammalian and fungal TET/JBP enzymes, 5gmC functions to counter the repressive effects of DNA cytosine methylation. Mutants lacking CMD1 were compromised for nonphotochemical quenching, a photoprotective mechanism required for growth under high-light conditions. This phenotype was traced to a decreased level of expression and an increased level of cytosine methylation at two genes encoding LHCSR3 (light-harvesting complex stress-related protein 3). *C. reinhartii* possesses two additional genes that are very closely related to CMD1 (>99% sequence identity at the protein level over the entire alignable length), possibly explaining why CMD1 mutants retain ~40% of the wild-type levels of 5gmC.

How does 5gmC promote cytosine demethylation? Unlike most other organisms of the Viridiplantae lineage, *C. reinhardtii*

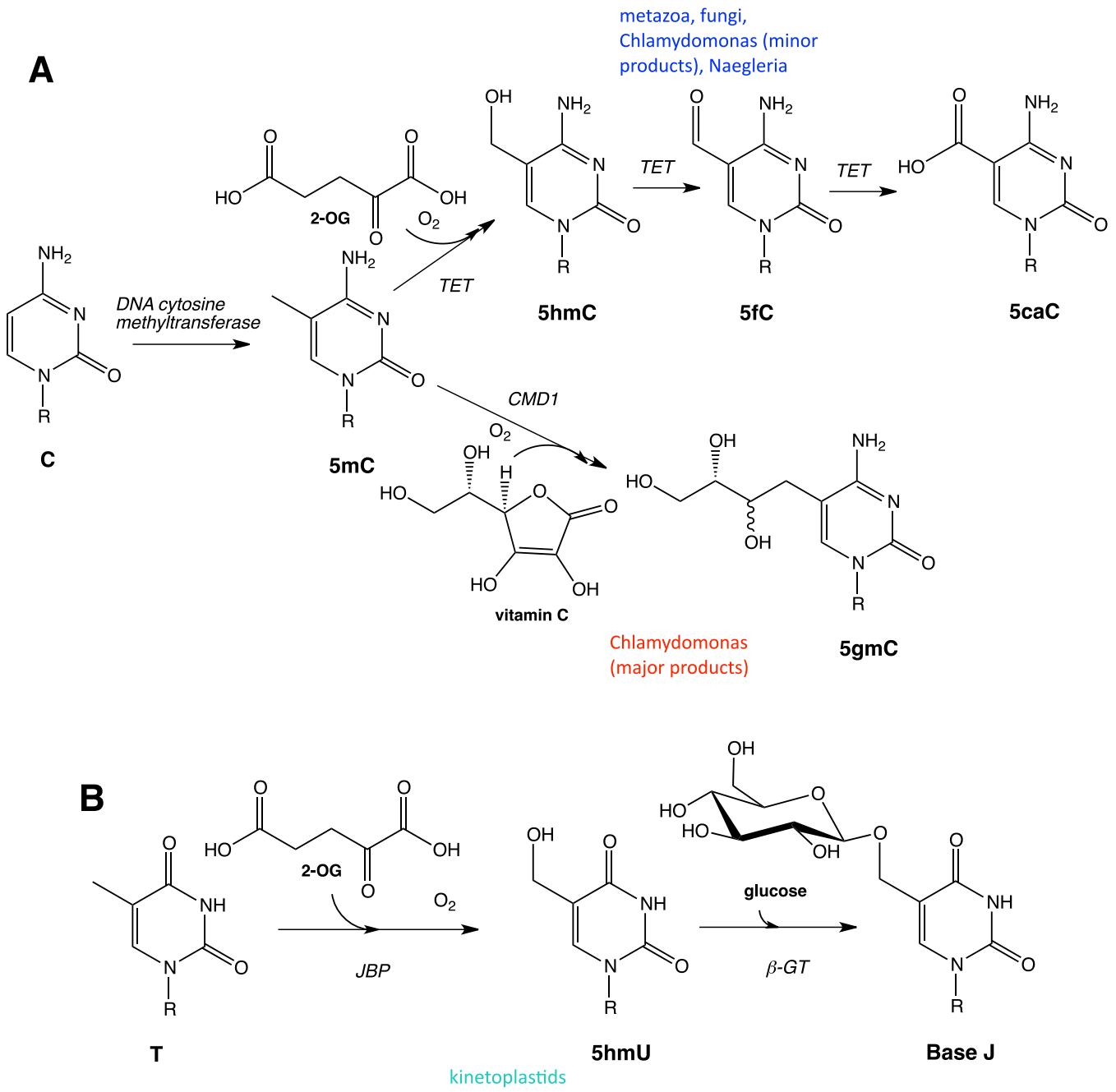

Figure 1. (A) Pathways for the transformations from C to 5caC or from C to 5gmC. (B) *JBP*-mediated oxidation of T to 5hmU followed by glycosylation to form base J.

lacks a member of the Demeter family of DNA glycosylases, the primary enzymes involved in cytosine demethylation in plants. However, it possesses a homologue of the TDG family, which has been shown to be involved in removing the 5fC and 5caC modifications from metazoan DNA. Hence, it is possible that the large adduct generated by CMD1 is a target for the cognate DNA glycosylase, which then helps demethylate regulatory regions of genes involved in photoadaptation to high-light conditions. This view is supported by the observation that in the CMD1 mutant in which global 5gmC levels drop by 40%, global 5mC levels are doubled. The same doubling of global 5mC occurs when ascorbate biosynthesis is removed. Because basal 5mC levels are 400-fold higher than 5gmC levels in wild-type cells, this phenomenon cannot be explained simply by conversion of 5mC to 5gmC. Rather, 5gmC formation is likely a necessary intermediate on a high-flux DNA demethylation pathway (whether replication-dependent or glycosylase-mediated) that turns over 5mC.

Certain eukaryotic lineages, including fungi such as *Coprinopsis cinereus*, show moderate to large lineage-specific expansions and frequent associations with DNA transposons of the KDZ (Kykuja–Dileera–Zisupton) family.[5] These DNA transposons have arguably played major roles in disseminating the associated TET/JBP enzymes across their host genomes and may utilize the enzymes to regulate both their own expression and that of other self-ish elements in the genome. CMD1 is related to these enzymes and was likely acquired by the Chlamydomonadales from such a source, especially given

that some versions in these algae are still linked to the transposons. Future studies will reveal whether the unusual catalytic activity reported for CMD1 is more widely distributed across the TET/JBP family.

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Notes

The authors declare the following competing financial interest(s): S.B. is a founder, and A.R. is a member of the scientific advisory board, of Cambridge EpiGenetix. L.A. declares no competing financial interests.

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