

# Ascorbic Acid Enhances Tet-Mediated 5-Methylcytosine Oxidation and Promotes DNA Demethylation in Mammals

Ruichuan Yin,<sup>†</sup> Shi-Qing Mao,<sup>‡</sup> Bailin Zhao,<sup>†</sup> Zechen Chong,<sup>§,⊥</sup> Ying Yang,<sup>§,⊥</sup> Chao Zhao,<sup>†</sup> Dapeng Zhang,<sup>†</sup> Hua Huang,<sup>†</sup> Juan Gao,<sup>‡</sup> Zheng Li,<sup>‡</sup> Yan Jiao,<sup>||</sup> Cuiping Li,<sup>†</sup> Shengquan Liu,<sup>†</sup> Danni Wu,<sup>†</sup> Weikuan Gu,<sup>||</sup> Yun-Gui Yang,<sup>§,⊥</sup> Guo-Liang Xu,<sup>‡</sup> and Hailin Wang<sup>\*,†</sup>

<sup>†</sup>The State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China

<sup>‡</sup>Group of DNA Metabolism, The State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200031, China

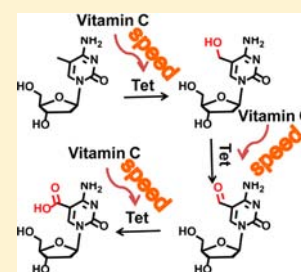
<sup>§</sup>Disease Genomics and Individualized Medicine Laboratory, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 100101, China

<sup>||</sup>Department of Orthopaedic Surgery, University of Tennessee Health Science Center, Memphis, Tennessee 38163, United States

<sup>⊥</sup>University of Chinese Academy of Sciences, Beijing 100039, China

## Supporting Information

**ABSTRACT:** DNA hydroxymethylation and its mediated DNA demethylation are critical for multiple cellular processes, for example, nuclear reprogramming, embryonic development, and many diseases. Here, we demonstrate that a vital nutrient ascorbic acid (AA), or vitamin C (Vc), can directly enhance the catalytic activity of Tet dioxygenases for the oxidation of 5-methylcytosine (5mC). As evidenced by changes in intrinsic fluorescence and catalytic activity of Tet2 protein caused by AA and its oxidation-resistant derivatives, we further show that AA can uniquely interact with the C-terminal catalytic domain of Tet enzymes, which probably promotes their folding and/or recycling of the cofactor Fe<sup>2+</sup>. Other strong reducing chemicals do not have a similar effect. These results suggest that AA also acts as a cofactor of Tet enzymes. In mouse embryonic stem cells, AA significantly increases the levels of all 5mC oxidation products, particularly 5-formylcytosine and 5-carboxylcytosine (by more than an order of magnitude), leading to a global loss of 5mC (~40%). In cells deleted of the *Tet1* and *Tet2* genes, AA alters neither 5mC oxidation nor the overall level of 5mC. The AA effects are however restored when Tet2 is re-expressed in the Tet-deficient cells. The enhancing effects of AA on 5mC oxidation and DNA demethylation are also observed in a mouse model deficient in AA synthesis. Our data establish a direct link among AA, Tet, and DNA methylation, thus revealing a role of AA in the regulation of DNA modifications.



## INTRODUCTION

DNA demethylation remarkably contributes to the dynamics of the epigenetic marker 5-methylcytosine (5mC) in mammals and is critical for multiple biological processes, including animal cloning,<sup>1</sup> nuclear reprogramming,<sup>2,3</sup> development,<sup>4–8</sup> and highly locus-specific regulation of gene activities.<sup>9–11</sup> DNA demethylation can be initiated by the oxidation of 5mC and the formation of 5-hydroxymethylcytosine (5hmC), which are catalyzed by ten eleven translocation (Tet) family dioxygenases.<sup>12–15</sup> The formed 5hmC can be diluted by DNA replication, suggesting a passive DNA demethylation pathway.<sup>16</sup> Moreover, the 5hmC can be further oxidized by Tet proteins to form 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC), which can be excised by thymine DNA glycosylase (TDG) followed by the reintroduction of unmethylated cytosine through the base-excision repair (BER) pathway.<sup>14,15</sup> This important pathway for active DNA demethylation has been thought to be involved in a number of prominent biological processes.<sup>5,6,10,11</sup> Early and recent studies suggested that active and replication-independent DNA demethylation

might be a rapid process.<sup>10,11</sup> The radically altered methylation, as observed in replication-independent demethylation of the paternal genome in zygotes, may complete within hours.<sup>5,6,17–19</sup> However, the observed levels of the active DNA demethylation intermediates, 5fC and 5caC in the cultured cells, were 100-fold less than the primary product 5hmC.<sup>13–15,20–22</sup>

Biochemically, the Tet-mediated DNA demethylation process should be irreversible because no replication-dependent mechanism is found to resynthesize 5mC oxidation products. However, the levels of both 5hmC and 5mC are relatively stable in normal mammalian tissues.<sup>23</sup> On the other hand, the landscapes of 5mC and 5hmC are altered in many diseases.<sup>23</sup> These observations implicate the biological importance of maintaining 5mC and 5hmC in various tissues. But, little is known on how cells maintain the levels of the 5mC oxidation products. The regulation mechanisms remain poorly under-

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stood. This prompted us to seek a new chemical factor that may regulate DNA methylation and 5hmC formation.

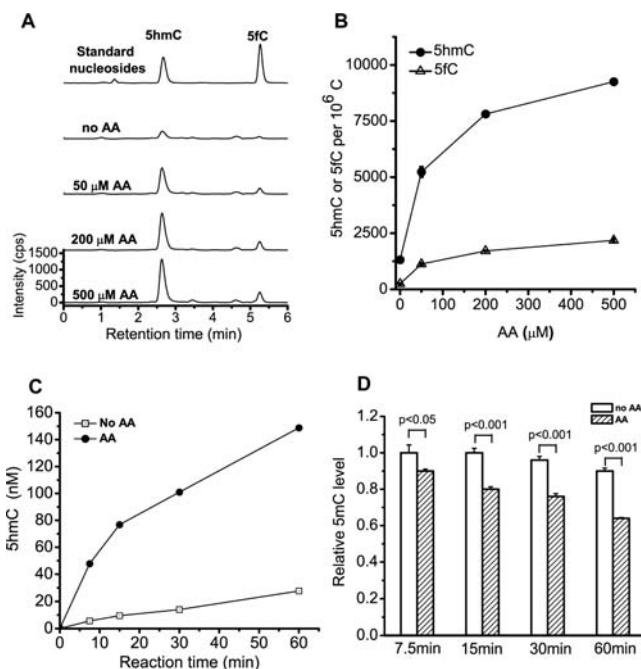
The catalytic activity of Tet dioxygenases for 5mC oxidation requires two cofactors, iron(II) and 2-ketoglutarate. Ascorbic acid (AA), serving for some nonheme iron dioxygenases (e.g., prolyl 4-hydroxylases) to rescue the enzyme by reducing the inactive iron(III) state,<sup>24</sup> might be another cofactor for Tet proteins. However, *in vitro* oxidation reactions previously suggested that the active Tet1 fragment would not act like prolyl 4-hydroxylase,<sup>12</sup> which may suddenly lose all the catalytic activity in the absence of AA,<sup>24</sup> and its catalytic activity is not significantly affected by AA.

Interestingly, AA could induce DNA demethylation at the cellular level.<sup>25–29</sup> Limited data suggested that this effect was probably associated with histone demethylases.<sup>28</sup> Since the histone-targeting demethylases cannot directly act on methylated DNA, we speculate that AA should be a better choice to be exploited by Tet dioxygenases to regulate DNA demethylation. The natural choice of nutrient AA would not sacrifice normal cellular functions in mammals. Chemically, the Tet oxidation activity could be efficiently regulated by changing the level of the known cofactor iron(II), but which may induce severe deleterious effects if not tightly controlled in mammals.<sup>30</sup>

Here, we hypothesize that AA may enhance Tet-mediated oxidation of 5mC and provide unique capacity of regulating the dynamics of DNA methylation. To test our hypothesis, we systematically investigated the effects of AA on the catalytic activity of Tet proteins, 5mC oxidation, and DNA demethylation and the underlying mechanisms. Our results provide new mechanistic insights into the biochemical role of AA and the change of DNA methylation in development, diseases, and regenerative therapies.

## RESULTS

**AA Directly Enhances *In Vitro* Oxidation Activity of Tet Dioxygenases.** We first purified the C-terminal catalytic domain (CD) of the Tet2 protein from overexpressing cells (Supporting Information, Figure S1A) and investigated the *in vitro* 5mC oxidation in genomic DNA. The levels of 5mC and 5mC oxidation products were detected by a newly developed assay using ultrahigh performance liquid chromatography-triple quadrupole mass spectrometry coupled with multiple-reaction monitoring (UHPLC-MRM-MS/MS, Supporting Information). Previously, AA was not shown to have any significant enhancement on the Tet1-mediated oxidation in *in vitro* reactions.<sup>12</sup> In those reactions, 100  $\mu\text{M}$   $\text{Fe}^{2+}$  was used. We re-examined the reactions by the use of  $\text{Fe}^{2+}$  at physiologically relevant concentration (10  $\mu\text{M}$ ).<sup>30</sup> Excess dithiothreitol (1.0 mM) was also included in all tested reaction solutions. Under such conditions, we observed the formation of 5hmC and 5fC in the presence of the purified Tet2 CD but without AA (Figure 1A and the Supporting Information, Figure S1B). Upon addition of AA (50–500  $\mu\text{M}$ ), the levels of 5hmC and 5fC increased in a dose-dependent manner by 4.0–7.0-fold and by 4.6–8.9-fold (Figure 1A,B), respectively. Time-course studies of 5hmC and 5fC formation showed that AA could accelerate the reactions (Figure 1C). The reaction rates for producing 5hmC and 5fC increased by 8.2- and 6.2-fold (Supporting Information, Figure S2), respectively. Accompanying with the increase in 5hmC and 5fC, there is a significant decrease in the 5mC level due to the oxidation of 5mC (Figure 1D).



**Figure 1.** AA stimulates Tet-catalyzed 5mC oxidation *in vitro*. (A) UHPLC-MRM-QQQ detection of 5hmC and 5fC in methylated lambda DNA oxidized by purified Tet2 CD. (B) AA dose-dependent formation of 5hmC and 5fC in methylated lambda DNA oxidized by purified Tet2 CD. (C) AA-induced stimulation of the reaction rate of Tet2 CD oxidizing 5mC to 5hmC. (D) Reduction in the 5mC level caused by AA-enhanced Tet oxidation.

Similarly, AA can stimulate the activity of the purified Tet1 CD to significantly increase the production of 5hmC and 5fC (Supporting Information, Figure S3).

As described above, AA-enhanced 5mC oxidation can be observed using the purified CD domains of Tet1 and Tet2. Since the CD domains of Tet proteins are conserved,<sup>12,13</sup> these results indicate that AA may directly interact with the CD domain of Tet proteins to enhance 5mC oxidation.

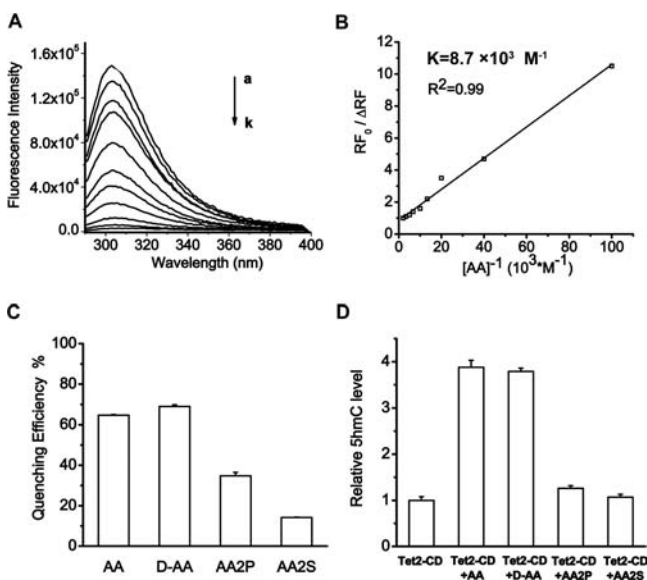
Interestingly, a number of strong reducing chemicals (spermidine, vitamin B1, vitamin E, glutathione, NADPH, and L-cysteine) cannot enhance the Tet-mediated oxidation of 5mC (Supporting Information, Figure S4). This suggests that AA is a unique cofactor of Tet dioxygenases, which cannot be simply replaced by other reducing chemicals. Alternatively, those reducing chemicals may not have an ability to reduce  $\text{Fe}^{3+}$  to  $\text{Fe}^{2+}$  for iron(II) recycling during Tet-mediated oxidation of 5mC.

To further understand the effect of AA, we tested the preincubation of Tet dioxygenases in the presence and absence of AA under aerobic and anaerobic conditions. We find that AA can stimulate Tet-mediated oxidation of 5mC and enhance formation of 5hmC and 5fC no matter whether Tet is preincubated with or without oxygen, further supporting the direct stimulation of Tet enzymes by AA (see the Supporting Information, Figure S4C,D).

On the other hand, the above preincubation results suggest that the reactions are enhanced even by the presence of AA during the preincubation of Tet enzymes (Supporting Information, Figure S4C,D), suggesting that AA is needed in very turnover of Tet-mediated oxidation of 5mC. This was also supported by the results obtained from the time course of Tet2CD-mediated oxidation of 5mC, showing the enhance-

ment effect of AA started from the first time point (Figure 1C and the Supporting Information, Figure S2).

**Binding of AA to CD Domain of Tet Enzymes.** It is not clear how AA enhances the catalytic activity of Tet proteins. To understand the underlying mechanism, we studied the interactions of AA with the conserved CD domains of Tet proteins. The Tet2 CD contains 5 tryptophan (W) and 35 tyrosine (Y) residues,<sup>12</sup> which are known to emit intrinsic fluorescence.<sup>31</sup> Indeed, we observed a strong fluorescence signal (maximum emission: 303 nm) with an excitation of 280 nm for the Tet2 CD at 20 nM (Figure 2A). Tryptophan-related



**Figure 2.** Interaction of AA with the Tet2 CD and its stimulation of the Tet2 CD catalytic activity. (A) Fluorescence quenching of the Tet2 CD caused by AA at varying concentrations (a, 0  $\mu\text{M}$ ; b, 10  $\mu\text{M}$ ; c, 25  $\mu\text{M}$ ; d, 50  $\mu\text{M}$ ; e, 75  $\mu\text{M}$ ; f, 100  $\mu\text{M}$ ; g, 150  $\mu\text{M}$ ; h, 200  $\mu\text{M}$ ; i, 300  $\mu\text{M}$ ; j, 400  $\mu\text{M}$ ; k, 500  $\mu\text{M}$ ). (B) Modified Stern–Volmer plot for estimating the binding constant of AA and the Tet2 CD. (C) Fluorescence quenching of the Tet2 CD caused by AA and its analogues at 100  $\mu\text{M}$ . (D) Stimulation of the Tet2 CD catalytic activity by AA and its analogues.

fluorescence (300–400 nm) was barely detected by an excitation at 295 nm. AA efficiently quenched the intrinsic fluorescence of the Tet2 CD, which is supposed to be mainly associated with the tyrosine residues, in a concentration-dependent manner. In contrast, the collision-caused quenching is negligible using acrylamide at a 100-times higher concentration (5.0 mM) (Supporting Information, Figure S5). This indicates that AA can directly interact with the CD domain of Tet proteins. The estimated binding constant is about  $8.7 \times 10^3 \text{ M}^{-1}$  (Figure 2B).

Interestingly, the quenching of the intrinsic fluorescence of the Tet2 CD is almost complete (98% for 500  $\mu\text{M}$  AA). Recent work using NMR and fluorescence anisotropy proved that similar fluorescence quenching caused by iron(II) and 2-ketoglutarate is linked to the change in the protein dynamics of a dioxygenase AlkB,<sup>32</sup> rendering the full catalytic activity to the enzyme. Therefore, our intrinsic fluorescence quenching data may also suggest that the unique interaction of AA with the CD domain of Tet enzymes probably promotes their folding, which favors the catalytic activity of Tet enzymes and accelerates the Tet-mediated oxidation reactions.

We further tested three AA analogues, one stereoisomer (D-AA, D-isoascorbic acid) and two oxidation-resistant derivatives (AA2P, L-ascorbic acid 2-phosphate; AA2S, L-ascorbic acid 2-sulfate). The chemical structures were shown in the Supporting Information, Figure S6. At the same tested concentration (100  $\mu\text{M}$ ), D-AA caused slightly higher fluorescence quenching efficiency than AA, indicating an interaction of D-AA with the Tet2 CD (Figure 2C). Meanwhile, D-AA also caused an enhancement (3.8 fold) for stimulating the Tet2 CD catalytic activity as high as AA (Figure 2D). In contrast, AA2P and AA2S caused 2- or 5-fold lower fluorescence quenching efficiency than D-AA (Figure 2C), suggesting a much weaker interaction with the Tet2 CD. Notably, AA2P and AA2S showed negligible stimulation for the catalytic activity of Tet2 (Figure 2D). All these results consistently support the important role of the interaction of AA with the CD of Tet2 in the stimulation of its catalytic activity.

Alternatively, the direct interaction of AA with Tet proteins may facilitate the recycling of the cofactor Fe<sup>2+</sup> by reducing the intermediate Fe<sup>3+</sup> to Fe<sup>2+</sup> during Tet-mediated oxidation of 5mC.

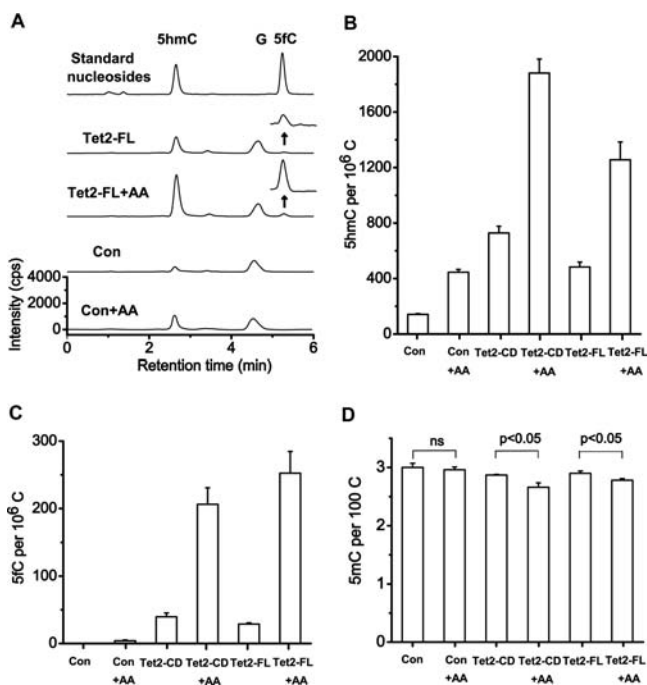
### AA Stimulates Formation of Active 5mC Oxidation Products in Tet-Transfected Cells.

The chemical environment in mammalian cells is very complex, and a multitude of small and bioactive molecules coexist. These small biomolecules might interfere with the stimulation effects of AA on Tet-mediated oxidation. Therefore, we examined the influence of AA on Tet-deficient cells transfected with an expression vector for either the full length Tet2 cDNA or cDNA for the Tet2 CD. Compared with untransfected cells, the level of 5hmC was significantly elevated (3.4- and 5.1-fold) in the cells complemented with full-length and the CD of Tet2 (Figure 3A,B and the Supporting Information, Figure S7), even in the absence of AA treatment. After 24 h exposure to 100  $\mu\text{M}$  AA, the level of 5hmC further increased 2.6-fold in the complemented cells (Figure 3B). Similarly, 5fC could be observed in the genomic DNA of the transfected cells, and its level increased 8.8- and 5.2-fold after AA treatment (Figure 3A,C and the Supporting Information, Figure S7). Accompanying with the increase in 5mC oxidation products, a significant decrease in 5mC was observed in cells transfected with the full length Tet2 cDNA and the Tet2 CD cDNA (Figure 3D). These results provide strong evidence that AA can enhance Tet-mediated oxidation and induce DNA demethylation at the cellular level.

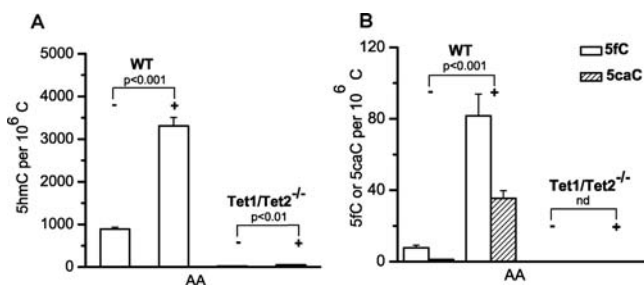
Interestingly, an increase in 5hmC in the control cells after addition of AA was also observed (Figure 3B). This is probably associated with the constitutive expression of residual Tet enzymes, by which AA may increase the 5hmC formation in the tested control cells.

### AA Stimulates Formation of Active 5mC Oxidation Products in ES Cells.

To investigate biological relevance of the observed AA enhancement on Tet activity, we then measured 5mC oxidation products in Tet-proficient embryonic stem (ES) cells. The frequency of 5hmC in genomic DNA of untreated mouse ES cells was about  $8.9 \times 10^2$  per million C. Treatment of cells with 100  $\mu\text{M}$  AA over 24 h increased the level of 5hmC 3.7-fold to  $3.31 \times 10^3$  per million C (Figure 4A). The identity of 5hmC was validated using high-resolution mass spectrometry analysis. The corresponding fractions collected from UHPLC separation of digested genomic DNA of the untreated and AA-treated cells had accurate mass/charge ratios of 258.1080 and 258.1092 (M + H), which match the theoretic



**Figure 3.** AA enhances Tet-mediated 5mC oxidation in transfected human HEK293T cells. (A) UHPLC-MRM-QQQ detection of 5hmC and 5fC in genomic DNA of Tet2-transfected HEK293T cells. (B) 5hmC in AA-treated (+) or untreated (-) HEK293T cells expressing full-length Flag-Tet2 (Tet2-FL) or the C-terminal CD of Tet2 (Tet2-CD). The HEK293T cells not expressing Flag-Tet2 were used as control cells (Con). (C) 5fC in AA-treated (+) or untreated (-) HEK293T cells expressing Flag-Tet2. (D) AA reduces the level of 5mC in genomic DNA of Tet2 CD-transfected HEK293T cells. Student *t* test was used for evaluation of statistical significance. “ns” indicates no significant difference.



**Figure 4.** (A) Increase of 5hmC in wild-type but not in Tet-deficient ES cells treated with AA. (B) Acceleration of active DNA demethylation by the increase of 5fC and 5caC in ES cells treated with AA. “nd” indicates “not detectable”. Student *t* test was used for evaluation of statistical significance.

monoisotopic mass of 5hmC (258.1084) with a deviation of 1.6–4.0 ppm (Supporting Information, Figure S8). We further examined 5hmC using T4-glucosyltransferase-catalyzed glucosylation,<sup>33</sup> which converts 5hmC in genomic DNA to glucosyl-5-hydroxymethylcytosine (5ghmC) detectable by UHPLC-MRM-QQQ analysis monitoring a transition pair of *m/z* 420 → 304. Indeed, the 5hmC peak completely disappeared, and a new peak corresponding to the glucosylated product (5ghmC) appeared for the genomic DNA from both AA-treated and untreated ES cells (Supporting Information, Figure S9A,B), but the level of glucosylated 5hmC in AA-treated ES cells was 4-fold higher than that in untreated ES cells (Supporting

Information, Figure S9C). These results consistently support the AA-induced increase of 5hmC in mouse ES cells.

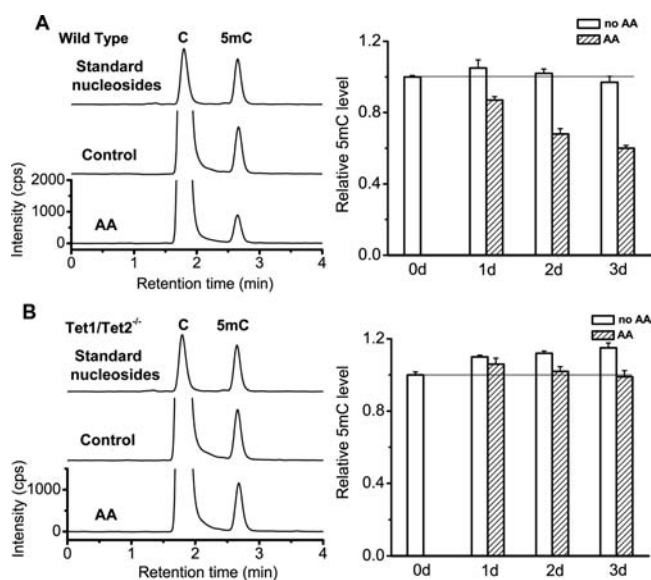
The primary 5mC oxidation product 5hmC does not lead to demethylation on its own, but it can cause replication-dependent passive demethylation.<sup>16</sup> On the other hand, the generated 5hmC can initiate active DNA demethylation by further oxidation, forming 5fC and 5caC.<sup>14,15,34</sup> The presence of 5fC and 5caC essentially indicates the occurrence of active DNA demethylation. Importantly, accompanying with significant increase in 5hmC (~4.0-fold), we also found that the level of 5fC in mouse ES cells was elevated from  $7.7 \pm 1.4$  (untreated) to  $81.7 \pm 12.2$  per million C after AA treatment (Figure 4B and the Supporting Information, Figure S10A), that is, a greater than 10.6-fold increase. Similarly, the level of 5caC was found to be elevated by 20-fold after AA treatment (Figure 4B and the Supporting Information, Figure S10B). The dramatic increase in 5fC and 5caC in AA-treated ES cells demonstrates a critical role of AA in active DNA demethylation.

We further posited that the large increase in the amount of 5hmC, 5fC, and 5caC induced by AA treatment may result from the enhanced Tet-mediated 5mC oxidation. In *Tet1/Tet2* double knockout mouse ES cells, 5hmC was barely detectable after AA treatment (Figure 4A and the Supporting Information, Figure S10A), as were 5fC and 5caC (Figure 4B and the Supporting Information, Figure S10).

To test whether AA changes the expression of Tet enzymes, we used real-time qPCR to evaluate the mRNA level of Tet1 in mouse ES cells. Tet1 is the predominant Tet enzyme in mouse ES cells.<sup>50</sup> No significant change in the expression of TET1 was observed (see the Supporting Information, Figure S11). These results further support that AA directly influences the catalysis of Tet.

Taken together, our data show that AA can directly enhance Tet-mediated 5mC oxidation and promote DNA hydroxymethylation by forming 5hmC and activate DNA demethylation by forming 5fC and 5caC in Tet-proficient ES cells.

**AA Induces Tet-Dependent DNA Demethylation in ES Cells.** To investigate the consequence of AA-enhanced Tet-mediated 5mC oxidation, we further measured the level of 5mC in the genomic DNA of mouse ES cells treated with or without AA. After treating ES cells with 100  $\mu$ M AA for 1 day, the level of 5mC decreased by 13%. By further treating cells for 2–3 days, the level of 5mC decreased by 32–40% (Figure 5A). These results indicate that AA can stimulate a dramatic erasure of 5mC in the genomic DNA of ES cells. The erasure of 5mC by AA was further validated by HPLC-UV analysis (Supporting Information, Figure S12). Previously, significant loss of 5mC has been reported at two developmental stages: erasure of somatic imprinting in primordial germ cells and rapid demethylation of the paternal pronucleus in zygotes.<sup>4</sup> However, these two large-scale demethylation events are linked with reprogramming of genomic methylation patterns. Alternatively, 5mC is largely erased by inhibiting or eliminating the activity of DNA methyltransferases.<sup>35</sup> Here, we show a global loss of the 5mC (~2/5) in ES cells without undergoing nuclear reprogramming and without inhibiting DNA methylation. This finding expands earlier observations that showed an AA-induced demethylation of specific genes.<sup>25,26</sup> In contrast to the wild-type of murine ES cells, the *Tet1/Tet2* double knockout showed minor reduction in 5mC following exposure to AA (Figure 5B). These results indicate that AA can induce DNA demethylation mainly by enhancing the Tet-mediated oxidation of 5mC.



**Figure 5.** AA enhances DNA demethylation in a Tet-dependent manner in mouse ES cells. (A) UHPLC-MRM-QQQ detection of 5mC (left) and the measured frequency of 5mC (right) in the genomic DNA of wild-type (WT) ES cells treated with AA or without AA (control) for 1–3 days (72 h). Only UHPLC-MS traces were shown for 3 days (72 h). (B) The *Tet1/Tet2* double knockout (*Tet1/Tet2*<sup>-/-</sup>) eliminates AA-induced 5mC erasure in ES cells. UHPLC-MRM-QQQ traces of 5mC (left) and the measured frequency of 5mC (right) were shown for 3 days culture and 1–3 days culture, respectively.

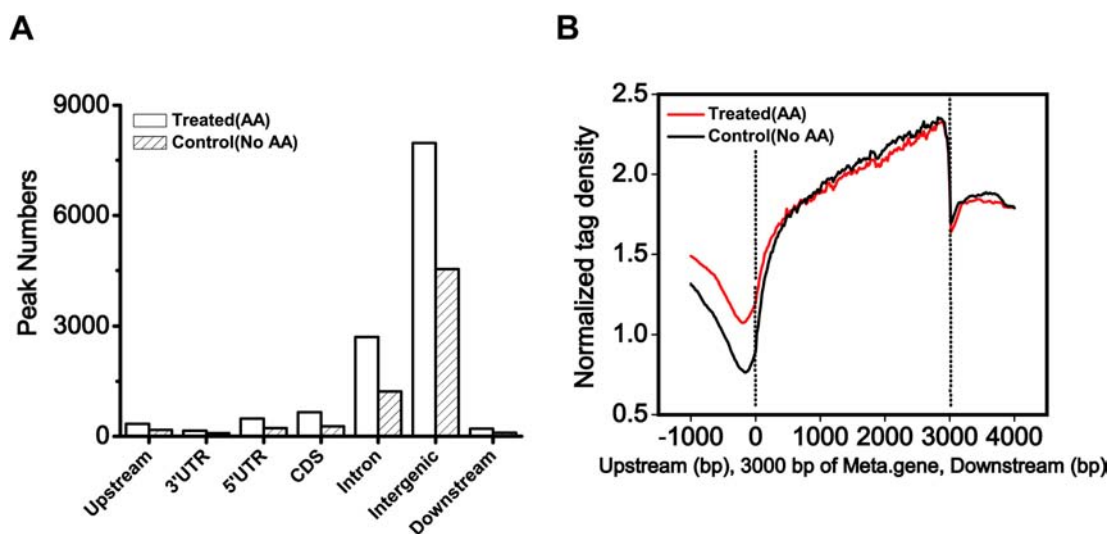
The observation of the slight reduction in 5mC caused by AA in *Tet1/Tet2*-deleted cells (Figure 5B) suggests one Tet-unrelated but minor mechanism for AA-induced DNA demethylation, which is probably related to histone demethylases<sup>28</sup>

**AA Regulates a Potential Broad Functional Pathway by 5hmC Formation.** To further explore the roles of AA on DNA modifications, we examined the genome-wide distribution and enrichment of 5hmC in mouse ES cells using a selective chemical labeling of 5hmC pull-down approach<sup>33</sup> coupled with deep-sequencing by Illumina HiSeq 2000 platform. Using MACS,<sup>33</sup> we found a total of 12 525 peaks in

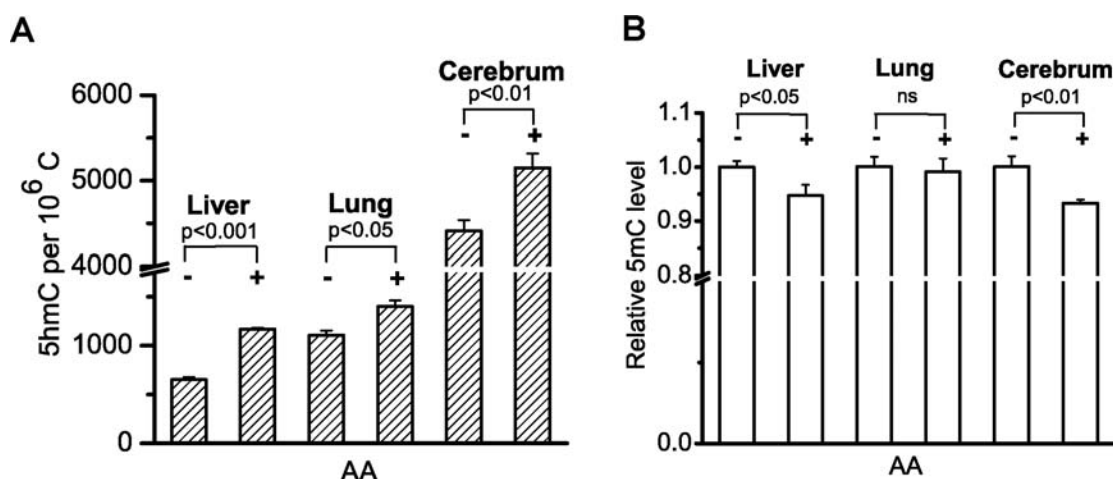
AA-treated cells and 6625 peaks in the control ( $p < 10^{-5}$ , fold enrichment > 10). In every genomic feature (Figure 6A), the enrichment of 5hmC is approximate to 2-fold in AA-treated cells as compared to that of the control. To explore the potential functions of the elevated 5hmC, we compared the distribution of 5hmC (Figure 6B) within the gene regions and their up- and downstream regions between the AA-treated and the control. In particular, we observed that AA increased the abundance of 5hmC near transcription start sites (TSS) (Figure 6B and the Supporting Information, Figure S13A), suggesting a mechanism for the regulation of gene expression by AA. On the basis of gene ontology (GO) and enrichment analysis (Supporting Information, Figure S13B), 545 genes were enriched in functional pathways related to differentiation and various organ development (including brain, muscle, heart, blood, epithelium, etc.), phosphorylation, protein biosynthesis and complex assembly, behavior, transport, cell cycle, and cell signaling, indicating a potential broad regulatory function of 5hmC formation by AA.

Interestingly, our previous work showed that *sfx/sfx* mice deficient in AA synthesis, if without AA supplement, suffered from a number of developmental problems, for example, a loss of body weight, larger brain and kidney, smaller spleen and thymus, abnormal red blood cells and white blood cells, and reduced osteocalcin.<sup>36</sup> Although the heart, liver, and testes were not affected in *sfx/sfx* mice, heart damage has been reported in animal models lacking *Gulo* activity.<sup>36</sup>

**AA Promotes 5mC Oxidation and Demethylation in Mice.** Having shown that AA can regulate the formation of 5hmC in cultured cells, we next examined whether AA can affect 5mC oxidation in mammals. Although most animals have an ability to synthesize AA by themselves, humans have to depend upon dietary AA (or Vc) due to an inherited mutation in the gene that encodes L-gulonolactone oxidase (*Gulo*), which is a key and ultimate enzyme of AA biosynthesis.<sup>37</sup> To simulate human dependence upon AA, we tested an animal model using homozygote *sfx/sfx* (spontaneous fracture) mice, which are deficient in AA synthesis because of a deletion in the *Gulo* gene.<sup>36</sup> We observed that AA supplementation significantly increased the level of 5hmC in adult *sfx/sfx* mouse tissues (Figure 7A). Moreover, AA reduced the level of 5mC in the



**Figure 6.** (A) Distribution of 5hmC in different gene features and (B) the average gene profile.



**Figure 7.** AA promotes oxidative DNA demethylation in a mouse model deficient in AA synthesis. The levels of (A) 5hmC and (B) 5mC in mice without and with dietary AA supplementation were compared. “ns” indicates no significant difference. (Student *t* test was used for evaluation of statistical significance.).

liver ( $P < 0.05$ , *t* test) and in the cerebrum ( $P < 0.01$ , *t* test) (Figure 7B). These results clearly show that AA regulates the levels of 5mC and 5hmC in mammals.

The measured 5hmC level in the cerebrum is 4–8 times higher than in other two tissues (liver and lung). This is consistent with previous work showing the highest abundance of 5hmC in the central nervous system.<sup>22</sup> Coincidentally, brain is the most enriched tissue for AA due to the high expression of AA-specific transporter SVCT2.<sup>38</sup> The level of AA is about 40 times higher in brain (1–2 mM) than that in plasma in normal mice.<sup>39</sup> Previous work showed that AA withdrawal (for three weeks) could not eliminate AA from brain and almost half of AA was retained.<sup>40</sup> Surprisingly, the decrease in the 5hmC level was also observed in the cerebrum of AA-withdrawn mice ( $P < 0.01$ , *t* test, Figure 7A), suggesting a sensitivity of 5hmC formation to the AA change. Our previous work showed that the brain size of *sfx/sfx* mice is 1.7 times as large as that of normal mice without AA supplement.<sup>36</sup> Here, our results of GO analysis showed the involvement of AA in cerebellum development by enriching 5hmC in genes of ATP7A, ATP2B2, DAB1, AGTPBP1, MTPN, ATG7, MYO16, GAS1, WNT7A, and KLHL1 (Supporting Information, Figure S13B and Supporting Information, Excel Tables S1 and S2).

Taken together, these results may suggest an important role of AA-regulated 5hmC formation in brain development.

## DISCUSSION

Here, we demonstrate that AA can remarkably improve the efficiency of iterative 5mC oxidation by directly enhancing the catalytic activity of Tet dioxygenases, thereby regulating the dynamics of DNA methylation. Mechanistically, AA may promote replication-dependent and passive DNA demethylation by enhancing 5hmC formation<sup>16</sup> and accelerate active DNA demethylation by enhancing formation of 5fC and 5caC. The mechanism on the acceleration of active DNA demethylation by AA may partially explain early and recent contradicting observations on rapid and active DNA demethylation.<sup>10,11,17–19,41–44</sup> By acceleration of DNA demethylation, AA can erase two-fifths of 5mC in the ES cells genome, which would not be achieved by active but AA-free Tet proteins. This suggests that the rate of DNA demethylation

is crucial for both the maintenance and the changes of stable but reversible 5mC landscape.

Since it is a vital nutrient widely distributed but with varying concentrations in various tissues, AA could be exploited by nature to regulate the dynamics of DNA methylation in mammals. To the best of our knowledge, this is the first report on the direct linkage of AA, Tet, and DNA methylation.

Aberrant DNA methylation occurs in a number of diseases, including cancers, neurodevelopmental disorders, neurodegenerative and neurological diseases, and autoimmune diseases.<sup>45</sup> Many genes involved in the main pathways (DNA repair, Ras signaling, cell cycle control, p53 network, and apoptosis) are transcriptionally inactivated by the hypermethylation at specific CpG islands (located in promoters) in cancer cells.<sup>45</sup> Recently, it was reported that the silencing of microRNA by promoter hypermethylation is not only linked to cancer but also to metastasis.<sup>46</sup> Interestingly, the reduced 5hmC level was observed in the hematopoietic malignancies and a broad range of solid tumors.<sup>23,47</sup> These observations may indicate a critical role of the impaired regulation machinery on 5mC oxidation and DNA demethylation in the disease development. Potentially, AA might be an important defense by reducing the risks of promoter hypermethylation and maintaining the 5hmC level through the mechanism revealed in this work.

Our findings may suggest some unexplored nutrition values of AA for affecting DNA methylation. Besides its well-known function as an important water-soluble antioxidant, AA may modulate gene expression, induce differentiation of several mesenchymal cell types, and promote proliferation.<sup>48</sup> Since these processes mostly require changes in methylation patterns, our findings on AA-enhanced oxidation of 5mC might account for these biological functions. Our GO and enrichment analysis also suggest that AA may regulate differentiation and organ development through its effect on 5hmC formation.

Notably, AA-regulated 5hmC formation may be critical for brain development as indicated by GO analysis showing 10 brain development-related genes with enriched 5hmC (Supporting Information, Figure S13B, Excel Table S1). This is also supported by the observations of reduced 5hmC in mouse cerebrum (this work) and phenotypically increased size of mouse brain caused by AA deficiency.<sup>36</sup> In fact, the dynamics of DNA methylation are altered in neurological disorders.<sup>49</sup>

Recently, by microarray analysis, Suzlwach et al. found no significant increase in Tet family gene expression during neurodevelopment although 5hmC markedly increased from the early postnatal stage to adulthood.<sup>49</sup> The observation hinted an additional factor required for regulating the 5hmC formation in neurons. Our work suggests that AA may partly contribute to the dynamics of DNA methylation in brain development.

We found that the enhancement of 5mC oxidation is due to the interaction of AA with the CD of Tet1 and Tet2 proteins. Therefore, AA can play an important role in ES self-renewal, normal myelopoiesis, myeloid leukemia and glomas, and the mammalian epigenome, for which the catalytic activities of Tet1 and Tet2 are crucial.<sup>23,50–52</sup> Since all three Tet proteins share a high degree of homology within their CDs,<sup>53,54</sup> our findings should be extendable to Tet3, which is critical for epigenetic reprogramming of fertilized zygotes and somatic nuclear programming in animal clones.<sup>19</sup> This may implicate the involvement of AA in the key functions of Tet3. Indeed, AA can improve the speed and efficiency of human and mouse iPSC generation by defined factors.<sup>25,27,28</sup> Furthermore, our *Tet1/Tet2* double knockout study clearly supports an important role of AA in the DNA demethylation of somatic cells and ES cells by enhancing Tet-mediated 5mC oxidation.

In conclusion, we discovered that AA can stimulate the oxidation of 5-methylcytosine by directly enhancing the catalytic activity of Tet dioxygenases probably through noncovalent interaction-induced change in enzyme dynamics and/or iron(II) recycling. AA thereby promotes replication-dependent (passive) DNA demethylation by increasing 5hmC formation and replication-independent active DNA demethylation by increasing 5fC and 5caC formation, regulating the dynamics of genome-wide DNA methylation in mammals. Through this mechanism, AA may potentially regulate a plethora of biological functions. The findings were also confirmed using *Tet*-transfected and deleted cells and using model animals deficient in ascorbic acid synthesis.

## METHODS

**UHPLC-MS/MS Analysis.** Genomic DNA was extracted from the cultured cells using a genomic DNA purification kit (Promega, Madison, WI) according to the manufacturer's instructions. The extracted DNA (10  $\mu$ g) was digested to nucleosides with 1.0 U DNase I, 2.0 U calf intestinal phosphatase, and 0.005 U snake venom phosphodiesterase I at 37 °C overnight. The digests were filtered by ultrafiltration tubes to remove the enzymes and then were subjected to UHPLC-MS/MS analysis for detection of 5mC, 5hmC, 5fC, and 5caC. The stable isotope 5'-(methyl-d3) 2'-deoxycytidine ([<sup>2</sup>D<sub>3</sub>] 5mC) was used as an internal standard for calibrating UHPLC-MS/MS quantitation of 5mC.

Analysis of 5mC and its oxidation products was performed with a Zorbax Eclipse plus C18 column (2.1 mm  $\times$  100 mm, 1.8  $\mu$ m, Agilent) for separation and electrospray MS/MS (Agilent 6410B, Santa Clara, CA) for detection in the positive ion mode.

**In Vitro Oxidation Reactions.** The methylated DNA (0.5  $\mu$ g) was incubated with the purified Tet2 CD or Tet1 CD (0.25  $\mu$ g) in 100  $\mu$ L of HEPES buffer (50 mM HEPES, pH 8.0, 50 mM NaCl, 1 mM 2-oxoglutarate, 10  $\mu$ M FeCl<sub>2</sub>, 1 mM ATP, 1 mM DTT, and 0–500  $\mu$ M AA) at 37 °C. After reaction for 2 h, the Tet2 CD was inactivated by heating for 5 min at 95 °C. The reacted DNA was then subjected to enzymatic digestion followed by UHPLC-MS/MS analysis.

**Binding Assay.** Interaction of AA with Tet proteins was studied by intrinsic fluorescence quenching assays. Samples were prepared as in vitro oxidation reactions except that no methylated DNA was added.

After incubation for 10 min, the samples were subjected to fluorescence analysis.

All fluorescence measurements were performed on a Fluoromax-4 spectrometer (Horiba Jobin Yvon Inc., Edison, NJ) at room temperature. Intrinsic fluorescence emission spectra of the Tet2 CD were monitored by exciting the sample at 280 nm and measuring the emission from 290 to 400 nm. Slits for both excitation and emission were set at 5 nm. The integration time was set as 0.1 s.

The binding constant was estimated according to the modified Stern–Volmer equation:<sup>55,56</sup>

$$\frac{RF_0}{\Delta RF} = \frac{1}{fK} \times [Q]^{-1} + \frac{1}{f}$$

where  $RF_0$  and  $RF$  are the relative fluorescence intensities of protein in the absence and presence of quencher, respectively.  $[Q]$  is the concentration of the quencher.  $\Delta RF$  is equal to  $RF_0 - RF$ .  $f$  is the fractional maximum fluorescence intensity of the protein.  $K$  is the quenching constant and is also considered as the binding constant.<sup>56</sup>

By plotting  $RF_0/\Delta RF$  versus  $[Q]^{-1}$ , a linear curve could be obtained with a slope of  $1/(fK)$  and an intercept of  $1/f$ . The binding constant was estimated from the quotient of the intercept and the slope.

## ASSOCIATED CONTENT

### Supporting Information

Details of the materials, methods, and experimental procedures as well as additional information as noted in the text. This material is available free of charge via the Internet at <http://pubs.acs.org>.

### Related Articles

During the revision of this manuscript, Dr. Gaofeng Wang's lab (at the University of Miami) reported a stimulation of Tet-mediated hydroxylation of 5mC by ascorbate at cellular level (<http://www.jbc.org/cgi/doi/10.1074/jbc.C113.464800>).

## AUTHOR INFORMATION

### Corresponding Author

hlwang@rcees.ac.cn

### Notes

The authors declare no competing financial interest.

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