

Article

Pharmacologic and Oncohistone Inhibition of SETD2 Converge on Genomic Instability

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Simple Summary

SETD2 is a tumor suppressor that trimethylates histone H3 at lysine 36 (H3K36me3). When inactivated, it drives the development of several cancers, most notably clear cell renal cell carcinoma (ccRCC). Our study looked beyond genetic loss of SETD2 activity, as occurs during oncogenesis, to other, clinically relevant means for inactivation of this methyltransferase: pharmacologic inhibition and a histone mutation that binds and sequesters SETD2. We found that both pharmacologic inhibition and sequestration made cells more genomically unstable. Identification of loss of genomic stability as a canonical feature of SETD2 inactivation is important as it reveals a potential mechanism for progression of cancers expressing oncohistone mutations, and potential liability associated with targeting this methyltransferase.

Abstract

Background/Objectives: SETD2 is a dual-function methyltransferase important for methylation of histone H3 at lysine 36 and α -tubulin in spindle microtubules. Genetic inactivation of SETD2 during oncogenesis drives loss of H3K36me3, genomic instability, and cancer progression. This study asked if disruption of genomic stability was a canonical feature of SETD2 inactivation via different pathways. **Methods:** We evaluated the impact of EPZ-719, a pharmacologic SETD2 inhibitor, and an H3.3K36M mutant histone (“oncohistone”) that binds and sequesters SETD2, on methylation activity and genomic stability in human cell lines. SETD2 activity was measured using *in vitro* methylation assays, H3K36me3 loss confirmed by Western analysis, and mitotic defects, specifically micronuclei and chromatin bridges, quantified with cytogenetic analysis. **Results:** EPZ-719 caused a dose- and time-dependent reduction in SETD2 activity on both histone and tubulin substrates, accompanied by significant increases in chromatin bridges and micronuclei in retinal pigmented epithelial (RPE-1) and 786-O ccRCC cells. Similarly, oncohistone expression markedly decreased SETD2 function, as determined by H3K36me3 levels, and induced comparable mitotic defects in 786-O cells, and aneuploidy in two chondrocyte cell lines expressing the



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H3K36M oncohistone. Combining EPZ-719 with H3K36M expression did not exacerbate mitotic defects beyond either oncohistone or pharmacologic inhibition alone, consistent with inhibition of SETD2 as their shared underlying mechanism of action. Conclusions: Pharmacologic inhibition and oncohistone-mediated sequestration of SETD2 converge on the induction of mitotic defects, underscoring SETD2's essential role in maintaining genomic stability. Identification of loss of genomic stability as a canonical feature of SETD2 inactivation points to a potential therapeutic liability associated with targeting SETD2 in cancers where it is overexpressed and reveals a mechanism that could contribute to the progression of cancers expressing oncohistone mutations.

Keywords: EPZ-719; methylation; micronuclei; chromatin bridges; mitosis; microscopy; H3K36M; cytoskeleton; epigenetics

1. Introduction

SETD2 (SET domain containing 2) is a dual-function methyltransferase with important functions on both chromatin and the cytoskeleton. On chromatin, SETD2 is required for the transcription-coupled deposition of histone H3 lysine 36 trimethylation (H3K36me3) and aids in transcriptional fidelity, regulation of RNA splicing, and DNA damage repair [1–6]. Beyond its activity on histones, SETD2 also trimethylates other substrates, including α -tubulin at lysine 40 (α -TubK40me3), a modification essential for stabilizing microtubules and supporting mitotic spindle function during cell division [7–11].

Because SETD2 regulates genomic and cytoskeletal integrity, its inactivation affects multiple aspects of cellular homeostasis. In the nucleus, inactivation of SETD2 leads to replication stress, defective DNA repair, and chromosome segregation errors [12–15]. Loss of H3K36me3 compromises the repair and resolution of replication-associated lesions and recombination intermediates, yielding persistent DNA linkages that manifest as chromatin bridges [15]. These defects culminate in chromosome mis-segregation, and the resulting lagging chromosomes and bridge fragments are often partitioned into micronuclei during the subsequent interphase, thereby propagating DNA damage and reinforcing genomic instability [7,14,16,17]. In parallel, disruption of SETD2-dependent microtubule methylation compromises spindle assembly and cytokinesis, converging on lagging chromosomes, micronuclei formation, chromatin bridges, and aneuploidy, which are hallmarks of genomic instability that fuel tumor evolution [7,16,18].

This instability is clinically relevant in tumors with inactivating SETD2 mutations, which are often truncating mutations, as well as loss of SETD2 due to chromosome 3p deletion. Prior studies [7,16,19] have demonstrated that clear cell renal cell carcinoma (ccRCC) tumors harboring SETD2 mutations exhibit increased chromatin bridges and micronuclei, reinforcing the link between SETD2 loss and genomic instability. These frequent defects found in ccRCC also correlate with higher-grade, more aggressive disease, and poor prognosis [20–23]. SETD2 mutations are not restricted to renal cancer but occur across a wide range of human malignancies, underscoring the global significance of SETD2-dependent pathways. Many large-scale cancer sequencing initiatives, including The Cancer Genome Atlas (TCGA), have shown SETD2 is recurrently mutated in ccRCC, lung adenocarcinoma, uterine endometrial carcinoma, bladder urothelial carcinoma, colorectal cancer, melanoma, and hepatocellular carcinoma, with mutation frequencies typically ranging from 5–15% depending on tumor type. Patient-derived datasets further indicate that SETD2-mutant tumors exhibit distinct clinical features, including elevated mutation burden and altered immune-related transcriptional programs across TCGA cohorts [24]. It

was also found that SETD2 mutations are associated with favorable responses to immune checkpoint inhibitors in treated patient populations [24].

Across these diverse lineages, SETD2 loss is associated with defects in DNA repair, replication stress, aberrant transcription, and mitotic errors, implicating SETD2 as a central guardian of genome stability [24,25]. These widespread alterations highlight the broad clinical importance of SETD2 inactivation and emphasize the need to understand how different modes of SETD2 disruption contribute to tumor development and progression across varied tumor contexts. Infrequently, SETD2 overexpression has also been observed in other malignancies, including advanced and castration-resistant prostate cancer and hepatocellular carcinoma, where elevated SETD2 protein levels are associated with worse survival and activation of oncogenic signaling pathways and increased cell proliferation and migration [26–28].

Beyond direct genetic inactivation, SETD2 inactivation due to a lysine 36 to methionine mutation (K36M) in histone H3.3 (H3.3K36M), termed an “oncohistone”, also can occur [29–31]. This mutation of the non-canonical histone variant H3.3 sequesters SETD2, acting as a dominant-negative inhibitor [32–35]. Importantly, the H3.3K36M mutation is highly prevalent in several cancers, including subsets of head and neck and approximately 90% of chondroblastomas [31,36,37]. H3.3K36M sequestration inhibits SETD2 activity on wild-type histone substrates, leading to a global reduction in H3K36me3 [32,35,38]. This epigenetic reprogramming by the H3.3K36M oncohistone impairs transcriptional fidelity and DNA repair, creating a permissive environment for tumorigenesis [38–40]. However, the impact of an H3.3K36M oncohistone on SETD2-dependent maintenance of genomic stability has not been examined.

Although SETD2 is classically regarded as a tumor suppressor due to its essential roles in preserving transcriptional fidelity and genomic stability, it has been suggested that its catalytic activity can be co-opted to sustain oncogenic programs in certain cancers. For example, in t(4;14) multiple myeloma, MMSET-driven accumulation of H3K36me2 creates a dependency on SETD2-mediated H3K36me3 deposition [41,42]. Similarly, SETD2 overexpression in prostate and liver cancers correlates with aggressive phenotypes, suggesting that elevated SETD2 activity may promote tumor progression [26–28]. These observations have motivated efforts to pharmacologically inhibit SETD2, despite its canonical tumor-suppressor role. EPZ-719 is a first-in-class, selective SETD2 inhibitor that serves as a critical chemical probe to dissect SETD2 biology and dependency mechanisms. EPZ-719 established SETD2 as a druggable target and informed the development of later clinical candidates such as EZM0414 [43]. EPZ-719 is a non-allosteric inhibitor that directly blocks SETD2 methyltransferase activity [44], aiming to exploit SETD2 dependency in tumors where its activity supports oncogenic processes, such as sustaining transcriptional programs or stabilizing mitotic structures. However, given SETD2’s dual role in chromatin and cytoskeletal regulation, systemic inhibition may pose risks for genomic instability, underscoring the need to define contexts where the SETD2 blockade is therapeutically advantageous versus detrimental. While EPZ-719 is not currently suitable for patient therapeutics, it serves as a valuable chemical probe to dissect SETD2 biology and dependency mechanisms in cancer and provide insights into SETD2 contributions to tumor progression.

We sought to determine whether clinically relevant routes for SETD2 inactivation, specifically pharmacologic inhibition and oncohistone-driven sequestration, pose a risk for heightened genomic instability. Accordingly, we investigated the impact of EPZ-719 mediated inactivation or H3.3K36M sequestration on genomic instability, focusing specifically on anaphase and telophase, the mitotic stages where previously reported mitotic defects that occur in response to inactivation of SETD2 have been reported. Here, we show that both pharmacologic and H3.3K36M-mediated sequestration of SETD2 converge on

genomic instability, identifying maintenance of genomic stability as a canonical function of this methyltransferase.

2. Materials and Methods

2.1. Cell Culture

All cell lines used were supplemented with 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA). The cell lines used in this study were routinely monitored for mycoplasma and confirmed to be negative. Screening was performed using DAPI (4', 6-Diamidino-2-phenylindole) staining. For DAPI staining, cells were fixed, then stained with DAPI and subsequently examined under fluorescence microscopy at the 60× objective to assess for extranuclear punctate signals indicative of mycoplasma. Only cell lines confirmed to be negative were used for experiments.

h-TERT RPE-1 cells (Cellosaurus CVCL_4388): Immortalized human retinal pigmented epithelial (hTERT RPE-1) cells (gift from Dr. Gregory Pazour, University of Massachusetts Medical School, Worcester, MA, USA) were maintained in DMEM/F-12 (Life Technologies, Carlsbad, CA, USA). 786-O cells (Cellosaurus CVCL_1051): Human *VHL*-deficient RCC 786-O cells were maintained in RPMI-1640 media (Life Technologies, Carlsbad, CA, USA). 786-O K36M cells: 786-O cells stably expressing H3.3K36M, along with matched control cells (gift from Dr. Josh Jang, Van Andel Institute, Grand Rapids, MI, USA), were maintained in RPMI-1640 medium (Life Technologies, Carlsbad, CA, USA). For the H3.3K36M-expressing cells, cells were always grown in media supplemented with 3 µg/mL of puromycin.

786-O histone mutant cell line generation: Lentiviral particles were produced in HEK293T cells using a standard third-generation packaging system (Addgene). HEK293T cells were seeded in 6 cm dishes to reach 50–80% confluence at the time of transfection and cultured in DMEM (ThermoFisher Scientific, Waltham, MA USA, 11965092) supplemented with 10% fetal bovine serum (MilliporeSigma, Burlington, MA, USA, 12306C-500ML) without antibiotics. The lentiviral histone mutant plasmid (H3.3K36M) was a generous gift from the laboratory of Scott Rothbart (Van Andel Institute, Grand Rapids, MI, USA), in which the coding sequence for human histone H3.3K36M was cloned into the pCDH-EF1-FHC lentiviral expression vector (Addgene, Cambridge, MA, USA, plasmid #64874). Cells were transfected using X-tremeGENE 360 (MilliporeSigma, Burlington, MA, USA, XTG360-RO) transfection reagent with a plasmid mixture containing the lentiviral H3.3K36M plasmid, psPAX2 packaging plasmid (Addgene plasmid #12260), and pMD2.G envelope plasmid (Addgene plasmid #12259) at a 4:3:1 ratio (total DNA, 2 µg per dish). Transfection complexes were incubated with cells for 12–15 h, after which the media was replaced with fresh DMEM containing 10% FBS and 1× Penicillin–Streptomycin (ThermoFisher Scientific, 15140122). Viral supernatants were collected at 72 h post-transfection, pooled, and clarified by centrifugation to remove cellular debris. Viral stocks were either used immediately or stored at −80 °C until use.

For the generation of stable histone mutant cell lines, 786-O cells were infected with viral supernatant with 8 µg/mL polybrene (MilliporeSigma, H9268). Viral media was replaced 24 h after transduction with RPMI-1640 (ThermoFisher Scientific, 11875093) supplemented with 10% fetal bovine serum and 1× Penicillin–Streptomycin. 72 h post-infection, cells were placed under 3 µg/mL puromycin (ThermoFisher Scientific, A1113803) selection and maintained with media changes every 2–3 days until all uninfected cells were eliminated. Stable cell populations were expanded under continuous puromycin selection and validated for the loss of targeted histone modifications by Western blotting.

Chondrocyte mutant cell line (Cellosaurus CVCL_6850): The parental chondrocyte cell line and clones stably expressing the H3.3K36M oncohistone were generously provided by the laboratory of Dr. Zhiguo Zhang (Columbia University, New York City, NY, USA).

The generation and validation of this cell line were performed as described previously [31]. Cells were maintained under the culture conditions reported in the original publication.

2.2. EPZ-719 Treatment

Cells were seeded for immunofluorescence and/or Western blotting and received EPZ-719 treatment concentrations of 250 nM, 500 nM, or 1000 nM of EPZ-719 for varying durations, including 0, 24, 48, and 72 h. For each time point, cells were treated every 24 h with fresh EPZ-719 at the appropriate concentration for the duration of the timeframe. EPZ-719 was obtained from MedChemExpress (Monmouth Junction, NJ, USA, HY-139626). A stock of 10 mM was made in dimethyl sulfoxide (DMSO).

2.3. Cell Synchronization

Cells were synchronized using an adapted protocol based on Vassilev et al. [45]. Briefly, at the 48 h time point of EPZ-719 treatment, cells were incubated with 6 μ M RO-3306 for 16 h to arrest them at the G2/M boundary. At 72 h, cells were released from the block by washing with fresh culture medium and allowed to progress into anaphase and telophase for 1.5 h. This synchronization strategy was chosen to enrich the population of cells specifically in late mitotic stages.

2.4. Immunocytochemistry

Coverslips were first UV-sterilized for 20 min in six-well dishes to eliminate potential contaminants and ensure optimal conditions for cell growth and imaging. Cells were then seeded onto the coverslips and cultured until they reached the desired confluence. Cells were fixed in 4% paraformaldehyde (PFA) diluted in PBS for 30 min at 37 °C, followed by a single wash with 1 $\times$ PBS (Phosphate-Buffered Saline). Permeabilization was performed using 0.5% Triton X-100 for 30 min at room temperature, after which cells were washed twice with 1 $\times$ PBS to remove residual detergent. Blocking was carried out for 1 h using 3.75% BSA in 1 $\times$ PBS. After blocking, primary antibody incubation was performed overnight at 4 °C with gentle rocking. The following morning, cells were washed three times with 1 $\times$ PBS. Secondary antibody incubation was conducted for 1 h at room temperature in 3.75% BSA in 1 $\times$ PBS, followed by three washes of 10 min each with 1 $\times$ PBS. A postfix step using 4% PFA in PBS was applied for 10 min at room temperature, then washed twice with 1 $\times$ PBS. Nuclear staining was performed using DAPI at a 1:4000 dilution in PBS for 10 min at room temperature, followed by two washes with 1 $\times$ PBS. Finally, coverslips were mounted on slides using Invitrogen ProLong Gold Antifade Mountant (Invitrogen, Waltham, MA USA, catalog #P10144).

2.5. Antibodies

Antibodies used: Histone H3 (Cell Signaling, Danvers, MA, USA, D2B12, rabbit, 1:5000), H3K36me3 (Abcam, Cambridge, UK, 9050, rabbit, 1:1000), H3.3K36M (Invitrogen, Waltham, MA, USA, MA5-24680, rabbit, 1:1000) which was selected as it underwent advanced verification to confirm there was no cross-reactivity with non-mutant histone (<https://www.thermofisher.com/antibody/product/H3-K36M-oncohistone-mutant-Antibody-clone-RM193-Recombinant-Monoclonal/MA5-24680> (accessed on 25 January 2026)), GAPDH (ProteinTech, Rosemont, IL, USA, 10494-1-AP, rabbit, 1:3000), α -tubulin (Santa Cruz Biotechnology, Dallas, TX, USA, sc-32293, DM1A, mouse, 1:1000), SETD2 (Sigma, St. Louis, MO, USA, HPA042451, rabbit, 1:1000), DAPI (Life Technologies, Carlsbad, CA, USA 62248, 1:4000), anti-rabbit Alexa Fluor 488 (Life Technologies, A110341:4000, 1:2000).

2.6. Microscopy

Fixed cells were imaged without a region of interest (ROI) using a CFI Plan Apochromat Lambda 60× oil, 1.4 numerical aperture (NA) objective, and DS-Qi2 camera and mounted on Nikon Eclipse Ti2-E inverted research microscope (Nikon Instruments, Melville, NY, USA) equipped for standard phase contrast and epifluorescence microscopy, as well as for deconvolution. Image acquisition was carried out using an Andor Zyla 4.2 + sCMOS high-sensitivity monochrome camera and was driven by Nikon NIS-Elements Advanced Research (AR) image acquisition and analysis software. Acquired images were processed for advanced three-dimensional (3D) and two-dimensional (2D) deconvolution modules for improved image quality. Eight-bit images were exported, and figures were prepared using Photoshop version CC software (Adobe Systems, Mountain View, CA, USA). Graphical representations and statistical analyses were performed using GraphPad Prism software version 10.3.1. Cell phenotypes (e.g., micronuclei and bridge formation) were scored visually by counting non-overlapping fields in a raster pattern across the coverslip using the Eclipse Nikon Ti2-E inverted research microscope (Melville, NY, USA).

2.7. In Vitro Methyltransferase Assay, Scintillation, and Autoradiography

Here, 2.8 µM recombinant His SETD2 (amino acids 1433 to 1711) was incubated with substrate, either histone H3.1 (4.2 µM) or tubulin purified from porcine brain (0.45 µM), in the presence of vehicle (dimethyl sulfoxide) or EPZ-719 in reaction buffer (50 mM Tris HCl pH 8.8, 5 mM MgCl₂, 4 mM DTT). Reactions were supplemented with the methyl donor S-adenosyl-L-methionine [methyl-³H] (~60.4 nCi/µL; specific activity 82.3 Ci/mmol) and incubated at room temperature for 17 h. Reactions were stopped with Laemmli buffer. Samples were resolved by SDS-PAGE, after which gels were fixed and stained with Coomassie blue (0.25% Coomassie blue, 50% methanol, 10% acetic acid) for 30 min, followed by destaining (30% methanol, 10% acetic acid) until protein bands were clearly visible. The protein bands of interest were excised from the gel and dissolved in 500 µL of 50% SOLVABLE™ (Revvity, Waltham, MA, USA) for 3 h at 50 °C in a thermomixer set to rotate at 300 rpm. The dissolved samples were then transferred to scintillation vials containing 4 mL of Ultima Gold™ (Revvity) and incubated overnight, protected from light. Radioactivity was quantified as counts per minute (CPM) using an LS 6500 liquid scintillation system (Beckman Coulter, Brea, CA, USA), with a tritium detection efficiency greater than 60%. For autoradiography, the 3H signal was enhanced by incubating the gels for 30 min with ENLIGHTNING™ Rapid Autoradiography Enhancer (Revvity, Waltham, MA, USA). Gels were then dried for 2 h at 80 °C and exposed to X-ray film prior to development.

2.8. Western Analysis

To make whole cell lysates, cells (70–80% confluent unless otherwise noted) were washed and collected in ice-cold PBS and pelleted by centrifugation at 4 °C. Pellets were either flash frozen in liquid nitrogen and stored at −80 °C for future use or resuspended in 1× lysis buffer (25 mM Tris, pH 8.0, 300 mM NaCl, 1 mM EDTA, 1% NP40). Protease inhibitor (1× complete protease inhibitor cocktail Roche, Cat. #04693132001), 1 mM sodium orthovanadate (Na₃VO₄, Sigma Aldrich, Cat. #S6508), and 1 mM PMSF (Sigma Aldrich, Cat. #P7626) were added fresh to the lysis buffer before use. The cellular extracts were sonicated for 13 cycles (30 s on and 30 s off per cycle) using a Diagenode Bioruptor 300, and the extracts were centrifuged at maximum speed for 10 min at 4 °C. The cell pellet was discarded, and the supernatant was collected as a whole cell extract. Whole cell extracts were subjected to BCA-protein assay to quantify and normalize protein levels. Soluble proteins were subject to immunoblot analysis using Criterion TGX precast 4–15% SDS-PAGE gels (Bio-

Rad, Cat. #64557025) followed by transfer to the PVDF membrane. Membranes were blocked for 1 h in 5% non-fat milk and incubated with primary antibodies overnight at 4 °C. Membranes were subsequently washed three times with Tris-buffered saline with 0.1% Tween[®] 20 detergent (TBST) for 10 min each, and primary antibodies were tagged with horseradish peroxidase (HRP) conjugated goat anti-mouse and goat anti-rabbit secondary antibodies by incubation for 1 h at room temperature. Membranes were then washed three times with TBST (10 min each) and visualization performed using ECL and ECL Prime (ThermoFisher Scientific, Cat. #32106 and Amersham/Cytiva, Buckinghamshire, UK, Cat. #RPN2232, respectively). Quantitation of immunoblots was performed via densitometric analysis using ImageJ 1.54g and the following protocol: <https://www.yorku.ca/yisheng/Internal/Protocols/ImageJ.pdf> (accessed on 25 January 2026). Original images and densitometry are provided in Supplemental Materials.

2.9. Flow Cytometry

This is an adapted protocol originally from Kim and Sederstrom [46] to perform flow cytometry to assess cell cycle stages in cells. First, to collect all cells, including precarious mitotic cells, cells were spun down in a centrifuge (Thermo Scientific #75004509: Sorvall™ ST 40 Centrifuge) at $300 \times g$ for 5 min at room temperature. After aspiration of media, the cell pellet was resuspended with PBS for another wash and spin down. PBS was discarded, and 20% ethanol was added dropwise while flicking the tube, and the cells underwent a two-hour incubation with ethanol and were flicked occasionally throughout the duration to ensure proper mixing. After the incubation period, cells were spun down at $300 \times g$ for 5 min at 4 °C. Next, the cells were washed with PBS prior to preparing for counting the number of cells using trypan blue with a hemocytometer. The aim was to have 2 million cells for FACs; if needed, cells were further diluted to reach the appropriate cell count. Cells were spun down at $300 \times g$ for 5 min and resuspended the pellet in 400 µL of PBS and 20 µL of propidium iodide (PI, Sigma Aldrich #P4864), flicking gently to mix. A portion of cells was reserved to serve as a negative control that did not have the addition of PI. The PI-stained cells were left for 20 min at room temperature prior to being transferred to FACs tubes for analysis.

Cell cycle samples were acquired using a BD FACSCantoII (BD Biosciences, Franklin Lakes, NJ USA) equipped with 488 nm, 561 nm, and 633 nm lasers. Samples were acquired at a low flow rate, and 10,000 total events were recorded per sample. Data analysis was performed using Flowjo 10.10.0. The built-in Cell Cycle analysis module in Flowjo failed to accurately model and represent the phases of the cell cycle; therefore, the percentages of cells in G1 and G2/M phase were determined by manual gating of propidium iodide staining (YG610-A parameter).

2.10. MTase-Glo Luminescence Methyltransferase Assay

The methyltransferase activity of recombinant SET-domain SETD2 protein (Cat. #HMT-11-129, Reaction Biology) was assessed using the MTase-Glo™ Methyltransferase Assay Kit (Promega) according to the manufacturer's protocol, with minor modifications. Bovine tubulin (Cytoskeleton Inc., Denver, CO, USA) and calf thymus histone (Cat. #10223565001, Roche/Sigma-Aldrich, St. Louis, MO USA) were used as substrates at final concentrations of 1 µM (2.2 µg per well) and 5 µM (5.5 µg per well), respectively. A higher histone concentration was used because SETD2 exhibits lower methylating activity toward histone than toward nucleosome in vitro. Recombinant human SETD2 (35.2 kDa) was used at a final concentration of 1 µM (0.7 µg per well). For each assay, 20 µL reaction mixtures were prepared in a 96-well PCR plate containing $1 \times$ methylation buffer (50 mM Tris-HCl, pH 8.8; 5 mM MgCl₂; 4 mM DTT; and protease inhibitor cocktail at $2 \times$ final concentration), 1 mM

S-adenosylmethionine (SAM), EPZ-719 (Cat #HY-139626, MedChemExpress) at various concentrations, the indicated substrate, and SETD2 enzyme. The plate was incubated overnight at room temperature. Following incubation, 8 μ L of each reaction was transferred to a new PCR plate containing 2 μ L of 5 $\times$ MTase-Glo reagent (prepared by diluting the 10 $\times$ stock 1:1 with distilled water) and incubated for 30 min at room temperature. MTase-Glo detection solution (10 μ L) was then added to each well and incubated for an additional 30 min. During this step, the total 20 μ L reaction mixture was transferred to a 96-well half-area white, flat-bottom plate (Cat. #3992, Corning, Manassas, VA USA) for luminescence measurement. Luminescence was measured using a Synergy H1 Hybrid reader (BioTek, Winooski VT, USA) with the following settings: gain 135, integration time 1 s, read height 6.5 mm, and 5 s of shaking prior to reading (no lid). Background signals were obtained from reactions lacking both SETD2 and substrate. Net relative luminescence units (RLU) were calculated by subtracting the corresponding background mean values from the raw signals. For IC₅₀ analysis, three independent experiments were performed for each condition. Background-corrected RLUs, normalized to substrate-only controls, were fitted to a four-parameter logistic (4PL) model using GraphPad Prism to determine IC₅₀ values. The mean luminescence values at 0 nM EPZ-719 were set to 100%. Results are presented as fitted mean curves with individual data points shown.

2.11. Statistics

All statistical analyses were performed using GraphPad Prism version 10.3.1 (GraphPad Software, San Diego, CA, USA). Data are presented as mean $\pm$ standard deviation (SD) unless otherwise indicated. For comparisons between two groups (e.g., treated vs. control), a two-tailed unpaired Student's *t*-test assuming equal variance was used. For experiments involving more than two groups (e.g., dose-response or combined treatment conditions), one-way ANOVA followed by Tukey's post hoc test was applied to assess pairwise differences. Significance thresholds were set at $p < 0.05$. Specific significance levels are indicated in figure legends as follows: $p < 0.05$ (*), $p < 0.01$ (**), $p < 0.001$ (***), and $p < 0.0001$ (****). For all experiments, the number of biological replicates (*n*) is specified in the corresponding figure legends. Micronuclei and chromatin bridge quantifications were based on scoring ≥ 500 –1000 cells per condition across non-overlapping fields in a raster pattern. For scintillation assays, counts per minute (CPM) were analyzed using one-way ANOVA with Tukey's post hoc test to determine dose-dependent effects. Effect size metrics were included where appropriate (Cohen's *d* for *t*-tests) to quantify the magnitude of observed effects and are provided in Supplemental Table S1.

3. Results

3.1. EPZ-719 Inhibits Histone and Tubulin Methylation

To optimize exposure conditions for EPZ-719, a time- and dose-dependent reduction in the SETD2-dependent methyl mark H3K36me3 was measured in both 786-O and RPE cells. Western analysis showed that exposure to 1000 nM of EPZ-719 decreased H3K36me3 over 24, 48, and 72 h in 786-O (Figure 1A) and RPE cells (Figure 1B). Total H3 levels were assessed as a loading control and confirmed that the observed changes were specific to loss of H3K36me3 and not due to global decreases in histone levels. Dose–Response experiments at 72 h showed a marked reduction in H3K36me3 at concentrations as low as 200 nM, with maximum loss at 1000 nM in both cell lines (Figure 1C,D). Quantification of normalized densitometry confirmed the expected significant suppression of H3K36me3 following EPZ-719 treatment compared to untreated controls (Figure 1E). We identified maximal inhibition of SETD2 activity at 72 h of exposure with 1000 nM of EPZ-719, which was selected as the time and dose for all further experiments.

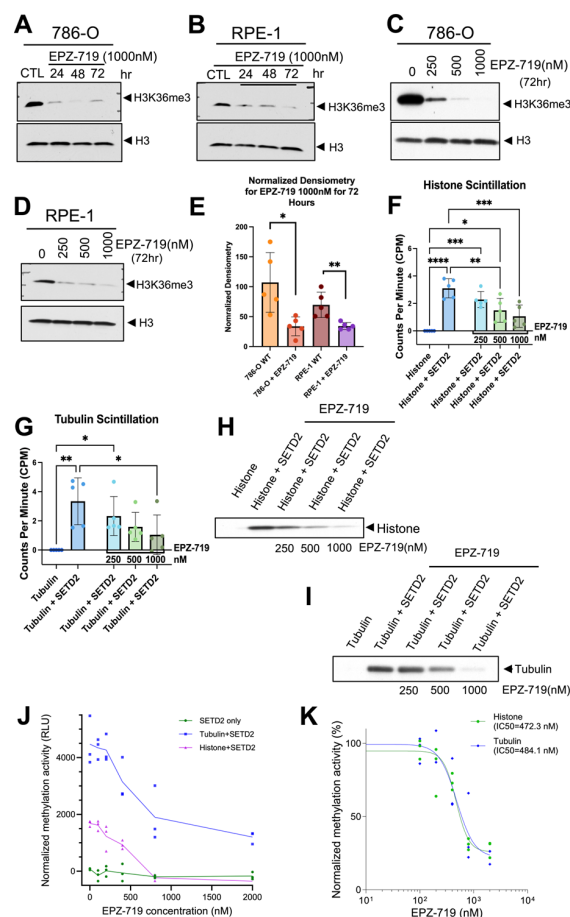


Figure 1. EPZ-719 inhibits SETD2 methyltransferase activity. (A,B) Western blot analysis of H3K36me3 levels in cells treated with EPZ-719 (1000 nM) for the indicated times compared to vehicle controls (CTL). (C,D) Dose–response to EPZ-719 at 72 h at the concentrations shown. (E) Quantification of H3K36me3 after 72 h treatment with EPZ-719 (1000 nM) in 786-O and RPE-1 cells ($n = 5$), comparing treated vs. control groups. Data are presented as mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, Student's t -test. (F,G) *In vitro* methylation assays using histone (F) and tubulin (G) substrates in the presence of EPZ-719 at 250, 500, and 1000 nM. Data are shown as counts per minute (CPM), mean $\pm$ SD; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, Student's t -test. (H,I) Recombinant SET domain was incubated with purified histone (H) or tubulin (I) substrates and increasing concentrations of EPZ-719 (250–1000 nM) in the presence of radiolabeled SAM. (J) Dose–response inhibition of SETD2-mediated methylation of tubulin using the MTase-Glo™ Methyltransferase Assay Kit. Luminescence signals (RLU, relative light units) were normalized to the mean RLU values from reactions lacking both substrate and SETD2 enzyme. (K) IC₅₀ values for EPZ-719 inhibition of SETD2-mediated methylation of tubulin and histone ($n = 3$). As shown in Supplementary Table S1, Cohen's d effect size estimates for panels (E–G) were all in the large range, indicating substantial differences between groups independent of sample size. Original images and densitometry available in the Supplemental Materials.

As an additional biochemical measure of SETD2 inhibition, we performed scintillation-based *In Vitro* methyltransferase assays using histone and tubulin substrates. As shown in Figure 1F, exposure to EPZ-719 produced a clear dose-dependent reduction in methyltransferase activity, with counts per minute (CPM) significantly decreased at 250 nM and further reduced at 500 nM and 1000 nM. At the highest concentration (1000 nM), SETD2 activity was nearly abolished compared with untreated controls ($p < 0.001$, one-way ANOVA with Tukey's Post Hoc test). EPZ-719 similarly inhibited SETD2 methylation of α -tubulin (Figure 1G), with modest reductions evident at 250 nM and progressively greater inhibition

seen at 500 nM and 1000 nM ($p < 0.05$ to $p < 0.01$). These findings confirm that EPZ-719 effectively suppresses SETD2-mediated methylation of both histone and tubulin substrates.

To further corroborate these results using an orthogonal approach, we employed an MTase-Glo methyltransferase assay with purified components. EPZ-719 again produced a robust dose-dependent decrease in SETD2 catalytic activity toward both substrates, with inhibition detectable at low nanomolar concentrations and near-complete loss of activity at approximately 1 μ M. Curve-fitted analyses revealed comparable IC_{50} values for histone and tubulin methylation, demonstrating that EPZ-719 consistently inhibits SETD2 enzymatic function across substrate contexts (Figure 1H,I).

3.2. EPZ-719 Induces Genomic Instability

To assess whether SETD2 inhibition by EPZ-719 promotes genomic instability, we performed cytogenetic analysis of synchronized cells at anaphase/telophase. Previous studies have shown that loss of SETD2 activity results in a significant increase in micronuclei and chromatin bridges detected at these stages of mitosis [7,15,16]. Representative DAPI (4',6-diamidino-2-phenylindole)-stained images of micronuclei detected in EPZ-719-treated cells are shown in Figure 2A,B. Quantification demonstrated a significant elevation in the percentage of cells containing micronuclei in both RPE and 786-O cells after EPZ-719 exposure (Figure 2C,D; $p < 0.05$, two-tailed unpaired Student's *t*-test) compared to vehicle controls. Specifically, micronuclei were observed in approximately 12–15% of RPE cells and 18–20% of 786-O cells following EPZ-719 treatment, compared to less than 5% in vehicle-treated matched controls.

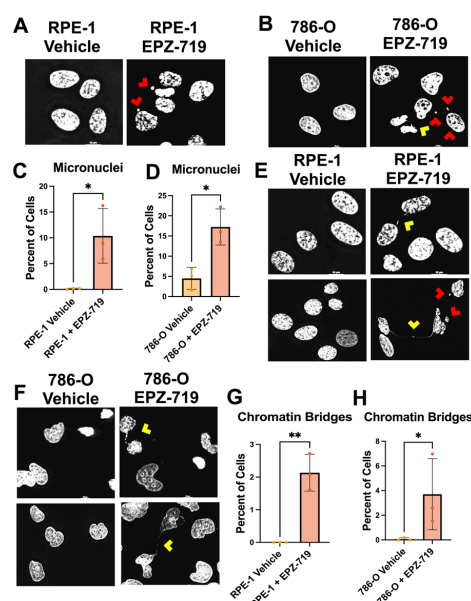


Figure 2. EPZ-719 treatment induces genomic instability. (A,B) Representative DAPI-stained images of RPE-1 (A) and 786-O (B) cells treated with vehicle or EPZ-719 (1000 nM) for 72 h, showing micronuclei (red arrowheads). Scale bar = 10 μ m (C,D) Quantification of micronuclei in RPE-1 (C) and 786-O (D) cells after EPZ-719 treatment. Data represent the percent of cells with micronuclei ($n = 1000$ cells per condition). Data are presented as mean $\pm$ SD. Statistical significance was determined using Student's *t*-test; * $p < 0.05$. (E,F) Representative DAPI-stained images of RPE-1 (E) and 786-O (F) cells treated with vehicle or EPZ-719, showing chromatin bridges (yellow arrowheads). Scale bar = 10 μ m (G,H) Quantification of chromatin bridges in RPE-1 (G) and 786-O (H) cells following EPZ-719 treatment ($n = 500$ cells per condition). Data are presented as mean $\pm$ SD. Statistical significance was determined using Student's *t*-test; * $p < 0.05$, ** $p < 0.01$. As shown in Supplementary Table S1, Cohen's *d* effect size estimates for panels (C,D,G,H) were all in the large range, indicating substantial differences between groups independent of sample size.

In addition to micronuclei, EPZ-719 treatment also induced chromatin bridges, a hallmark of defective DNA repair and a mitotic segregation defect occurring during anaphase [47]. Representative images illustrate the presence of chromatin bridges in EPZ-719-treated RPE and 786-O cells (Figure 2E,F). Quantification confirmed a low, but significant increase in chromatin bridge formation in both cell lines after exposure to EPZ-719 (Figure 2G,H; $p < 0.05$ to $p < 0.01$, two-tailed unpaired Student's *t*-test). Approximately 2% of RPE cells and 4% of 786-O cells exhibited chromatin bridges following EPZ-719 treatment, whereas these structures were detected in less than 1% of vehicle-treated controls. Collectively, these data show that pharmacologic inhibition of SETD2 by EPZ-719 compromises genomic stability, as evidenced by the presence of micronuclei and chromatin bridges.

3.3. Expression of an H3.3K36M Oncohistone Induces Genomic Instability

We next focused on another clinically relevant mechanism of SETD2 inactivation, the H3.3K36M oncohistone (Figure 3A). We expressed a modified H3.3 histone expression construct containing a K36M mutation in 786-O cells to examine its impact on genomic instability. Western analysis confirmed a near-complete loss of H3K36me3 in H3.3K36M-expressing cells compared to parental control expressing WT H3.3, while total histone H3 and GAPDH levels remained unchanged (Figure 3B). An H3.3K36M-specific antibody with little cross-reactivity to WT histone (see Section 2) validated the continued expression of the oncohistone in these cells. Quantification of H3K36me3 densitometry normalized for H3 corroborated the significant reduction in the SETD2-dependent histone methyl mark in cells expressing the oncohistone (Figure 3C).

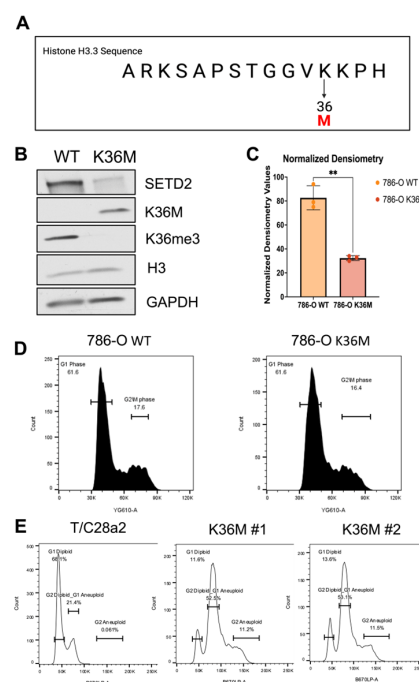


Figure 3. H3.3K36M oncohistone expression inactivates SETD2. (A) Schematic representation of the histone H3.3 sequence around the K36M mutation. (B) Western analysis of 786-O cells expressing wild-type H3.3 (WT) or H3.3K36M. Blots were probed for SETD2, H3.3K36M, H3K36me3, and total H3; GAPDH served as a loading control. (C) Quantification of densitometry for H3K36me3 normalized to total H3 in WT versus K36M-expressing cells. Data represent mean $\pm$ SD ($n = 3$ biological replicates). Statistical significance was determined using a two-tailed unpaired Student's *t*-test; ** $p < 0.01$. (D) Flow cytometry analysis of cell cycle distribution in WT and K36M-expressing 786-O cells to determine equivalent mitotic indices. (E) Flow cytometry analysis of DNA content in WT T/C-28a2 chondrocytes and two independent H3.3K36M-expressing clones using propidium iodide (PI) to

quantify diploid and aneuploid populations. As shown in Supplementary Table S1, Cohen's *d* effect size estimates for panel (C) were all in the large range, indicating substantial differences between groups independent of sample size. Uncropped Western blots found in Supplemental Materials.

To ensure that differences in the detection of cytogenetic abnormalities were not due to differences in the mitotic index of parental 786-O versus oncohistone-expressing 786-O cells, we performed flow cytometry to assess the cell cycle distribution (Figure 3D; gating strategy in Supplemental Figure S1). Comparable fractions of cells in G2/M were present in both WT and oncohistone-expressing 786-O cells (Table 1).

Table 1. Cell Cycle Distribution Percentages.

	786-O WT	786-O K36M
G1	61.6	61.6
S	17.4	21.3
G2/M	17.6	16.4

To determine whether the presence of the H3.3K36M oncohistone also induces genomic instability, we quantified micronuclei and chromatin bridges in 786-O cells expressing H3.3K36M and normal parental 786-O cells as controls. Representative DAPI-stained images for micronuclei are shown in Figure 4A. Quantification confirmed a significant elevation in the percentage of cells containing micronuclei in H3.3K36M-expressing cells (Figure 4B; $p < 0.05$, two-tailed unpaired Student's *t*-test). Approximately 18–22% of H3.3K36M cells exhibited micronuclei, whereas less than 5% of WT cells contained micronuclei. The percentage of H3.3K36M expressing cells with chromatin bridges was low but was significantly elevated relative to parental controls. Representative images of chromatin bridges are shown in Figure 4C and quantification provided in Figure 4D ($p < 0.05$, two-tailed unpaired Student's *t*-test). Together, these findings demonstrate that the H3.3K36M oncohistone promotes genomic instability, characterized by increased micronuclei and formation of chromatin bridges, consistent with inhibition of SETD2 activity.

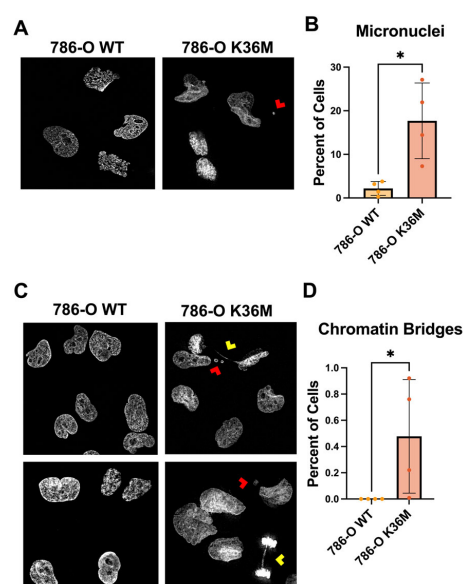


Figure 4. H3.3K36M expression induces genomic instability. (A) Representative DAPI-stained images of 786-O WT cells with wild-type H3.3 (WT) or expressing H3.3K36M, showing increased micronuclei (red arrowheads) in K36M-expressing cells. Scale bar = 10 μ m. (B) Quantification of micronuclei formation in parental and H3.3K36M-expressing cells. Data represent the percentage of cells with micronuclei ($n \geq 500$ cells per condition). (C) Representative DAPI-stained images of parental and

H3.3K36M-expressing cells highlighting chromatin bridges (yellow arrowheads). Scale bar = 10 μ m. (D) Quantification of chromatin bridges in parent and H3.3K36M-expressing cells ($n \geq 500$ cells per condition). Data are presented as mean $\pm$ SD. Statistical significance determined using Student's t -test; * $p < 0.05$. As shown in Supplementary Table S1, Cohen's d effect size estimates for panels (B,D) were all in the large range, indicating substantial differences between groups, independent of sample size.

To determine whether these effects extend to the oncogenic settings in which H3.3K36M most commonly occurs, we turned to human chondrocytes expressing the H3.3K36M oncohistone [31]. Flow cytometry analysis revealed that expression of the H3.3K36M oncohistone led to an increase in aneuploid DNA content relative to the parental T/C-28a2 cells. Parental chondrocytes displayed predominantly diploid G1 and G2/M peaks with minimal aneuploid populations (2.6% of G2/M aneuploid). In contrast, two independent K36M-expressing clones (K36M #1 and K36M #2) showed substantially expanded aneuploid fractions with 11.2% and 11.1% aneuploid G2/M cells, respectively. These K36M DNA content profiles exhibited broadened and shifted peaks characteristic of chromosome abnormalities. Because aneuploidy is a recognized indicator of genomic instability, the increased aneuploid cell populations observed in both K36M clones demonstrate that the introduction of the H3.3K36M mutation in chondrocytes is sufficient to disrupt normal maintenance of genomic stability in this cell lineage.

3.4. No Increase in Genomic Instability When Oncohistone and Pharmacologic SETD2 Inhibition Is Combined

Finally, given that SETD2 has both chromatin and cytoskeletal targets, it is possible that pharmacologic inhibition via EPZ-719 versus H3.3K36M oncohistone sequestration could have non-overlapping targets and mechanisms underlying their effect on genomic instability. To address this possibility, we asked if adding EPZ-719 to H3.3K36M-expressing cells would increase micronuclei over pharmacologic or oncohistone sequestration alone. Representative images of micronuclei using immunofluorescence of DAPI-stained cells are shown in Figure 5A. Importantly, adding EPZ-719 to oncohistone-expressing cells did not increase micronuclei over either condition alone. As shown in Figure 5B, quantification of micronuclei showed no significant increase when EPZ-719 and H3.3K36M expression were combined (Figure 5B).

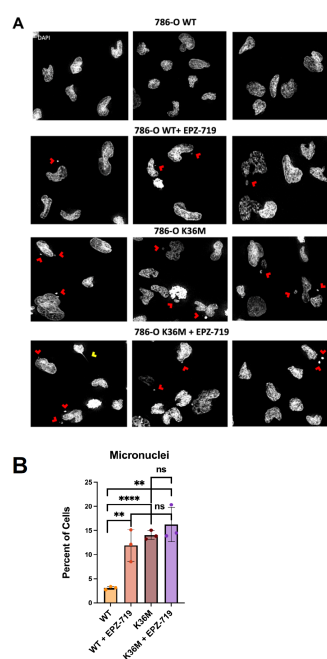


Figure 5. Combined pharmacologic and genetic SETD2 inhibition does not increase genomic instability in 786-O cells. (A) Representative DAPI-stained images of parental or 786-O cells K36M-expressing,

treated with vehicle or EPZ-719 (1000 nM) for 72 h. Micronuclei are indicated by red arrowheads. Yellow arrowhead indicates a chromatin bridge. Scale bar = 10 μ m. **(B)** Quantification of micronuclei across four conditions: WT, WT + EPZ-719, H3.3K36M, and H3.3K36M + EPZ-719. Data represent the percentage of cells with micronuclei ($n \geq 500$ cells per condition). Bars show mean $\pm$ SD. Statistical significance was determined using one-way ANOVA with Tukey's post hoc test; ** $p < 0.01$, *** $p < 0.0001$, ns = not significant.

4. Discussion

Our findings show that SETD2 inactivation, whether through pharmacologic inhibition or oncohistone-mediated sequestration, converges on a common outcome of increased genomic instability. Under both conditions, we observed increased chromatin bridges and micronuclei, consistent with previously reported chromosomal segregation defects associated with genetic inactivation of SETD2 [7,15,16]. The presence of chromatin bridges suggests unresolved DNA replication intermediates or improper chromosome segregation, whereas micronuclei formation is associated with chromosome mis-segregation and breakage events [47,48]. Both defects are hallmarks of defective mitotic fidelity and cancer progression [49]. Together, these observations underscore the vulnerability of pathways governed by SETD2 and highlight the broader biological consequences of disrupting its methyltransferase activity.

EPZ-719 treatment mimics the mutational loss-of-function of SETD2 by broadly inhibiting SETD2's catalytic activity. In contrast to EPZ-719, oncohistone mutations physically sequester methyltransferases, such as the H3.3K36M oncohistone sequestration of SETD2. These lysine-to-methionine mutations are thought to prevent access of methyltransferases to their wild-type histone targets [32,50,51]. Despite having distinct mechanisms for SETD2 inhibition- pharmacologic versus sequestration- the downstream consequences converge on genomic instability, reinforcing the central role of SETD2 in maintaining chromatin and mitotic integrity.

While pharmacologic inhibition and oncohistone-mediated sequestration have overlapping phenotypic consequences, i.e., the induction of genomic instability, these perturbations act through distinct upstream mechanisms and therefore, have the potential to differentially affect SETD2's substrate repertoire. Pharmacologic inhibition broadly suppresses SETD2 catalytic activity, reducing methylation across multiple substrates including histone H3 K36, α -tubulin K40, and actin K68. In contrast, the H3.3K36M oncohistone primarily interferes with histone methylation by sequestering SETD2 away from chromatin, and direct impact on non-histone targets has not been evaluated. Despite this divergence in biochemical impact, both perturbations ultimately converge at the level of defective chromatin maintenance and genome integrity and induce similar downstream effects on chromatin bridges and micronuclei formation. This relationship highlights that phenotypic convergence reflects shared cellular consequences rather than identical mechanistic pathways, and it underscores the need for future substrate-specific studies, such as rescue approaches or separation-of-function SETD2 variants, to delineate the relative contributions of histone and cytoskeletal methylation loss to mitotic defects.

While our findings in RPE-1 and 786-O cells, along with a chondrocyte model, demonstrate a clear requirement for SETD2 in maintaining genomic stability, it is important to note that SETD2 function can be highly context-dependent. Prior studies have shown that SETD2 loss can produce divergent phenotypes across tumor types, influenced by factors such as cell-of-origin, co-occurring mutations (including *VHL* and *PBRM1*), chromatin state, and oncogenic signaling environment [20,52–54]. Thus, although we expect certain aspects of the SETD2 dependency observed here to be broadly relevant, the magnitude and downstream consequences of SETD2 loss may vary across different backgrounds. Future

studies incorporating additional tumor models and diverse mutational contexts will be essential to determine the extent to which these findings generalize across cancer types.

This context-dependent behavior also aligns with a growing recognition that SETD2 can exhibit both tumor-suppressive and oncogenic roles depending on lineage and mutational background. SETD2 has historically been characterized as a tumor suppressor, particularly in tumor types such as clear cell renal cell carcinoma, where its loss promotes genomic instability, replication stress, and transcriptional dysregulation. However, increasing evidence indicates that in certain genetic or lineage settings, SETD2 activity may support oncogenic transcriptional programs or provide a proliferative advantage, suggesting that SETD2 dependency varies across tumor backgrounds. Distinguishing contexts in which SETD2 activity is tumor suppressive versus those in which it may be oncogenic is therefore essential for interpreting the impact of SETD2 disruption and for anticipating the consequences of pharmacologic SETD2 inhibition. These considerations highlight the importance of careful patient selection and mechanistic characterization as SETD2-targeted strategies move toward clinical evaluation.

Although EPZ-719 is a useful and selective tool compound for interrogating SETD2 function, it is not representative of clinically advanced SETD2-targeting agents. While EPZ-719 provides a valuable tool for interrogating SETD2 catalytic function, it remains a chemical probe rather than a clinically optimized inhibitor. As with other early-stage tool compounds, potential off-target effects cannot be fully excluded, and our study was not designed to comprehensively profile EPZ-719 selectivity beyond known SETD2 substrate loss. Although the convergence between EPZ-719 treatment and H3K36M-mediated sequestration supports an on-target mechanism, additional controls, such as orthogonal inhibitors, rescue constructs, or chemoproteomic profiling, will be important in future work to more definitively resolve on-target versus off-target contributions. Incorporating such approaches will be essential for interpreting how small-molecule SETD2 inhibition translates across experimental and therapeutic contexts. Given that SETD2 inhibition can induce forms of genomic instability, understanding how these effects shape toxicity profiles and therapeutic windows will be essential as next-generation inhibitors move toward clinical application.

In contrast to tool compounds such as EPZ-719, inhibitors such as EZM0414, currently being evaluated in clinical settings, are designed with therapeutic selectivity, pharmacokinetic properties, and safety considerations appropriate for patient use. Nevertheless, our findings have relevance for the broader landscape of ongoing efforts to develop selective SETD2 inhibitors. EZM0414, an oral, selective SETD2 inhibitor, advanced to a phase 1/1b first-in-human study in relapsed/refractory multiple myeloma (MM) and diffuse large B-cell lymphoma (DLBCL), with dose-escalation followed by expansion cohorts (including t(4;14) MM) to test the therapeutic hypothesis that specific cancers exhibit SETD2 dependency. Although ClinicalTrials.gov lists NCT05121103 as terminated as of 25 June 2025, due to a strategic business decision, our work emphasizes the need for rigorous biomarker selection and translational endpoints to define a safe therapeutic window and patient subsets most likely to benefit. Within this context, our data, showing that SETD2 inhibition perturbs both chromatin and the cytoskeletal targets and increases genomic instability, argue for close monitoring of mitotic fidelity in future protocols evaluating direct SETD2 inhibitors.

This need for precision is further underscored by the complex biology of SETD2, which belongs to a growing class of dual-function methyltransferases that act on both chromatin and the cytoskeleton, a paradigm increasingly recognized as “chromatocytoskeletal” regulation. In addition to catalyzing H3K36 tri-methylation, SETD2 also tri-methylates α -tubulin at lysine 40 and actin at lysine 68 [7,55]. Other methyltransferases with similar dual roles have emerged. One example is SMYD2, which methylates histone H3 at H3K4 and H3K36

as well as non-histone substrates, such as α -tubulin at lysine 394, and affects both transcriptional regulation and cytoskeletal dynamics [56]. Another example is EZH2, which methylates H3K27 and has emerging evidence of non-histone substrates, along with the broader class of PRMT family enzymes (such as SET8) that methylate tubulin [57,58]. These findings suggest that inhibition of the catalytic activity of histone methyltransferases could have on-target and off-target consequences by simultaneously disrupting the chromatin and cytoskeletal functions. For SETD2, this complexity means that systemic inhibition may impair DNA repair and transcriptional fidelity, while destabilizing microtubules (and possibly other cytoskeletal elements such as actin), amplifying the impact on genomic instability. These considerations demonstrate the need for a comprehensive evaluation of SETD2 inhibitors in preclinical models, not only for chromatin-related effects but also for cytoskeletal integrity.

Study Limitations

Several limitations must be acknowledged related to the present study. Our analyses primarily relied on synchronization to enrich for anaphase and telophase, the stages in which chromatin bridges and micronuclei are most readily observable. While this strategy minimizes artifacts inherent to asynchronous populations and increases our ability to detect these specific phenotypes, it also limits the scope of genomic instability features we can evaluate. In particular, lagging chromosomes and cytokinesis defects were not systematically assessed and therefore represent an important limitation of the current work. Additionally, this approach precludes evaluation of other mitotic abnormalities, such as multipolar spindles, centrosome amplification, and defects occurring in earlier phases of mitosis, which may also contribute to the genomic instability observed in these models. Thus, this study may underestimate the impact of EPZ-719 and expression of the H3.3K36M oncohistone on genomic instability, which future studies could build upon.

Another limitation is that, although we quantified micronuclei and chromatin bridges, we did not assess downstream consequences of these mitotic errors, such as activation of DNA damage signaling pathways, potential chromothripsis events, or long-term cell fate outcomes. These are critical downstream manifestations of mitotic instability and would provide additional mechanistic insight into how SETD2 loss shapes genome integrity over time. Future studies incorporating DNA damage response markers, genome-wide structural variation profiling, and extended live-cell fate mapping will be needed to define the full spectrum of consequences arising from SETD2 disruption.

Additionally, our analysis was primarily limited to two cell lines, RPE and 786-O, which may not fully recapitulate SETD2 biology across diverse tumor contexts. Although we have addressed this in part by adding a chondrocyte cell line expressing the H3.3K36M oncohistone for DNA-content assessment, which represents the physiologically relevant lineage for H3.3K36M-driven chondroblastoma. The increased aneuploidy in K36M-expressing chondrocytes supports the idea that this oncohistone disrupts genome stability in its native cellular state. Despite this, our overall model diversity remains constrained. Evaluating additional cancer types, including those that retain wild-type SETD2 or over-express SETD2, will be important in determining whether the phenotypes observed here represent universal or lineage-specific consequences of SETD2 inactivation. Such studies will also help clarify how co-occurring mutations and cellular context shape dependency on SETD2-mediated chromatin and cytoskeletal functions.

Furthermore, EPZ-719 exposure was short-term (72 h), and the long-term consequences of sustained SETD2 inhibition were not evaluated. EPZ-719 selectivity was not comprehensively evaluated, and off-target effects cannot be fully excluded. These considerations are particularly relevant because cells may develop compensatory mechanisms for the loss of

cytoskeletal methylation that allows them to survive the strong drive toward apoptosis that accompanies acute loss of SETD2 [59]. Such mechanisms may indeed exist in the 786-O cells expressing the H3.3K36M oncohistone, and could confound results. These adaptive processes could influence the observed phenotypes and represent an additional area for future investigation.

A further limitation of this work is the inability to resolve the relative contributions of histone versus non-histone SETD2 substrates to the observed genomic instability. Future studies employing substrate-specific rescue strategies or separation-of-function SETD2 variants will be required to define the distinct roles of chromatin- versus cytoskeleton-directed methylation in maintaining mitotic integrity. In line with this, a related limitation concerns the broader cellular consequences of SETD2 inhibition. Although mitotic indices were comparable across conditions as assessed by flow cytometry, we did not evaluate cell-cycle-independent stress responses that may be triggered by prolonged EPZ-719 exposure. SETD2 inhibition has the potential to induce broader cellular stress signaling, such as oxidative stress, ER stress, or p53-mediated pathways, that could influence cellular phenotypes independently of mitotic defects. Because these pathways were not investigated, we cannot exclude the possibility that non-mitotic stress responses contributed to the observed phenotypes. Future studies incorporating assays for stress-response activation will be necessary to define the cellular consequences of acute and sustained SETD2 inhibition more fully.

An additional limitation of this work is the lack of mechanistic validation underlying the non-additive phenotype observed when EPZ-719 treatment is combined with H3.3K36M expression. While both perturbations converged on similar degrees of genomic instability, we did not directly examine SETD2 chromatin occupancy, residual catalytic activity, or substrate engagement under these combined conditions. As a result, the molecular basis for the absence of further genomic destabilization remains unresolved. Future studies incorporating SETD2 ChIP-based occupancy mapping or substrate-specific rescue approaches will be necessary to distinguish complete pathway saturation from more nuanced mechanistic interactions between pharmacologic inhibition and oncohistone-mediated sequestration.

Future studies should address these limitations by expanding cell line diversity, quantifying additional mitotic abnormalities, and evaluating the cellular response to chronic EPZ-719 treatment. Future clinical studies of direct SETD2 inhibitors (e.g., EZM0414) should incorporate pharmacodynamic assays for cytoskeletal modifications, cytoskeletal integrity metrics, and mitotic error quantification to prospectively monitor genomic instability risks and define responsive subsets (e.g., t(4;14) MM) in expansion cohorts.

5. Conclusions

Our study revealed that SETD2 disruption, whether through pharmacologic inhibition or oncohistone-mediated sequestration, compromises genome integrity through convergent mechanisms. These findings extend the understanding of SETD2 beyond its canonical role as the H3K36me3 methyltransferase, highlighting its integrated functions as a regulator of transcriptional and mitotic fidelity. The dual substrate specificity of SETD2 introduces unique vulnerabilities when its activity is perturbed, with implications for therapeutic strategies targeting this enzyme. Moving forward, systematic evaluation of SETD2 (and other methyltransferase) inhibitors should incorporate both chromatin and cytoskeletal endpoints to capture their biological impact fully. Such efforts will be critical for balancing the potential benefits of SETD2-targeted therapies against the risk of exacerbating genomic instability, a hallmark of cancer and potential driver of tumor evolution.

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/cancers18050819/s1>. Supplemental Table S1: Cohen's *d* effect sizes. Supplemental Westerns: All uncropped Westerns blots. Supplemental Densitometry of all Westerns shown. Supplemental Figure S1: Representative flow cytometry profiles illustrating the gating strategy used for propidium iodide (PI)-based cell cycle analysis in wild-type and K36M mutant-expressing cells (both 786-O and chondrocytes). Cells were first gated on forward scatter area versus forward scatter height (FSC-A vs. FSC-H) to exclude doublets and aggregates, retaining FSC singlets (left panels). Singlet populations were further refined using side scatter width versus side scatter height (SSC-W vs. SSC-H) to remove residual multiplets and debris (second panels). A final singlet gate was applied using PI fluorescence area versus height (YG610-A vs. YG610-H) to ensure inclusion of single cells only (third panels). DNA content histograms were generated from the final singlet population based on PI fluorescence intensity (YG610-A), and cell cycle phases were quantified according to DNA content distribution, with G1 and G2/M phases indicated (right panels). Percentages denote the proportion of cells within each gate or cell cycle phase.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

786-0	Renal cell carcinoma
ANOVA	Analysis of variance
BCA	Bicinchoninic acid assay
BSA	Bovine Serum albumin
CPM	Counts per minute
Ctl	Control
DAPI	4',6-diamidino-2-phenylindole
DLBCL	Diffuse Large B-Cell Lymphoma
DNA	Deoxyribonucleic acid
FACs	Fluorescence-activated cell sorting
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
H3	Histone H3
H3K36me3	Histone H3 lysine 36 tri-methylation
HCC	Hepatocellular carcinoma
IHC	Immunohistochemistry
K40me3	Tubulin lysine 40 tri-methylation
MM	Multiple Myeloma
MMSET	Multiple Myeloma SET domain
NA	Numerical aperture
PBS	Phosphate-buffered saline
PFA	Paraformaldehyde
PI	Propidium iodide
PRMT	Protein arginine methyltransferase
RPE	Retinal pigmented epithelial
RT	Room temperature
TBST	Tris-buffered saline with Tween-20
UV	Ultraviolet radiation
WT	Wild-type

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