JACS
JOURNAL OF THE AMERICAN CHEMICAL SOCIETY

Article

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

Ruichuan Yin, Shi-Qing Mao, Bailin Zhao, Zechen Chong, Ying Yang, Chao Zhao, Dapeng Zhang, Hua Huang, Juan Gao, zheng li, Yan Jiao, Cuiping Li, Shengquan Liu, Danni Wu, Weikuan Gu, Yungui Yang, Guo-Liang Xu, and Hailin Wang

J. Am. Chem. Soc., **Just Accepted Manuscript** • DOI: 10.1021/ja4028346 • Publication Date (Web): 14 Jun 2013

Journal of the American Chemical Society is published by the American Chemical Society. 1155 Sixteenth Street N.W., Washington, DC 20036

	and Ecotoxicology

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

Ruichuan Yin[1], Shi-Qing Mao[2], Bailin Zhao[1], Zechen Chong[3,5], Ying Yang[3,5], Chao Zhao[1], Dapeng Zhang[1], Hua Huang[1], Juan Gao[2], Zheng Li[2], Yan Jiao[4], Cuiping Li[1], Shengquan Liu[1], Danni Wu[1], Weikuan Gu[4], Yungui Yang[3,5], Guoliang Xu[2], Hailin Wang[1,*]

[1]The State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China.

[2]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.

[3]Disease Genomics and Individualized Medicine Laboratory, Beijing Institute of Genomics, Chinese Academy of Sciences, Beijing 100101, China

[4]Department of Orthopaedic Surgery, University of Tennessee Health Science Center, Memphis, TN 38163, USA

[5]University of Chinese Academy of Sciences, Beijing 100039, China

KEYWORDS : Vitamin C; Tet family dioxygenases; 5-Hydroxymethylcytosine; 5-Formylcytosine; Active DNA demethylation.

ABSTRACT: DNA hydroxymethylation and its mediated-DNA demethylation are critical for multiple cellular processes, e.g., 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^{2+}. 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 nor 5mC oxidation or 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 of 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 [1], nuclear reprogramming [2,3], development [4-8], and highly locus-specific regulation of gene activities [9-11]. The DNA demethylation can be initiated by the oxidation of 5-methylcytosine (5mC) and the formation of 5-hydroxymethylcytosine (5hmC), which are catalyzed by Ten Eleven Translocation (Tet) family dioxygenases [12-15]. The formed 5hmC can be diluted by DNA replication, suggesting a passive DNA demethylation pathway [16]. 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 re-introduction of unmethylated cytosine through the base-excision repair (BER) pathway [14,15]. This important pathway for active DNA demethylation has been thought to be involved in a number of prominent biological processes [5,6,10,11]. Early and recent studies suggested that the active and replication-independent DNA demethylation might be a rapid process [10,11]. The radically altered methylation, as observed in the replication-independent demethylation of paternal genome in zygotes, may complete within hours [5,6, 17-19]. 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 [13-15, 20-22].

Biochemically, the Tet-mediated DNA demethylation process should be irreversible because no replication-dependent mechanism is found to re-synthesize 5mC oxidation products. However, the levels of both 5hmC and 5mC are relatively stable in normal mammalian tissues [23]. On the other hand, the landscapes of 5mC and 5hmC are

altered in many diseases [23]. These observations implicate the biological importance of maintaining 5mC and 5hmC in various tissues. But little is known on how the cells maintain the levels of the 5mC oxidation products. The regulation mechanisms remain poorly understood. 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 non-heme iron dioxygenases (e.g., prolyl 4-hydroxylases) to rescue the enzyme by reducing inactive iron (III) state [24], 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 [12], which may suddenly lose all the catalytic activity in the absence of AA [24], and its catalytic activity is not significantly affected by AA.

Interestingly, AA could induce DNA demethylation at cellular level [25-29]. Limited data suggested that this effect was probably associated with histone demethylases [28]. 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 [30].

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 C-terminal catalytic domain (CD) of Tet2 protein from overexpressing cells (SI, Figure S1A) and investigated the *in vitro* 5-mC oxidation in genomic DNA. The levels of 5mC and 5mC oxidation products were detected by a newly developed assay using ultra-high performance liquid chromatography-triple quadrupole mass spectrometry coupled with multiple-reaction monitoring (UHPLC-MRM-MS/MS, Supplementary Methods). Previously, AA was not shown to have any significant enhancement on the Tet1-mediated oxidation in *in vitro* reactions [12]. In those reactions, 100 μM Fe^{2+} was used. We re-examined the reactions by the use of Fe^{2+} at physiologically relevant concentration (10 μM) [30]. 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 SI, Figure S1B). Upon addition of AA (50 - 500 μM), the levels of 5hmC and 5fC increased in a dose-dependent manner by 4.0-7.7 fold and by 4.6-10.0 fold (Figure 1A and 1B), 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 (SI, 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).

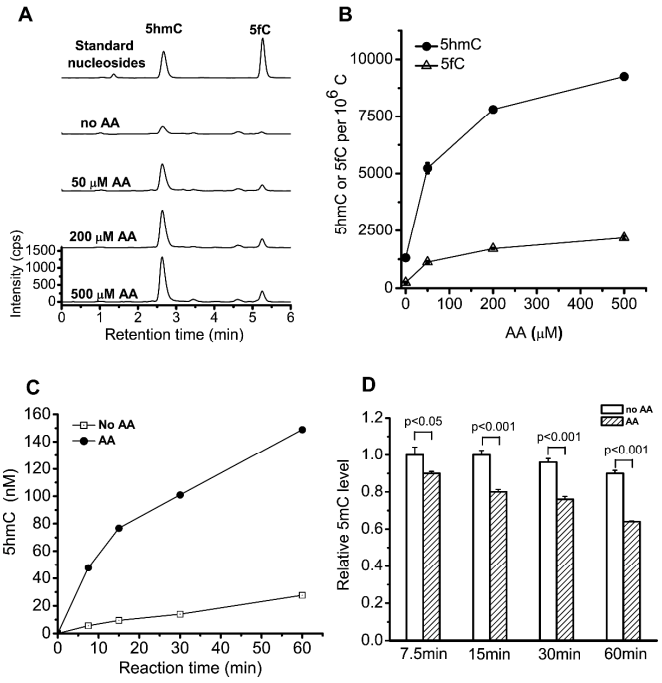

Figure 1. AA stimulates Tet-catalyzed 5mC oxidation *in vitro*. (A) UHPLC-MRM-QQQ detection of 5hmC and 5fC in methylated lambda DNA oxidized by purified Tet2 CD. (B) AA-dose-dependent formation of 5hmC and 5fC in methylated lambda DNA oxidized by purified Tet2 CD. (C) AA-induced stimulation of the reaction 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 (SI, Figure S3).

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

Interestingly, a number of strong reducing chemicals (spermidine, vitmin B1, vitamin E, glutathione, NADPH, L-cysteine) cannot enhance the Tet-mediated oxidation of 5mC (SI, Figure S4). This suggests that AA is a unique cofactor of Tet dioxygenases, which cannot be simply re-

placed by other reducing chemicals. Alternatively, those reducing chemicals may not have an ability to reduce Fe^{3+} to 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 the Tet-mediated oxidation of 5mC and enhance the formation of 5hmC and 5fC no matter whether Tet is pre-incubated with or without oxygen, further supporting the direct stimulation of Tet enzymes by AA (See SI Figure S4C and 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 (SI Figure S4C and S4D), 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-medatied oxidation of 5mC, showing the enhancement effect of AA started from the first time point (Figure 1C and SI, Figure S2).

The 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 [12], which are known to emit intrinsic fluorescence [31]. Indeed, we observed a strong fluorescence signal (maximum emission: 303 nm) with an excitation of 280 nm for Tet2 CD at 20 nM (Figure 2A). Tryptophan-related fluorescence (300-400 nm) was barely detected by an excitation at 295 nm. AA efficiently quenched the intrinsic fluorescence of 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) (SI, Figure S5). This indicates that AA can directly interact with the CD domain of Tet proteins. The estimated binding constant is about $8.7\times10^3\ M^{-1}$ (Figure 2B).

Interestingly, the quenching of the intrinsic fluorescence of Tet2 CD is almost complete (98% for 500 μM 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 [32], 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 analogs, 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 SI, Figure S6. At the same tested concentration (100 μM), D-AA caused slightly higher fluorescence quenching efficiency than AA, indicating an interaction of D-AA with Tet2 CD (Figure 2C). Meanwhile, D-AA also caused an enhancement (3.8 folds) for stimulating Tet2 CD catalytic activity as high as AA (Figure 2D). In contrast, AA2P and AA2S caused two- or five-fold lower fluorescence quenching efficiency than D-AA (Figure 2C), suggesting a much weaker interaction with Tet2 CD. Notably, AA-2P and AA2S showed negligible stimulation for the catalytic activity of Tet2 (Figure 2D). All these results consistently support the important role of AA's interaction with CD domain 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^{2+} by reducing the intermediate Fe^{3+} to Fe^{2+} during the Tet-mediated oxidation of 5mC.

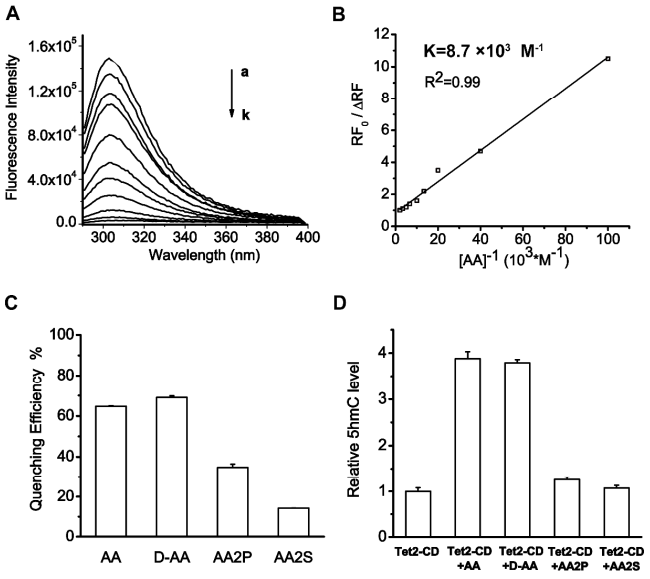

Figure 2. The interaction of AA with Tet2 CD and its stimulation of Tet2 CD catalytic activity. (A) The fluorescence quenching of Tet2 CD caused by AA at varying concentrations (a, 0 μM; b, 10 μM; c, 25 μM; d, 50 μM; e, 75 μM; f, 100 μM; g, 150 μM; h, 200 μM; i, 300 μM; j, 400 μM; k, 500 μM). (B) The modified Stern-Volmer plot for estimating binding constant of AA and Tet2 CD. (C) The fluorescence quenching of Tet2 CD caused by AA and its analogs at 100 μM. (D) The stimulation of Tet2 CD catalytic activity by AA and its analogs.

AA stimulates the 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 co-exist. 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 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 and 3B and SI, Figure S7), even in the absence of AA treatment. After 24-h exposure to 100 μM AA, the level of 5hmC further increased 2.6-fold in the complemented cells (Figure 3B). Similarly, 5fC could be observed in genomic DNA of the transfected cells and its level increased 8.8 and 5.2-fold after AA treatment (Fig 3A and 3C and SI, 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 Tet2 CD cDNA (Figure 3D). These results provide strong evidence that AA can enhance the Tet-mediated oxidation and induce DNA demethylation at cellular level.

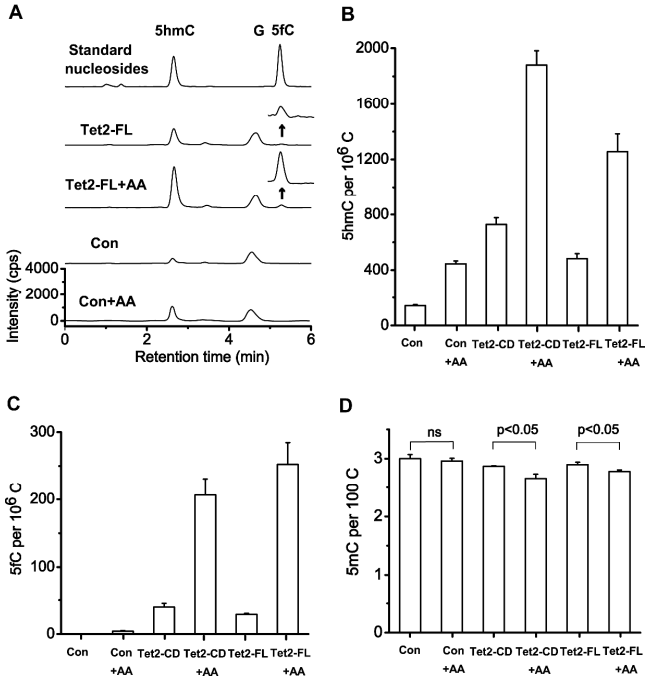

Figure 3. AA enhances Tet-mediated 5mC oxidation in transfected human HEK293T cells. (A) UHPLC-MRM-QQQ detection of 5hmC and 5fC in genomic DNA of Tet2-transfected HEK293T cells. (B) 5hmC in AA-treated (+) or untreated (-) HEK293T cells expressing full length Flag-Tet2 (Tet2-FL) or C-terminal CD domain 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.

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 the 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 the Tet-proficient embryonic stem (ES) cells. The frequency of 5hmC in genomic DNA of untreated mouse ES cells was about 8.9×10^2 per million C. Treatment of cells with 100 μM AA over 24 h increased the level of 5hmC 3.7-fold to 3.31×10^3 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 monoisotopic mass of 5hmC (258.1084) with a deviation of 1.6 - 4.0 parts per million (SI, Figure S8). We further examined 5hmC using T4 -glucosyltransferase-catalyzed glucosylation [33], which converts 5hmC in genomic DNA to glucosyl-5-hydroxymethylcytosine (5ghmC) detectable by UHPLC-MRM-QQQ analysis monitoring a transition pair of m/z 420.1 → 304.0. Indeed, the 5hmC peak completely disappeared and a new peak corresponding to the glucosylated product (5ghmC) appeared for genomic DNA from both AA-treated and -untreated ES cells (SI, Figure S9 A and B), but the level of glucosylated 5hmC in AA-treated ES cells was 4-fold higher than that in untreated ES cells (SI, Figure S9C). These results consistently support the AA-induced increase of 5hmC in mouse ES cells.

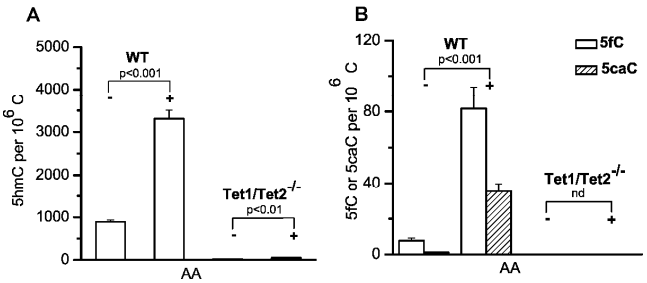

Figure 4 (A) Increase of 5hmC in wild-type but not in Tet-deficient ES cells treated with AA. (B) Acceleration of active DNA demethylation by the increase of 5fC and 5caC in ES cells treated with AA. "nd" indicates "not detectable". Student t test was used for evaluation of statistical significance.

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

crease 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 SI, Figure S10A), nor 5fC and 5caC (Figure 4B and SI, 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 [50]. No significant change in the expression of TET1 was observed (See Figure SI11). 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 hydoxymethylation by forming 5hmC and activate DNA demethylation by forming 5fC and 5caC in Tet-proficient ES cells.

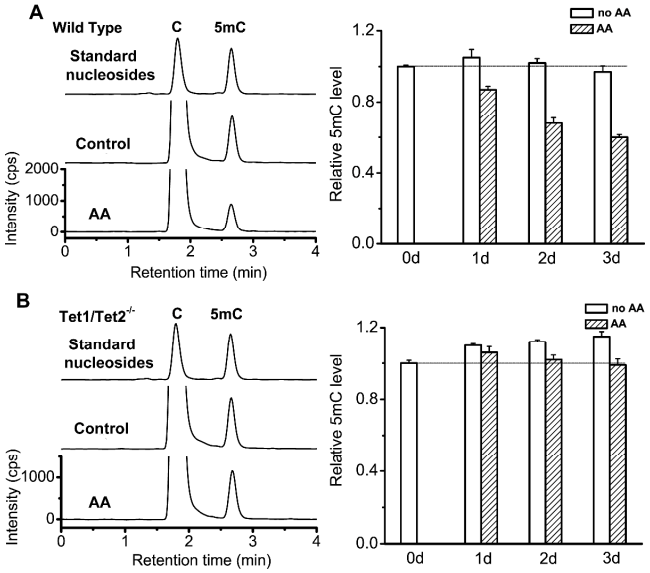

Figure 5. AA enhances DNA demethylation in a Tet-dependent manner in mouse ES cells. (A) UHPLC-MRM-QQQ detection of 5mC (left) and the measured frequency of 5mC (right) in genomic DNA of wild-type (WT) ES cells treated with AA or without AA (Control) for 1-3 days. Only UHPLC-MS traces were shown for 3 days (72 h). (B) The *Tet1*/*Tet2* double knockout (*Tet1*/*Tet2*$^{-/-}$) 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.

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 genomic DNA of mouse ES cells treated with or without AA. After treating ES cells with 100 μM 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 genomic DNA of ES cells. The erasure of 5mC by AA was further validated by HPLC-UV analysis (SI, 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 [4]. 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 [35]. 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 [25,26]. In contrast to the wild type of murine ES cells, *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.

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 [28].

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 [33] coupled with deep-sequencing by Illumina HiSeq 2000 platform. Using MACS [33], we found a total of 12525 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 AA treated and Control. In particular, we observed that AA increased the abundance of 5hmC near transcription start sites (TSS) (Figure 6B and SI, Figure S13A), suggesting a mechanism for the regulation of gene expression by AA. Based on Gene Ontology (GO) and enrichment analysis (SI, Figure S13B), 545 genes were enriched in functional pathways related to differentiation and various organ development (including brain, muscle, heart, blood, epithelium...), phosphorylation, protein biosynthesis and complex assembly, behavior, transport, cell cycle, 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, e.g. a

loss of body weight, larger brain and kidney, smaller spleen and thymus, abnormal red blood cells and white blood cells, and reduced osteocalcin [36]. Although the heart, liver, and testes were not affected in sfx/sfx mice, heart damage has been reported in animal models lacking Gulo activity [36].

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 [37]. 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 [36]. 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 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 mammal.

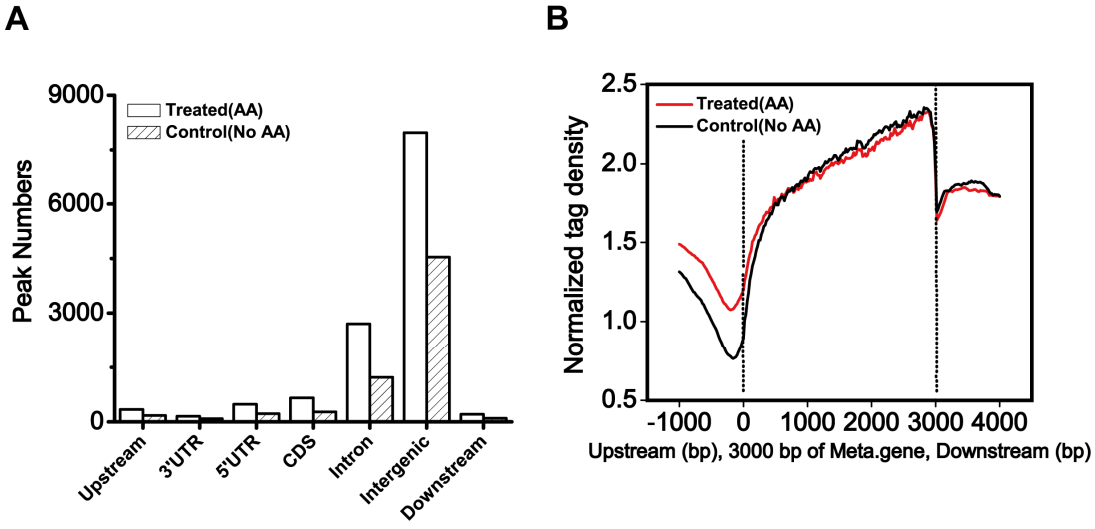

Figure 6. The distribution of 5hmC in different gene features (A) and the average gene profile (B).

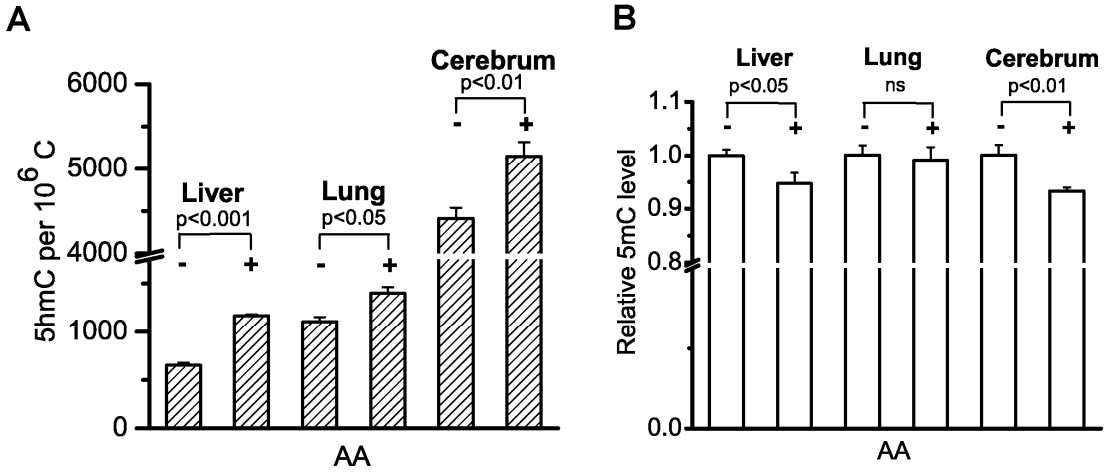

Figure 7. AA promotes oxidative DNA demethylation in a mouse model deficient in AA-synthesis. The levels of 5hmC (A) and 5mC (B) in mice without and with dietary AA supplementation were compared. "ns" indicates no significant difference. (Student t test was used for evaluation of statistical significance.)

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 central nervous system [22]. Coincidently, brain is the most enriched tissue for AA due to the high expression of AA-specific transporter SVCT2 [38]. The level of AA is about 40 times higher in brain (1-2 mM) than that in plasma in normal mice [39]. Previous work showed that AA withdrawal (for three weeks) could not eliminate AA from brain and almost half of AA retained [40]. Surprisingly, the decrease in 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 [32]. 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, KLHL1 (SI, Figure S13B and SI, Excel Table 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 the 5hmC formation [16]; and accelerate active DNA demethylation by enhancing the 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 [10,11,17-19,41-44]. By acceleration of DNA demethylation, AA can erase two-fifth 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, autoimmune diseases [45]. 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 [45]. Recently, it reported that the silencing of microRNA by the promoter hypermethylation is not only linked to cancer but also to metastasis [46]. Interestingly, the reduced 5hmC level was observed in the hematopoietic malignancies and a broad range of solid tumors [23,47]. 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 [48]. Since these processes mostly require the 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 (Figure 5B and SI, 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 [36]. In fact, the dynamics of DNA methylation are altered in neurological disorders [49]. Recently, by microarray analysis, Suzlwach et al. found no significant increase in Tet family gene expression during neurodevelopment although 5-hmC markedly increased from the early postnatal stage to adulthood [49]. 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 domain of Tet1 and Tet2 proteins. Therefore, AA can play an important role in ES self-renewal, normal myelopoiesis, myeloid leukemia and glomas, and mammalian epigenome, for which the catalytic activities of Tet1 and Tet2 are crucial [23,50-52]. Since all three Tet proteins share a high degree of homology within their CD domains [53,54], our findings should be extendable to Tet3, which is critical for epigenetic reprogramming of fertilized zygotes and somatic nuclear programming in animal clone [19]. 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 [25,27,28]. 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 non-covalent 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 animal 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 μg) 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 ultra-filtration 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 ([$^2D_3$] 5mC) was used as an internal standard for calibrating UHPLC-MS/MS quantitation of 5mC.

5mC and its oxidation products analysis was performed with a Zorbax Eclipse Plus C18 column (2.1×100mm, 1.8 μm, 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 μg) was incubated with the purified Tet2-CD or Tet1 CD (0.25 μg) in a 100 μl HEPES buffer (50 mM HEPES, pH 8.0, 50 mM NaCl, 1 mM 2-oxoglutarate, 10 μM $FeCl_2$, 1 mM ATP, 1 mM DTT, and 0-500 μM 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.

The 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 Tet2-CD were monitored by exciting the sample at 280 nm and measuring the emission from 290 - 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 [55,56].

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

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

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 are given in the Supporting Information. This material is available free of charge via the Internet at http://pubs.acs.org."

AUTHOR INFORMATION

Corresponding Author

hlwang@rcees.ac.cn.

Notes

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).

ACKNOWLEDGMENT

We thank Prof. Michael Weinfeld at the University of Alberta for his discussion and comments. This study was supported by grants from the Ministry of Science and Technology of China (2011CB936001 and 2009CB421065) and National Science Foundation of China (21077129 and 21125523) to W. H.

REFERENCES

(1) Wilmut, I.; Shnieke, A. E.; McWhir, J. et al. *Nature* 1997, 385, 810.
(2) Mikkelsen, T.S.; Hanna, J.; Zhang, X. et al. *Nature* 2008, 454, 49.
(3) Jullien, J.; Pasque, V.; Halley-Stott, R. P. et al. *Nat Rev Mol Cell Biol* 2011, 12, 453.
(4) Reik, W.; Dean, W.; Walter, J. *Science* 2001, 293, 1089.
(5) Mayer, W.; Niveleau, A.; Walter, J.; et al. *Nature* 2000, 403, 501.
(6) Oswald, J.; Engemann, S.; Lane, N. et al. *Curr Biol* 2000, 10, 475.
(7) Rideout, I. W.; Eggan, K.; Jaenisch, R. *Science* 2001, 293, 1093.
(8) Simonsson, S.; Gurdon, J. *Nat Cell Biol* 2004, 6, 984.
(9) Guo, J. U.; Su, Y.; Zhong, C. et al. *Cell* 2011, 145, 423.
(10) Kangaspeska, S.; Stride, B.; Métivier, R. et al. *Nature* 2008, 452, 112.
(11) Métivier, R.; Gallais, R.; Tiffoche, C. et al *Nature* 2008, 452, 45.
(12) Tahiliani, M.; Koh, K. P.; Shen, Y. et al *Science* 2009, 324, 930.
(13) Wu, H.; Zhang, Y. *Genes Dev* 2011, 25, 2436.
(14) He, Y. F.; Li, B. Z.; Li, Z. et al *Science* 2011, 333, 1303.
(15) Ito, S.; Shen, L.; Dai, Q. et al. *Science* 2011, 333, 1300.
(16) Inoue, A.; Zhang, Y. *Science* 2011, 334, 194.
(17) Iqbal, K.; Jin, S. G.; Pfeifer, G. P. et al. *Proc Natl Acad Sci USA* 2011, 108, 3642.

(18) Wossidlo, M.; Nakamura, T.; Lepikhov, C. et al. *Nat Commun* 2011, 2, 241.
(19) Gu, T. P.; Guo, F.; Yang, H. et al. *Nature* 2011, 477, 606.
(20) Pfaffeneder, T.; Hachner, B.; Truß, M. et al. *Angew Chem Int Ed* 2011, 123, 7146.
(21) Bhutani, N.; Burns, D. M.; Blau, H. M. *Cell* 2011, 146, 866.
(22) Globisch, D.; Münzel, M.; Müller, M. et al. *PLoS ONE* 2011, 5, e15367.
(23) Tan, L.; Shi, Y. G. *Development* 2012, 139, 1895.
(24) Gorres, K. L.; Raines, R. T. *Crit Rev Biochem Mol Biol* 2010, 45, 106.
(25) Stadtfeld, M.; Apostolou, E.; Ferrari, F. et al. *Nat Genet* 2012, 44, 398.
(26) Chung, T. L.; Brena, R. M.; Kolle, G. et al. *Stem Cells* 2010, 28, 1848.
(27) Esteban, M. A.; Wang, T.; Qin, B. M.; et al. *Cell Stem Cell* 2010, 6, 71.
(28) Wang, T.; Chen, K. S.; Zeng, X. M. et al. *Cell Stem Cell*, 2011, 9, 575.
(29) Chung, T. L.; Turner, J. P.; Thaker, N. Y.; Kolle, G.; Cooper-White, J. J.; Grimmond, S. M.; Pera, M. F.; Wolvetang, E. J. *Stem Cells* 2010, 28, 1782.
(30) Mackenzie, E. L.; Iwasaki, E.; Tsuji, Y. *Antioxid Redox Signal* 2008, 10, 997.
(31) Lakowicz, J. R. Principles of Fluorescence Spectroscopy, 3rd ed.; Springer-Verlag: Berlin Heidelberg, 2006.
(32) Bleijlevens, B.; Shivarattan, T.; van den Boom, K. S.; de Haan, A.; van der Zwan, G.; Simpson, P. J.; Matthews, S. J. *Biochemistry,* 2012, 51, 3334.
(33) Song, C. X.; Szulwach, K. E. ; Fu, Y. et al. *Nat Biotechnol* 2011, 29, 68.
(34) Maiti, A.; Drohat, A. C. *J Biol Chem* 2011, 286, 35334.
(35) Jackson, M. ; Krassowska, A. ; Gilbert, N. et al. *Mol Cell Biol* 2004, 24, 8862.
(36) Jiao, Y.; Li, X. M.; Beamer, W. G. et al. *Mamm Genom* 2005, 16, 20.
(37) Nishikimi, M.; Yagi, K. *Am J Clin Nutr* 1991, 54, 1203S.
(38) May, J. M. (2012) Chapter 6 Vitamin C transport and its role in the central nervous system. In "Water soluble vitamins, subcellular biochemistry 56 (Stanger, D. ed.) Springer Science+Business Media B.V. p85-99.
(39) Maeda, N.; Hagihara, H.; Nakata, Y. et al. *Proc Natl Acad Sci USA* 2000, 97, 841.
(40) Kim, H.; Bae, S.; Yu, Y. et al. *Immune Network* 2012, 12, 18.
(41) Bruniquel, D.; Schwartz, R. H. *Nat Immunol* 2003, 4, 235.
(42) Paroush, Z.; Keshet, I.; Yisraeli, J.; Cedar, H. *Cell* 1990, 63, 1229.
(43) Zhang, F.; Pomerantz, J. H.; Sen, G. et al. *Proc Natl Acad Sci USA* 2007, 104, 4395.
(44) Martinowich, K.; Hattori, D.; Wu, H. et al. *Science* 2003, 302, 890.
(45) Portela, A.; Esteller, M. *Nat Biotech* 2010, 28, 1057.
(46) Lujambio, A.; Ropero, S.; Ballestar, E. et al. *Cancer Res* 2007, 67, 1424.
(47) Jin, S. G.; Jiang, Y.; Qiu, R.; Rauch, T. A.; Wang, Y.; Schackert, G.; Lu, Q.; Pfeifer, G. P. *Cancer Res*, 2011, 71, 7360.
(48) De Tullio, M. C. (2012) Chapter 4 Beyond the antioxidants: Double life of vitamin C. Water Soluble Vitamins (O. Stangered ed.), Subcellular Biochemistry 56, Springer Science+Business Media B.V. p49-65.
(49) Szulwach, K. E.; Li, X. K.; Li, Y. J. et al. *Nat Neurosci* 2011, 14, 1607.
(50) Koh, K. P.; Yabuuchi, A.; Rao, S. et al. *Cell Stem Cell* 2011, 8, 200.
(51) Ito, S.; D'Alessio, A. C.; Taranova, O. V. et al. *Nature* 2010, 466, 1129.
(52) Branco, M. R.; Ficz, G.; Reik, W. *Nat Rev Genet* 2012, 3, 7.
(53) Iyer, L. M.; Tahilian, M.; Rao, A.; Aravind, L. *Cell Cycle* 2009, 8, 1698.
(54) Loenarz, C.; Schofield, C. *J Chem Biol* 2009, 16, 580.
(55) Eftink, M. R.; Ghiron, C. A. *Anal biochem* 1981, 114, 199.
(56) Sułkowska, A. *J Mol Struct* 2002, 614, 227.

TOC