SPOP mutation induces replication over-firing by impairing Geminin ubiquitination and triggers replication catastrophe upon ATR inhibition

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Geminin and its binding partner Cdt1 are essential for the regulation of DNA replication. Here we show that the CULLIN3 E3 ubiquitin ligase adaptor protein SPOP binds Geminin at endogenous level and regulates DNA replication. SPOP promotes K27-linked non-degradative poly-ubiquitination of Geminin at lysine residues 100 and 127. This poly-ubiquitination of Geminin prevents DNA replication over-firing by indirectly blocking the association of Cdt1 with the MCM protein complex, an interaction required for DNA unwinding and replication. SPOP is frequently mutated in certain human cancer types and implicated in tumorigenesis. We show that cancer-associated SPOP mutations impair Geminin K27-linked poly-ubiquitination and induce replication origin over-firing and re-replication. The replication stress caused by SPOP mutations triggers replication catastrophe and cell death upon ATR inhibition. Our results reveal a tumor suppressor role of SPOP in preventing DNA replication over-firing and genome instability and suggest that SPOP-mutated tumors may be susceptible to ATR inhibitor therapy.
Genomic stability relies on precise genome replication. Tens of thousands of DNA replication start sites must be established during each cell cycle to ensure the accurate and complete duplication of more than 3 billion base pairs of DNA in the human genome. To ensure accurate progress in DNA replication, licensing of this process is initiated by assembly of the pre-replicative complex (pre-RC) on replication origins at G1 phase. After the G1/S transition, origins are not to be relicensed or reactivated for the remainder of the cell cycle. In yeast, origin reactivation is a driver of gene amplification, copy number variation, and aberrant chromosome segregation. In mammalian cells, it causes chromosomal breaks and activation of the DNA damage response.

The pre-RC is composed of the origin recognition complex (ORC) including Cdc6, Cdt1, and the mini-chromosome maintenance (MCM) proteins. ORC binds origins of replication and recruits Cdc6 at the M/G1 transition. Cdc6-bound ORC recruits Mcm2-7 in complex with Cdt1 at the origins of DNA replication. Once the pre-RC is assembled, origins are licensed for replication in S phase and are ready to be fired. Cdt1 activity is limited to G1 through the control of its synthesis, degradation, and activity. The low level of Cdt1 in the early S phase is thought to result from targeted degradation whereas its higher level in G2 phase is caused by its stabilization. However, the increase of Cdt1 in late S and early G2 poses a potential risk of replication origin over-firing and re-replication, which could occur if there were residual activity of the DNA-replicating enzymes in G2. The activity of Cdt1 is tightly controlled by Geminin, a replication inhibitory factor, which directly binds to and suppresses the replication-stimulating function of Cdt1. Geminin is an unstable protein that is targeted for degradation by the anaphase-promoting complex (APC). Both Geminin and Cdt1 are expressed at high levels in late S and G2 phases, where Geminin binds Cdt1 and prevents DNA re-replication. Furthermore, Geminin controls the basal level of Cdt1 and inhibits its accumulation during mitosis by inhibiting its ubiquitin-dependent proteolysis. Thus, it is proposed that Geminin has both negative and positive roles in pre-RC formation, indicating that the protein level of Geminin may not be the sole key regulatory mechanism in controlling its function to ensure proper DNA replication.

Two Geminin molecules self-associate via a coiled-coil domain to form a homodimer, or possibly a tetramer as suggested by crosslinking experiments. The Geminin dimer forms a heterotrimer with Cdt1, which is required for the inhibition of Cdt1 function. However, the Geminin/Cdt1 complex may exist in different states since Geminin/Cdt1 can be replication-active or inactive depending on the stage of the cell cycle. A Geminin/Cdt1 complex heterotrimer-heterohexamer transition model was proposed to explain the active and inactive states of Cdt1. Even if Cdt1/Geminin has been extensively studied, there is no conclusive understanding of how this complex is regulated.

The SPOP gene encodes a substrate-binding adaptor subunit of the CUL13 (CUL3)-RING box 1 (RBX1) E3 ubiquitin ligase (CRL) complex. SPOP is implicated in oncogenesis since it is frequently mutated in human cancers such as prostate and endometrial cancers. Notably, almost all SPOP mutations detected in prostate cancer patient samples are hemizygous mutations, suggesting that prostate cancer-derived SPOP mutants function in a dominant-negative manner, which is consistent with the findings from biochemical and structural studies showing that the SPOP protein can form a dimer or oligomer via its BTB domain and BACK domain. Several cancer-relevant proteins have been identified as the substrates of SPOP, such as androgen receptor (AR), SRC-3, TRIM24, and BRD4, and these proteins are aberrantly upregulated in SPOP-mutated PCa cells and patient tissues. SPOP is also implicated in regulating genomic stability. However, how SPOP precisely controls genomic stability remains poorly understood.

In the present study, we demonstrate that SPOP functions as an E3 ubiquitin ligase that binds to Geminin abundantly at S phase and catalyzes K27-linked non-degradative poly-ubiquitination of Geminin. We show that SPOP-dependent poly-ubiquitination of Geminin blocks MCM binding to Cdt1. This process prevents over-firing of DNA replication. Cancer-associated SPOP mutations impair DNA replication surveillance and cause replication origin over-firing and re-replication, thereby increasing replication stress and sensitizing cancer cells to ATR inhibition.

Results
Identification of the DNA replication factor Geminin as a SPOP-binding protein. Both the Cancer Genome Atlas (TCGA) prostate cancer dataset and the whole-genome sequencing data of an independent cohort show that SPOP mutant tumors display higher genome alterations than SPOP wild-type (WT) tumors. While a handful of previous studies suggest that SPOP deregulation may lead to genomic instability, no study has directly examined the impact of SPOP mutations on DNA replication. Hjorth-Jensen reported that SPOP knockdown led to reduced transcription of genes important for DNA repair and replication including BRCA2, ATR, CHK1, and RAD51. However, the TCGA data show that none of these genes are downregulated in SPOP-mutated prostate cancer patient samples. The mRNA levels of these genes are negatively correlated to SPOP mRNA expression in prostate cancers.

The majority (>97%) of SPOP mutations detected in prostate cancer patient samples is localized in the substrate-binding MATH domain, suggesting that tumorigenesis linked to SPOP mutations originates from deregulation of SPOP substrate ubiquitination. To determine how many of the proteins that directly bind SPOP are DNA replication and repair factors, we performed cluster analysis of the yeast two-hybrid interactionome we generated through tandem affinity purification and mass spectrometry. We found that Geminin is the only DNA replication factor among the SPOP-interacting proteins identified by the two independent methods, implying that Geminin is a SPOP-interacting protein. Co-immunoprecipitation (co-IP) assays confirmed that both ectopically expressed and endogenous SPOP interacted with Geminin in 293T cells and PC-3 prostate cancer cells.

Together, our data demonstrate that SPOP directly binds Geminin and that their interaction occurs at the endogenous level. Proteins bound by SPOP usually harbor a SPOP-binding consensus sequence (SBC, \( \Phi\cdot\pi-S/S/T-S/T \) where \( \Phi \) is a nonpolar
1H-15N HSQC NMR spectrum of 15N-labeled SPOP proteins in PC-3 cells using the indicated antibodies. In 293T cells transiently transfected with Myc-SPOP-WT or upon titration with unlabeled WT (peptide and SPOP residues at the binding interface. Putative intermolecular hydrogen bonds are shown in black dashes. The potential electrostatic surface overlap of yeast two-hybrid screen data from Fudan University (Shanghai), mass spectrometry-based SPOP interactome from Mayo Clinic, and the DNA replication gene set.

Deletion of these clusters of Y2H positive clones contain this motif (Fig. 1f).

199VSSST203 is a functional SBC motif in Geminin.

199VSSST203 is the only putative SBC motif in Geminin. All three pi clusters of Y2H positive clones contain this motif (Fig. 1f).

Nuclear magnetic resonance (NMR) spectroscopy confirmed this binding motif and better defined the binding interface in SPOP. The Geminin WT SBC-containing peptide (195AEGTVSSSTDAKPC1208) and the SBC-alanine mutant counterpart (195AEGTVAAAADAKPC1209) were tested for interaction with the recombinant SPOP MATH domain (amino acids 28–166) purified from E. coli. Upon addition of non-labeled Geminin WT peptides to 15N-labeled SPOP-MATH domain, there were multiple chemical shift perturbations in the 1H-15N heteronuclear single quantum coherence (HSQC) spectrum of SPOP MATH (Fig. 1h). In contrast, there were almost no changes in the 1H-15N HSQC spectrum of SPOP MATH upon addition of unlabeled Geminin peptide (amino acids 195–209). j Crystal structure of SPOP-MATH (surface representation) in complex with the Geminin peptide (amino acids 195–209) in stick representation. Peptide residues 196–204 were modeled in the electron density. The red surface corresponds to SPOP residues for which chemical shift perturbations were detected in the 1H-15N HSQC NMR spectrum of 15N-labeled SPOP MATH titrated with unlabeled Geminin peptide. Selected SPOP residues frequently mutated in cancer patients are labeled. k Representation of Geminin peptide and SPOP residues at the binding interface. Putative intermolecular hydrogen bonds are shown in black dashes. The potential electrostatic surface of SPOP is shown in blue and red for positive and negative charges, respectively. Source data are provided in this paper or Mendeley database (10.17632/Bn7xt5skhc.1).

Similar results for (d), (e), (g) panels were obtained in two independent experiments.
SPOP promotes K27-linked non-degradative poly-ubiquitination of lysine residues 100 and 127 in Geminin. Increased expression of SPOP WT largely enhanced Geminin poly-ubiquitination in 293T cells, and this ubiquitination had no effect on Geminin protein level (Fig. 2a). Geminin ubiquitination by SPOP was confirmed by different methods (Supplementary Fig. 2a–c). SPOP could not induce poly-ubiquitination of Geminin SBC deletion mutant (Supplementary Fig. 2d). Knockout of endogenous SPOP by CRISPR-Cas9 greatly attenuated Geminin poly-ubiquitination in 293T cells (Fig. 2b and Supplementary Fig. 2e) and such effect was reversed by restored expression of SPOP (Fig. 2b), suggesting a specific effect of SPOP on Geminin poly-ubiquitination. Given that SPOP overexpression or knockdown did not affect Geminin protein level (Fig. 2a and Supplementary Fig. 2f–i), we sought to determine the ubiquitin chain linkage type(s) of SPOP-mediated poly-ubiquitination of Geminin. To this end, we expressed WT ubiquitin, single lysine residue-only or lysine-null mutants in 293T cells and showed that SPOP specifically augmented K27-linked poly-ubiquitination of Geminin (Fig. 2c). To determine which lysine residues of Geminin are ubiquitinated by SPOP, we transfected 293T cells with Flag-tagged Geminin in combination with SPOP and ubiquitin and collected cells for mass spectrometry. Ubiquitination at lysine residues 27, 50, and 100 and 127 in Geminin was detected by mass spectrometry (Fig. 2d). Mutagenesis analysis showed that only mutation of Lys100 and Lys127 substantially reduced SPOP-dependent Geminin poly-ubiquitination (Fig. 2e). Mutations of both Lys100 and Lys127 completely abolished SPOP-induced poly-ubiquitination of Geminin (Fig. 2f). These data indicate that SPOP promotes K27-linked poly-ubiquitination on Geminin Lys100 and Lys127.

Table 1 Data collection and refinement statistics for SPOP-MATH-Geminin peptide (PDB entry 7KLZ).

| Data collection | Space group | P 41 2 2 |
|----------------|-------------|-----------|
| Cell dimensions | α, β, γ (Å) | 103.06, 103.06, 131.81 |
| α, β, γ (%) | 90.00, 90.00, 90.00 |
| Resolution (Å) | 47.99–3.40 (3.52–3.40) |
| Rmerge (Å) | 0.096 (1.27) |
| Completeness (%) | 95.8 (100) |
| Redundancy | 37.0 (38.5) |
| Refinement | Resolution (Å) | 47.99–3.40 |
| No. reflections | 10264 |
| Rwork/Rfree | 0.21/0.24 |
| No. atoms | 2307 |
| Protein | 2297 |
| Ligand/ion | 10 |
| Water | 0 |
| B-factors | Protein | 89.0 |
| Ligand/ion | 147.6 |
| Water | 0 |
| R.m.s. deviations | Bond lengths (Å) | 0.002 |
| Bond angles (°) | 0.48 |

*One crystal was used for structure determination. Values in parentheses are for highest-resolution shell.

SPOP-mediated poly-ubiquitination of Geminin inhibits MCM protein binding to Cdt1. To investigate how SPOP-mediated ubiquitination influences the DNA replication regulatory function of Geminin, we examined whether Geminin ubiquitination affects its interaction with Cdt1. Co-IP data showed that transient prostate cancer-derived SPOP mutants fail to promote Geminin poly-ubiquitination. SPOP mutations in prostate cancers mainly occur in the MATH domain, which is responsible for substrate binding. Using co-IP assays, we showed that SPOP AMATH mutant lost the ability to bind to and promote ubiquitination of Geminin while CUL3-binding-deficient mutant ΔBTB, which cannot ubiquitinate substrates, retained the ability to bind Geminin (Fig. 3b). As expected, SPOP ΔBTB failed to promote poly-ubiquitination of Geminin (Fig. 3c). Based upon our NMR spectroscopy and X-ray crystallography results that Geminin interacts with SPOP-MATH domain on a surface frequently mutated in prostate cancer patients (Fig. 1h–k), we examined whether prostate cancer–associated mutations in SPOP would impair its ability to promote Geminin poly-ubiquitination. We generated 11 prostate cancer–associated SPOP mutants. Co-IP assays demonstrated that the Geminin binding ability of all 11 SPOP mutants was largely impaired compared with SPOP WT (Fig. 3d). SPOP-mediated poly-ubiquitination of Geminin was also markedly attenuated by these mutations (Fig. 3e). Overexpressing SPOP-WT or SPOP mutants have no influence on Geminin protein level (Supplementary Fig. 2g). Furthermore, we showed that in vitro Geminin poly-ubiquitination by SPOP was abolished in the context of SPOP F102C and F133V, two SPOP mutations most frequently found in prostate cancer patient specimens (Fig. 3f). Thus, prostate cancer–associated SPOP mutants fail to promote Geminin poly-ubiquitination. Almost all the SPOP mutations detected so far (with one exception) are hemizygous mutations and act in a dominant-negative fashion. To mimic the pathophysiological conditions in patients, we introduced the mutated allele of SPOP into prostate cancer cell lines that do not contain endogenous mutated SPOP. We transfected SPOP mutant F133V into BPH1 and DU145 cell lines and treated the cells with or without hydroxyurea (HU), a replication inhibitory agent, to see whether Geminin protein level changes in a different context. Expression of F133V did not alter the protein level of Geminin or its partner Cdt1 (Supplementary Fig. 2f). Thus, SPOP mutations impair non-degradative poly-ubiquitination of Geminin.
expression of SPOP mutants F102C and F133V had no effect on the interaction between ectopically expressed Geminin and Cdt1 (Supplementary Fig. 3a). Similarly, stable expression of F133V failed to affect the Geminin-Cdt1 interaction in BPH1 and DU145 cell lines at endogenous level (Supplementary Fig. 3b). Mutation of Geminin ubiquitination sites Lys100 and Lys127 also had no effect on Geminin binding to Cdt1 (Supplementary Fig. 3b). Since binding of the MCM complex to Cdt1 is critical for regulation of DNA replication firing, we further investigated whether SPOP-mediated Geminin ubiquitination impacts MCM complex access to Cdt1. As expected, Geminin knockdown decreased Cdt1 binding with pre-RC proteins such as MCM2, CDC6, and ORC2, and this process was reversed by expression of shRNA-resistant WT Geminin but not the K100R/K127R mutant (Fig. 4a and Supplementary Fig. 3d). Similar to the effect of the ubiquitination-resistant mutant of Geminin, expression of cancer-derived SPOP mutants also increased Cdt1 binding to MCM2, CDC6, and ORC2 but had no influence on Geminin binding to Cdt1 (Fig. 4b and Supplementary Fig. 3e).

Current structural knowledge of the replication origin and origin recognition complex formation is mostly derived from studies in budding yeast46,47. A single-particle cryo-EM structure of a pre-insertion loading intermediate of ORC-Cdc6-Cdt1-MCM (OCCM) complex from yeast was recently obtained by truncating the C-terminal WD domain of Mcm6 (PDB 6WGG)47. This structural intermediate, which precedes OCCM formation during
the helicase loading process, shows how Cdt1 associates with the MCM complex. Despite low sequence similarities, Cdt1 from human (hCdt1) and yeast (yCdt1) both have conserved winged-helix domains (WHD) in their middle (M-WHD) and C-terminal (C-WHD) regions (Supplementary Fig. 3f). Because of these structural similarities, we built a model by using the crystal structure of the Geminin-bound M-WHD region of hCdt1 (PDB 2WVR) to replace the corresponding yCdt1 region in the aforementioned OCCM intermediate complex (PDB 6WGG). The hCdt1 and yCdt1 M-WHD structures can be overlaid without any steric interference on the MCM complex (Supplementary Fig. 3g). The C-terminal part of bound Geminin, however, creates a steric hindrance. Molecular dynamics simulations based on the hCdt1-Geminin crystal structure revealed large amplitude motions of the C-terminal helical regions of the Geminin homodimer construct used for crystallography (Supplementary Fig. 3h). Taking this flexibility into account, we adjusted and optimized the conformation of Geminin C-terminal regions to eliminate any steric clash. Our structural model for Geminin-bound OCCM (Fig. 4c) predicts that K27-linked poly-ubiquitination at Geminin Lys127 would cause a steric clash with the MCM complex that would displace Cdt1 from MCM. This analysis provides a plausible explanation as to how SPOP-mediated ubiquitination of Geminin affects the Cdt1-MCM interaction, while not directly affecting the Geminin-Cdt1 interaction. In agreement with our model in Fig. 4c, co-IP and mass spectrometry assays showed that Geminin could pull down MCM proteins and that SPOP-mediated poly-ubiquitination of Geminin reduced Geminin-MCM complex formation (Supplementary Fig. 3l), indicating that Geminin indirectly associates with MCM proteins.

We further performed fluorescence-activated cell sorting (FACS) analysis to determine the effect of SPOP ubiquitination of Geminin on cell re-replication. Geminin knockdown increased

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**Fig. 3 Prostate cancer-derived SPOP mutants fail to promote Geminin poly-ubiquitination.**

**a** Schematic of domain organization of SPOP and SPOP mutations detected in 1013 cases of prostate cancers. b 293T cells transfected with Flag-Geminin in combination with empty vector (EV), SPOP WT, or mutants were harvested for co-IP and WB with indicated antibodies. c 293T cells were transfected with the indicated plasmids and harvested for IP to detect the ubiquitination of Geminin. d 293T cells were co-transfected with Flag-Geminin and SPOP WT or mutants and harvested for co-IP and WB. e 293T cells were co-transfected with Flag-Geminin and SPOP WT or mutants and harvested for co-IP and WB to detect Geminin poly-ubiquitination. f In vitro ubiquitination assay was performed by incubating the reconstituted E1 and E2 enzymes with CUL3/RBX1, Myc-SPOP WT, or mutants and optimized the conformation of Geminin C-terminal regions to detect the ubiquitination of Geminin. 293T cells were co-transfected with Flag-Geminin and SPOP WT or mutants and harvested for co-IP and WB. g Co-IP analysis of indicated proteins and ubiquitination in 293T cells transiently transfected with Flag-Geminin-WT or S202F mutant. Source data are provided in this paper or Mendeleev database (10.17632/8n7x5krkc.1). Similar results for (b–h) panels were obtained in two independent experiments.
Prostate cancer-derived SPOP mutants increase replication origin firing, re-replication, and genome instability, especially upon ATR inhibition. The Cdt1-inhibitory function of Geminin is activated at the S and early G2 phases of the cell cycle to prevent the assembly of pre-RC16,19,26,49. We sought to determine whether SPOP regulates Geminin poly-ubiquitination in a cell-cycle-dependent manner. To this end, PC-3 cells were synchronized through thymidine and L-mimosine double block (Fig. 5a). Cells were pulse-labeled with 30 μM BrdU prior to WB analysis (d), FACS analysis (e), or quantification (f). Data are presented as the mean ± SD of three independent experiments. Two-tailed unpaired Student’s t-test; ***P < 0.001. Source data are provided in this paper or Mendeley database (10.17632/8n7xt5rkhc.1). Similar results for (a), (b), and (d) panels were obtained in three independent experiments.

Fig. 4 SPOP-mediated poly-ubiquitination of Geminin inhibits MCM proteins binding to Cdt1. a PC-3 cells were infected with lentivirus expressing indicated shRNAs or WT or mutated Geminin and harvested for co-IP and WB analysis. b PC-3 cells were infected with lentivirus expressing empty vector or different SPOP mutants and harvested for co-IP and WB. c The structure of human Cdt1-Geminin is overlaid to that of yeast Cdt1-MCM after conformational optimization. A modeled K27-linked poly-ubiquitin chain attached to Geminin Lys127 (light blue) is incompatible with Cdt1-Geminin binding to the MCM complex. d-f PC-3 cells were infected with lentivirus expressing control shRNA or Geminin-specific shRNA in combination with empty vector (EV), Geminin WT, or K100/127R mutant. Cells were pulse-labeled with 30 μM BrdU prior to WB analysis (d), FACS analysis (e), or quantification (f). Data are presented as the mean ± SD of three independent experiments. Two-tailed unpaired Student’s t-test; ***P < 0.001. Source data are provided in this paper or Mendeley database (10.17632/8n7xt5rkhc.1). Similar results for (a), (b), and (d) panels were obtained in three independent experiments.

Prostate cancer-derived SPOP mutants increase replication origin firing, re-replication, and genome instability, especially upon ATR inhibition. The Cdt1-inhibitory function of Geminin is activated at the S and early G2 phases of the cell cycle to prevent the assembly of pre-RC16,19,26,49. We sought to determine whether SPOP regulates Geminin poly-ubiquitination in a cell-cycle-dependent manner. To this end, PC-3 cells were synchronized through thymidine and L-mimosine double block (Fig. 5a). We observed a slight increase in a population with DNA content started to increase from S phase after a decrease at G1 phase in EV cells (Fig. 5b), similar to what was previously reported15,49–52. However, when chromatin binding of pre-RC proteins such as MCM2 began to decrease after completion of replication and entry into early G2 phase in control cells, MCM2 association with chromatin remained high at S phase and early G2 phase in SPOP-F133V mutant cells (Fig. 5b, c), indicating that the replication machinery remains active at this stage of the cell cycle in SPOP F133V mutant cells. Both SPOP and Geminin protein levels fluctuated during the cell cycle and plateaued at S and early G2 phases (Fig. 5b), implying that these two proteins might work in concert at these stages of the cell cycle.

To determine the impact of cell cycle-dependent oscillation of SPOP protein on Geminin poly-ubiquitination, we synchronized PC-3 cells expressing HA-tagged K27-only ubiquitin (Ub) (HA-Ub-K27) by nocodazole (Supplementary Fig. 4a). A dramatic increase in Geminin K27-linked poly-ubiquitination was observed in control cells at S and early G2 phases compared to cells at other phases (Fig. 5d). Notably, both the substrate (Geminin) and the E3 ligase (SPOP) also reached the highest protein level at these two phases in control PC-3 cells (Fig. 5b). However, the cell-cycle-dependent K27-linkage poly-ubiquitination of Geminin was completely abolished in SPOP-F133V mutant-expressing PC-3 cells even though Geminin protein level retained a similar oscillation pattern as in control cells (Fig. 5d). Notably, the total poly-ubiquitination of Geminin was also decreased at S and early G2 phases in F133V mutant cells transfected with WT ubiquitin (Supplementary Fig. 4b). Furthermore, FACS analysis with BrdU-
Fig. 5 Prostate cancer-derived SPOP mutants increase replication origin firing, re-replication, and genome instability, especially upon ATR inhibition.

a PC-3 cells stably expressing empty vector (EV) and SPOP-F133V mutant were synchronized by thymidine and L-mimosine double blockade. Cells were released and harvested at the indicated time points for FACS-based cell cycle analysis. b PC-3 cells expressing EV and SPOP F133V were synchronized as in (a) and harvested for WB. Similar results were obtained in three independent experiments. c Protein bands for chromatin-associated MCM2 from the experiment shown in (b) and the other two repeats were quantified using ImageJ and normalized to total MCM2 protein level. Data are presented as the mean ± SD (n = 3). The P value was calculated by two-way ANOVA analysis. d EV- and F133V-expressing PC-3 cells transfected with HA-Ub-K27-only were synchronized with nocodazole and released at different time points and harvested for IP and WB. e, f PC-3 cells infected with lentivirus expressing EV or SPOP F133V in combination with control or Geminin-specific shRNAs. Cells were pulse-labeled with 30 μM BrdU for 30 min and harvested for FACS analysis (e) and quantitative data are shown in (f). Data are shown as the mean ± SD of three independent experiments. Two-tailed unpaired Student’s t-test. g PC-3 cells infected with lentivirus expressing empty vector (EV) or F133V in combination with control or Geminin-specific shRNAs were treated with DMSO or 100 nM VE-822 for 8 h followed by DNA fiber assay. Inter-origin distances between 200 replication firing origins were measured (n = 200). Data are shown as the mean ± SD of three independent experiments. Two-tailed unpaired Student’s t-test. h Stable PC-3 cells as indicated were treated with DMSO or 100 nM VE-822 for 8 h followed by DNA fiber assay. DNA re-replication was quantified from 200 DNA tracks and relative re-replication fold changes are presented. Data are shown as the mean ± SD of three independent experiments (n = 3). Two-tailed unpaired Student’s t-test. i PC-3 cells infected with lentivirus expressing the indicated shRNAs or EV or SPOP F133V were treated with DMSO or increased doses of VE-822 for 24 h and harvested for WB with indicated antibodies. j Stable PC-3 cells as indicated were treated with VE-822 (100 nM) for 24 h. Cells were harvested for karyotyping. Quantification of chromosome breaks per cell are shown. More than 70 metaphases from four biological replicates were counted. Data are mean ± SEM. Two-tailed unpaired Student’s t-test. Source data are provided in this paper or Mendeley database (10.17632/8n7xt5rkhc.1). Similar results for (b), (d), and (i) panels were obtained in two independent experiments.

labeled cells showed that SPOP-F133V expression resulted in an increase of re-replicated cells (>4N) in PC-3 cells (Fig. 5e, f and Supplementary Fig. 4c). A similar result was obtained in Geminin knockdown cells regardless of SPOP-F133V expression (Fig. 5e, f). These data demonstrate that SPOP mutant cells undergo an aberrant replication process including overloading of the pre-RC proteins onto chromatin (Fig. 5b, c) and ultimately DNA re-replication (Fig. 5e, f).

To further assess the effect of SPOP mutation on re-replication, we employed DNA fiber assay to gauge abnormal replication. The ATR-CHK1 pathway plays a key role in suppressing dormant origin over-firing and preventing RPA depletion in nuclear pool which protects DNA replication forks from collapse at S phase. We included VE-822, an ATR inhibitor in our DNA fiber assay to determine whether ATR inhibition has any additional effects on DNA re-replication in SPOP mutant cells.
As a positive control, we found that the distance between two adjacent origins (inter-origin distance) was decreased in cells treated with VE-822 (Fig. 5g and Supplementary Fig. 4d). However, this phenomenon was also observed in Geminin knockdown or SPOP-F133V mutant-expressing cells without VE-822 treatment (Fig. 5g). Importantly, the inter-origin distance in F133V and/or Geminin knockdown cells was lower than that in control cells and was further reduced after co-treatment of VE-822 (Fig. 5g). The decreased inter-origin distance indicates that there is increased origin firing in SPOP-mutated cells, providing a plausible explanation as to why there is increased binding of pre-RC proteins to chromatin (Fig. 5b). DNA re-replication also increased by several folds in PC-3 cells expressing SPOP mutant or Geminin shRNA compared to control cells (Fig. 5h and Supplementary Fig. 4e), and their effects were further enhanced by ATR inhibitor treatment (Fig. 5i). Both increased replication stress and re-replication burden sensitize ATR inhibition-caused replication catastrophe and double-strand breaks and ultimately lead to cell death. We assessed whether this would also be the case in SPOP mutant cells. As expected, treatment with ATR inhibitor alone increased the level of γ-H2AX in control PC-3 cells and this effect was largely enhanced by expression of SPOP mutant F133V or Geminin knockdown (Fig. 5i). However, combination of SPOP-F133V expression and Geminin knockdown failed to induce more DNA breaks than each condition alone in PC-3 cells treated with ATR inhibitor (Fig. 5i). These data suggest that SPOP and Geminin work in the same pathway in prohibition of ATR inhibition-caused replication catastrophe and double-strand breaks. We also checked the numbers of intra-chromosomal breaks per cell after these groups of cells were treated or not with ATR inhibitor (Fig. 5j and Supplementary Fig. 4e, f). The results were consistent with changes in γ-H2AX level (Fig. 5i). Therefore, prostate cancer-associated SPOP mutation F133V impairs DNA re-replication checkpoint, promotes chromosomal instability, and leads to replication catastrophe when ATR is inhibited.

**SPOP mutant cells are hypersensitive to ATR inhibition.** Because SPOP mutant cells acquire a marked increase in replication stress and re-replication burden (Fig. 5g, h), and encounter replication catastrophe upon ATR inhibition (Fig. 5i, j), we hypothesized that SPOP-mutated cells are hypersensitive to ATR inhibitors due to replication catastrophe. To test this hypothesis, we measured the viability of SPOP mutant-expressing BPH1, C4-2, 22RV1, DU145, and PC-3 cells treated with two different ATR inhibitors (AZD6738 and VE-822). A dose surviving assay demonstrated that expression of SPOP mutant F133V in all five cell lines resulted in decreased IC50 of both inhibitors compared with EV control cell lines (Fig. 6a and Supplementary Fig. 5a–c). ATR knockdown markedly inhibited growth of SPOP mutant cells with formation of fewer and smaller colonies while ATR knockdown in control cells only slightly decreased the size and number of colonies (Fig. 6b, c and Supplementary Fig. 5d, e). We also examined the effect of ATR inhibition by VE-822 in a clinically-relevant setting by using the SPOP Q165P mutant patient-derived xenograft (PDX) model established from a prostate cancer metastatic lesion. Similar to the other SPOP mutants, such as F102C and F133V, Q165P lost the ability to bind to and ubiquitinate Geminin (Supplementary Fig. 5f, g). Similar to the results in F133V-expressing cells, both Q165P mutant-expressing DU145 and PC-3 cells had much lower IC50 doses of VE-822 (Fig. 6d, e and Supplementary Fig. 5h) and VE-822 treatment resulted in much smaller colony numbers compared to vehicle-treated cells (Fig. 6f–i). We also observed organoids from Q165P PDX tumors. We showed that the diameter of mock-treated SPOP Q165P organoids was much larger than that of the WT counterpart, but the diameter of SPOP Q165P organoids was much smaller than that of the control organoids when they were treated with VE-822 (Fig. 6j, k). We also treated SPOP-WT and Q165P PDX tumors with VE-822 in mice. Similar to the finding in organoid culture, SPOP Q165P mutant PDX tumors were more sensitive to VE-822 compared to SPOP-WT PDX tumors (Fig. 6j, m). Our data demonstrate that SPOP-mutated prostate cancer cells are hypersensitive to ATR inhibition in vitro and in vivo.

**Discussion**

Although SPOP is broadly recognized as a tumor suppressor in prostate cancer and SPOP mutations are associated with high frequency of genomic rearrangements, the molecular mechanisms by which SPOP mutations promote genome instability remain poorly understood. In the present study, we identify a role for SPOP in guarding DNA replication and chromosomal stability. Based on our findings, we envision a model wherein SPOP mutant cells have increased replication origin firing and re-replication burden, causing replication catastrophe and cell death upon ATR inhibition (Fig. 7). Therefore, our work uncovers a previously unrecognized tumor suppressor role of SPOP in preventing DNA from over-replication and genome instability.

Geminin plays a pivotal role in regulating replication licensing, ensuring only one replicate of DNA per cell cycle. Accordingly, the expression level of Geminin protein is low or undetectable in G1 and surges through S to early G2 phases of the cell cycle. However, Geminin activity does not entirely correlate with its expression level, implying that there exist additional mechanisms regulating Geminin activity beyond its protein level. Intriguingly, a previous study identified a 98YWKA100 motif in Geminin that is critical for Geminin to prevent DNA replication from over-firing, although the underlying mechanism was unexplored. Our data reveal that the Lys100 in the 98YWKA100 motif is one of the SPOP ubiquitination sites. Most importantly, we show that while Geminin K100R mutant retains its ability to bind to Cdt1, it is similar to the ability of previously reported 98AWA100 mutant. SPOP-mediated ubiquitination of Geminin K100R mutant is substantially reduced. Thus, our findings provide a mechanistic explanation for the critical function of Geminin 98YWKA100 motif and its poly-ubiquitination in preventing DNA over-replication.

It has long been suggested that different replication licensing statuses of Geminin/Cdt1 exist. However, how the equilibrium between a licensing permissive to a licensing inhibitory status shifts during the cell cycle remains an open question. Our work suggests that SPOP-dependent Geminin poly-ubiquitination indirectly blocks MCM access to Cdt1 and that this effect might be achieved through steric hindrance as our work suggests that SPOP-dependent Geminin poly-ubiquitination provides a functional switch for the Geminin-Cdt1 complex. We acknowledge that other mechanisms are possible, but our proposed model, consistent with experimental results, provides the simplest possible explanation for how SPOP regulates DNA replication via Geminin ubiquitination (Fig. 7b). Among other explanations, one could be a general conformational change in Geminin-Cdt1-MCM complex caused by SPOP-induced poly-ubiquitination of Geminin. Consistent with the finding that K27-linked poly-ubiquitination of Geminin does not cause protein degradation and that such modification is reversible, our work also suggests that oscillations in SPOP protein levels during the cell cycle drive fluctuations in Geminin poly-ubiquitination, which allows re-formation of the permissive status for the replication firing in the next cell cycle.
Fig. 6 SPOP mutant cells are sensitive to ATR pathway inhibition. a IC50 analysis of two ATR inhibitors in five prostate cell lines expressing EV or SPOP-F133V mutant. b, c Colony formation assays were performed in DU145 and PC-3 cell lines infected with lentivirus expressing control or ATR-specific shRNA or empty vector (EV) or SPOP mutant F133V. The number of colonies was counted. Representative colonies are shown in (b) with quantification data shown in (c). Data are presented as the mean ± SD of three independent experiments. Two-tailed unpaired Student’s t-test. d, e Dose-response survival curves of EV, SPOP F133V, and SPOP Q165P cells exposed to increasing concentrations of VE-822 in DU145 (d) and PC-3 (e) cells. Data are shown as the mean ± SD of three independent experiments (n = 3 replicates/group). Two-tailed unpaired Student’s t-test. f–h Colony formation assay was performed in DU145 (f, g) and PC-3 (h, i) cell lines treated with DMSO or VE-822. The number of colonies was counted. Representative colonies are shown in (f, g) with quantification data shown in (h, i). Data are shown as the mean ± SD of three independent experiments (n = 3). Two-tailed unpaired Student’s t-test. j, k SPOP WT and Q165P organoid lines derived from Q165P PDX tumors were cultured for 5 days, followed by treatment with DMSO or VE-822 (200 nM) for five more days. The representative images of organoids after the treatment are shown in (j) and the quantified data of the organoid diameter are shown in (k). All data are shown as mean ± SD (n = 200). The P value was calculated using unpaired two-tailed Student’s t-test.

m SPOP-WT or SPOP Q165P PDX tumors were transplanted subcutaneously into SCID mice and treated with VE-822 (60 mg/kg, 5 times weekly by oral gavage) or vehicle. Mice were treated for 3 weeks and then sacrificed. Xenograft tumors were isolated and are shown in (l). Log of quantified volumes of the tumors from (l) (n = 5) are shown in (m). All data are shown as mean ± SD. The P value was calculated by two-way ANOVA analysis. Source data are provided in this paper.
resistant to BET inhibitor due to elevated expression of BET family
strategies for SPOP-mutated patients. Indeed, it has been shown
indicates that SPOP targets a large spectrum of protein substrates
endogenous SPOP is present (Fig. 3d, e). Increasing evidence
dominant-negative effect of SPOP mutations. Almost all mutants
mutations may be susceptible to treatment with ATR inhibitors.
ATR inhibition highlights that prostate cancers harboring SPOP
replication catastrophe, massive DNA breaks, and cell death upon
result in Geminin inactivation and undesired replication over-
prevention of aberrant DNA re-replication at these stages of the
constraining DNA replication over-
mutants are unable to activate the inhibitory function of Geminin in
SPOP mutations trigger replication
catastrophe and cancer cell death upon ATR inhibition.
Whole-genome and exome sequencing of cancer patient samples
have shown that SPOP is the most frequently mutated gene in
primary prostate cancer37,28, suggesting that patients with SPOP
mutations represent an important subtype of prostate cancer. SPOP
mutations typically occur in a heterozygous state with a retained
wild-type allele and are able to dysregulate known substrates in a
dominant-negative manner32,23,58. Our finding also confirmed
the dominant-negative effect of SPOP mutations. Almost all mutants
lost the ability to bind and ubiquitinate Geminin even though
endogenous SPOP is present (Fig. 3d, e). Increasing evidence
indicates that SPOP targets a large spectrum of protein substrates
for degradation, which implies that dysregulation of different
downstream signaling pathways would require different therapeutic
strategies for SPOP-mutated patients. Indeed, it has been shown
that cells expressing prostate cancer-derived SPOP mutants are
resistant to BET inhibitor due to elevated expression of BET family
proteins BRD2, BRD3, and BRD423,24. In contrast, given that SPOP
mutants are unable to activate the inhibitory function of Geminin in
constraining DNA replication over-firing (Fig. 7c), SPOP mutation
triggers replication catastrophe upon ATR inhibition (Fig. 7d). This
model is further supported by our finding that SPOP-mutated
prostate cancer cells are hypersensitive to ATR inhibition. Thus,
our findings shed new light on the development of new therapeutics
for patients with SPOP-mutated prostate cancer.
In conclusion, we demonstrate that SPOP plays an important
role in ensuring the normal process of DNA replication by
controlling Geminin poly-ubiquitination and the switch of the
Geminin/Cdt1 complex from the replication licensing-competent
to the licensing-defective state. We further show that SPOP
protein expression plateaued at S and early G2 phases, thereby
triggering the highest level of poly-ubiquitination of Geminin and
prevention of aberrant DNA re-replication at these stages of the
cell cycle in normal cells. We also reveal that mutations in SPOP
result in Geminin inactivation and undesired replication over-
firing and re-replication. Our finding that SPOP mutation triggers
replication catastrophe, massive DNA breaks, and cell death upon
ATR inhibition highlights that prostate cancers harboring SPOP
mutations may be susceptible to treatment with ATR inhibitors.
BM beamline of the Advanced Photon Source at Argonne National Laboratory, IL. The diffraction data were processed with HKL2000[1]. Starting phases were obtained by molecular replacement using PHARE[2]. The initial model was adjusted using COOT[3] and refined using PHENIX[4]. Because of our low resolution (3.4 Å) diffraction data, we used previously reported higher resolution structures of other SPO-P-MATH-SBC complexes[5] to determine the polarity of the SBC-containing Geminin peptide we co-crystallized. Across different species and proteins, the SBC motif is highly conserved in sequence and in structure, with the nonpolar residue (Φ in the Φ-π-S/T-Σ/S BC motif) surrounded by aromatic residues. All molecular representations were generated using PyMOL[6].

Model building and molecular dynamics simulations. The structure of human Geminin-Cdt1 complex (PDB code 2WVB[7]) was docked onto that of budding yeast OCCM (PDB code 6WG0[8]) by best-fit overlay of the structure of human Cdt1 with that of Cdt1. The Geminin C-terminal helical region was altered to avoid structural clash with the structure of MCM2 using COOT[9]. This Gemini-OCCM structural model was optimized by molecular dynamics (MD) simulations. A model of a chain of three K27-linked ubiquitin molecules (PDB code 1UBQ[10]) attached to Geminin Lys127 via an isopeptide bond was generated using PyMol and optimized by MD simulations. The most abundant ubiquitin chain configuration was then.graphed to Geminin Lys127 in the above Gemini-OCCM model.

The MD simulations, model optimizations, and data analyses were carried out using GROMACS (version 2020.2) with the all-atom CHARMM36 force field[11]. The proteins were in triclinic boxes and solvated with explicit TIP3P water molecules. Charges were neutralized with Cl− ions and NaCl solution. All systems were equilibrated at 1 bar and 300 K using a velocity-rescaling thermostat[12]. This was followed by equilibration for 10 ns to a 1 bar constant pressure bath using the Berendsen weak coupling method. The above equilibration steps were performed with protein molecules positioned restrained using a force of 1000 kJ mol−1 nm−1. MD productions used periodic boundary conditions with a time step of 2.0 fs. The particle mesh Ewald method[13] with a Fourier grid spacing of 0.12 nm was used to mimic the long-range electrostatic interactions. A leap-frog integration algorithm was used for the MD simulations[14] and bond lengths were restrained using the LINCS algorithm[15]. Trajectories were written every 20 ps.

In vitro ubiquitination assay. Myc-tagged CUL3, RBX1 expression vectors were co-transfected with empty vector, SPOP-WT, or two mutants in 293T cells and proteins immunoprecipitated from cell lysate were mixed with GST-Geminin purified from E. coli. The mixed protein was incubated with 5 μg Ub, 500 ng E1, 750 ng E2, 0.6 μl 100 mM ATP, 3 μl 10X ubiquitin reaction buffer (500 mM Tris-HCl pH 7.5, 50 mM KCl, 50 mM NaF, 50 mM MgCl2, and 5 mM DTT), 3 μl 10X energy regeneration mix (200 mM creatine phosphate and 2 μg/ml creatine phosphokinase) and 3 μl 10X protease inhibitor cocktail at 30 °C for 2 h, followed by western blot (WB) analysis. The Ub, E1, and E2 were purchased from UBQUIGENT.

Cell proliferation assay. BrdU/FIPI flow cytometry was used. Cells were first incubated with 30 μM BrdU for 30 min. After cells were digested with trypsin and washed in PBS, cells were resuspended in 70% ice-cold ethanol and stored at −20 °C. Before flow cytometry analysis, cells were washed three times in PBS and incubated in 2.0 M HCl and 0.5% Triton X-100 for 30 min at room temperature. Cells were next washed with washing buffer (PBS, 1% BSA and 0.2% Triton X-100), and consecutively blocked in blocking buffer (PBS, 5% BSA and 0.2% Triton X-100) and without with anti-BrdU antibody for 1 h each. Cells were again washed three times and then incubated in blocking buffer with mouse-IgG FITC conjugate. Finally, cells were resuspended in 0.5 ml PBS containing 10 μg/ml RNase A and 20 μg/ml PI for flow cytometry analysis. Analysis was done using the FlowJo 10.4 analysis software (FlowJo LL).
in the Orbitrap with HCD fragmentation (isolation window 1.6–15.000 resolutions; NCE 30%). The results were processed with the UniProt human protein database (75,004 entries, download on 07-12-2020) using Protein Discoverer (Version 2.4.11.5, Thermo Fisher Scientific) and Mascot (Version 2.7.0, Matrix Science). The mass tolerances were 10 ppm for precursor and fragment Mass Tolerance 0.05 Da. Up to two missed cleavages were allowed. The search engine set cysteine carbamidomethylation as a fixed modification and N-acetylation in the proteins and oxidation of methionine as variable modifications. False discovery rate (FDR) thresholds for protein were at 0.05 and peptide were at 0.01. The minimum peptide length was 6 and the minimum number of peptide sequences was 1.

Karyotype analysis. PC-3 cells were treated with DMSO or 200 nM VE-822 for 24 h and Colcemid for 1 h before harvest. Cells were washed two times in PBS, and then resuspended in 0.075 M KCl at 37 °C for 15 min. Cells were fixed with fixative (3:1 methanol/glacial acetic acid) twice, for 15 min each time. Small drops of cell suspension were placed onto a slide surface and stained with Diff-Quick staining for 1 min. Approximately 100 cells with well-spread chromosomes were photographed and analyzed and counted in each group.

MTS dose-dependent survival assay and clonogenic survival assay. For MTS dose-dependent survival assay, the cells were plated at a density of 1000 cells/well in 96-well plates. After 24 h, the cells were treated with different concentrations of drugs and harvested at 48 h post-treatment. The OD value was read at a wavelength of 490 nm. For clonogenic survival assay, an appropriate number of cells for different doses of drugs were plated onto 6-well plates. After 12 days of culture, colonies were fixed and stained with crystal violet 0.5% (w/v) for 1 h. The number of colonies in each group was counted and analyzed.

Drug treatment of PDx tumors. All mice were housed under standard pathogen-free conditions with a 12 h light/dark cycle and access to food and water ad libitum. We comply with all relevant ethical regulations for animal care and use, and the animal studies received ethical approval by the Institutional Animal Care and Use Committee (IACUC) at the Mayo Clinic. The PDx tumors including SPOP-WT and Q165P mutant were expanded by passaging tumor pieces (~1 mm3) into the animal studies received ethical approval by the Institutional Animal Care and was counted and analyzed.

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Author contributions H.H. conceived the study. J.M., Q.S., G.C., H.S. and M.V.B. performed experiments, data collection, and analysis. Y.W. provided the SPOP Q16SP DXD model and Y.Z., Y.Y., and Y.H. expanded PDX tumors for drug treatment. L.W. performed bioinformatics analysis. H.H., G.M., C.W., and D.Y. wrote the manuscript.

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