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RESEARCH PAPER

Fusaric acid-induced promoter methylation of DNA methyltransferases triggers DNA hypomethylation in human hepatocellular carcinoma (HepG2) cells

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ABSTRACT

Fusaric acid (FA), a mycotoxin contaminant of maize, displays toxicity in plants and animals; however, its epigenetic mechanism is unknown. DNA methylation, an epigenetic modification that regulates gene expression, is mediated by DNA methyltransferases (DNMTs; DNMT1, DNMT3A, and DNMT3B) and demethylases (MBD2). The expression of DNMTs and demethylases are regulated by promoter methylation, microRNAs (miR-29b) and post-translational modifications (ubiquitination). Alterations in these DNA methylation modifying enzymes affect DNA methylation patterns and offer novel mechanisms of FA toxicity. We determined the effect of FA on global DNA methylation as well as a mechanism of FA-induced changes in DNA methylation by transcriptional (promoter methylation), post-transcriptional (miR-29b) and post-translational (ubiquitination) regulation of DNMTs and MBD2 in the human hepatocellular carcinoma (HepG2) cell line. FA induced global DNA hypomethylation ($p < 0.0001$) in HepG2 cells. FA decreased the mRNA and protein expression of DNMT1 ($p < 0.0001$), DNMT3A ($p < 0.0001$), and DNMT3B ($p < 0.0001$) by upregulating miR-29b ($p < 0.0001$) and inducing promoter hypermethylation of *DNMT1* ($p < 0.0001$) and *DNMT3B* ($p < 0.0001$). FA decreased the ubiquitination of DNMT1 ($p = 0.0753$), DNMT3A ($p = 0.0008$), and DNMT3B ($p < 0.0001$) by decreasing *UHRF1* ($p < 0.0001$) and *USP7* ($p < 0.0001$). FA also induced *MBD2* promoter hypomethylation ($p < 0.0001$) and increased MBD2 expression ($p < 0.0001$). Together these results indicate that FA induces global DNA hypomethylation by altering *DNMT* promoter methylation, upregulating miR-29b, and increasing MBD2 in HepG2 cells.

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Introduction

Fusaric acid (FA; 5-butylpicolinic acid), a ubiquitous mycotoxin and secondary metabolite produced by pathogenic fungi of the genus *Fusarium*, contaminates agricultural foods and exhibits low to moderate toxicity [1]. Previously, feed samples were reported to contain an average of 643 µg/kg FA [2] and approximately 2.5–18 µg/kg FA were reported to contaminate commercial foods and feeds [3]. These foods, especially maize, form an essential part of the human and animal diet; and the consumption of FA-contaminated commodities may have serious health implications. Studies evaluating the effects of FA are limited and understanding the molecular and epigenetic effects of FA exposure is important in decreasing FA contamination and lowering the risk of FA-related adverse health outcomes.

FA is phytotoxic to several plants by inhibiting root and leaf cell function [4] and has been implicated in the pathogenesis of wilt diseases [4–7]; it is a highly lipophilic toxin that traverses cellular membranes and induces toxicity by altering various biochemical processes. Known mechanisms of FA toxicity include alterations in membrane permeability [5,7], oxidative stress [8,9], mitochondrial dysfunction [6,10,11], DNA damage [12,13], and apoptosis [10,12,14,15]. It is also immunotoxic to peripheral blood mononuclear cells (PBMCs) and human monocytic (THP-1) cells [14]. FA has tumouristatic and tumouricidal effects in several mammalian tumour cell lines, thereby, displaying anti-cancer activity [13,16]. It has neurochemical effects in mice brain and reduced aggressive behaviour and motor activity [17]. FA also attenuates isoproterenol induced heart failure by preventing the development of cardiac hypertrophy and fibrosis [18].

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Supplemental data for this article can be accessed here.

FA is a chelating agent and the removal of essential divalent cations such as calcium affects bone ossification [19] and blood coagulation [20]; it also chelates copper causing hypotension [21,22] and notochord malformation [23]. The toxicity of FA may also be attributed to synergistic interactions with other co-occurring mycotoxins such as fumonisin B_1 (FB_1) [24], deoxynivalenol (DON) [25], and 4,15-diacetoxyscirpenol (DAS) [26].

DNA methylation is a common epigenetic modification that regulates gene expression and plays a major role in cell signalling pathways that are essential in the normal growth and development of higher organisms. Dysregulation in the DNA methylation pattern has been observed in several human diseases such as cancer [27] and neurodegeneration [28]. DNA methylation is catalyzed by DNA methyltransferases (DNMTs) such as DNMT1, DNMT3A, and DNMT3B. DNMT1 is a maintenance DNMT that binds specifically to hemi-methylated DNA and is responsible for conserving the methylation pattern from one generation to the next [29]. DNMT3A and DNMT3B are *de novo* DNMTs that target unmethylated cytosine bases to initiate methylation [29]. DNMTs are the major regulators of DNA methylation and alterations in their expression and activity affects DNA methylation patterns and cellular function. The activity and stability of DNMTs are regulated by promoter methylation, microRNAs, and post-translational modifications (PTMs).

Promoter methylation, methylation of CpG islands within the promoter region of specific genes, is important in regulating gene transcription; promoter hypermethylation prevents binding of transcription factors and inhibits gene transcription, whereas promoter hypomethylation activates gene transcription.

MicroRNAs are small non-coding RNA molecules that post-transcriptionally regulate gene expression by binding to the 3′ untranslated region (3′UTR) of the target messenger RNA (mRNA) and negatively regulating the processing, stability, and translation of the mRNA [30]. MiR-29 plays a major role in cell proliferation, differentiation, and apoptosis [31,32]. The miR-29 family consists of two clusters: cluster 1, located on chromosome 7q32.3, consists of miR-29a and miR-29b-1; and cluster 2, located on chromosome 1q32.2, consists of miR-29b-2 and miR-29c. MiR-29b-1 and miR-29b-2 have identical mature sequences and are collectively referred to as miR-29b. Several effects of miR-29b have been identified such as activating the tumour suppressor protein, p53 and regulating cell proliferation, and apoptosis by targeting *p85α* and the *cell division cycle 42* (*CDC42*) [31,32]. It prevents liver fibrosis by targeting the PI3K/AKT signalling pathway [33], and targets *AKT2* and *AKT3* to regulate the Warburg effect in ovarian cancer cells [34]. MiR-29b can also regulate the DNA methylation status of the cell in a negative feedback loop by directly targeting *DNMT3A* and *DNMT3B* [35,36]. Furthermore, the expression of miR-29b is itself epigenetically regulated and thus inversely correlated with the DNA methylation status of the cell.

PTMs also regulate the expression and activity of DNMTs. These modifications occur in the N- and C-terminal regions of the protein and include acetylation and ubiquitination [29]. The acetylation of DNMTs is regulated by the acetyltransferase, Tip60 and the deacetylases, HDAC1 and HDAC2 [29,37,38]. The ubiquitination of DNMTs is triggered by DNMT acetylation and is regulated by the E3 ligase, ubiquitin-like and ring finger domain 1 (UHRF1), and the deubiquitylating enzyme, ubiquitin specific peptidase 7 (USP7) [29,37,38]. The ubiquitination of DNMTs play a major role in inhibiting DNMT stability and promoting proteasomal degradation.

DNA methylation forms a platform for several methyl binding proteins. Methyl-CpG binding domain proteins (MBDs) are a family of nuclear proteins that play an important role in regulating DNA methylation and gene transcription by recruiting chromatin remodelling complexes to regions of methylated DNA. Several MBDs have been identified (MBD1-6); however, MBD2 is the major MBD that binds specifically to methylated CpG islands and acts as a methylation-dependent transcriptional repressor and DNA demethylase [39].

Although several effects of FA have been described, the effect of FA on epigenetic regulation has not been determined. This study aimed to determine an epigenetic effect of FA in the human hepatocellular carcinoma (HepG2) cell line, as a mechanism of FA-induced toxicity. The effect of FA on global DNA methylation as well as the mechanism of FA-induced changes in DNA methylation by transcriptional (promoter methylation), post-transcriptional (miR-29b), and post-translational (ubiquitination) regulation of DNMTs and MBD2 was determined.

Results

Fusaric acid induced global DNA hypomethylation in HepG2 cells

We first determined the effect of FA on global DNA methylation in liver (HepG2) cells. 5-methylcytosine, a common marker of global DNA methylation, was quantified using a commercialized kit (Abcam, ab117128) and 5-aza-2-DC was used as a negative control. The percentage of 5-methylcytosine in the 5-aza-2-DC and FA-treated HepG2 cells were decreased compared to the control ($p < 0.0001$; Figure 1). This suggested that FA induced a dose-dependent decrease in global DNA methylation in HepG2 cells.

Fusaric acid decreased the expression of DNMT1, DNMT3A, and DNMT3B in HepG2 cells

The DNMTs, DNMT1, DNMT3A, and DNMT3B, play a major role in initiating and maintaining DNA methylation patterns. Due to the FA-induced global DNA hypomethylation in the HepG2 cells, we evaluated the mRNA and protein expressions of DNMT1, DNMT3A, and DNMT3B. FA significantly decreased the mRNA expression of *DNMT1* ($p < 0.0001$; Figure 2(a)), *DNMT3A* ($p < 0.0001$; Figure 2(a)), and *DNMT3B* ($p < 0.0001$; Figure 2(a)) in HepG2 cells compared to the control. The protein expression of DNMT1 ($p < 0.0001$; Figure 2(b)), DNMT3A ($p < 0.0001$; Figure 2(b)), and DNMT3B ($p < 0.0001$; Figure 2(b)) was also significantly decreased in the FA-treated HepG2 cells compared to the control.

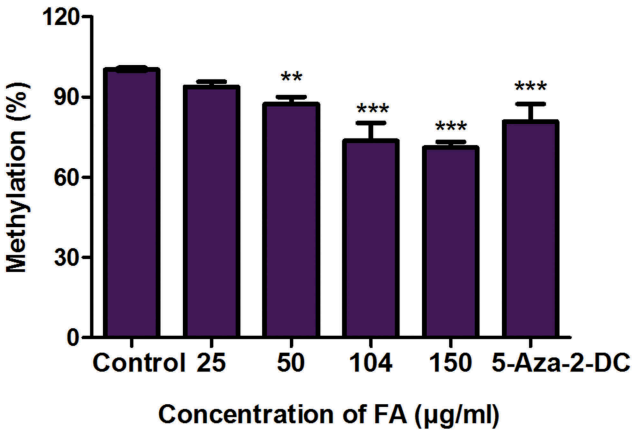

Figure 1. Fusaric acid induced global DNA hypomethylation in HepG2 cells. DNA isolated from control and FA-treated HepG2 cells were assayed for global DNA methylation by quantifying 5-methylcytosine using a Colorimetric Methylated DNA Quantification Kit. Fusaric acid decreased the percentage of 5-methylcytosine in HepG2 cells compared to the control. Results are represented as mean fold-change ± SD ($n = 3$). Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons test (**$p < 0.005$, ***$p < 0.0001$).

Fusaric acid altered DNMT promoter methylation in HepG2 cells

The methylation of gene promoters plays a major role in determining transcriptional activity and gene expression. We determined if the decrease in the mRNA expression of *DNMT1, DNMT3A*, and *DNMT3B* observed in the FA-treated HepG2 cells were a result of promoter methylation. FA significantly increased promoter methylation of *DNMT1* ($p < 0.0001$; Figure 3) and *DNMT3B* ($p < 0.0001$; Figure 3) in HepG2 cells compared to the control; however, the promoter methylation of *DNMT3A* was decreased in the lower FA concentrations (25, 50, and 104 µg/ml) and increased in the higher FA concentration (150 µg/ml) ($p < 0.0001$; Figure 3).

Fusaric acid decreased miR-29b promoter methylation, upregulated miR-29b, and decreased the expression of Sp1 in HepG2 cells

The expression of miR-29b is regulated by DNA methylation; miR-29b is silenced by DNA hypermethylation whereas DNA hypomethylation is known to upregulate miR-29b [36]. Since FA induced DNA hypomethylation in HepG2 cells, we determined the effect of FA on the promoter methylation and expression of miR-29b. FA significantly decreased the promoter methylation of miR-29b ($p < 0.0001$; Figure 4(a)) and increased the expression of miR-29b ($p < 0.0001$; Figure 4(b)) in HepG2 cells compared to the control. The expression of miR-29b was also significantly increased by 5-aza-2-DC ($p < 0.0001$; Figure 4(b)).

MiR-29b is also a known regulator of *DNMT* expression. MiR-29b was previously shown to directly target *DNMT3A* and *DNMT3B* and indirectly target *DNMT1* via repression of the transcriptional activator, *Sp1* [35,36,40]. This was confirmed using the bioinformatics prediction algorithm software, TargetScan version 7.1. MiR-29b was found to have complementary base pairs with *DNMT3A* at

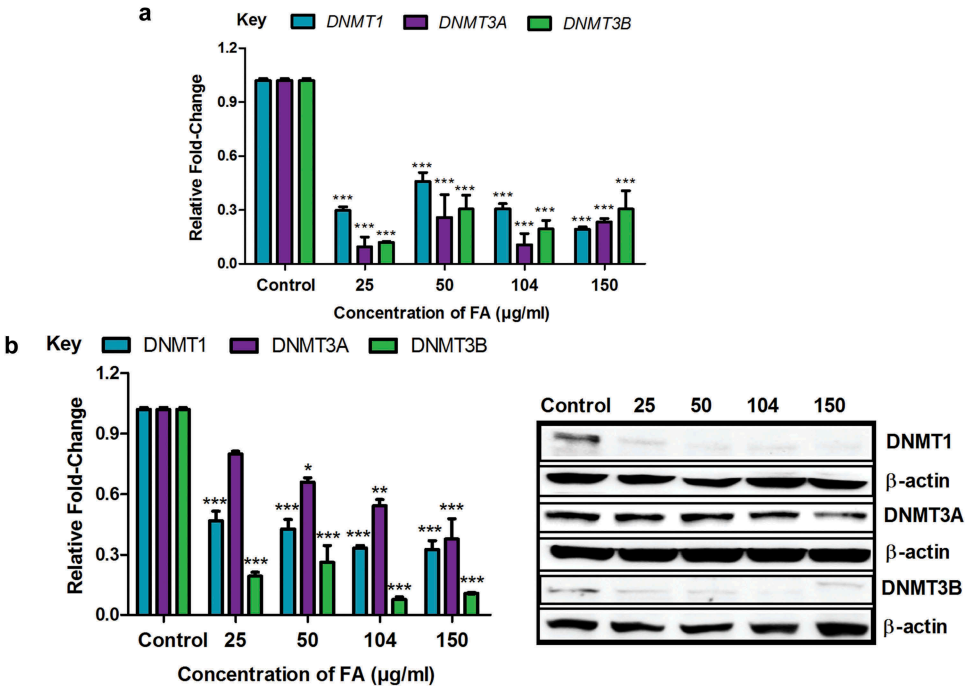

Figure 2. The effect of FA on DNA methyltransferases in HepG2 cells. (a) RNA isolated from control and FA-treated HepG2 cells were reverse transcribed into cDNA and analyzed by qPCR. Fusaric acid significantly decreased the mRNA expression of *DNMT1, DNMT3A,* and *DNMT3B* in HepG2 cells. (b) Protein expression of DNMT1, DNMT3A, and DNMT3B were determined by Western blot. Fusaric acid decreased the protein expression of DNMT1, DNMT3A, and DNMT3B in HepG2 cells. Results are represented as mean fold-change ± SD ($n = 3$). Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$).

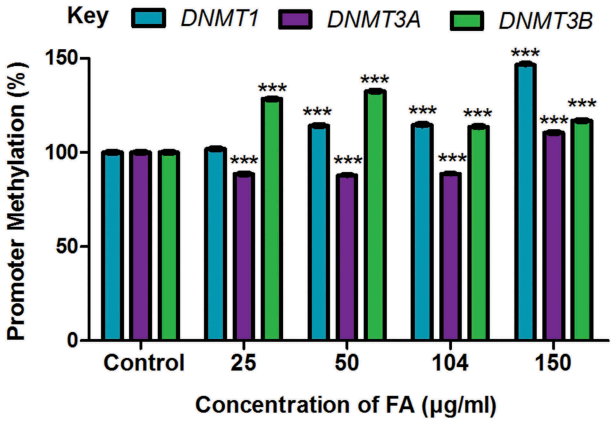

Figure 3. The effect of FA on the promoter methylation of *DNMT1, DNMT3A,* and *DNMT3B* in HepG2 cells. DNA isolated from control and FA-treated HepG2 cells were assayed for *DNMT* promoter methylation using the OneStep qMethyl Kit. Fusaric acid induced promoter hypermethylation of *DNMT1* and *DNMT3B*, and altered promoter methylation of *DNMT3A* in HepG2 cells. Results are represented as mean fold-change ± SD ($n = 3$). Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons test (***$p < 0.0001$).

positions 862-868, 1305-1311, and 5559-5565; *DNMT3B* at position 1202-1209; and *Sp1* at position 3584-3591 (Figure 4(c)). *DNMT1* was not a direct target of miR-29b. Due to the increase in miR-29b and decrease in *DNMT* expression by FA, we then determined the effect of FA on the mRNA expression of *Sp1*. FA significantly decreased the expression of *Sp1* ($p < 0.0001$; Figure 4(d)) in HepG2 cells compared to the control. These data suggest that the decrease in the mRNA expression of *DNMT1, DNMT3A*, and *DNMT3B* may be influenced by miR-29b.

Fusaric acid decreased the ubiquitination of DNMT1, DNMT3A, and DNMT3B by decreasing the expression of UHRF1 and USP7 in HepG2 cells

PTMs such as acetylation and ubiquitination regulate the activity and expression of DNMTs. The acetylation of DNMTs triggers the ubiquitination of DNMTs leading to proteasomal degradation. We determined if the decrease in the protein expression of DNMT1, DNMT3A, and DNMT3B in the FA treatments were a result of the ubiquitination and proteasomal degradation of the DNMTs. FA significantly decreased the ubiquitination of DNMT1 ($p = 0.0753$; Figure 5(a)),

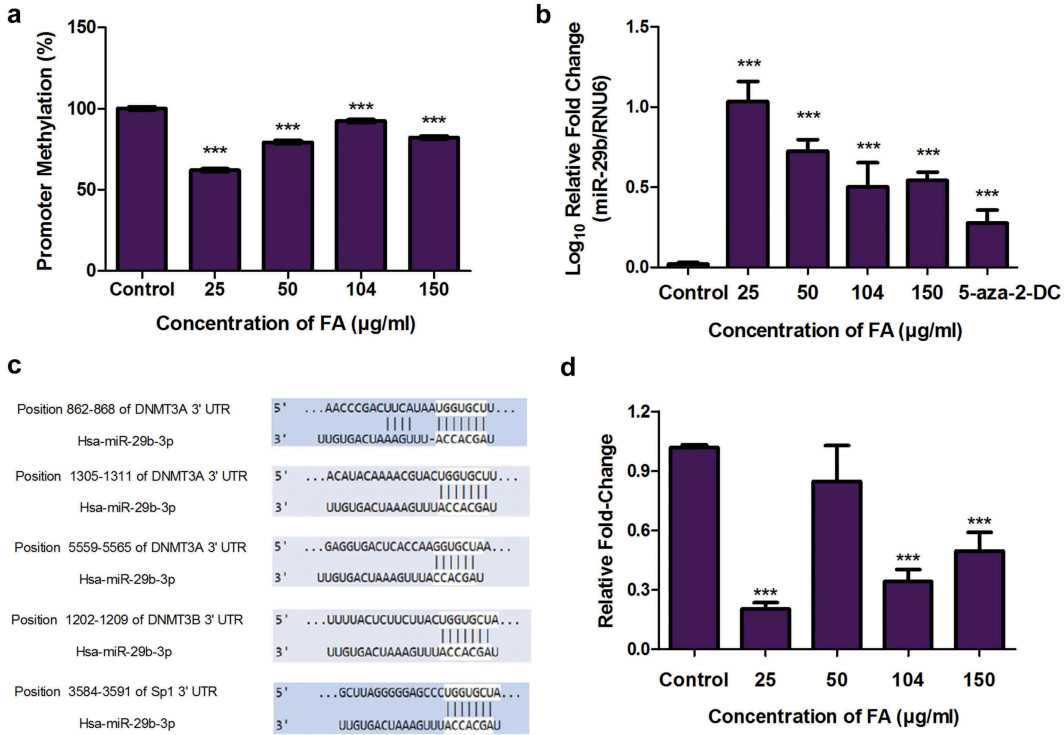

Figure 4. The effect of FA on miR-29b and *Sp1* in HepG2 cells. (a) DNA isolated from control and FA-treated HepG2 cells were assayed for miR-29b promoter methylation using the OneStep qMethyl Kit. Fusaric acid induced promoter hypomethylation of miR-29b in HepG2 cells. (b) RNA isolated from control and FA-treated HepG2 cells were reverse transcribed into cDNA and analyzed by qPCR. Fusaric acid significantly increased the expression of miR-29b in HepG2 cells. (c) TargetScan analysis of miR-29b to the 3UTRs of *DNMT3A, DNMT3B,* and *Sp1*. (d) RNA isolated from control and FA-treated HepG2 cells were reverse transcribed into cDNA and analyzed for *Sp1* expression by qPCR. Fusaric acid decreased the mRNA expression of *Sp1* in HepG2 cells. Results are represented as mean fold-change ± SD ($n = 3$). Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons test (***$p < 0.0001$).

DNMT3A ($p = 0.0008$; Figure 5(a)), and DNMT3B ($p < 0.0001$; Figure 5(a)) in HepG2 cells compared to the control. However, at 150 µg/ml FA the ubiquitination of DNMT1 and DNMT3B were increased.

The ubiquitination regulators, UHRF1 and USP7, are the major enzymes responsible for ubiquitinating and deubiquitinating DNMTs, respectively. The FA-induced decrease in the ubiquitination of DNMT1, DNMT3A, and DNMT3B led to the assessment of *UHRF1* and *USP7*. FA significantly decreased the mRNA expression of *UHRF1* ($p < 0.0001$; Figure 5(b)) and *USP7* ($p < 0.0001$; Figure 5(b)) in HepG2 cells compared to the control. These results indicate that the decrease in the protein expression of DNMT1, DNMT3A, and DNMT3B observed in the FA-treated cells is not due to the ubiquitination and proteasomal degradation of DNMTs.

Fusaric acid induced MBD2 promoter hypomethylation and increased the expression of MBD2 in HepG2 cells

Methyl CpG binding domain protein 2 (MBD2), a major MBD, promotes global DNA hypomethylation by binding specifically to methylated DNA and functioning as a methylation-dependent transcriptional repressor and DNA demethylase. We determined if the FA-induced decrease in global DNA methylation occurred as a result of MBD2. FA significantly decreased *MBD2* promoter methylation ($p < 0.0001$; Figure 6(a)) and increased the protein expression of MBD2 ($p < 0.0001$; Figure 6(b)) in HepG2 cells compared to the control. The mRNA expression of *MBD2* ($p < 0.0001$), and other MBDs such as *MBD1* ($p < 0.0001$), *MBD3* ($p < 0.0001$), *MBD4* ($p < 0.0001$), *MBD5* ($p < 0.0001$), and *MBD6* ($p < 0.0001$) were significantly

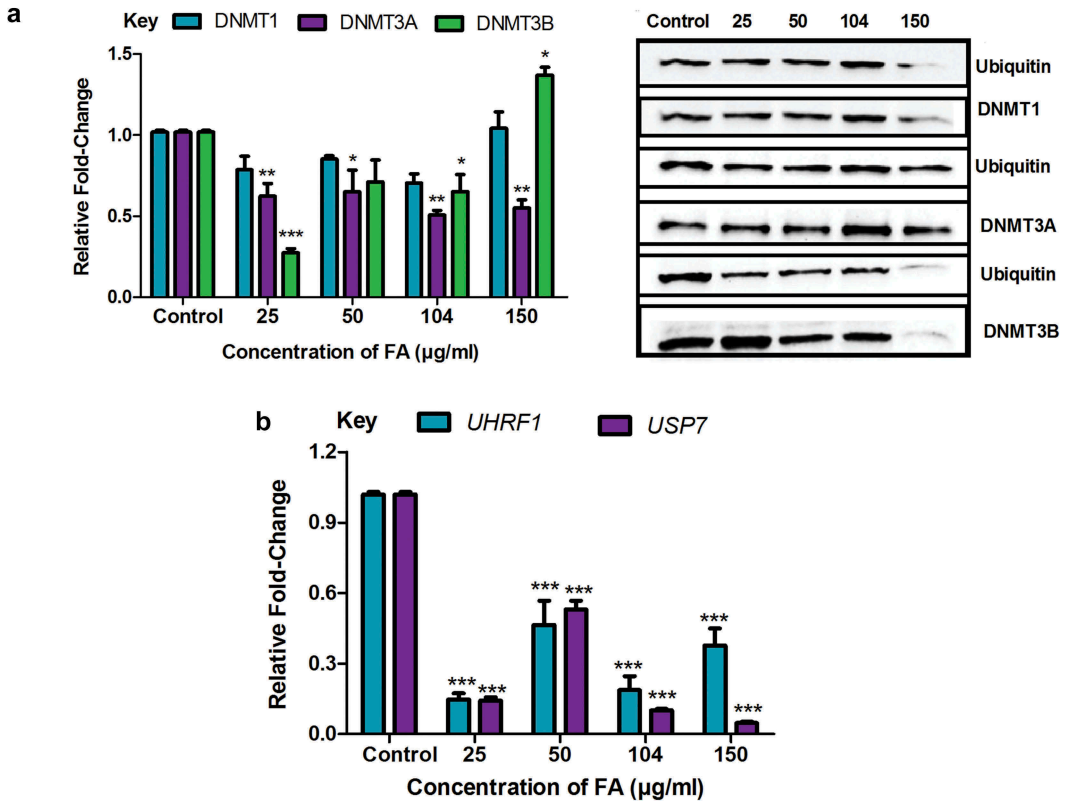

Figure 5. The effect of FA on the ubiquitination of DNMT1, DNMT3A, and DNMT3B in HepG2 cells. (a) The ubiquitination of DNMT1, DNMT3A, and DNMT3B were detected by immuno-precipitation and Western blot. Fusaric acid altered the ubiquitination of DNMT1, DNMT3A, and DNMT3B in HepG2 cells. (b) RNA isolated from control and FA-treated HepG2 cells were reverse transcribed into cDNA and analyzed by qPCR. Fusaric acid significantly decreased the expression of *UHRF1* and *USP7* in HepG2 cells. Results are represented as mean fold-change ± SD ($n = 3$). Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons test (*$p < 0.05$, **$p < 0.005$, ***$p < 0.0001$).

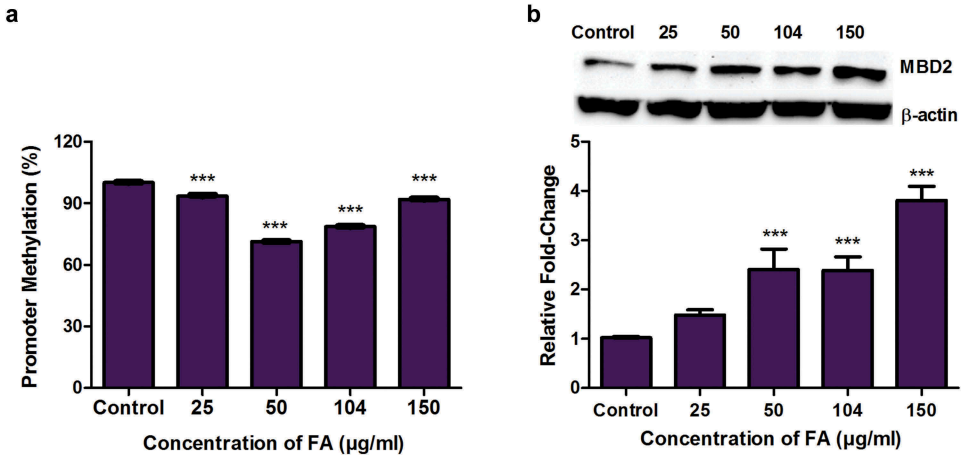

Figure 6. The effect of FA on *MBD2* promoter methylation and MBD2 expression in HepG2 cells. (a) DNA isolated from control and FA-treated HepG2 cells were assayed for *MBD2* promoter methylation using the OneStep qMethyl Kit. Fusaric acid significantly induced promoter hypomethylation of *MBD2* in HepG2 cells. (b) Protein expression of MBD2 was determined by Western blot. Fusaric acid significantly increased the protein expression of MBD2 in HepG2 cells. Results are represented as mean fold-change ± SD ($n = 3$). Statistical significance was determined by one-way ANOVA with the Bonferroni multiple comparisons test (***$p < 0.0001$).

decreased in the FA-treated cells compared to the control (Supplementary Table S1).

Discussion

FA, a neglected mycotoxin found in agricultural foods, alters biological pathways causing toxicity in various plant and animal models. To date several mechanisms of FA toxicity have been described [10,12,14,15,20,21,23]; however, the effect of FA on epigenetic modifications is unknown. DNA methylation is an important epigenetic modification that regulates chromatin structure and alters gene expression and thus may play a crucial role in FA toxicity. In this study, we provide evidence that FA alters global DNA methylation in HepG2 cells by modulating the expression of DNMTs and demethylases in a mechanism that involves alterations in promoter methylation and miR-29b expression, but not the ubiquitination of DNMTs.

FA induced global DNA hypomethylation in HepG2 cells as evidenced by the significant decrease in 5-methylcytosine content (Figure 1); this global DNA hypomethylation is due to a concomitant decrease in the expression of the *de novo* methyltransferases, DNMT3A and DNMT3B, and the maintenance methyltransferase, DNMT1 (Figure 2(a,b)) as well as an increase in the demethylase, MBD2 (Figure 6(b)). Furthermore, FA altered the mRNA expression of *DNMT1* and *DNMT3B* by inducing promoter hypermethylation (Figure 3). This is in agreement with previous studies in which promoter hypermethylation of *DNMT1* and *DNMT3B* decreased the mRNA expression of *DNMT1* and *DNMT3B*, respectively [41,42]. Although promoter hypomethylation of *DNMT3A* is associated with an increase in the transcription of *DNMT3A*, the decrease in *DNMT3A* mRNA transcript levels observed in the FA-treated HepG2 cells suggests possible regulation at the post-transcriptional level.

MicroRNAs regulate gene expression at the post-transcriptional level. This occurs in a sequence specific manner and leads to either the degradation of the target mRNA or inhibition of translation. MiR-29b, regulated by DNA methylation, was previously shown to repress DNA methylation by directly targeting *DNMT3A* and *DNMT3B*, and indirectly targeting *DNMT1* by inhibiting the transcriptional activator, *Sp1* [35,36]. This was further confirmed using TargetScan version 7.1 (Figure 4(c)). FA significantly upregulated the expression of miR-29b in HepG2 cells (Figure 4(b)) and the expression of miR-29b was inversely correlated with the DNA methylation status in the FA-treated HepG2 cells, as evidenced by the significant decrease in miR-29b promoter methylation (Figure 4(a)). The upregulation of miR-29b also corresponds with the decrease in the mRNA expression of *Sp1, DNMT1, DNMT3A*, and *DNMT3B* in the FA-treated cells. This is in agreement with previous studies where overexpression of miR-29b was found to downregulate the expression of *DNMT3A* and *DNMT3B*, and induce global DNA hypomethylation in acute myeloid leukaemia (AML) and lung cancer cells [35,36]. Overexpression of miR-29b in AML was also shown to downregulate the expression of *Sp1* causing a subsequent decrease in *DNMT1* expression and global DNA hypomethylation [35,40]. Therefore, these results indicate that the FA-induced increase in miR-29b expression may be an alternative mechanism for the reduced *DNMT3A* mRNA expression and an additional mechanism for the reduced *DNMT1* and *DNMT3B* mRNA expressions.

The protein expression of DNMT1, DNMT3A, and DNMT3B was also significantly decreased in the FA-treated HepG2 cells (Figure 2(b)). PTMs such as acetylation and ubiquitination play a major role in influencing the catalytic activity, stability, and protein-protein interactions of DNMTs. The acetylation of DNMTs is mediated by Tip60 and primes DNMTs for UHRF1-mediated ubiquitination and proteasomal degradation [29,37,38]. The DNMTs are deacetylated by HDAC1 and HDAC2, and deubiquitinated by USP7.

The role of acetylation and ubiquitination on the regulation of DNMT1 is well understood. The acetylation of DNMT1 on lysine (K) residues, K1349 and K1415, in the catalytic domain decreases DNMT1 activity whereas the acetylation of K1111, K1113, K1115, and K1117 in the lysine-glycine rich (KG)-repeat increases the transcriptional repressor activity of DNMT1 [43]. The acetylation of lysine residues in the KG-repeat also increases the DNMT1-UHRF1 interaction and impairs the DNMT1-USP7 interaction, thereby, promoting the ubiquitination and degradation of DNMT1 [44,45]. The overexpression of UHRF1 was also shown to increase the ubiquitination of DNMT1 and decrease DNMT1 expression [44]. Previous studies also indicate that UHRF1

physically interacts with DNMT3A and DNMT3B, thereby, inhibiting the activity of both DNMT3A and DNMT3B and promoting proteasomal degradation [46].

The decrease in the protein expression of DNMT1, DNMT3A, and DNMT3B in the FA-treated HepG2 cells suggested that FA may also decrease the protein expression of DNMTs by ubiquitination and proteasomal degradation. In fact, FA actually decreased the ubiquitination of DNMT1, DNMT3A, and DNMT3B in HepG2 cells (Figure 5(a)). The expression of *UHRF1* and *USP7* was also significantly decreased in the FA-treated cells (Figure 5(b)), suggesting that the decrease in the ubiquitination of DNMT1, DNMT3A, and DNMT3B was a result of *UHRF1* and *USP7*. Thus, the FA-induced decrease in the protein expression of DNMT1, DNMT3A, and DNMT3B was due to the increased *DNMT* promoter methylation and/or miR-29b expression and a subsequent inhibition of translation, and not the ubiquitination and proteasomal degradation of the DNMT protein.

UHRF1 also contains a methyl DNA-binding domain, SRA (SET and RING associated) domain, that binds preferentially to hemi-methylated DNA and functions to recruit DNMT1 to hemi-methylated CpG islands to facilitate maintenance of DNA methylation [47]. The observed decrease in global DNA methylation in the FA-treated HepG2 cells may also occur as a result of the decrease in UHRF1 and DNMT1 leading to a loss in the maintenance of DNA methylation.

In addition to alterations in the expression of DNMTs and UHRF1, FA may also induce global DNA hypomethylation by targeting the transcriptional repressor and demethylase, MBD2. MBD2 plays an essential role in hypomethylation and was previously shown to activate gene expression by promoting demethylation of several target genes. Our results indicate that FA induced *MBD2* promoter hypomethylation (Figure 6(a)) and increased the protein expression of MBD2 (Figure 6(b)) in HepG2 cells. This occurred despite the significant decrease in the mRNA expression of *MBD2* (Supplementary Table S1), and suggests that the FA-induced expression of MBD2 may contribute to global DNA hypomethylation. Previous studies indicate *MBD2* promoter hypomethylation to be associated with active gene transcription and an increase in *MBD2* expression. Although, MBD2 is associated with gene activation, overexpression of MBD2, and global DNA hypomethylation leads to genomic instability in several human cancers [48,49].

Global DNA hypomethylation is considered a hallmark of cancer as it leads to genomic instability and increases the frequency of mutations [50]. Global DNA hypomethylation also inhibits cellular differentiation [51] and induces apoptosis [51–54]. Previously, FB_1, a *Fusarium*-derived mycotoxin often co-produced with FA, was shown to induce global DNA hypomethylation (by modulating the expression of DNMTs and MBD2) and histone demethylation, possibly leading to chromatin instability and liver tumourigenesis [55]. FB_1 also alters promoter methylation of tumour suppressor genes (*c-myc, p15, p16*, and *e-cadherin*) [56,57], inhibits miR-27b and increases cytochrome P450 1B1 [58] leading to hepatic neoplastic transformation. Zearalenone also induces global DNA hypomethylation and reduces the viability of human bronchial epithelial cells via DNA damage, cell cycle arrest, and apoptosis [59]. In contrary, other *Fusarium* produced mycotoxins such as deoxynivalenol and T2 toxin induce global DNA hypermethylation and histone demethylation [60,61]. The toxicity of FA has been mainly attributed to oxidative stress, DNA damage, and apoptosis [10,12–15,62], and the FA-induced global DNA hypomethylation may provide an alternative mechanism by which FA induces its genotoxic and cytotoxic effects.

In conclusion, this study provides an alternative mechanism of FA-induced genotoxicity and cytotoxicity at the epigenetic level. The results indicate that FA induces global DNA hypomethylation in HepG2 cells by decreasing the expression of DNMT1, DNMT3A, and DNMT3B and increasing the expression of MBD2 (Figure 7). The results further indicate that FA decreases the expression of DNMT1, DNMT3A, DNMT3B, and MBD2 proteins by increasing promoter methylation and/or by upregulating miR-29b. It has also been shown that miR-29b itself can be regulated by DNA methylation, and that reduced methylation as seen globally following treatment with FA may lead to increased expression of miR-29b. These findings suggest that FA-induced changes in DNA methylation may potentially be used as a biomarker for FA exposure and toxicity. Finally, targeting the DNA methylation pathway via epigenetic modulation of DNMTs and miR-29b may provide a therapeutic intervention against FA toxicity; this is particularly

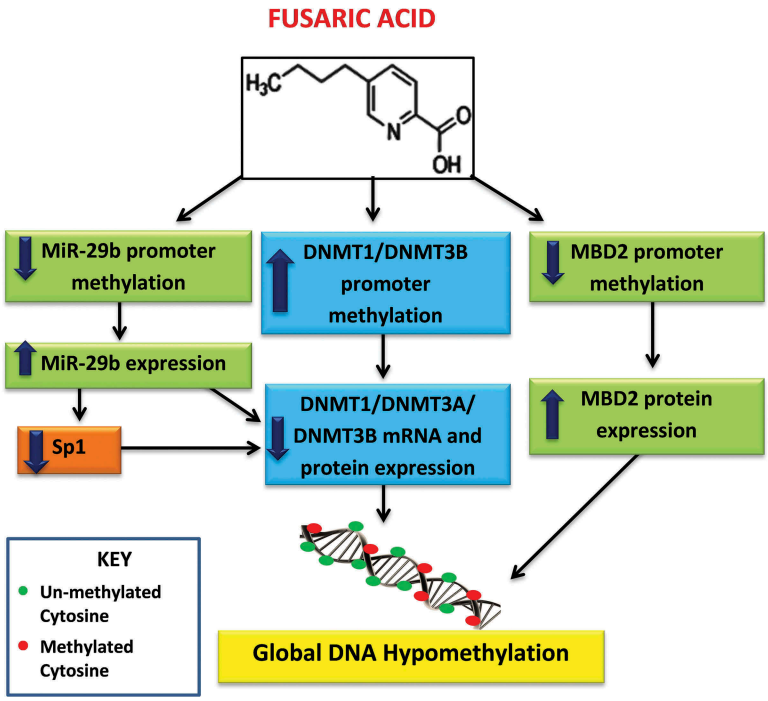

Figure 7. Proposed mechanism of FA-induced global DNA hypomethylation in HepG2 cells. FA induces global DNA hypomethylation by decreasing the mRNA and protein expression of DNMT1, DNMT3A, and DNMT3B. The decrease in DNMTs is caused by promoter hypermethylation of *DNMT1* and *DNMT3B*, and promoter hypomethylation and upregulation of miR-29b. MiR-29b negatively regulates the mRNA expression of *DNMT1, DNMT3A,* and *DNMT3B*. In addition, FA may also induce global DNA hypomethylation by causing promoter hypomethylation and upregulation of MBD2.

important in poverty stricken areas where maize forms a staple diet and the risk of FA contamination is high.

Materials and methods

Materials

FA (*Gibberella fujikuroi*, F6513) and the DNA methylation inhibitor, 5-aza-2-deoxycytidine (5-aza-2-DC; A3653) were purchased from Sigma-Aldrich. The HepG2 cell line was purchased from Highveld Biologicals. Cell culture consumables were obtained from Lonza Biotechnology. Western Blot reagents were purchased from Bio-Rad. All other reagents were purchased from Merck.

Cell culture and treatment

HepG2 cells (1.5×10^6) were cultured (37°C, 5% CO_2) in complete culture media (CCM; Eagle's Minimum Essentials Medium (EMEM) containing 10% foetal calf serum, 1% penicillin-streptomycin fungizone, and 1% L-glutamine), until 90% confluent. Stocks of FA (1 mg/ml) were prepared in 0.1 M PBS and the cells were incubated (37°C, 5% CO_2, 24 h) with various concentrations of FA (25, 50, 104, and 150 µg/ml). These FA concentrations were obtained from literature [10] and represented 90%, 75%, 50%, and 40% cell viabilities, respectively. The 5-aza-2-DC (50 mM) stock was prepared in 100% DMSO. The concentration of 5-aza-2-DC (10 µM, 24 h) inducing DNA hypomethylation in HepG2 cells was obtained from literature [63] and used as a negative control. An untreated control (CCM only) was also prepared. Cell viability was determined using the trypan blue cell exclusion method. All results were verified by performing two independent experiments in triplicate.

DNA isolation and quantification of DNA methylation

Genomic DNA was isolated from control and FA-treated HepG2 cells. Briefly, HepG2 cells were incubated in cell lysis buffer (600 µl, 15 min, RT; 0.5 M EDTA (pH 8.0), 1 M Tris-Cl (pH 7.6), 0.1% SDS) and potassium acetate buffer (600 µl, 8 min, RT;

5 M potassium acetate, glacial acetic acid) before centrifugation (13,000×g, 5 min, 24°C). The supernatant containing genomic DNA was transferred into fresh 1.5 ml micro-centrifuge tubes and 100% isopropanol (600 µl) was added to precipitate the DNA which was recovered by centrifugation (13,000×g, 5 min, 24°C). The DNA was washed in 100% ethanol (300 µl) and centrifuged (13,000×g, 5 min, 24°C). The DNA pellets were air dried (30 min, RT), resuspended in DNA hydration buffer (40 µl; 10 mM EDTA (pH 8.0), 100 mM Tris-Cl (pH 7.4)), and heated (65°C, 15 min). DNA concentration was determined using the Nanodrop2000 spectrophotometer (Thermo-Fischer Scientific) and standardized to 100 ng/µl. DNA purity was assessed using the A260/A280 absorbance ratios.

The DNA was used to quantify global DNA methylation using the Colorimetric Methylated DNA Quantification Kit (Abcam, ab117128), as per manufacturer's instructions. The percentage 5-methylcytosine (5-mC) content was calculated using the supplied formula (Supplementary Information) and represented as fold-change relative to the control.

Promoter methylation of miR-29b, DNMTs, and MBD2

Genomic DNA was isolated from control and FA-treated HepG2 cells using the Quick-g-DNA MiniPrep Kit (Zymo Research, D3007), as per manufacturer's instructions. The isolated DNA was then eluted in nuclease-free water and purified using the DNA Clean and Concentrator™-5 Kit (Zymo Research, D4003), as per manufacturer's instructions. The DNA was quantified using the Nanodrop2000 spectrophotometer and standardized to 4 ng/µl. The promoter methylation of *DNMT1, DNMT3A, DNMT3B, MBD2,* and miR-29b was assessed using the OneStep qMethyl Kit (Zymo Research, 5310), as per manufacturer's instructions. Briefly, 20 ng DNA was subject to a test and reference reaction containing specific primers (Supplementary Table S2). Cycling conditions were as follows: digestion by methyl sensitive restriction enzymes (AccII, HpaII, and HpyCH4IV) (37°C, 2 h), initial denaturation (95°C, 10 min), followed by 45 cycles of denaturation (95°C, 30 s), annealing (Supplementary Table S2, 60 s), extension (72°C, 60 s), final extension (72°C, 60 s), and a hold at 4°C. The percentage methylation was calculated using the supplied formula (Supplementary Information) and represented as fold-change relative to the control.

RNA isolation and quantitative polymerase chain reaction (qPCR)

Total RNA was extracted from control and FA-treated HepG2 cells using Qiazol Reagent (Qiagen, 79306). Briefly, HepG2 cells were rinsed in 0.1 M PBS and incubated (5 min, RT) in 500 µl Qiazol and 500 µl 0.1 M PBS before extraction with a cell scraper. Cellular lysates were incubated overnight (−80°C). Thereafter, chloroform (100 µl) was added and centrifuged (12,000×g, 4°C, 15 min). The aqueous phase containing RNA was transferred to fresh 1.5 ml micro-centrifuge tubes and 100% cold isopropanol (250 µl) was added to each sample before overnight incubation (−80°C). Samples were centrifuged (12,000×g, 4°C, 20 min) and the RNA pellets were washed in 75% cold ethanol (500 µl). Finally, samples were centrifuged (7,400×g, 4°C, 15 min), RNA pellets were air dried (30 min, RT), resuspended in nuclease-free water (15 µl), and incubated (3 min, RT). The RNA was quantified using the Nanodrop2000 spectrophotometer and standardized to 1,000 ng/µl. The A260/A280 absorbance ratio was used to assess RNA purity.

The RNA was used to prepare cDNA using the miScript II RT Kit (Qiagen, 218161), as per manufacturer's instructions. The expression of miR-29b was analyzed using the miScript SYBR Green PCR Kit (Qiagen, 218073) and specific 10X miScript primer assay [Hs_miR-29b_1, Qiagen, MS00006566], as per manufacturer's instructions. Human RNU6 (Qiagen, MS000033740) was used as the housekeeping gene to normalize microRNA expression.

For mRNA expression, cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad, 1708891), as per manufacturer's instructions. The expression of *DNMT1, DNMT3A, DNMT3B, MBD1-MBD6, Sp1, UHRF1,* and *USP7* was determined using the Sso Advanced™ Universal SYBR Green Supermix (Bio-Rad, 1725270), as per manufacturer's instructions. *GAPDH* was used as the housekeeping gene to normalize mRNA expression. Primer sequences and annealing temperatures are listed in Supplementary Table S2. All qPCR experiments were conducted using the CFX96 Real Time PCR System (Bio-Rad) and analyzed using the Bio-Rad CFX Manager™

Software version 3.1. The comparative threshold cycle (Ct) method was used to determine relative changes in expression [64].

Protein isolation and Western blot

The protein expression of DNMT1, DNMT3A, DNMT3B, and MBD2 was determined using Western blot. Briefly, crude protein extracts were isolated from control and FA-treated HepG2 cells using cytobuster reagent (200 µl; Novagen, 71009) supplemented with protease and phosphatase inhibitors (Roche; 05892791001 and 04906837001, respectively). The protein was quantified using the bicinchoninic acid (BCA) assay, standardized to 1 mg/ml and boiled (100°C, 5 min) in a 1:1 dilution with 1X Laemmli buffer [dH_2O, 0.5 M Tris-HCl (pH 6.8), glycerol, 10% SDS, 5% β-mercaptoethanol, 1% bromophenol blue]. Thereafter, the proteins were separated using sodium dodecyl sulphate polyacrylamide gel electrophoresis (10% resolving gel, 4% stacking gel; 1 h, 150 V) and transferred onto nitrocellulose membranes using the Bio-Rad Trans-Blot® Turbo Transfer System (20 V, 30 min). Following transfer, the membranes were blocked in 5% BSA in Tris buffered saline with 0.05% Tween 20 [TTBS; 150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.05% Tween 20, dH_2O, pH 7.5; 1 h, RT] and probed overnight (4°C) with primary antibody [DNMT1 (Cell Signalling Technology, #5032S; 1:250), DNMT3A (Cell Signalling Technology, #3598S; 1:500), DNMT3B (Santa Cruz, sc-130740; 1:250), and MBD2 (Santa Cruz, sc-271562; 1:500)]. The membranes were rinsed five times with TTBS (10 min, RT) and probed with a horse-radish peroxidase (HRP)-conjugated secondary antibody [goat anti-rabbit (Cell Signalling Technology, #7074S; 1:10,000) and goat anti-mouse (Cell Signalling Technology, #7076P2; 1:5,000); 1 h, RT]. The membranes were rinsed five times in TTBS (10 min, RT). The Clarity™ Western ECL Substrate Kit (Bio-Rad, #170-5060) was used to detect specific protein bands and the images were captured using the ChemiDoc™ XRS+ Molecular Imaging System (Bio-Rad). The membranes were then quenched in hydrogen peroxide (5%, 37°C, 30 min), rinsed once in TTBS (10 min, RT) and probed with the housekeeping protein, anti-β-actin (Sigma-Aldrich, A3854; 1:5,000; 30 min, RT) to normalize protein expression. Densitometric analysis was performed using the Bio-Rad Image Lab Software version 5.1 and the results were represented as a fold-change in band density (RBD) relative to the control.

Immuno-precipitation

Immuno-precipitation was used to determine ubiquitinated DNMT1, DNMT3A, and DNMT3B levels. Briefly, crude protein extracts were isolated from control and FA-treated HepG2 cells using 1X cell lysis buffer [500 µl; 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100]. The protein was quantified using the BCA assay and standardized to 1.5 mg/ml. Thereafter, the protein lysates (200 µl) were incubated with primary antibody [DNMT1 (Cell Signalling Technology, #5032S); DNMT3A (Cell Signalling Technology, #3598S); and DNMT3B (Santa Cruz, sc-130740); 1:100] overnight (4°C) and the antigen-antibody complex was precipitated using protein A beads (20 µl 50% bead slurry; Cell Signalling Technology, #9863) for 1–3 h at 4°C. The immuno-precipitates were recovered by centrifugation (14,000×g, 4°C, 30 s), washed five times in 1X cell lysis buffer (500 µl), resuspended in 3X Laemmli buffer (20 µl) and boiled (100°C, 5 min). The samples were then analyzed by Western blotting using the following antibodies: primary antibody [ubiquitin (BD BioSciences, BD550944; 1:1,000), DNMT1 (Cell Signalling Technology, #5032S; 1:1,000), DNMT3A (Cell Signalling Technology, #3598S; 1:1,000), and DNMT3B (Santa Cruz, sc-130740; 1:500)] and secondary antibody [goat anti-rabbit (Cell Signalling Technology, #7074S) and goat anti-mouse (Cell Signalling Technology, #7076P2); 1:5,000]. The protein expression of ubiquitin was divided by the total protein expressed to determine the ratio of ubiquitinated protein.

Statistical analysis

GraphPad Prism version 5.0 (GraphPad Prism Software Inc.) was used to perform all statistical

analyses. The one-way analysis of variance (ANOVA) with the Bonferroni multiple comparisons test was used to analyze the data. The results were expressed as the mean fold-change ± standard deviation (SD) (n = 3), unless otherwise indicated. Statistical significance was considered at $p < 0.05$.

Author contributions

TG, SN, PN, and AC conceptualized and designed the study. TG conducted all laboratory experiments, analyzed the data, and wrote the manuscript. PN assisted in conducting laboratory experiments. SN, PN, and AC revised the manuscript. All authors have read the manuscript prior to submission.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability

All datasets generated in this study are available in Supplementary Information and from the corresponding author on reasonable request.

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