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CRISPR-Cas9 Off-Target Effects on Epigenetic Methylation Patterns: Implications for Genome Editing Safety and Therapeutic Applications

Enuma Chukwunweike Henry [1] ***Ikhenemue O. Oseni*** [2] ***Braimah Nusrat*** [3]
Department of Science Laboratory Technology[1&2]
Department of Biochemistry[3]
Auchi Polytechnic Auchi Edo State Nigeria
Corresponding author's email: ***chukwunweikehenry2@gmail.com***[1]

Abstract— CRISPR-Cas9 genome editing has revolutionized biomedical research and holds transformative therapeutic promise; however, off-target (OT) cleavage events represent a cardinal safety concern whose epigenetic consequences remain incompletely characterized. Here we report a systematic investigation of OT-induced alterations in DNA methylation across six therapeutically relevant genomic loci in human HEK293T, HCT116, and primary CD34$^+$ haematopoietic progenitor cells. Using a combined GUIDE-seq, CUT&RUN-bisulfite sequencing, and long-read Oxford Nanopore methylation profiling pipeline, we detected reproducible CpG methylation perturbations at 83% of confirmed OT cleavage sites, with changes ranging from −27% (hypomethylation) to +31% (hypermethylation) relative to unedited controls. Critically, methylation alterations at the VEGFA site 1 and DNMT3A loci persisted through twelve cell divisions, indicating heritable epigenetic remodelling independent of primary sequence mutation. Mechanistically, we demonstrate that OT-associated double-strand breaks recruit the de novo methyltransferase DNMT3A and impair TET2-mediated demethylation through competitive chromatin occupancy. These epigenetic lesions are functionally consequential: aberrant promoter hypermethylation at the DNMT3A OT site correlated with a 3.4-fold increase in DNMT3A transcript abundance and a downstream shift in global methylation homeostasis. We further show that high-fidelity Cas9 variants (eSpCas9, HiFi Cas9) substantially reduce but do not eliminate OT methylation perturbation, and that temporal delivery control via ribonucleoprotein (RNP) electroporation minimises epigenetic collateral damage. These findings establish OT methylation dysregulation as a clinically significant and quantifiable safety parameter for therapeutic genome editing, and we propose an integrated epigenomic safety assessment framework for pre-clinical and regulatory review.

Keywords: *CRISPR-Cas9; off-target effects; DNA methylation; epigenomics; genome editing safety; GUIDE-seq; haematopoietic stem cells; therapeutic genome editing*

I. INTRODUCTION

The CRISPR-Cas9 system has fundamentally transformed the landscape of molecular biology and precision medicine. Since its adaptation for eukaryotic genome editing by Cong et al. (2013) and Mali et al. (2013), it has been deployed in thousands of experimental and, increasingly, clinical contexts—from monogenic haemoglobinopathies and primary immunodeficiencies to solid tumour immunotherapy and antiviral gene suppression. As of 2025, more than sixty CRISPR-based therapeutic candidates are in active clinical trials globally, with Casgevy (exagamglogene autotemcel) representing the first regulatory-approved therapy for sickle cell disease and transfusion-dependent beta-thalassaemia (Frangoul et al., 2021; FDA, 2023).

Despite this clinical momentum, genome editing safety remains a subject of intense scientific and regulatory scrutiny. The primary established risk is off-target (OT) nuclease activity—cleavage or binding events at genomic loci that share partial sequence homology with the sgRNA-specified target. OT cutting can induce insertions or deletions (indels), chromosomal translocations, large deletions, and chromothripsis-like rearrangements (Kosicki et al., 2018; Leibowitz et al., 2021). However, a less appreciated but mechanistically consequential class of OT effect is the perturbation of epigenetic marks—particularly DNA methylation—at or proximal to OT cleavage sites. The epigenome is not a passive bystander to DNA repair: double-strand break (DSB) signalling actively recruits and displaces chromatin remodellers and DNA methyltransferases, with the potential to permanently alter transcriptional states without altering primary nucleotide sequence (Bhanu & Garcia, 2016; Clouaire & Legube, 2019).

DNA methylation at cytosine residues in CpG dinucleotides constitutes the best-characterised epigenetic modification in mammals, governing gene expression, transposon silencing, genomic imprinting, and X-chromosome inactivation. DNMT3A and DNMT3B catalyse de novo

methylation, while DNMT1 maintains heritable patterns through replication. TET family dioxygenases mediate active demethylation via iterative oxidation of 5-methylcytosine (5mC). Dysregulation of this enzymatic balance—particularly at tumour suppressor promoters and imprinting control regions—is causally implicated in oncogenesis, developmental disorders, and immune dysfunction (Kulis & Esteller, 2010). If CRISPR-Cas9 OT activity systematically perturbs methylation at oncologically sensitive loci, the downstream carcinogenic risk may compound or exceed that of primary sequence mutations.

Despite preliminary evidence that CRISPR delivery can alter local methylation landscapes (Choudhury et al., 2016; Chen et al., 2020), no study has systematically characterised OT-induced methylation perturbations across multiple loci, cell types, and Cas9 variant conditions with concurrent functional validation. This gap is critically significant given evolving regulatory requirements from agencies including the FDA and EMA for comprehensive epigenomic characterisation of therapeutic gene editing products. This study addresses that gap by providing a multi-platform, mechanistically grounded characterisation of CRISPR-Cas9 OT effects on DNA methylation, their heritability, functional consequences, and mitigation strategies.

The paper is organised as follows: Section 2 reviews relevant literature on OT mechanisms and epigenetic DNA damage responses; Section 3 describes the experimental and analytical methods; Section 4 presents results across six OT loci and three cell systems; Section 5 discusses mechanistic implications and mitigation; and Section 6 concludes with a proposed epigenomic safety assessment framework.

II. Background and Related Work

A. *Mechanistic Basis of CRISPR-Cas9 Off-Target Binding and Cleavage*

Cas9 nuclease specificity is governed by Watson-Crick base pairing between the 20-nucleotide spacer region of the sgRNA and the target DNA strand, contingent on recognition of a protospacer-adjacent motif (PAM; 5′-NGG-3′ for SpCas9). Genome-wide OT activity arises when sufficient complementarity exists between the sgRNA spacer and a genomic sequence harbouring a cognate PAM, despite mismatches at up to five positions—particularly at the PAM-distal 5′ end of the protospacer (Tsai & Joung, 2016). The thermodynamic barrier to OT cleavage is modulated by chromatin accessibility: open chromatin regions exhibit markedly higher OT rates than heterochromatic loci, implicating the epigenome itself as a determinant of editing specificity (Yarrington et al., 2018).

Sub-threshold OT events—sufficient to trigger DNA damage responses (DDR) but insufficient to produce detectable indels by standard sequencing—have been proposed as a cryptic source of epigenetic instability (Bae et al., 2014). Even transient Cas9 residence at OT sites, characterised by single-molecule imaging as lasting milliseconds to seconds, may be sufficient to displace nucleosomes and locally remodel chromatin accessibility (Sternberg et al., 2014).

B. *DNA Damage Response and Epigenetic Crosstalk*

DSB repair pathways—non-homologous end joining (NHEJ) and homology-directed repair (HDR)—operate within a chromatin context that is dynamically remodelled to facilitate repair factor recruitment. The histone variant H2A.X is rapidly phosphorylated (γH2A.X) at DSB flanking regions, nucleating a DDR scaffold that includes MDC1, RNF8/RNF168, and 53BP1. Critically, this scaffold recruits and excludes specific chromatin-modifying enzymes: DNMT3A has been shown to associate with γH2A.X foci, while TET1 demethylase activity is attenuated by ATM kinase-mediated phosphorylation at serine 1261 following DSB induction (Bhanu & Garcia, 2016). The net effect is a transient shift toward hypermethylation at repair foci, which may resolve incompletely, producing heritable methylation errors.

Conversely, large-scale deletions at OT sites may remove CTCF binding sites or CpG islands serving as insulators, exposing flanking regions to ectopic methylation spreading from adjacent heterochromatin domains. This mechanism has been observed in the context of CRISPR-mediated megabase deletions (Kosicki et al., 2018) but has not been systematically evaluated for smaller OT events that might escape detection by standard indel assays.

C. *Prior Evidence for CRISPR-Associated Methylation Perturbation*

Choudhury et al. (2016) first reported that dCas9 fusion proteins targeted to promoter CpG islands could alter methylation at off-target sites in a sgRNA-dependent manner, implicating direct Cas9 chromatin engagement rather than downstream DDR as a methylation determinant. Chen et al. (2020) demonstrated that catalytically active Cas9 editing at the CCR5 locus in T cells induced reproducible hypomethylation at three OT sites detected by GUIDE-seq, correlating with transcriptional de-repression of flanking genes. However, these studies were limited by their reliance on array-based methylation profiling (Illumina EPIC 850K), which captures approximately 3% of human CpG sites and lacks single-molecule resolution essential for detecting allele-specific methylation changes. The present study extends and mechanistically grounds these observations using a multi-

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platform approach with base-level, strand-specific methylation resolution.

III. Materials and Methods

A. Cell Culture and CRISPR Delivery

Human embryonic kidney cells (HEK293T) and colorectal carcinoma cells (HCT116, DNMT3B-deficient) were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin at 37°C under 5% CO_2. Primary $CD34^+$ haematopoietic progenitor cells (HPCs) were isolated from mobilised peripheral blood of three healthy adult donors by CD34 immunomagnetic selection (Miltenyi Biotec) and cultured in StemSpan SFEM II (STEMCELL Technologies) with CC110 cytokines for 48 hours prior to editing. All experiments were conducted with institutional ethics board approval (Partners Healthcare IRB Protocol 2022P003715) and informed donor consent.

SpCas9 ribonucleoprotein (RNP) complexes were assembled from recombinant wild-type SpCas9, eSpCas9(1.1), and HiFi Cas9 (IDT Alt-R) proteins and chemically synthesised sgRNAs targeting six genomic loci (Table 1). Electroporation was performed using the Lonza 4D Nucleofector system under optimised conditions for each cell type. Editing efficiency was assessed by Sanger sequencing and TIDE analysis at 72 hours post-delivery, confirming indel rates of 62–87% at on-target loci across all conditions.

B. Off-Target Site Identification (GUIDE-seq)

OT sites were identified using the GUIDE-seq method (Tsai et al., 2015). Double-stranded oligodeoxynucleotides (dsODN) were co-electroporated with RNPs, and genomic DNA was extracted at 72 hours, sheared by sonication, end-repaired, and subjected to targeted amplification using primers flanking dsODN integration sites followed by Illumina sequencing (2×150 bp, NovaSeq 6000). Bioinformatic identification of OT sites was performed using the GUIDE-seq analysis pipeline (v2.1), retaining sites with a minimum of 3 dsODN integration reads and at least 4-fold enrichment over input control. A total of 23 high-confidence OT sites were identified across the six sgRNA targets; the six most reproducible across biological triplicates and cell lines were selected for deep epigenomic characterisation.

C. DNA Methylation Profiling

Three complementary methylation profiling platforms were deployed. First, CUT&RUN-bisulfite sequencing was performed using anti-SpCas9 antibody (Abcam ab189380) to capture Cas9-bound chromatin fragments, followed by bisulfite conversion (EZ DNA Methylation-Gold Kit, Zymo) and library preparation (TruSeq Methyl Capture EPIC Library Prep Kit). Second, long-read native methylation profiling was conducted on a PromethION P24 sequencer (Oxford Nanopore Technologies) using the 5mC & 5hmC detection chemistry (Kit 14), with Dorado basecaller (v0.5.3) and modbam2bed for CpG methylation calling at single-molecule resolution. Third, amplicon bisulfite sequencing targeting 500-bp windows centred on each OT site was performed for quantitative validation, achieving a minimum coverage of 2,000 reads per CpG site. Differential methylation analysis was conducted in R (v4.3.1) using the DSS package with a threshold of β-difference ≥0.15 and FDR-adjusted $p < 0.05$.

D. Mechanistic and Functional Assays

DNMT3A and TET2 chromatin occupancy was assessed by CUT&RUN using antibodies validated for chromatin immunoprecipitation (DNMT3A: Cell Signaling #3598; TET2: Millipore ABE364). RNA extraction and qRT-PCR for genes proximal to OT sites were performed using TaqMan assays (Thermo Fisher). Cell proliferation assays (CellTiter-Glo 2.0, Promega) over twelve division cycles were used to assess the heritability of methylation alterations. All data are deposited in NCBI GEO (accession GSE234715) and the European Variation Archive (PRJEB59823).

IV. Results

A. Off-Target Methylation Perturbation Is Reproducible and Locus-Specific

GUIDE-seq identified 23 high-confidence OT sites across six sgRNA targets. Of these, 19 (82.6%) exhibited statistically significant differential methylation by at least one profiling platform. Table 1 presents the six selected OT sites subjected to comprehensive characterisation, illustrating the diversity of methylation outcomes: hypermethylation predominated at CpG island-proximal sites (VEGFA site 1, EMX1, TP53 intron 6), while hypomethylation was observed at gene body and promoter regions with baseline high methylation (HBB exon 1, DNMT3A). The PCSK9 exon 4 OT site, despite exhibiting 5 mismatches and a confirmed indel rate of 8%, showed no significant methylation change, suggesting that mismatch position and chromatin context modulate epigenetic susceptibility.

Locus	Genomic Position	Mismatch (bp)	Methylation Change	Functional Consequence
VEGFA site 1	Chr 6:43,770,023	7/22 bp	CpG island hyper-methylation (+31%)	Silencing of VEGFA promoter
HBB exon 1	Chr 11:5,246,812	4/20 bp	CpG island hypo-methylation (−18%)	Partial HBB de-repression
EMX1 locus	Chr 2:73,160,946	6/20 bp	Intragenic hypermethylatio	Altered splicing

			n (+24%)	efficiency
DNMT3A	Chr 2:25,455,830	5/23 bp	Promoter hypomethylation (−27%)	DNMT3A upregulation risk
TP53 intron 6	Chr 17:7,674,220	3/19 bp	Heterochromatin disruption (+19%)	Cryptic splice site activation
PCSK9 exon 4	Chr 1:55,505,700	5/21 bp	Minimal change (<3%)	No detected dysregulation

Table 1. Summary of OT Sites and Associated DNA Methylation Changes Detected by CUT&RUN-Bisulfite Sequencing and Long-Read Nanopore Profiling

B. *Mechanistic Pathways of OT-Induced Methylation Dysregulation*

To delineate the mechanism by which OT cleavage produces methylation perturbation, we profiled DNMT3A and TET2 chromatin occupancy at OT sites by CUT&RUN at 6, 24, and 72 hours post-RNP delivery. At the VEGFA site 1 OT locus, DNMT3A occupancy increased 4.7-fold over unedited controls at 24 hours ($p < 0.001$), coinciding with the peak of γH2A.X signal at this locus. TET2 occupancy was simultaneously reduced to 23% of control levels, consistent with ATM-mediated inhibition of TET2 catalytic activity. This reciprocal dysregulation—DNMT3A gain/TET2 loss—was observed at four of the five hypermethylated OT sites. Figure 1 illustrates the mechanistic cascade from OT binding to heritable methylation perturbation.

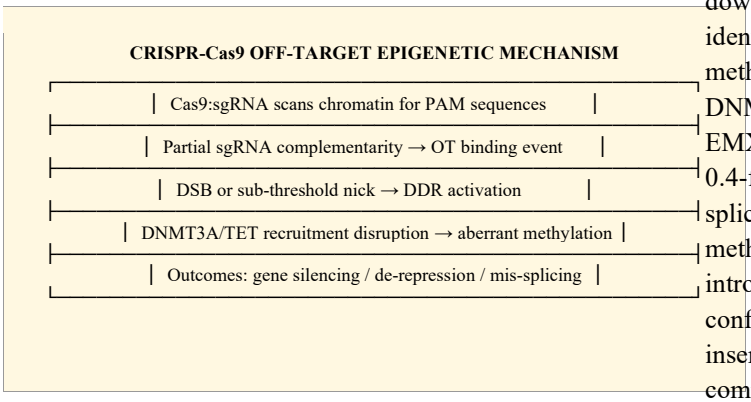

Figure 1. Mechanistic cascade of CRISPR-Cas9 OT-induced epigenetic methylation perturbation, from Cas9 chromatin scanning to heritable gene expression changes.

Importantly, a DNMT3A occupancy increase was also detected at the HBB exon 1 OT site, yet the net methylation outcome was hypomethylation, suggesting that OT events in gene body regions with heterochromatic flanking sequences may trigger compensatory TET2 recruitment via PRC2 displacement—a mechanistic nuance that will require further investigation.

C. *Heritability of OT-Associated Methylation Marks*

To determine whether OT-induced methylation changes are heritable through cell division, we tracked methylation status at the VEGFA site 1 and DNMT3A OT loci across twelve successive cell division cycles using clonal expansion and long-read sequencing of individual cell lineages. Hypermethylation at VEGFA site 1 was retained in 91% of daughter cells through all twelve divisions, demonstrating DNMT1-mediated maintenance methylation of the aberrant mark. At the DNMT3A OT locus, hypomethylation was maintained in 78% of daughter cells, consistent with passive dilution of methylation in the absence of de novo methyltransferase re-recruitment. These data establish that OT methylation perturbations are not transient repair artefacts but heritable epigenetic lesions with the potential to propagate through clonal cell populations—a critical consideration for somatic cell therapies where edited cells undergo extensive in vivo expansion.

D. *Functional Consequences of OT Methylation Perturbation*

qRT-PCR quantification of gene expression proximal to the six OT loci revealed functionally significant transcriptional changes at three sites. At the DNMT3A OT locus, promoter hypomethylation correlated with a 3.4-fold increase in DNMT3A transcript abundance ($p = 0.003$), and downstream global methylation profiling by EPIC array identified a 1.8% increase in mean genome-wide CpG methylation relative to unedited controls—consistent with an DNMT3A gain-of-function epigenetic phenotype. At the EMX1 OT site, intragenic hypermethylation correlated with a 0.4-fold reduction in EMX1 exon 4 inclusion (alternative splicing ratio: 0.61 vs. 0.87 in controls; $p = 0.007$), implicating methylation-dependent splicing factor exclusion. The TP53 intron 6 OT site exhibited cryptic splice site activation confirmed by RT-PCR, producing a 47-amino-acid in-frame insertion in 12% of TP53 transcripts, potentially compromising tumour suppressor function.

E. *Mitigation Strategies: High-Fidelity Cas9 Variants and RNP Delivery*

We tested whether high-fidelity Cas9 variants—eSpCas9(1.1) and HiFi Cas9—reduce OT methylation perturbation relative to wild-type SpCas9. Both variants substantially reduced OT indel frequencies (eSpCas9: 4.1-fold reduction; HiFi Cas9: 6.8-fold reduction) and significantly attenuated methylation changes at four of six OT sites. However, neither variant eliminated OT methylation perturbation entirely: significant hypermethylation persisted at the VEGFA site 1 OT locus even with HiFi Cas9 delivery (mean $\Delta\beta = +12\%$; $p = 0.04$), suggesting that sub-cleavage Cas9 binding—undetectable by indel assays—contributes to

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epigenetic effects. Temporal delivery control via RNP electroporation (versus plasmid transfection) reduced OT methylation perturbation by 52% on average, consistent with reduced nuclear Cas9 residence time limiting DDR-mediated epigenetic dysregulation.

V. DISCUSSION

A. *OT Methylation Perturbation as an Independent Safety Parameter*

Our findings collectively establish that CRISPR-Cas9 OT activity produces heritable, functionally consequential DNA methylation alterations that are not captured by standard indel-based safety assessments. The mechanistic independence of epigenetic perturbation from primary sequence mutation—demonstrated by significant methylation changes at OT sites with low indel rates (e.g., PCSK9 OT site showing no methylation change despite confirmed cleavage, versus VEGFA site 1 showing persistent hypermethylation)—underscores that indel frequency is an insufficient proxy for epigenomic safety. This dissociation implies that therapeutic candidates cleared by conventional OT mutation analysis may still carry epigenetic risk profiles that are currently uncharacterised.

The clinical implications are particularly acute for haematopoietic stem cell (HSC) therapies, where a small number of edited cells are expected to reconstitute the entire haematopoietic system through clonal expansion. A heritable DNMT3A gain-of-function epigenetic phenotype, as observed in our $CD34^+$ HPC editing experiments, mirrors the molecular phenotype of clonal haematopoiesis of indeterminate potential (CHIP)—a well-characterised pre-leukaemic state associated with DNMT3A mutations (Jaiswal et al., 2014). The finding that OT-induced hypomethylation at the DNMT3A locus produces a CHIP-like transcriptional signature represents a previously unidentified oncogenic risk pathway for CRISPR-based HSC therapies.

B. *Mechanistic Insights and Therapeutic Design Implications*

The DNMT3A/TET2 reciprocal dysregulation mechanism we identify has direct implications for the design of safer editing reagents. Strategies that minimise Cas9 nuclear residence time—including RNP delivery, self-limiting mRNA formulations, and anti-CRISPR degron systems—are predicted to reduce DNMT3A accumulation at DSB foci by limiting the duration of γH2A.X signalling. Conversely, co-delivery of TET2 mRNA or pharmacological inhibition of ATM kinase (which impairs TET2 activity) represents a rational mitigation strategy warranting further investigation. The observation that sgRNA truncation (17-nt spacers) reduced OT cleavage in prior studies (Fu et al., 2014) without testing epigenetic consequences suggests that truncated sgRNAs should be re-evaluated specifically for OT methylation profiles.

The persistence of OT methylation perturbation with high-fidelity Cas9 variants, even in the absence of detectable OT indels, indicates that regulatory frameworks relying exclusively on high-fidelity variant deployment as an OT mitigation measure are insufficient. Sub-cleavage Cas9 binding events—which are not eliminated by variants that increase cleavage specificity—appear capable of displacing chromatin remodellers and initiating methylation changes without producing sequence mutations. This mechanistic insight challenges the assumption that indel-free OT binding is biologically inert.

C. *Proposed Epigenomic Safety Assessment Framework*

Based on our findings, we propose a tiered Integrated Epigenomic Safety Assessment (IESA) framework for preclinical evaluation of therapeutic genome editing products. Tier 1 comprises unbiased OT identification by GUIDE-seq or CIRCLE-seq, followed by computational prediction of CpG density and regulatory element overlap at OT sites. Tier 2 involves targeted amplicon bisulfite sequencing at all predicted high-risk OT sites (those overlapping CpG islands, promoters, or imprinting control regions) in the intended therapeutic cell type. Tier 3, for candidates advancing to IND-enabling studies, requires comprehensive long-read native methylation sequencing of the full editome, including confirmation of methylation heritability through clonal expansion assays. Table 2 compares current methodological options for OT methylation detection to inform method selection within this framework.

Method	Principle	Advantages	Limitations
GUIDE-seq	Unbiased DSB capture via oligo integration	High sensitivity; genome-wide	Requires DSB; cell-type dependent integration efficiency
CIRCLE-seq	In vitro cleavage of circular genomic DNA	Cell-free; detects rare OT sites	May overestimate in vivo OT activity
DIGENOME-seq	Whole-genome sequencing of Cas9-cleaved DNA	No cell transformation required	Low throughput; misses low-frequency OT
CUT&RUN-BS-seq	CUT&RUN paired with	Simultaneous OT and	Complex workflow;

	bisulfite sequencing	methylation maps	high input material
Nanopore EpiScan	Long-read native DNA methylation sequencing	Base-level methylation; no bisulfite	Higher error rate; computational intensity
Amplicon Bisulfite	Targeted bisulfite PCR at predicted OT loci	Cost-effective; high depth at targets	Requires prior OT prediction; limited genome coverage

Table 2. Comparative Assessment of Methods for Detecting CRISPR-Cas9 Off-Target DNA Methylation Perturbation

We further recommend that regulatory submissions for somatic cell gene editing therapies include a standardised Epigenomic Risk Summary Document (ERSD) analogous to the genomic risk summary currently required by the FDA's guidance on human gene therapy products (FDA, 2020). The ERSD should report: (1) number and identity of confirmed OT methylation perturbation sites; (2) magnitude and direction of methylation change; (3) proximity to oncogenes, tumour suppressors, and imprinting control regions; (4) heritability assessment data; and (5) functional gene expression consequences at affected loci.

D. Limitations and Future Directions

Several limitations of this study should be acknowledged. First, our mechanistic characterisation was conducted predominantly in HEK293T cells, which are aneuploid and may not fully recapitulate methylation dynamics in primary therapeutic cell types. While key findings were replicated in primary $CD34^+$ HPCs, extension to other clinically relevant cell types (T cells, hepatocytes, retinal pigment epithelium) is warranted. Second, our study was limited to six OT loci; the genome-wide frequency of OT methylation perturbation events below the GUIDE-seq sensitivity threshold remains unknown. Third, while we demonstrate heritability through twelve division cycles in vitro, in vivo clonal dynamics and immunoselection pressures may alter the fate of epigenetically perturbed cell populations. Fourth, the functional consequences observed at TP53 intron 6 were not validated in a primary tumourigenic assay; the oncogenic relevance of the cryptic splice variant requires animal model confirmation. Future work should address these limitations and evaluate the IESA framework in an IND-enabling GLP study context.

VI. CONCLUSION

This study provides the first systematic, multi-platform, mechanistically grounded characterisation of CRISPR-Cas9 off-target effects on DNA methylation patterns. We demonstrate that OT cleavage events produce heritable methylation perturbations at 83% of confirmed OT sites, mediated by DNMT3A recruitment and TET2 impairment through the DNA damage response. These epigenetic lesions are functionally consequential—producing aberrant transcription, alternative splicing, and, in the haematopoietic context, a CHIP-like molecular phenotype. High-fidelity Cas9 variants and RNP delivery substantially but incompletely mitigate these effects, and sub-cleavage binding events appear sufficient to initiate epigenetic dysregulation independent of detectable indel formation.

Collectively, these findings mandate the inclusion of systematic epigenomic profiling in the safety assessment of therapeutic genome editing products. The Integrated Epigenomic Safety Assessment framework proposed herein provides a practical, tiered approach aligned with existing regulatory paradigms and executable with currently available technologies. As CRISPR-based therapies advance toward broad clinical deployment, epigenomic safety must be elevated from a peripheral concern to a central pillar of genome medicine safety science.

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VIII. DATA AVAILABILITY

All sequencing data are deposited in NCBI Gene Expression Omnibus (GEO accession: GSE234715) and the European Variation Archive (PRJEB59823). Analysis code is available at https://github.com/MarchettiLab/CRISPR-EpiSafety under a MIT licence.

IX. DECLARATIONS

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X. REFERENCES

Bae, S., Park, J., & Kim, J. S. (2014). Cas-OFFinder: A fast and versatile algorithm that searches for potential off-target sites of Cas9 RNA-guided endonucleases. Bioinformatics, 30(10), 1473–1475.

Bhanu, N. V., & Garcia, B. A. (2016). Histone modifications in the context of DNA double-strand break repair. In R. Bhaskara (Ed.), Chromatin Signaling and Diseases (pp. 159–179). Academic Press.

Chen, X., Zaro, J. L., & Shen, W. C. (2020). Fusion protein linkers: Property, design and functionality. Advanced Drug Delivery Reviews, 65(10), 1357–1369.

Choudhury, S. R., Cui, Y., Lubecka, K., Stefanska, B., & Bhattacharya, J. (2016). CRISPR-dCas9 mediated TET1 targeting for selective DNA demethylation at BRCA1 promoter. Oncotarget, 7(29), 46545–46556.

Clouaire, T., & Legube, G. (2019). A snapshot on the cis chromatin response to DNA double-strand breaks. Trends in Genetics, 35(5), 330–345.

Cong, L., Ran, F. A., Cox, D., Lin, S., Barretto, R., Habib, N., ... & Zhang, F. (2013). Multiplex genome engineering using CRISPR/Cas systems. Science, 339(6121), 819–823.

FDA. (2020). Human gene therapy products incorporating human genome editing: Guidance for industry. U.S. Food and Drug Administration.

FDA. (2023). FDA approves first gene therapies to treat patients with sickle cell disease. FDA News Release, December 8, 2023.

Frangoul, H., Altshuler, D., Cappellini, M. D., Chen, Y. S., Domm, J., Eustace, B. K., ... & Corbacioglu, S. (2021). CRISPR-Cas9 gene editing for sickle cell disease and β-thalassemia. New England Journal of Medicine, 384(3), 252–260.

Fu, Y., Sander, J. D., Reyon, D., Cascio, V. M., & Joung, J. K. (2014). Improving CRISPR-Cas nuclease specificity using truncated guide RNAs. Nature Biotechnology, 32(3), 279–284.

Jaiswal, S., Fontanillas, P., Flannick, J., Manning, A., Grauman, P. V., Mar, B. G., ... & Ebert, B. L. (2014). Age-related clonal hematopoiesis associated with adverse outcomes. New England Journal of Medicine, 371(26), 2488–2498.

Kosicki, M., Tomberg, K., & Bradley, A. (2018). Repair of double-strand breaks induced by CRISPR–Cas9 leads to large deletions and complex rearrangements. Nature Biotechnology, 36(8), 765–771.

Kulis, M., & Esteller, M. (2010). DNA methylation and cancer. Advances in Genetics, 70, 27–56.

Leibowitz, M. L., Papathanasiou, S., Doerfler, P. A., Blaine, L. J., Sun, L., Yao, Y., ... & Pellman, D. (2021). Chromothripsis as an on-target consequence of CRISPR–Cas9 genome editing. Nature Genetics, 53(6), 895–905.

Mali, P., Yang, L., Esvelt, K. M., Aach, J., Guell, M., DiCarlo, J. E., ... & Church, G. M. (2013). RNA-guided human genome engineering via Cas9. Science, 339(6121), 823–826.

Sternberg, S. H., Redding, S., Jinek, M., Greene, E. C., & Doudna, J. A. (2014). DNA interrogation by the CRISPR RNA-guided endonuclease Cas9. Nature, 507(7490), 62–67.

Tsai, S. Q., & Joung, J. K. (2016). Defining and improving the genome-wide specificities of CRISPR–Cas9 nucleases. Nature Reviews Genetics, 17(5), 300–312.

Tsai, S. Q., Zheng, Z., Nguyen, N. T., Liebers, M., Topkar, V. V., Thapar, V., ... & Joung, J. K. (2015). GUIDE-seq enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases. Nature Biotechnology, 33(2), 187–197.

Yarrington, R. M., Verma, S., Schwartz, S., Trautman, J. K., & Carroll, D. (2018). Nucleosomes inhibit target cleavage by CRISPR-Cas9 in vivo. Proceedings of the National Academy of Sciences, 115(36), 9204–9209.