

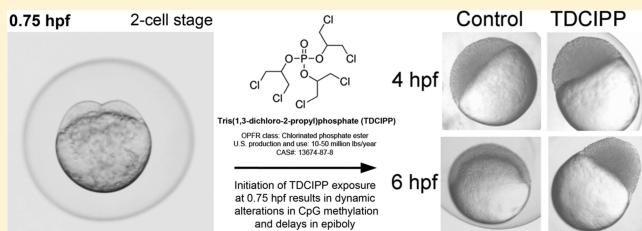
Dynamic Alterations in DNA Methylation Precede Tris(1,3-dichloro-2-propyl)phosphate-Induced Delays in Zebrafish Epiboly

Allison Kupsco, Subham Dasgupta, Christine Nguyen, and David C. Volz*^{ID}

Department of Environmental Sciences, University of California, Riverside, Riverside, California 92521, United States

S Supporting Information

ABSTRACT: Tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) is an organophosphate flame retardant that impacts zebrafish epiboly, an effect that may be associated with genomewide hypomethylation. Using zebrafish as a model, the objectives of this study were (1) to quantify concentration-dependent impacts of TDCIPP on epiboly, (2) to determine whether co-exposure with folic acid (FA), a methyl donor, mitigates TDCIPP-induced impacts, and (3) using 10 previously identified TDCIPP-susceptible loci, to rely on bisulfite amplicon sequencing (BSAS) to monitor CpG methylation dynamics across multiple TDCIPP concentrations in the presence or absence of FA. Embryos were exposed to TDCIPP from 0.75 h postfertilization (hpf) to 2, 4, 6, or 24 hpf in the presence or absence of 1 mM FA. Although TDCIPP delayed epiboly by up to 3 h by 6 hpf and induced malformations by 24 hpf, FA was unable to mitigate TDCIPP-induced effects at any stage that was evaluated. Moreover, while no differences in global methylation were detected using a 5-methylcytosine DNA enzyme-linked immunosorbent assay, BSAS revealed that TDCIPP-induced effects on CpG methylation were dependent on concentration and developmental stage and that early effects on methylation do not persist despite continuous exposure. Our findings demonstrate that TDCIPP delays zebrafish epiboly, a phenotype that is preceded by complex, dynamic alterations in DNA methylation.



INTRODUCTION

Early zebrafish development is characterized by a series of coordinated cell divisions and movements that prepare the embryo for proper differentiation and patterning. Beginning at the end of the blastula period, cells amassed on the animal pole of the yolk spread over the surface in a process called epiboly. This represents the onset of gastrulation and cell fate determination. Proper cell migration during epiboly is critical for normal development, and epiboly mutants induce embryo mortality and severe abnormalities.¹ Similarly, chemicals that delay or arrest epiboly may lead to downstream abnormalities later in development.

Key early developmental events such as epiboly are preceded by reprogramming of the DNA methylome. Cytosine methylation of DNA is an epigenetic mark that regulates transcription and differentiation. During cleavage within zebrafish embryos, parentally inherited 5-methylcytosines (5-mCs) are passively erased and, beginning at 2 h postfertilization (hpf), patterns are reestablished to reach paternal methylation levels by gastrulation.^{2,3} Treatment of early zebrafish embryos with 5-azacytidine (5-azaC), a DNA methyltransferase inhibitor, decreased the level of DNA methylation and induced abnormal somite patterning,⁴ demonstrating that DNA methylation is critical for patterning and differentiation.

Tris(1,3-dichloro-2-propyl)phosphate (TDCIPP) is a high-production volume flame retardant found within indoor environments, including house dust, sofas, and baby furniture.^{5,6} Metabolites of TDCIPP have been detected in

urine from mother–toddler pairs,⁷ and human exposure has increased over the past 10 years.⁸ TDCIPP has been linked to impacts on behavior,^{9–11} reproduction,^{12,13} receptor signaling,¹⁴ and gene expression.¹⁵ Furthermore, previous studies demonstrated that TDCIPP exposure within the first 2 h of zebrafish embryogenesis leads to enhanced toxicity later in development,¹⁶ and others have reported that early exposure to TDCIPP delays epiboly and impacts segmentation.¹⁵ However, the mechanisms of these effects during early embryonic development within zebrafish remain unknown. Therefore, as chronic human exposure to TDCIPP following migration from treated products is likely common within the United States, additional studies with animal models are needed (1) to provide a foundation for understanding the impacts of TDCIPP exposure on early embryonic development and (2) to help in the formulation of mechanistic-based hypotheses for prenatal developmental toxicity studies within rodents and epidemiological studies within human populations.

Using a restriction enzyme-based approach, our earlier studies demonstrated that the cleavage period (0.75–2.25 hpf) during embryogenesis is susceptible to TDCIPP-induced delays in zygotic genome methylation,¹⁶ suggesting that downstream effects on embryonic development may be

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associated with impacts on methylation during cleavage. Therefore, we conducted whole-genome bisulfite sequencing (WGBS) to identify the magnitude and extent, if any, of 5-azaC- and TDCIPP-induced effects on the entire DNA methylome at 2 hpf (following exposure from 0.75 to 2 hpf) at single-base pair resolution.¹⁷ Within this study, we found that rapid embryonic uptake of 250 μ M 5-azaC or 2 μ M TDCIPP from 0.75 to 2 hpf resulted in chemical- and chromosome-specific alterations in cytosine methylation at 2 hpf, and that TDCIPP-induced impacts on the zebrafish DNA methylome at 2 hpf are complex and primarily localized to regions outside of classical CpG islands.¹⁷ Importantly, this study yielded positional information about altered CpG methylation within TDCIPP-susceptible loci, data that may be useful for identifying future biomarkers of TDCIPP exposure within zebrafish and humans.

Interestingly, TDCIPP did not inhibit DNA methyltransferase activity *in vitro* up to 500 μ M,¹⁷ suggesting that TDCIPP may induce CpG hypomethylation via depletion of methyl donors (e.g., S-adenosyl-L-methionine). Methyl supplementation has the potential to reverse chemically induced DNA methylation,^{18,19} and maternal supplementation with methyl donors (such as folic acid) has been shown to alter methylation profiles in offspring.^{20–22} Therefore, the objectives of the current study were (1) to quantify TDCIPP-induced impacts on epiboly as a function of concentration, (2) to determine whether co-exposure with folic acid mitigates TDCIPP-induced impacts on epiboly, and (3) on the basis of TDCIPP-susceptible loci identified from our previous WGBS study, to rely on bisulfite amplicon sequencing (BSAS), a cost-effective, targeted sequencing-based strategy,²³ to monitor CpG methylation dynamics across multiple TDCIPP concentrations and developmental stages in the presence or absence of folic acid.

MATERIALS AND METHODS

Animals. Adult 5D zebrafish (*Danio rerio*) were maintained and bred on a recirculating system as previously described by Vliet et al.²⁴ All embryos were collected immediately after spawning and staged according to previously described methods.²⁵ Adult breeders were handled and treated in accordance with an Institutional Animal Care and Use Committee (IACUC)-approved animal use protocol (20150035) at the University of California, Riverside.

Chemicals. TDCIPP (98% pure) and folic acid (FA) ($\geq 97\%$) were purchased from ChemService (West Chester, PA) and Sigma-Aldrich (St. Louis, MO), respectively. TDCIPP stocks and working solutions were prepared in embryo medium (EM) as previously described.¹⁶ FA treatments were prepared in EM immediately prior to each experiment to minimize degradation.

Epiboly and Deformity Assessments. Viable 5D embryos (30 initial embryos per replicate beaker) were staged within 30 min after spawning, randomly distributed to clean 50 mL glass beakers (three beakers per treatment for 4 and 6 hpf time points; nine beakers per treatment for the 24 hpf time point), and then exposed to 10 mL of vehicle [0.1% dimethyl sulfoxide (DMSO)] or TDCIPP (0.78, 1.56, or 3.12 μ M) in the presence or absence of 1 mM FA. Thirty initial embryos per replicate beaker were exposed to ensure that a sufficient number of embryos remained for the imaging procedures described below. TDCIPP concentrations were selected on the basis of our previous work,¹⁶ a study published by Fu et al.,¹⁵

and additional range-finding exposures using epiboly progression as a readout. The FA concentration selected for this study was based on a previous study that successfully mitigated selenite-induced cardiac and neural abnormalities using 1 mM FA supplementation.¹⁹ In addition, as 1 mM FA (~ 0.44 mg/mL FA) approached the aqueous solubility at room temperature and pH 7 (all FA treatment solutions in this study exhibited a yellowish-orange color), this concentration represented the maximum allowable concentration for determining whether FA mitigates TDCIPP-induced effects.

All exposures were conducted under static conditions in the dark (to prevent photolysis of FA) at 28 °C from 0.75 hpf to 4, 6, or 24 hpf. At 4 or 6 hpf, embryos were chilled for 30 min at 4 °C to cease development for imaging, oriented in lateral recumbency within 4% methyl cellulose (10 embryos per beaker), and imaged using a Leica MZ10 F stereomicroscope equipped with a DMC2900 camera. Epiboly progression was assessed by quantifying cell height above the yolk at 4 and 6 hpf, and as percent epiboly at 6 hpf; all measurements were performed within ImageJ (<https://imagej.nih.gov/ij/>). At 24 hpf, embryos were imaged directly in EM (20 embryos per beaker) and scored as normal, deformed, or dead.

Genomic DNA Extractions. Embryos were exposed to vehicle (0.1% DMSO) or TDCIPP (0.78 or 3.12 μ M) in the presence or absence of 1 mM FA as described above (60 initial embryos per replicate beaker; 12 beakers per treatment for 2 hpf; four beakers per treatment for 4 and 6 hpf). Sixty initial embryos per replicate beaker were exposed to ensure that 50 embryos per replicate remained for genomic DNA extractions. At 2 hpf, three beakers containing 50 embryos each were pooled into a single 2 mL cryovial and snap-frozen in liquid nitrogen, resulting in four replicate pools containing 150 embryos per pool; at 4 and 6 hpf, 50 embryos from each beaker were transferred to four 2 mL cryovials and snap-frozen in liquid nitrogen, resulting in four replicate pools containing 50 embryos per pool. Genomic DNA was extracted from 2, 4, or 6 hpf embryos using a slightly modified version of the Wizard Genomic DNA Purification Kit (Promega, Madison, WI). Following homogenization, each homogenate was spiked with 10 μ L of proteinase K (>600 mAU/mL) and 1 μ L of 100 mg/mL RNase A, incubated at 55 °C while being shaken at 300 rpm, and then incubated with an additional 1 μ L of RNase A for 4 h at 37 °C. Samples were cleaned and concentrated using a Genomic DNA Clean & Concentrator-10 Kit (Zymo Research Corp., Irvine, CA) per the manufacturer's instructions. DNA was quantified using a Qubit 2.0 Fluorometer, and DNA quality was confirmed on a 1% agarose gel.

Bisulfite Amplicon Sequencing (BSAS). BSAS was adapted on the basis of previously published protocols.²³ Genomic DNA samples (50 ng for 2 hpf, 150 ng for 4 hpf, or 150 ng for 6 hpf) were bisulfite-treated using an EZ DNA Methylation-Lightning Kit (Zymo). Ten regions of interest (ROIs) were identified on the basis of TDCIPP-susceptible loci identified from our previous WGBS study (Table S1).¹⁷ These 10 ROIs were selected on the basis of five CpGs that were also impacted by FA within humans,²¹ while the five remaining ROIs were selected to represent exonic and intergenic regions with a range of methylation differences relative to vehicle controls. Primers were designed with Zymo's Bisulfite Primer Seeker (<http://www.zymoresearch.com/tools/bisulfite-primer-seeker>) to amplify 300 to 400 bp ROIs (Table S1). ROIs were amplified within a 25 μ L reaction mixture containing ZymoTaq PreMix, 5 ng of bisulfite-treated DNA, and primers at 2.5 μ M

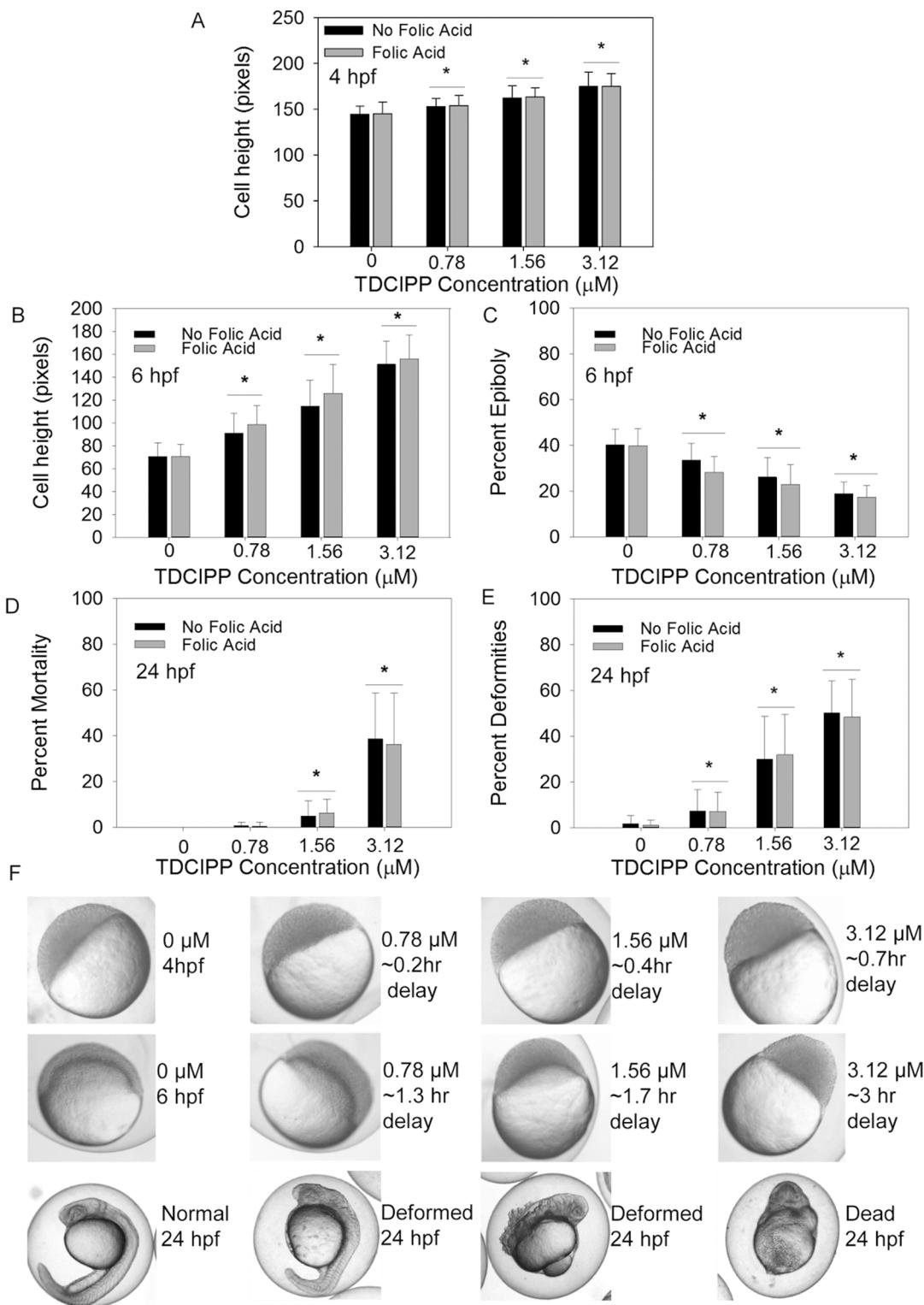


Figure 1. Developmental impacts of vehicle (0.1% DMSO) or TDCIPP (0.78, 1.56, or 3.12 μM) in the presence or absence of 1 mM FA on (A) cell height at 4 hpf, (B) cell height at 6 hpf, (C) percent epiboly at 6 hpf, (D) percent mortality at 24 hpf, and (E) percent deformities at 24 hpf. (F) Representative images of epiboly delays at 4 and 6 hpf and deformities at 24 hpf (embryos are approximately 0.7 mm in diameter). Data are presented as means \pm the standard deviation. Black bars represent data for TDCIPP-only treatments in EM, while gray bars represent data for TDCIPP treatments containing 1 mM FA. Asterisks denote significant differences ($p \leq 0.05$) within TDCIPP treatments (with or without FA) relative to respective vehicle controls. For 4 and 6 hpf data, a total of 30 total embryos were imaged across three replicate beakers (10 embryos per beaker; for 24 hpf data, mean percentages are based on nine beakers containing 20 imaged embryos per beaker).

each using an Eppendorf Mastercycler Nexus Thermocycler. Polymerase chain reaction (PCR) conditions were as follows: 95 °C for 10 min, followed by 35 cycles of 95 °C for 30 s, 51–

65 °C (depending on the ROI) for 30 s, and 72 °C for 30 s, with a final extension step at 72 °C for 7 min. PCR products were cleaned and purified using a QIAquick 96 PCR

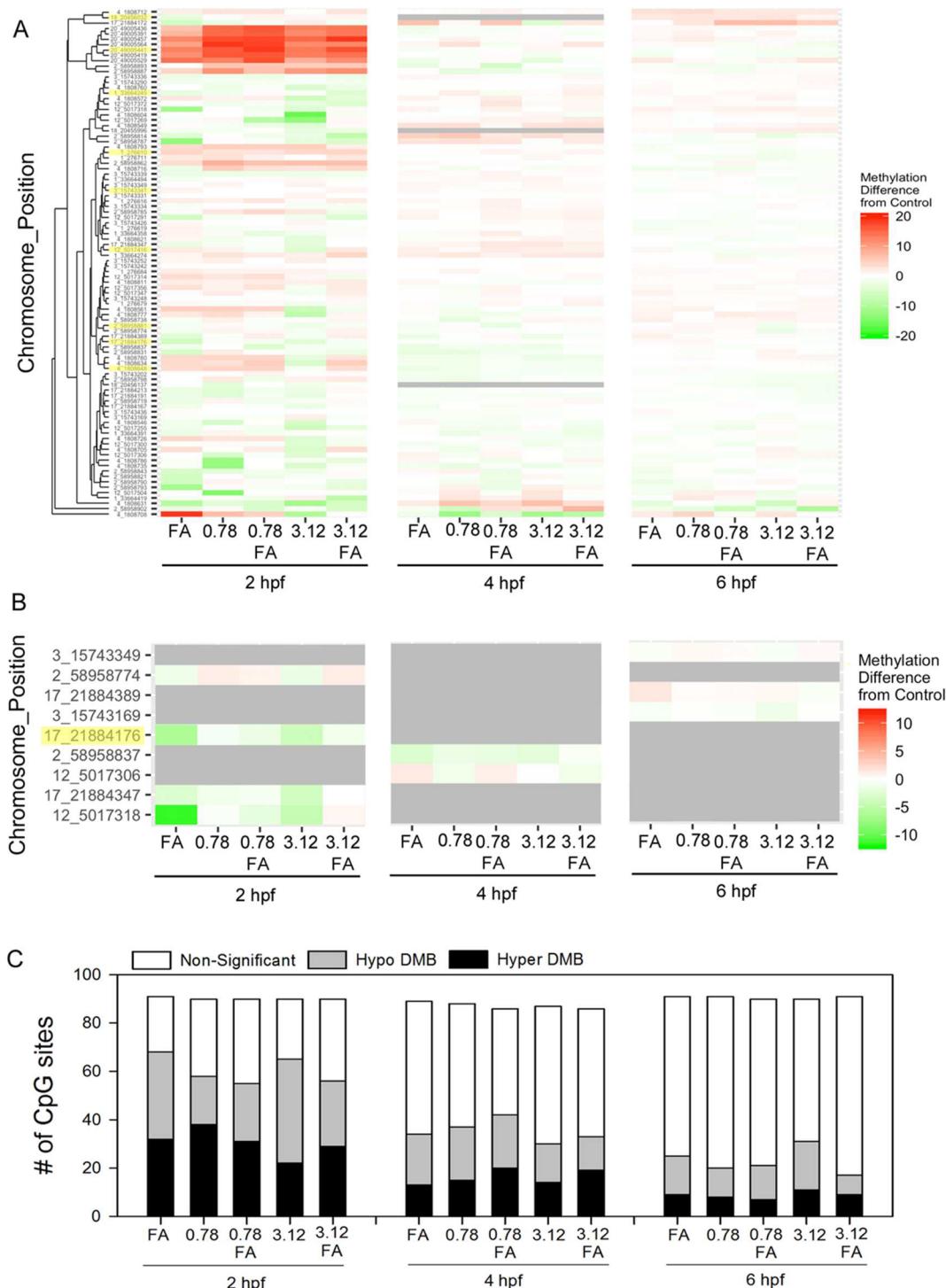


Figure 2. Bisulfite amplicon sequencing of 10 amplicons from embryos exposed to vehicle (0.1% DMSO) or TDCIPP (0.78 or 3.12 μ M) in the presence or absence of 1 mM FA from 0.75 hpf to 2, 4, or 6 hpf. Complete linkage-based clustering of methylation differences (relative to stage-matched vehicle controls) by position for (A) all CpG sites or (B) CpG sites with a significant interaction ($p \leq 0.05$) between TDCIPP and FA at 2, 4, or 6 hpf. In panels A and B, positions highlighted in yellow represent CpG sites that were previously identified on the basis of whole-genome bisulfite sequencing. (C) Summary of MethylKit-based pairwise comparisons between treatments and stage-matched vehicle controls. White, gray, and black bars represent the number of nonsignificant, hypomethylated, and hypermethylated CpG sites, respectively ($q \leq 0.01$). $N =$ four genomic DNA samples per treatment.

Purification Kit (Qiagen), and amplicons were quantified using a Qubit 2.0 Fluorometer. Amplicon quality was confirmed using an Agilent 2100 Bioanalyzer system and high-sensitivity DNA kit. Amplicons were pooled prior to library preparation at a concentration of 0.1 ng per amplicon. Library preparation was

performed with the Nextera XT DNA Library Prep Kit (Illumina, San Diego, CA) and indexed by treatment replicate. Library quality and quantity were confirmed using a Qubit 2.0 Fluorometer and 2100 Bioanalyzer system. Libraries were then pooled by stage (2, 4, or 6 hpf), diluted to a concentration of

1.3 pM (with 40% PhiX control), and paired-end (2×150) sequenced on our Illumina MiniSeq Sequencing System using a 300-cycle Mid Output Reagent Kit.

All sequencing data were uploaded to Illumina's BaseSpace in real time for downstream analysis of quality control and potential differences in methylation. All three sequencing runs resulted in $>92\%$ of reads with $\geq Q30$. Reads were aligned, and duplicate reads were removed using a ROI-based enrichment manifest within MethylSeq. MethylKit was used on positions with $>10X$ coverage to assess significant methylation differences of $>1\%$ relative to stage-matched vehicle controls; $q \leq 0.01$ was used to minimize false positives associated with multiple comparisons. To identify potential interactions between TDCIPP and FA, a quasi-binomial model was used to run logistic regression analysis on all CpG sites with $>10X$ coverage using RStudio (<https://www.rstudio.com>).

DNA Methylation Quantitation. Global 5-methylcytosine (5-mC) was measured using 10 ng of genomic DNA (three samples per treatment) and a 5-mC DNA enzyme-linked immunosorbent assay (ELISA) kit (Zymo) per the manufacturer's instructions. Only two replicates were used for 2 hpf samples, as there was <10 ng of genomic DNA within two of four replicate samples following BSAS.

Statistical Analyses. All statistical analyses were performed within RStudio (<https://www.rstudio.com>). Two-way analysis of variance (ANOVA) was performed on 5-mC ELISA data, log-transformed cell height data, and arcsin-transformed percent epiboly data to meet assumptions for normality and equal variance. Pairwise comparisons were analyzed using Tukey-based post hoc tests. Percent mortality and deformity data at 24 hpf were analyzed using binomial logistic regressions. Significance was determined at $p \leq 0.05$.

Accession Number. Raw Illumina (fastq.gz) sequencing files (144 files totaling 2.59 GB) are available via NCBI's BioProject database under BioProject ID PRJNA395080.

RESULTS AND DISCUSSION

During early zebrafish embryogenesis, cells proliferate and aggregate on the animal pole until 3.25 hpf prior to epiboly initiation and progression. As TDCIPP was previously shown to delay epiboly at a single concentration ($3 \mu\text{M}$),¹⁵ we quantified the effects of TDCIPP on epiboly as a function of TDCIPP concentration. Moreover, we investigated whether co-exposure with a methyl donor (FA) mitigated the effects of TDCIPP on epiboly. Initiation of TDCIPP exposure at 0.75 hpf resulted in an increase in average cell height in a concentration-dependent manner at 4 and 6 hpf (Figure 1A,B), and embryos exposed to $3.12 \mu\text{M}$ TDCIPP were delayed by as much as 3 at 6 hpf relative to vehicle controls (Figure 1C). Embryo mortality at 24 hpf was significantly increased following exposure to 1.56 and $3.12 \mu\text{M}$ TDCIPP (Figure 1D), and TDCIPP exposure resulted in an increase in the number of abnormalities at all test concentrations following exposure from 0.75 to 24 hpf (Figure 1E,F). Interestingly, while exposure to FA alone from 0.75 to 24 hpf did not affect epiboly progression at 4 or 6 hpf or result in deformities or mortality at 24 hpf, FA was unable to mitigate TDCIPP-induced effects at all three developmental stages evaluated (Figure 1A–E).

Folate is a cofactor in the formation of S-adenosyl-L-methionine, a substrate required for cytosine methylation. FA supplementation is recommended for women of reproductive age through 12 weeks of gestation to decrease the incidence of neural tube defects,²⁶ and these impacts are thought to be

mediated by DNA methylation. Within animal models, FA has also been used as a methyl donor to rescue chemically induced adverse developmental phenotypes. For example, a FA-containing diet fed to female agouti mice restored *Avg* locus methylation levels and offspring coat color altered by bisphenol A exposure.¹⁸ Similarly, co-exposure with 1 mM FA significantly decreased the effect of selenite on zebrafish embryo hatching and survival.¹⁹ However, in our study, co-exposure to 1 mM FA was unable to block or mitigate TDCIPP-induced effects on zebrafish epiboly, suggesting that TDCIPP-induced impacts on embryonic development may not be due to methyl donor depletion resulting from TDCIPP exposure.

To determine whether FA mitigated TDCIPP-induced effects on DNA methylation, we examined global methylation levels in genomic DNA from embryos exposed to TDCIPP in the presence and absence of FA. Consistent with previously published data,² the level of global methylated cytosine ranged between 4 and 6% for all treatments (Figure S1). However, similar to our previous study that relied on a liquid chromatography–tandem mass spectrometry-based method,¹⁷ no significant differences in global methylation were detected across all treatments using a 5-mC DNA ELISA, suggesting that global methylation assays are not sensitive enough to detect base resolution changes induced by TDCIPP in zebrafish embryos. Therefore, using our existing WGBS data set for TDCIPP,¹⁷ we relied on BSAS as a targeted sequencing strategy to monitor differentially methylated CpGs within TDCIPP-susceptible loci.

Of 10 amplicons analyzed, nine amplicons were aligned and 94 total CpG sites were detected, resulting in a 12–13M reads passing filter (Table S2) with an average of 143000 targeted unique aligned reads and a mean target coverage of 4038 reads (Table S3). Overall, consistent with previous studies,² CpG methylation averaged between 81 and 88% (Figure S2) while non-CG methylation ranged from 0.6 to 1.09% (Table S3), indicating that bisulfite conversion was successful across all samples. Interestingly, clustered heat maps revealed variable impacts of TDCIPP and FA at different stages (Figure 2A and Table S4); raw percent methylation data for all treatments (including vehicle controls) and stages are available in Tables S5 (2 hpf), S6 (4 hpf), and S7 (6 hpf). Relative to vehicle controls, the magnitude of the effect on methylation decreased from 2 to 6 hpf despite being continuous immersion in TDCIPP, suggesting that effects of TDCIPP on methylation are reversible within a 4 h exposure period even though internal TDCIPP doses increase from 2 to 6 hpf.¹⁷ In addition, the direction of response (hyper- vs hypomethylation) was not consistent from 2 to 6 hpf within each treatment. Logistic regression analysis was used to detect the potential interaction of FA and TDCIPP on CpG methylation at each stage (Figure 2B and Figure S3). While a significant interaction between TDCIPP and FA ($p \leq 0.05$) was detected for nine CpG positions at 2, 4, or 6 hpf (Figure 2B), none of these positions were shared among stages. Finally, on the basis of pairwise comparisons relative to stage-matched vehicle controls, the number of differentially methylated bases decreased from 2 to 6 hpf (Figure 2C) and included both hypo- and hypermethylated positions. Overall, our BSAS data suggest that methylation dynamics are strongly dependent on TDCIPP concentration and developmental stage, and that the majority of TDCIPP-induced effects on methylation at 2 hpf do not persist at 4 and 6 hpf despite continuous exposure.

In conclusion, our findings demonstrate that TDCIPP significantly delays zebrafish epiboly following initiation of exposure at 0.75 hpf, a phenotype that is preceded by complex, dynamic alterations in DNA methylation. In the study presented here, TDCIPP impacted DNA methylation in a highly stochastic manner where the magnitude and direction of alterations in DNA methylation were not consistent throughout epiboly, suggesting that (1) these epigenetic marks are not stable during this brief window of embryonic development within zebrafish and (2) if these experiments were repeated multiple times, the magnitude and direction of the response for TDCIPP-induced effects on these same CpG sites at 2, 4, and 6 hpf may vary from experiment to experiment. Thus, although a subset of CpG sites were methylated in a TDCIPP-dependent manner, we were unable to identify positions that may serve as biomarkers of TDCIPP exposure, raising questions about the utility and reliability of CpG methylation as biomarkers of exposure within animal models and human populations. Nevertheless, this study demonstrates the importance of follow-up, targeted approaches such as BSAS for monitoring DNA methylation dynamics following identification and prioritization of susceptible loci using WGBS. In addition, this study further provides insight into chemically induced impacts on zebrafish methylation during embryogenesis that, given the strong level of variability within a highly controlled laboratory study, may help explain potential sources of uncertainty within human population-based environmental epigenetic studies.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: [10.1021/acs.estlett.7b00332](https://doi.org/10.1021/acs.estlett.7b00332).

BSAS amplicons, sequencing quality, and sequencing results ([XLSX](#))

5-mC DNA ELISA data and logistic regression results ([PDF](#))

AUTHOR INFORMATION

Corresponding Author

*E-mail: david.volz@ucr.edu. Phone: (951) 827-4450. Fax: (951) 827-3993.

ORCID

David C. Volz: [0000-0001-8620-2575](https://orcid.org/0000-0001-8620-2575)

Notes

The authors declare no competing financial interest.

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