

## BRIEF COMMUNICATION

# In vitro relationships of galactic cosmic radiation and epigenetic clocks in human bronchial epithelial cells

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**Abstract**

Ionizing radiation is a well-appreciated health risk, precipitant of DNA damage, and contributor to DNA methylation variability. Nevertheless, relationships of ionizing radiation with DNA methylation-based markers of biological age (i.e. epigenetic clocks) remain poorly understood. Using existing data from human bronchial epithelial cells, we examined in vitro relationships of three epigenetic clock measures (Horvath DNAmAge, MiAge, and epiTOC2) with galactic cosmic radiation (GCR), which is particularly hazardous due to its high linear energy transfer (LET) heavy-ion components. High-LET <sup>56</sup>Fe was significantly associated with accelerations in epiTOC2 ( $\beta = 192$  cell divisions, 95% CI: 71, 313,  $p$ -value = .003). We also observed a significant, positive interaction of <sup>56</sup>Fe ions and time-in-culture with epiTOC2 (95% CI: 42, 441,  $p$ -value = .019). However, only the direct <sup>56</sup>Fe ion association remained statistically significant after adjusting for multiple hypothesis testing. Epigenetic clocks were not significantly associated with high-LET <sup>28</sup>Si and low-LET X-rays. Our results demonstrate sensitivities of specific epigenetic clock measures to certain forms of GCR. These findings suggest that epigenetic clocks may have some utility for monitoring and better understanding the health impacts of GCR.

**KEYWORDS**

DNA methylation, epigenetic age, Fe, mitotic clock, radiation, Si, X-ray

## 1 | INTRODUCTION

Various forms of radiation are widely appreciated contributors to human cancer. For instance, ultraviolet solar radiation has been identified as the main causal factor in developing skin neoplasms (Narayanan et al., 2010). Meanwhile, ionizing radiation has been implicated in a host

of solid and liquid cancers (Brenner et al., 2003). One of the main mechanisms by which radiation exerts its harmful effects is through DNA damage, including double-stranded breaks (Sanders et al., 2020). Still, there is evidence that radiation may also alter phenotypes through DNA-based epigenetic modifications, particularly DNA methylation alterations (Miousse et al., 2018). One 2018 in vitro study, used a human bronchial epithelial cell line to explore the relationships of high linear energy transfer (LET) galactic cosmic radiation (GCR) (<sup>56</sup>Fe and <sup>28</sup>Si ions) as well as terrestrial low-LET X-rays on genome-wide DNA

**Abbreviations:** DNAmAge, Horvath DNA methylation age; EpiTOC2, epigenetic time to cancer-2; GCR, galactic cosmic radiation; LET, linear energy transfer; MiAge, mitotic age.

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methylation patterns (Kennedy et al., 2018). The authors were able to identify signature CpGs that were associated with each type of radiation and were able to use the  $^{56}\text{Fe}$  ion methylation signatures to identify tumor versus normal lung tissue. Despite this epigenome-wide evidence, studies examining the relationships between radiation exposure and epigenetic clocks in humans have been more limited. Some of the existing evidence in this area comes from an analysis of childhood cancer survivors using a three CpG measure of biological aging. The study found that survivors who received total body irradiation and a hematopoietic stem cell transplant were biologically older than childhood cancer survivors who did not receive radiation (Daniel et al., 2018). Two studies of head/neck cancer and early-stage breast cancer patients found significant acceleration in blood epigenetic clocks following patients' treatment with radiation therapies (Sehl et al., 2020; Xiao et al., 2021). However, when considering results beyond observational data, an earlier *in vitro* study interested in the relationship of epigenetic clocks and DNA damage-related senescence found no significant relationships between Horvath DNAmAge and 10Gy X-ray exposure even after 14 days of culture (Lowe et al., 2016).

In the present analysis, we use the aforementioned human bronchial epithelial cell dataset (Kennedy et al., 2018) to explore *in vitro* relationships of three types of radiation exposure (high-LET  $^{56}\text{Fe}$ , high-LET 28Si, and low-LET X-rays) with three epigenetic clocks (Horvath DNA methylation age [DNAmAge], Epigenetic Time to Cancer-2 [epiTOC2], and Mitotic Age [MiAge]). These markers were selected given their strong relationships with cancer, which was of specific interest in the original study of Kennedy et al., 2018. The Horvath DNAmAge biomarker is a largely tissue-independent predictor of chronological age and an estimate of biological aging calculated from 353 CpG sites (Horvath, 2013). In addition, accelerated DNAmAge is associated with adverse environmental exposures and increased cancer risk (Dhingra et al., 2018; Lau & Robinson, 2021). The epiTOC2 biomarker is calculated from 163 CpG sites and estimates the cumulative number of stem cell divisions (mitotic counts) in a tissue. Acceleration of the epiTOC2 biomarker has been associated with numerous cancers and pre-invasive cancer lesions including lung adenocarcinoma and carcinoma *in situ* (Teschendorff, 2020). MiAge is calculated from 268 CpG sites and estimates the total number of lifetime cell divisions of a tissue. Prior research comparing tumors to normal adjacent tissues has shown that MiAge is universally accelerated in 13 cancer types (including lung squamous cell cancer) and is associated with worse cancer survival (Youn & Wang, 2018). We hypothesized that ionizing radiation exposure, particularly the hazardous high-LET heavy-ion components, would accelerate epigenetic clocks, thus, reflecting increased cancer and disease risk. Another important impetus for this study is that radiation can also be offered as a cancer therapeutic. Hence, building a more comprehensive understanding of radiation toxicity and potential biomarkers may be of immense public health benefit.

## 2 | MATERIALS AND METHODS

### 2.1 | Study data and radiation exposure

Our analyses used a publicly available NCBI GEO Methylation450K BeadChip human bronchial epithelial cell dataset (Series GSE108187

on "<https://www.ncbi.nlm.nih.gov/geo/>") also available in the NASA GeneLab repository (<https://genelab-data.ndc.nasa.gov/genelab/accession/GLDS-317/>). The dataset is comprised of 102 samples. Based on information in the original publication (Kennedy et al., 2018), the immortalized human bronchial epithelial cells (HBEC3-KT) were grown in 5%  $\text{CO}_2$  at 37°C and passaged (1:4) twice a week for 3 months. From each passage, cell pellets were collected, flash-frozen, and stored at  $-80^\circ\text{C}$  for later DNA extraction. Three biological replicate cultures were independently irradiated.  $^{56}\text{Fe}$  doses were 0, 0.1, 0.3 or 1.0 Gy (Beam energy: 600 MeV/u; dose rate for the 0.1 Gy dose was 0.1 Gy/min, for the 0.3 Gy dose, 0.3 Gy/min, and for the 1.0 Gy dose, 1 Gy/min). 28Si doses were 0.0, 0.3, 1.0 Gy (Beam energy: 300 MeV/u; dose rate for the 0.3 Gy dose was 0.28 Gy/min, and for the 1.0 Gy dose, 0.63 Gy/min). X-ray doses were 0 and 1.0 Gy (beam energy 320 kV; dose rate  $\sim 1$  Gy/min). The 0.0 Gy mock irradiated samples were seeded at the same time as the experimental samples but were not placed in the radiation beam line. Immediately after irradiation, cultures were returned to their 37°C incubator for 48 h before pellets were collected. The remaining cells continued to be passaged for additional time points for a 3-month period. Additional detail regarding cell handling, DNA extraction, and methylation processing can be found in the original publication (Kennedy et al., 2018). Final 102 samples include the following exposures:  $^{56}\text{Fe}$  ions (4 doses  $\times$  4-time points  $\times$  3 replicates [4 removed in QC and not uploaded to NCBI] = 44 samples); 28Si ions (3 doses  $\times$  4-time points  $\times$  3 replicates = 36 samples); X-ray (2 doses  $\times$  4-time points  $\times$  3 replicates [2 removed in QC and not uploaded to NCBI] = 22 samples).

### 2.2 | Calculation of epigenetic clocks

We identified three cancer-relevant epigenetic clocks that could be applied to a human bronchial epithelial cell line: DNAmAge (Horvath, 2013), EpiTOC2 (Teschendorff, 2020), and MiAge (Youn & Wang, 2018). DNAmAge was calculated by uploading the downloaded methylation Beta values to a publicly available online calculator (<http://dnamage.genetics.ucla.edu>). EpiTOC2 was calculated from the downloaded methylation Beta values using instructions and R code available at <https://doi.org/10.5281/zenodo.2632938>. MiAge was calculated from the downloaded methylation Beta values using instructions and R code available at <http://www.columbia.edu/~sw2206/software.htm>.

### 2.3 | Statistical analysis

In our primary analysis, we used linear models and all available data points to model the relationships between doses of each of the three radiation exposures and each of the three epigenetic clocks. These models were also adjusted for time-in-culture. We next ran models that included an interaction term between radiation dose and time-in-culture. As a final assessment of modeling assumptions,

**TABLE 1** Relationships of ionizing radiation with epigenetic clocks

Model	Radiation	Clock	Difference in epigenetic clock	<i>p</i> -value	95% CI low	95% CI high	Adj. <i>p</i> -value (Holm)
No Interaction	56Fe	DNAmAge	-56.448	0.866	-730.107	617.210	1.000
No Interaction	56Fe	MiAge	107.362	0.061	-4.987	219.711	0.969
No Interaction	56Fe	epiTOC2	192.248	0.003	71.220	313.276	0.047
No Interaction	28Si	DNAmAge	275.559	0.356	-323.171	874.288	1.000
No Interaction	28Si	MiAge	30.022	0.454	-50.564	110.608	1.000
No Interaction	28Si	epiTOC2	-16.205	0.839	-177.087	144.677	1.000
No Interaction	X-ray	DNAmAge	-72.308	0.860	-915.918	771.302	1.000
No Interaction	X-ray	MiAge	-18.266	0.681	-109.761	73.228	1.000
No Interaction	X-ray	epiTOC2	66.392	0.162	-29.063	161.848	1.000
Interaction	56Fe	DNAmAge	-366.219	0.508	-1474.651	742.213	1.000
Interaction	56Fe	MiAge	102.993	0.270	-83.032	289.018	1.000
Interaction	56Fe	epiTOC2	241.427	0.019	42.011	440.843	0.321
Interaction	28Si	DNAmAge	-9.134	0.983	-894.332	876.063	1.000
Interaction	28Si	MiAge	21.556	0.718	-98.990	142.101	1.000
Interaction	28Si	epiTOC2	13.900	0.907	-226.448	254.247	1.000
Interaction	X-ray	DNAmAge	-516.243	0.392	-1753.600	721.114	1.000
Interaction	X-ray	MiAge	50.864	0.422	-79.049	180.778	1.000
Interaction	X-ray	epiTOC2	105.842	0.134	-35.745	247.430	1.000

Notes: Summary of tests assessing the associations between various radiation types and epigenetic clocks. Estimates and tests for the “No Interaction” models correspond to the slope coefficients of the radiation dose in the linear models with no interaction between radiation dose and time-in-culture. Estimates and tests for the “Interaction” models correspond to the slopes of the radiation dose and time-in-culture interactions in the interaction models. All nominal *p*-values were adjusted at once using Holm's procedure; only one test was found to be significant at the 5% FWER level.

we ran a sensitivity analysis testing the relationships between time-in-culture and the epigenetic clocks. Lastly, we looked for overlaps between epigenetic clock component CpGs and CpGs previously associated with radiation exposures (Kennedy et al., 2018). All statistical analyses were performed using R Version 4.1.0 (R Core Team, Vienna, Austria) and a *p*-value <.05 was used as the threshold for statistical significance. We further adjusted for multiple testing using the Holm method to control for the family-wise error rate (FWER) at .05.

### 3 | RESULTS

#### 3.1 | Direct radiation dose and epigenetic clock relationships

Table 1 presents the results from the models examining relationships between radiation doses and epigenetic clocks. Increases in high-LET 56Fe were significantly associated with increases in epiTOC2 ( $\beta = 192$  cell divisions, 95% CI: 71, 313, *p*-value = .003). This relationship remained statistically significant after adjusting for multiple hypothesis testing using the Holm method (adjusted *p*-value = .047). High-LET 56Fe was not significantly associated with DNAmAge or MiAge. Furthermore, there were no significant associations of high-LET 28Si or low-LET X-rays with any of the epigenetic clocks.

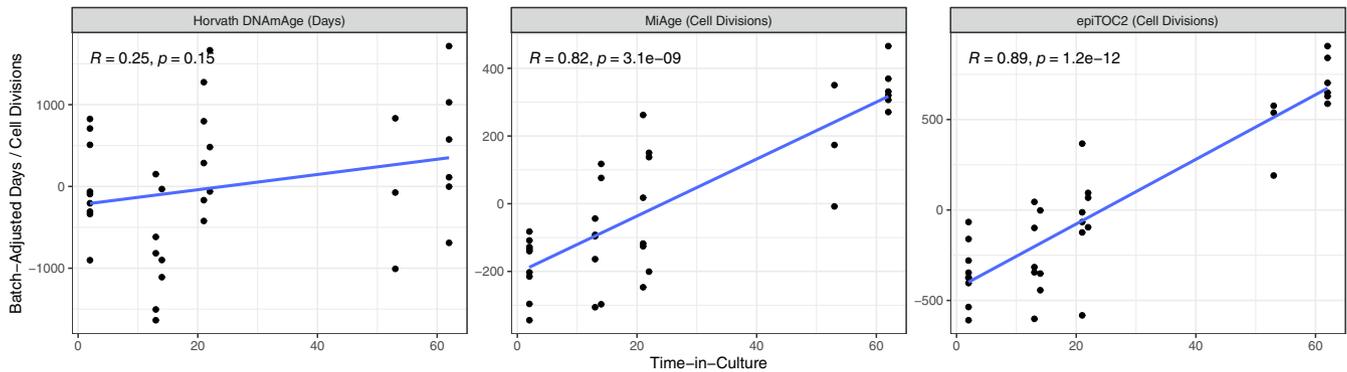
#### 3.2 | Radiation dose and time-in-culture interactions

Table 1 also presents relationships between radiation dose and time-in-culture interactions with each of the epigenetic clocks. We observed a significant, positive interaction of high-LET 56Fe and time-in-culture with epiTOC2 (95% CI: 42, 441, *p*-value = .019). However, this association was no longer statistically significant after Holm adjustment (adjusted *p*-value = .321). High-LET 56Fe and time-in-culture interactions were not significantly associated with DNAmAge or MiAge. There were no significant interactions of high-LET 28Si or low-LET X-rays with any of the epigenetic clocks. In our sensitivity analysis, in the absence of radiation, time-in-culture had the strongest relationships with batch-adjusted epiTOC2 ( $r = 0.89$ ) and MiAge ( $r = 0.82$ ) values (Figure 1). The relationship with batch-adjusted DNAmAge was weaker ( $r = 0.25$ ).

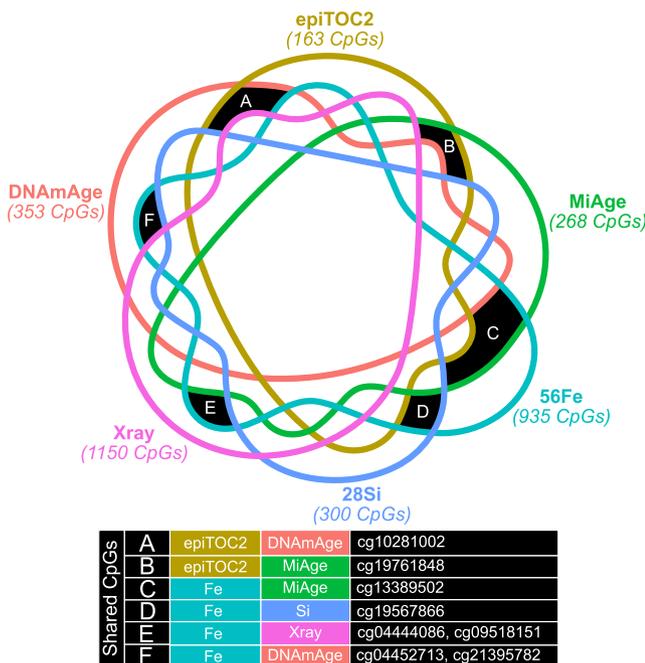
#### 3.3 | Overlapping CpG relationships

To help explain our results, we examined if there was any overlap between the CpGs that make up the epigenetic clocks and the CpGs previously associated with the three radiation types (high-LET 56Fe, high-LET 28Si, and low-LET X-rays) (Figure 2). There was no overlap between epiTOC2 component CpGs and CpGs associated with high-

Association of Time-in-Culture and Clock Outputs in the Absence of Radiation



**FIGURE 1** Association of Time-in-Culture and Epigenetic Clocks in the absence of radiation. Presents the time-in-culture and batch-adjusted epigenetic clock outputs' (DNAmAge, MiAge, and epiTOC2) correlation coefficients. Radiation-type-specific batch effects were mitigated by de-meaning these groups' epigenetic clock outputs prior to estimating the correlation coefficients.



**FIGURE 2** Overlap between epigenetic clock component CpGs and CpGs associated with radiation. Venn diagram demonstrating overlaps between CpGs associated with radiation types (high-LET 56Fe, high-LET 28Si, and low-LET X-rays) and epigenetic clock (DNAmAge, MiAge, and epiTOC2) component CpGs. One CpG is shared between epiTOC2 and DNAmAge (a), one CpG is shared between epiTOC2 and MiAge (b), one CpG is shared between 56Fe and MiAge (c), one CpG is shared between 56Fe and 28Si (d)], two CpGs are shared between 56Fe and X-rays (e), and two CpGs are shared between 56Fe and DNAmAge (f)

LET 56Fe. The DNAmAge and epiTOC2 biomarkers share the component site cg10281002. MiAge and epiTOC2 shared the component site cg19761848. MiAge component site cg13389502 was associated with high-LET 56Fe. Site cg19567866 was associated with both high-LET 56Fe and high-LET 28Si. Sites cg04444086 and cg09518151 were associated with both high-LET 56Fe and low-LET X-rays. Finally,

DNAmAge sites cg04452713 and cg21395782 were associated with high-LET 56Fe.

#### 4 | DISCUSSION

In this experimental study of human bronchial epithelial cells, we examined relationships of high- and low-LET radiation exposures with three epigenetic clocks (DNAmAge, epiTOC2, and MiAge). The interaction between time-in-culture and high-LET 56Fe exposure along with high-LET 56Fe exposure alone was significantly associated with increases in epiTOC2 estimated stem cell divisions. The direct high-LET 56Fe and epiTOC2 association remained statistically significant following adjustments for multiple hypothesis testing. Neither high-LET 28Si nor low-LET X-rays were associated with any of the epigenetic clocks tested. Furthermore, we found no overlap when comparing CpGs previously associated with 56Fe ion exposure (Kennedy et al., 2018) and component CpGs of epiTOC2.

We used publicly available data to build upon the work of Kennedy et al., 2018. The authors were interested in building a better understanding of the biological health risks of galactic cosmic radiation (GCR) to better inform human space travel. Thus, they examined the impact of high linear energy transfer GCR (56Fe and 28Si ions) as well as terrestrial low linear energy transfer X-rays on genome-wide DNA methylation patterns in human bronchial epithelial cells in culture. Their experiments demonstrated that 56Fe ions primarily induced hypermethylation, X-rays primarily induced hypomethylation, and 28Si had mixed effects. They identified 935 CpG sites that were associated with 56Fe ions, 300 CpGs associated with 28Si ions, and 1150 CpGs associated with X-rays. To determine the relevance of the methylation changes to human lung cancer, the authors then used the significant CpGs to build signatures to distinguish primary lung tumor for normal tissues available in the Cancer Genome Atlas Project. Only the 56Fe ion sites reasonably distinguished between tumor and normal specimens in human adenocarcinoma and squamous cell carcinoma of the lung (Kennedy et al., 2018).

To meaningfully add to these results, our analyses focused on epigenetic clocks, which have been demonstrated to have robust associations with cancer but whose relationships with radiation exposure remain understudied (Lau & Robinson, 2021). Given the adverse nature of radiation exposure, we hypothesized that radiation exposures, primarily the high-LET ions, would be associated with the acceleration of the epigenetic clocks. In line with findings from Kennedy et al., 2018, our most robust associations were with 56Fe ions. In our study, 56Fe ions were the only radiation type associated with an epigenetic clock. In line with our hypothesis, increases in the dose of 56Fe ion exposure were associated with increases in epiTOC2, suggesting increased cancer risk from this exposure. We observed no associations with 28Si ions or X-rays. Given that the cells were cultured for ~3 months, Kennedy et al., 2018 were also able to explore radiation-induced DNA methylation changes over time. They found that radiation-induced methylation changes occurred early but did not change over time. This finding is also in line with our observed statistically significant direct 56Fe association, but the time-in-culture and 56Fe ion interaction no longer being statistically significant after adjusting for multiple hypothesis testing. Our findings together with the inability of 28Si or X-ray associated CpGs to delineate between tumor and normal tissues suggest that DNA methylation and epigenetic clocks are more sensitive to 56Fe ions and their cancer-related risks. We found no overlap between CpGs previously associated with 56Fe exposure (Kennedy et al., 2018) and component CpGs of epiTOC2 to help explain this differential sensitivity.

We explicitly considered why epiTOC2, and not DNAmAge or MiAge, was sensitive to 56Fe ions. Especially, since CpGs associated with 56Fe ions are also MiAge (cg13389502 [*HIC1*]) and DNAmAge (cg04452713 [*DST*], cg21395782 [*NDUFA13*]) component CpGs. The *HIC1* gene is a tumor repressor and growth regulatory gene (Pinte et al., 2004) while *DST* and *NDUFA13* encode a cytoskeletal linker protein (Brown et al., 1995) and a subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Angebault et al., 2015), respectively. It seems unlikely that it is due to correlations given that epiTOC2 was only marginally better correlated with time-in-culture than MiAge. These differences in exposure sensitivity are more likely due to robust differences in component CpGs. For example, epiTOC2 only shares one CpG with DNAmAge (cg10281002 [*TBX5*]) and MiAge (cg19761848 [*GBX2*]), respectively. *TBX5* and *GBX2* encode transcription factors involved in development (Lin et al., 1996; McDermott et al., 2005).

Importantly, there was also very little overlap between radiation exposure associated CpGs suggesting that the exposures themselves generated very different DNA methylation changes. Only one CpG was shared between 56Fe and 28Si ion exposures (cg19567866 [*SPOCK1*]). The *SPOCK1* gene encodes a protein thought to regulate epithelial to mesenchymal transitions in cancer cells (Sun et al., 2020). Two CpGs were shared between 56Fe and X-ray exposures (cg04444086 [*SSTR5-AS1*], cg09518151 [*LYG1*]). *SSTR5-AS1* is an antisense RNA gene associated with cancer (Wang et al., 2019) while *LYG1* is involved in lysozyme activity (Liu et al., 2021).

Despite our novel findings, this study has some important limitations. First this is an experimental study based on cell culture data and physiological relationships may differ in in vivo contexts or with primary tissues. Second, cells were exposed to radiation at 0, 0.1, 0.3, and 1.0 Gy. These exposures are likely to differ in real-life contexts. For instance, a single chest X-ray has an approximate radiation dose of 0.1 mSv (0.0001 Gy) or 10 days of natural background radiation exposure (Diederich & Lenzen, 2000). Some may argue that our study then speaks to more chronic radiation exposure but this is relevant for long space missions. Nevertheless, additional studies will be necessary to test that assertion and test if the observed associations persist at lower acute radiation exposures.

In conclusion, our study uses epigenetic clocks to provide novel evidence that DNA methylation may be a sensitive biomarker of high-LET 56Fe ion exposure. This finding adds to the body of literature suggesting that epigenetic clocks may be useful in monitoring the health of humans exposed to radiation including those whose exposures happen during spaceflight (Nwanaji-Enwerem et al., 2020). Still, future studies will be important for further characterizing these relationships and improving our understanding of epigenetic clock and radiation relationships.

#### AUTHOR CONTRIBUTIONS

Jamaji C. Nwanaji-Enwerem performed data analysis, visualization, developed analytical methodology, original writing, review & editing of final manuscript. Philippe Boileau performed data curation, data analysis, visualization, developed analytical methodology, original writing, review & editing of final manuscript. Andres Cardenas conceived the original study, supervised and reviewed statistical analyses, and contributed to writing/editing of the manuscript. Jonathan M. Galazka supervised and reviewed statistical analyses, and contributed to writing/editing of the manuscript.

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#### CONFLICT OF INTEREST

The authors declare no conflict of interests.

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