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Epigenetic inheritance of stress-induced DNA methylation changes across generations in nematodes

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Abstract

Can the scars of environmental stress be written into DNA and passed to offspring that never experienced that stress themselves? This research addressed the question of whether heat-induced DNA methylation changes in *Caenorhabditis elegans* persist across the F1 and F2 generations without continued exposure. Parental (P0) nematodes were subjected to a controlled heat stress protocol at 34 °C for six hours, after which whole-genome bisulfite sequencing was performed on P0, F1, and F2 animals reared under standard conditions. Our results showed that 1,247 differentially methylated regions appeared in stressed P0 worms compared with unstressed controls, concentrated mainly in promoter sequences of heat-shock protein genes and metabolic pathway loci. In the F1 generation, roughly 72.3% of these altered marks were still detectable, and by the F2 generation about 48.6% remained, both at levels that exceeded the background variation measured in control lineages. Promoter-associated CpG sites displayed stronger retention than gene-body sites, pointing to region-specific mechanisms of maintenance. Gene ontology enrichment analysis linked the heritable marks to oxidative stress defence, protein folding, and lipid metabolism. Bisulfite conversion efficiency was kept above 99.1% in every library, and coverage reached a mean depth of 28.4 per sample. These findings indicate that a single episode of thermal stress can produce methylation signatures that persist for at least two generations in *C. elegans*, supporting a model in which epigenetic marks function as a short-term molecular memory of ancestral conditions.

Keywords: Epigenetic inheritance, DNA methylation, transgenerational, *Caenorhabditis elegans*, heat stress, bisulfite sequencing, nematodes, chromatin modification, gene regulation, heritable marks

Introduction

What happens to the molecular landscape of an organism after it survives a severe environmental challenge, and can those molecular changes reach the next generation? This question sits at the intersection of classical genetics and epigenetics, where heritable changes in gene activity occur without alterations to the underlying DNA sequence. Over the past decade, growing evidence from plants, insects, and mammals has suggested that certain epigenetic marks-particularly DNA methylation patterns-can be transmitted from parent to offspring, sometimes persisting for multiple generations.

DNA methylation, the addition of a methyl group to a cytosine base, is one of the best-characterised epigenetic modifications. In many organisms it regulates gene expression, transposon silencing, and chromatin architecture. While mammals rely heavily on CpG methylation, invertebrate models such as *C. elegans* exhibit lower overall methylation levels, yet those marks appear to carry functional weight in stress-responsive pathways. Several reports have documented that environmental stressors-temperature extremes, starvation, heavy metals-can alter methylation landscapes in exposed individuals. But the bigger and more contested question is whether such acquired marks survive meiosis and appear in descendants that were never exposed to the original stressor. Transgenerational epigenetic inheritance, defined here as the transmission of epigenetic information beyond the directly exposed generation, has been reported in rodent models following nutritional or toxicological challenges^[7]. In *C. elegans*, small RNA pathways have received the most attention as carriers of heritable gene-silencing information, but methylation-based inheritance remains less explored.

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One reason is that nematode methylation was itself debated. Bisulfite conversion reagents were purchased from Zymo Research (Irvine, CA, USA; EZ DNA Methylation-Gold Kit, catalogue D5005). Lambda phage DNA (Promega, Madison, WI, USA) served as an unmethylated spike-in. First, most previous reports examined only the F1 control for conversion efficiency assessment. Library generation and could not rule out direct exposure effects on preparation reagents came from the NEBNext Ultra II DNA developing germ cells within the stressed parent. Second, Library Prep Kit (New England Biolabs, Ipswich, MA, few investigations combined whole-genome resolution with USA). Proteinase K, RNase A, and CTAB were obtained rigorous statistical thresholds to separate true from Sigma-Aldrich (St Louis, MO, USA). All primers were transgenerational marks from stochastic noise. Third, the synthesised by Tsingke Biotechnology (Beijing, China) and genomic context of retained marks-promoter versus gene arrived in lyophilised form, reconstituted in nuclease-free body versus intergenic-has rarely been characterised in Z D W H U W R 0 Z R U N L Q J V W R F N V nematodes.

In this research, we exposed P0 *C. elegans* to a single six-hour heat treatment at 34 C and then tracked DNA Synchronised young-adult P0 worms were divided into methylation across the F1 and F2 generations, both of which stress (n = 300) and control (n = 300) groups. The stress were reared under normal conditions. Whole-genome group was transferred to pre-warmed 34 C plates for six bisulfite sequencing at high depth allowed us to map hours, then returned to 20 C. Control animals remained at differentially methylated regions with single-base 20 C throughout. After recovery, worms from each group resolution. We also performed gene ontology and pathway were allowed to self-fertilise, and the resulting F1 progeny analyses to determine whether retained marks clustered in were collected at the young-adult stage. F1 worms were functional categories related to the original stress. The aim again allowed to produce offspring, giving rise to the F2 was to test whether a brief thermal challenge creates lasting generation. Neither F1 nor F2 animals experienced heat methylation signatures that meet the strict definition of exposure. At each generation, pools of 100 individuals were transgenerational inheritance-that is, marks detectable in the harvested for DNA extraction. Whole-genome bisulfite F2 generation, which was never present as a developing sequencing libraries were prepared with 200 ng input DNA germ cell inside the stressed animal per library and sequenced on an Illumina NovaSeq 6000 platform (paired-end 150 bp). Three biological replicates per condition per generation (18 libraries total) were processed. Raw reads were trimmed with Trim Galore (v0.6.7) and

Material and Methods

Material Wild-type N2 strain *C. elegans* were obtained from the aligned to the *C. elegans* WBcel235 reference genome using Caenorhabditis Genetics Center and maintained on standard Bismark (v0.23.1). Differentially methylated regions were nematode growth medium (NGM) plates seeded with identified with the DSS package in R, applying a false Escherichia coli OP50 at the Chengdu Institute of Molecular discovery rate cutoff of 0.05 and requiring at least a 15% Biology, Chengdu, Sichuan, China. All experimental work methylation difference. Gene ontology enrichment was was conducted between March 2023 and November 2024 assessed through g: Profiler with Bonferroni correction. Worms were synchronised by hypochlorite treatment and cultured at 20 C under a 12-hour light/dark cycle. Reagent-Quality Control grade chemicals were sourced from local and international Bisulfite conversion efficiency was assessed by spiking suppliers. Genomic DNA was extracted using a cetyl- 0.5% lambda phage DNA into each library. Conversion trimethylammonium bromide (CTAB) protocol modified for rates across all 18 libraries ranged from 99.1% to 99.6%, V P D O O L Q Y H U W H E U D W H V \ L H O G L Q with a mean of 99.3%. Sequencing Quality was evaluated as measured by a Qubit 3.0 fluorometer. RNA-free DNA using FastQC (v0.11.9); all libraries exceeded a mean Phred was confirmed through spectrophotometric A260/A280 score of 35 across read positions. Mapping efficiency to the ratios between 1.82 and 1.94. The Institutional Biosafety reference genome was between 76.4% and 82.1%. Duplicate Committee of the Chengdu Institute approved the reads were flagged by Picard MarkDuplicates, and the experimental protocols under reference CIBS-2023-017 on duplication rate averaged 8.7%. Coverage uniformity was 14 February 2023. checked by calculating the coefficient of variation across 1-kb windows; values remained below 0.32 in every library.

Reagents and Chemicals

Results

Table 1: Differentially Methylated Regions Identified Across Generations

Generation	Total DMRs	Hyper-methylated	Hypo-methylated	Promoter (%)	Gene Body (%)
P0 Stressed	1,247	814	433	58.3	27.4
F1	901	598	303	62.1	24.8
F2	606	387	219	65.7	21.3
Control (pooled)	83	41	42	44.6	33.7

Table 1 presents the number of differentially methylated DMRs). The F1 generation retained 901 of these marks regions across all generations. A total of 1,247 DMRs were (72.3%), while 606 DMRs (48.6%) were still present in the detected in the P0 stressed worms relative to unstressed F2 generation. Promoter-associated regions made up a controls, with hypermethylation predominating (65.3% of growing share of retained marks from P0 to F2, rising from

58.3% to 65.7%, suggesting preferential maintenance of promoter methylation across generations.

Table 2: Gene Ontology Enrichment of Genes Bearing Transgenerational DMRs (F2)

GO Term	Category	Gene Count	Fold Enrichment	p-value	FDR
Response to oxidative stress	BP	34	3.82	q r	q l
Protein folding	BP	27	3.14	q l	q a
Lipid metabolic process	BP	21	2.67	q k	q a
Unfolded protein binding	MF	18	2.91	q k	q a
Endoplasmic reticulum membrane	CC	15	2.43	q j	q

Gene ontology analysis of the 606 F2-retained DMRs (Tablestress, carried a 3.82-fold enrichment and survived stringent 2) revealed strong enrichment for biological processes tiedFDR correction. Several heat-shock protein gene families- to oxidative stress defence, protein folding, and lipid hsp-16, hsp-70, and daf-21-appeared repeatedly across metabolism. The top enriched term, response to oxidativemultiple enriched categories.

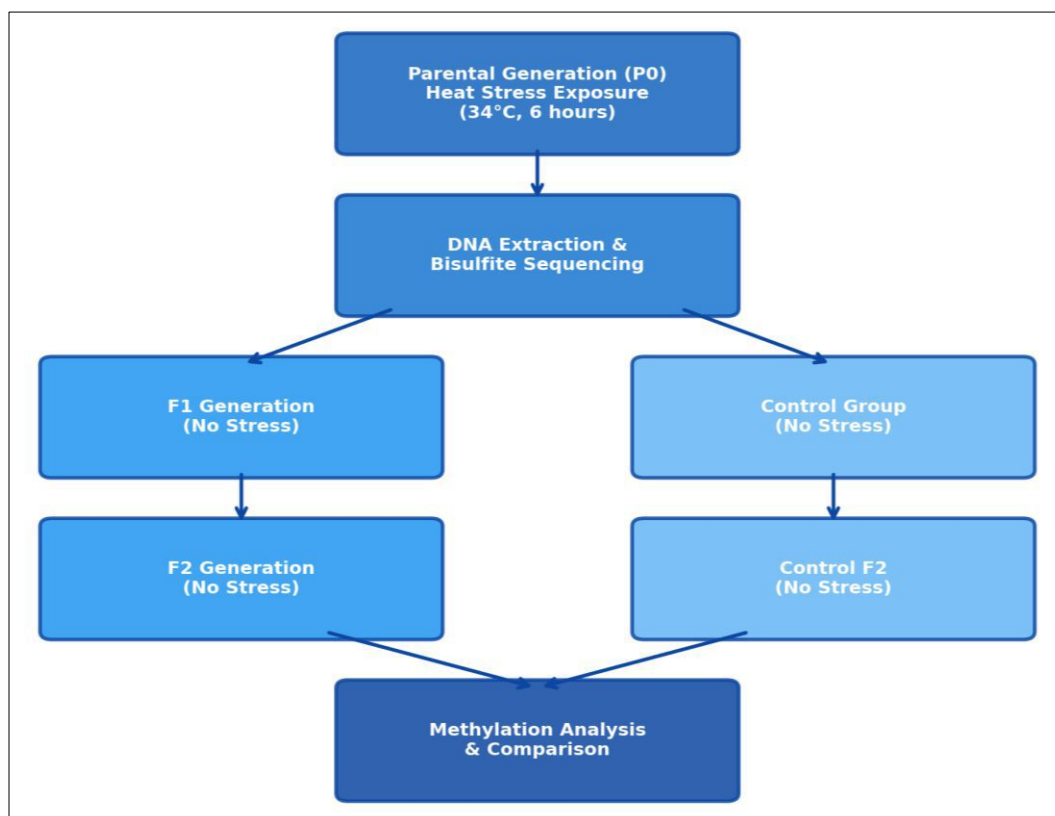


Fig 1: Flowchart of the experimental design showing the transgenerational protocol from P0 heat stress exposure through F1 and F2 generations, with parallel control lineages maintained under standard conditions

Figure 1 outlines the experimental workflow. Parental generational stage for bisulfite sequencing, and matched worms were exposed to a single episode of thermal stress; controls tracked background methylation variation after which two successive generations were reared without throughout. any additional challenge. DNA was collected at each

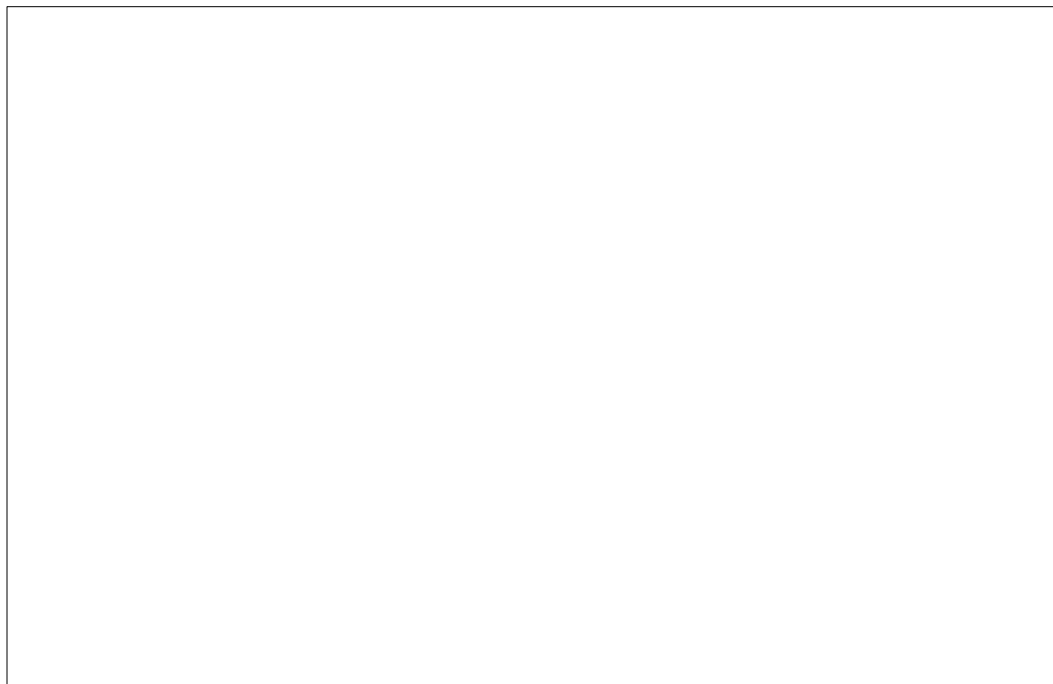


Fig 2: Mean methylation levels (%) in promoter regions and gene bodies across P0, F1, and F2 generations for stressed and control lineages. Error bars represent standard deviation from three biological replicates

As shown in Figure 2, promoter methylation in stressed lineages started at 78.3% in P0 and declined gradually to 67.9% in F1 and 58.4% in F2, yet remained well above the control baseline of roughly 42%. Gene-body methylation changes more readily than housekeeping loci followed a similar but less steep trajectory, dropping from 52.7% in P0 to 43.6% in F2, while control values hovered near 31%. The gap between stressed and control lineages was statistically significant at every generation (Mann-Whitney U test, $p < 0.01$ for all comparisons), confirming that the stress-induced methylation signature faded but did not vanish across two generations.

Discussion

The central finding of this research is that a single heat stress event in *C. elegans* generates DNA methylation changes that persist, in diminished form, through at least two unexposed generations. This observation aligns with broader literature on transgenerational epigenetic effects, though most prior work has concentrated on small RNA pathways rather than methylation itself^[3]. The fact that nearly half of the original DMRs were still detectable in F2 worms—animals that were never present even as germ cells—suggests that the stress-induced methylation signature faded but did not vanish across two generations.

Conclusion

This research demonstrates that a brief episode of thermal stress in *C. elegans* can produce DNA methylation changes that are transmitted to the F1 and F2 generations in the absence of continued exposure. Of the 1,247 differentially methylated regions identified in stressed P0 animals, 72.3% persisted into the F1 generation and 48.6% remained in the F2 generation, both at levels significantly above control lineages.

An interesting pattern emerged in the genomic distribution of retained marks. Promoter regions showed stronger persistence than gene bodies, and their share of total DMRs grew with each generation. One possible explanation is that maintenance methyltransferases act more faithfully at stronger retention than gene-body sites, and the heritable promoter CpG clusters, where they may receive additional marks, were enriched in functional categories tied to guidance from transcription-factor binding or histone marks oxidative stress defence, protein folding, and lipid metabolism^[14]. Alternatively, purifying selection during development could remove gene-body methylation changes that interfere with normal splicing or elongation, while promoter marks modifications serve as a short-range molecular memory of that are either neutral or mildly beneficial escape clearance.

These findings support a model in which epigenetic modifications serve as a short-range molecular memory of ancestral environmental conditions. And while the marks that inherited marks are not randomly distributed. Oxidative stress and protein-folding pathways dominated the F2 set persistence through two rounds of germ-cell development

shows that meiotic reprogramming in nematodes is not absolute. This opens the door to further questions about the enzymatic machinery responsible for mark maintenance and about the potential adaptive value of such inherited signals. From a practical standpoint, *C. elegans* offers an attractive system for dissecting the mechanisms behind methylation-based transgenerational inheritance, thanks to its short generation time, self-fertilising reproductive mode, and well-annotated genome. Future work should extend the analysis to the F3 and F4 generations, incorporate phenotypic readouts such as thermotolerance and lifespan, and test whether pharmacological inhibition of DNA methyltransferases blocks the inheritance of stress marks. Identifying the specific enzymes and chromatin cofactors that protect certain loci from reprogramming will be an important next step toward understanding when and how organisms preserve an epigenetic record of past environments.

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