Protein phosphatase 2A controls ongoing DNA replication by binding to and regulating cell division cycle 45 (CDC45)

Received for publication, July 31, 2019, and in revised form, September 20, 2019 Published, Papers in Press, September 27, 2019, DOI 10.1074/jbc.RA119.010432

Abbey L. Perl†, Caitlin M. O’Connor‡, Pengyan Fa§, Franklin Mayca Pozo¶, Junran Zhang#, Youwei Zhang‡, and Goutham Narla¶

From the Departments of †Pharmacology and ‡Internal Medicine, Case Western Reserve University School of Medicine, Cleveland, Ohio 44106, the §Department of Radiation Oncology, Ohio State University James Comprehensive Cancer Center and College of Medicine, Columbus, Ohio 43210, and the ‡Department of Internal Medicine, Division of Genetic Medicine, University of Michigan, Ann Arbor, Michigan 48105

Edited by Wolfgang Peti

Genomic replication is a highly regulated process and represents both a potential benefit and liability to rapidly dividing cells; however, the precise post-translational mechanisms regulating genomic replication are incompletely understood. Protein phosphatase 2A (PP2A) is a serine/threonine phosphatase that regulates a diverse array of cellular processes. Here, utilizing both a gain-of-function chemical biology approach and loss-of-function genetic approaches to modulate PP2A activity, we found that PP2A regulates DNA replication. We demonstrate that increased PP2A activity can interrupt ongoing DNA replication, resulting in a prolonged S phase. The impaired replication resulted in a collapse of replication forks, inducing dsDNA breaks, homologous recombination, and a PP2A-dependent replication stress response. Additionally, we show that during replication, PP2A exists in complex with cell division cycle 45 (CDC45) and that increased PP2A activity caused dissociation of CDC45 and polymerase α from the replisome. Furthermore, we found that individuals harboring mutations in the PP2A Aα gene have a higher fraction of genomic alterations, suggesting that PP2A regulates ongoing replication as a mechanism for maintaining genomic integrity. These results reveal a new function for PP2A in regulating ongoing DNA replication and a potential role for PP2A in the intra-S-phase checkpoint.

Controlled and efficient replication of cellular DNA is essential for all dividing cells to maintain genomic stability and cell survival, with rapidly dividing cells such as cancer cells being particularly sensitive to alterations in this process. This has resulted in the development of numerous cancer chemotherapeutics targeting replication to induce cancer cell death by inhibiting continued replication (1–3). The replisome, the machinery responsible for coordinating the replication process, is tightly regulated to ensure accurate DNA replication (4, 5). If the replisome becomes decoupled, DNA replication will stall. Replication stalling creates strain on the unmoving replication forks that can lead to fork collapse, resulting in the formation of dsDNA breaks (2, 6). Collapsed replication forks can either reinitiate replication through coordinated activation of the homologous recombination and the replication stress pathways or initiate signaling toward apoptosis. This process enables the cell to rapidly adjust to changes occurring during S phase to ensure accuracy during the replication process.

The serine/threonine protein phosphatase 2A (PP2A) is a heterotrimeric holoenzyme composed of a scaffolding subunit (A) with two isoforms encoded by two genes (α and β), a catalytic subunit (C) with two isoforms encoded by two genes (α and β), and one of 15 different regulatory subunit genes (B) that confer the enzymes substrate specificity (7, 8). The diversity in PP2A’s holoenzyme composition allows it to specifically regulate a broad range of cellular processes (9–11). PP2A has been implicated in inhibiting replication initiation and mitotic entry as well as in the negative regulation of components of the DNA damage response pathway, but PP2A’s function has never been studied in the context of ongoing DNA replication (12, 13). Our study provides the first evidence for PP2A-dependent regulation of DNA replication and highlights the translational potential for PP2A-induced replication stress as a cancer-specific therapeutic strategy.

In this present study, we utilized both a gain-of-function chemical biology approach and loss-of-function genetic approaches to specifically explore the effects of PP2A on DNA replication. Previous publications have shown that a series of small molecule activators of PP2A (SMAPs) induce PP2A-de-
PP2A controls DNA replication through regulation of CDC45

Dependent dephosphorylation of PP2A substrates and tumor growth inhibition through binding to the PP2A scaffold (14–21). The development, characterization, and pharmaceutical validation of the molecule used for our studies have been extensively described previously (12–18). Using indirect approaches to measure the site of drug binding, namely hydroxy radical footprinting and photoaffinity labeling using the PP2A AC dimer, SMAPs were identified to bind to the scaffold subunit of PP2A, as measured by the induction of highly protected regions on the Aα-subunit upon SMAP addition. Further studies using more direct high-resolution structural approaches, such as cryo-EM or X-ray crystallography, are still required to map the exact site of drug binding. The PP2A specificity of these molecules to PP2A has been validated using scaffold subunit mutations, the viral SV40 small T antigen, and the PP2A chemical inhibitor okadaic acid. Phosphatase activity assays, combined with in vitro binding to recombinant PP2A, have further confirmed the ability of SMAPs to bind to and activate PP2A specifically.

Here, SMAPs have been used as a tool to identify PP2A-dependent signaling that is altered when PP2A activity is acutely increased. Additionally, studies by our group and others have shown that recurrent patient-derived mutations in the Aα scaffold subunit of PP2A inhibit PP2A by disrupting holoenzyme formation. The Aα R183W mutation disrupts PP2A regulatory subunit binding to the scaffold resulting in inactivation of PP2A in a nearly identical manner by which the viral small T antigen from the DNA tumor virus (SV40) inactivates PP2A (22, 23). Additionally, the second most recurrent mutation, P179R, primarily disrupts binding of the catalytic subunit to the PP2A scaffold, thereby preventing holoenzyme formation, resulting in nearly complete loss of PP2A activity (22, 24). In our study, we leveraged our knowledge of these recurrent mutations and use them as genetic model systems to study the role of inactivated PP2A in the regulation of DNA replication. Using these complementary approaches, we show a new regulatory function for PP2A in the process of DNA replication and validate its importance in modulating key processes integral to the intra-S-phase checkpoint and chromosomal stability.

Using both chemical and genetic approaches, our study identified that PP2A activity resulted in an accumulation of cells in S phase and arrested DNA replication. Chemical activation of PP2A resulted in DNA replication fork stalling and collapse, causing an accumulation of dsDNA breaks. Additionally, both genetic and chemical biology approaches for modeling PP2A activation resulted in significant induction in Rad51 foci and the activation of an ATR-Chk1–dependent replication stress response in both cellular and in vivo model systems. Additionally, we present a unique PP2A-dependent mechanism for PP2A’s control of replication through the regulation of the replisome. Our data show that PP2A exists in complex with the replisome scaffold protein CDC45 during S phase, and active PP2A induces CDC45 to decouple from the replisome, resulting in the destabilization of the replisome. Finally, comparing the genome of patients harboring loss-of-function mutations in the Aα scaffold subunit of PP2A with those with functional PP2A, loss-of-function mutations in PP2A correlated with significantly greater global alterations to the overall genome. In total, our data present the first evidence for a role of PP2A as a key regulator of an intra-S-phase checkpoint by inhibiting ongoing replication through directly regulating the replisome, thus allowing cells to maintain accurate DNA replication.

Results

PP2A activation delays progression through S phase by altering DNA replication

Initially, we observed that three genetically distinct cancer cell lines, H358 (lung cancer), SW620 (colon cancer), and U20S (osteosarcoma), treated with the PP2A activator, DT-061, for 12 h resulted in a significant increase in the population of cells in S phase as analyzed by propidium iodide (PI) staining (Fig. S1, A–C). To further explore the effects of PP2A activation on DNA replication, a double thymidine block and release protocol was used to study the effects of PP2A activation during S phase. For this experiment, cells arrested at the G1–S transition were released and treated with DT-061 for varying times (Fig. 1A). Following DT-061 treatment, cell cycle profiles were analyzed using PI staining. We found that PP2A activation prolonged the time in S phase by over 5 h in all three cell lines tested (Fig. 1B–D) and Table S1).

Genetic inhibition of PP2A was used to test the role of endogenous PP2A in regulating S-phase progression. Two model systems containing the most recurrent, cancer-associated, loss-of-function mutations in PP2A were chosen for further study. First, SW620 cells expressing only V5-tagged WT-Aα or R183W mutant Aα PP2A scaffolding subunits were generated by sequential CRISPR-mediated knockout of both endogenous scaffold subunit genes, Aα and Aβ, coupled with stable overexpression of exogenous PP2A Aα scaffold subunit (Fig. S2, A and B). The Aα R183W mutation disrupts PP2A regulatory subunit binding to the scaffold, resulting in incompetent PP2A holoenzyme formation (21). Therefore, a cell expressing only mutant R183W Aα has markedly diminished PP2A activity. A patient-derived uterine cancer cell line, UT42, harboring the inactivating P179R Aα mutation, was also used. The P179R mutation primarily disrupts binding of the catalytic subunit to the PP2A scaffold, thereby preventing holoenzyme formation and decreasing total PP2A activity (22, 24). Utilizing this UT42 cell line, we overexpressed either V5-tagged EGFP or WT-Aα, thereby reconstituting fully competent PP2A into the UT42 cell line (Fig. S2, C and D).

Leveraging these genetic models for modulating PP2A activity, cell cycle progression was measured in double-thymidine–synchronized cells. Cell cycle profiles of synchronized SW620 cells containing WT-Aα transitioning through S phase showed delayed transition through the cell cycle when compared with mutant Aα–expressing cells (Fig. 1E). Cell cycle analysis of UT42 isogenic cells similarly showed that WT-Aα–expressing cells were delayed in their ability to complete S phase (Fig. 1F). These data suggest the PP2A activity interrupts cells progression through S phase.

PP2A activity results in impaired nucleotide incorporation

Prolonged S phase typically results from issues in the process of DNA replication; therefore, we began by measuring the effects of PP2A activation on ongoing replication by measuring
nucleotide incorporation into DNA with a BrdU incorporation assay. Double-thymidine–synchronized cells were pulsed with BrdU for 30 min 4 and 8 h after release and treatment with SMAP (Fig. 2A). As an internal control, we tested the response of cells to both the active SMAP, DT-061, and an inactive SMAP, 766. The inactive SMAP-766 shares a similar chemical structure with the active SMAP molecules but has no effects on cell viability or PP2A activation as measured by in vitro activity assays (15, 16).

All three cell lines tested showed significantly fewer BrdU-positive cells after 4 h of DT-061 treatment (Fig. 2, B–D). After 8 h of treatment, both H358 and U2OS cells continued to have a significantly lower percentage of BrdU-positive cells compared with control cells. By 8 h, only 17% of SW620 control cells

Figure 1. Small-molecule activation of PP2A results in prolonged S phase. A, schematic of the double-thymidine synchronization method. B–D, cell cycle profiles of synchronized H358 (B), SW620 (C), and U2OS (D) cells treated with DMSO vehicle control (blue) or 20 μM DT-061 (red) and collected at specified time points after release and drug addition. E, cell cycle profiles of synchronized SW620 cells expressing WT-Ace (red) or mutant Ace (blue) collected at specified time points after thymidine release. F, cell cycle profiles of synchronized UT42 cells with overexpression of EGFP control (blue) or WT-Ace (red).
were actively replicating, as the majority of the cell population had entered M phase; therefore, no differences were seen with treatment (Figs. 1C and 2 (B–D)). In contrast with DT-061–treated cells, no differences in BrdU incorporation were seen between DMSO- and 766-treated samples after 4 or 8 h of treatment in all three cell lines tested (Fig. 2, E–J). These data suggest that chemical activation of PP2A across multiple cellular contexts results in the arrest of ongoing DNA replication.

Next, the effects of genetic inhibition of PP2A on DNA replication were tested using the SW620 cells expressing only V5-tagged WT-Aα or R183W mutant Aα PP2A scaffolding subunits. Using a BrdU incorporation assay, WT-Aα–expressing SW620 cells continued to undergo replication 6 and 8 h after release from G1 at significantly higher levels than mutant Aα–expressing cells, indicating that WT-Aα cells were taking longer to transition through S phase (Fig. 2K and Fig. S3 (A and B)). Three hours after release, significantly more WT-Aα–expressing cells were in early S phase and significantly fewer cells in late S phase compared with R183W-Aα cells, despite equal levels of total BrdU-positive cells (Fig. 2H).
and Fig. S3 (A and B)). This delay in S-phase progression, with no change in the total number of replicating cells, suggests that the delayed transition through the cell cycle is due to impaired replication elongation rather than replication initiation. WT-Aα—expressing cells at the 6- and 8-h time points had more cells in both early and late phases of the cell cycle due to fewer total BrdU-positive R183W-Aα cells (Fig. S3, B and C). In addition to the SW620 genetic model, we utilized the HEC50B endometrial adenocarcinoma cell line harboring the R183W Aα mutation as an additional model to test the effects of PP2A activation (Cancer Cell Line Encyclopedia). We stably overexpressed either V5-tagged EGFP or WT-Aα in the HEC50B cells, thereby reconstituting fully competent PP2A (Fig. S2, E and F). Using the BrdU incorporation assay 3 h after release, we found significantly more BrdU-positive EGFP cells compared with WT-Aα—overexpressing cells, suggesting that cells expressing fully competent PP2A had less efficient DNA replication (Fig. 2L). Taken together, these data suggest that active PP2A inhibits ongoing DNA replication, resulting in an accumulation of cells in S phase.

Activated PP2A results in the collapse of ongoing DNA replication forks

We were next interested in analyzing the effects of PP2A activation on DNA replication fork dynamics. A DNA fiber–combing assay was performed on double-thymidine—synchronized cells treated with DT-061 for 4 h upon thymidine release followed by continuous 30-min ClIdU and IdU pulses (Fig. 3A). A 4-h incubation was chosen, as it is the time point at which the first signs of altered replication were noted (Fig. 1B). H358 cells treated with the PP2A activator DT-061 had both a lower number of continuous ClIdU-IdU fibers and decreased fiber length of both ClIdU and IdU fibers, suggesting discontinuous DNA replication (Fig. 3, B–D). These data support the hypothesis that PP2A activation induces replication fork stall- ing and possible replication fork collapse, resulting in dsDNA breaks and activation of the homologous recombination pathway to repair the resultant double-stranded breaks (Fig. 3E). To identify whether PP2A activation induced stalled forks to collapse, comet assays were performed in unsynchronized H358 cells treated with DT-061 for 12 h. PP2A activation significantly increased both the tail olive moment and tail moment, both indicators of the presence of dsDNA damage (Fig. 3, F–H). Immunofluorescence analysis of synchronized H358, SW620, and U2OS cells upon 2 h of release and PP2A activation showed significant induction of RAD51 foci formation, an early step in the processing of dsDNA breaks during replication (Fig. 3, I–M). Furthermore, SW620 cells expressing an inactivating R183W mutant Aα had significantly fewer cells containing Rad51 foci compared with WT-Aα—expressing cells, indicating that PP2A induces DNA forks to collapse during normal DNA replication in the absence of chemical activation (Fig. 3N). Additionally, immunofluorescence analysis of unsynchronized H358 cells treated with DT-061 for 12 h showed a significant increase in markers of replication stress and dsDNA breaks, as demonstrated by increased γ-H2AX and phosphorylated Ser-4/Ser-8 RPA2 (Fig. S4). In aggregate, these data provided evidence that active PP2A is sufficient to induce DNA forks to collapse, resulting in subsequent initiation of homologous recombination.

PP2A-mediated replication fork collapse activates an ATR-Chk1 replication stress response

To study the signaling effects resulting from PP2A-induced replication fork collapse, Western blot analysis of DNA damage markers was performed on synchronized H358, U2OS, and SW620 cells upon release and 4 h of DT-061 treatment. PP2A activation resulted in the induction of γ-H2AX and activated Chk1 coupled with increased levels of phosphorylated Thr-1989 ATR in all three cell lines tested (Fig. 4, A–C). Additionally, synchronized SW620 cells expressing only WT-Aα or R183W mutant Aα PP2A scaffolding subunits were analyzed 3 h after thymidine release. Western blot analysis showed that WT-Aα—expressing cells had increased levels of phosphorylated ATR, Chk1, and γ-H2AX when compared with the mutant Aα cells, indicating that the ATR-Chk1 signaling was only activated in cells with fully competent PP2A (Fig. 4D). These data suggest that competent PP2A more efficiently initiates homologous recombination, resulting in a replication stress response, an important processes for a protein playing a role in monitoring DNA replication.

SMAP-induced replication stress is PP2A-dependent

To verify that the effects of small molecule–driven PP2A activation with DT-061 on DNA replication were mediated by PP2A, we utilized H358 cells expressing the small T antigen of the SV40 tumor virus (Small T), a viral antigen characterized as a highly specific PP2A inhibitor (23, 25–27). Cells were synchronized as shown in Fig. 2A and treated with DT-061. PP2A activation in the isogenic Small T–expressing cells demonstrated no significant delay in S phase upon PP2A activator (DT-061) treatment, confirming the PP2A dependence of the observed effects on DNA replication (Fig. 5A and Table S2). Additionally, Small T–expressing cells had no significant increase in γ-H2AX and phosphorylated Chk1 upon DT-061 treatment (Fig. 5B). To further validate the PP2A dependence of the observed effects of DT-061 treatment, SW620 cells expressing only WT-Aα or R183W mutant Aα PP2A scaffolding subunits were treated with DT-061, and BrdU incorporation assays were performed after 2 h. Mutant Aα—expressing cells showed no significant difference in the percentage of BrdU-positive cells upon DT-061 treatment, whereas treatment induced a significant decrease in the BrdU-positive population in WT-Aα—expressing cells (Fig. 5C). Additionally, WT-Aα—expressing cells had a significant induction of phosphorylated Chk1 upon DT-061 treatment that was not induced in mutant Aα—expressing cells (Fig. 5D). Collectively, these data suggest that SMAPs regulate replication dynamics specifically through PP2A activation and support an endogenous role for PP2A in signaling toward DNA replicative stress.

PP2A activation induces replication stress response in vivo

To measure the effects of replication stress in tumors, a xenograft mouse model was treated with 5 mg/kg SMAP DT-061 twice daily. The dosing schedule is based on previous optimization that determined an in vivo half-life of DT-061 to be about
**Figure 3.** Activated PP2A results in collapse of ongoing replication forks. **A,** schematic of DNA fiber combing assay in synchronized cells treated with vehicle control or DT-061. **B,** representative images of DNA fibers after 4 h of treatment of H358 cells with either DMSO (left) or 20 μM DT-061 (right). Overlay of CldU fibers (green) and IdU fibers (red) is shown. **C,** quantification of the number of continuous CldU-IdU fibers from B. 100 total fibers were recorded from at least 13 individual images. **D,** quantification of mean ± S.D. (error bars) DNA fiber lengths after 4 h of treatment from B. 100 total fibers were recorded from at least 13 individual images. **E,** schematic of signaling cascades resulting from stalled DNA replication forks. **F,** comet assays were performed in unsynchronized H358 cells treated with vehicle control of 20 μM DT-061 for 12 h. **G** and **H,** tail olive moment and tail moment calculated from the comet assay in F using TriTek CometScore software. **Bar graphs,** mean ± S.D. of three biological replicates. **I,** schematic of the double-thymidine synchronization method used in **J**–**N.** Immunofluorescence analysis of RAD51 foci in H358 (**J**), SW620 (**K**), and U2OS (**L**) 2 h after release from thymidine block and treatment with either DMSO or 20 μM DT-061. **M,** quantification of **J**–**L** from 15 individual images taken from three biological replicates. Bar graphs are representative of the mean ± S.D. **N,** immunofluorescence analysis of RAD51 foci in SW620 cells expressing WT AXX (red) or mutant AXX (blue) 3 h after release from thymidine block. The bar graph shows mean ± S.D. of at least 12 individual images taken from three biological replicates. A 1-mm scale bar is shown on all immunofluorescence images. A two-tailed Student’s t-test statistical analysis was performed for all statistical analysis.
PP2A controls DNA replication through regulation of CDC45

A

Thymidine
13 Hours

Release
10 Hours

Thymidine
13 Hours

Release with SMAP
4 Hours

Harvest

H358

DT-061
− +

pT1989 ATR
268 55

total ATR
268 55

pS345 CHK1
268 55

total CHK1
55 15

γH2AX
15 40

GADPH
5

Phosphorylated ATR Levels

Phospho/Total Chk1 Levels

γH2AX Levels

Relative Expression

DMSO
DT-061

DMSO
DT-061

DMSO
DT-061

B

SW220

DT-061
− +

pT1989 ATR
268 55

total ATR
268 55

pS345 CHK1
268 55

total CHK1
55 15

γH2AX
15 40

Vinculin
130

Phosphorylated ATR Levels

Phospho/Total Chk1 Levels

γH2AX Levels

Relative Expression

DMSO
DT-061

DMSO
DT-061

DMSO
DT-061

C

U2OS

DT-061
− +

pT1989 ATR
268 55

total ATR
268 55

pS345 CHK1
268 55

total CHK1
55 15

γH2AX
15 40

GADPH
5

Phosphorylated ATR Levels

Phospho/Total Chk1 Levels

γH2AX Levels

Relative Expression

DMSO
DT-061

DMSO
DT-061

DMSO
DT-061

D

WT
Mutant

V5
55 268

pT1989 ATR
55 268

total ATR
55 268

pS345 CHK1
55 268

total CHK1
55 268

γH2AX
55 15

Vinculin
130 40

Phosphorylated ATR Levels

Phospho/Total Chk1 Levels

γH2AX Levels

Relative Expression

WT
Mutant

Relative Expression

WT
Mutant

Relative Expression

WT
Mutant
Figure 4. PP2A mediated replication fork collapse activates an ATR-Chk1 replication stress response. A–C, Western blot analysis of DNA damage response pathway proteins in synchronized H358 (A), SW620 (B), and U2OS (C) cells treated with DMSO or 20 μM DT-061 for 4 h upon release. Bar graphs, quantitative representation of the mean ± S.D. (error bars) from three biological replicates. For all bar graphs, a two-tailed Student’s t test statistical analysis was performed. D, Western blot analysis of ATR, Chk1, and γH2AX in SW620 WT-Aα and R183W mutant Aα cells 3 h after thymidine release. Bar graphs are quantitative representation of the mean ± S.D. from three biological replicates. A two-tailed Student’s t test statistical analysis was performed for all statistical analysis.

Figure 5. SMAP-induced replication stress is PP2A-dependent. A, cell cycle profiles of synchronized H358 control (top) or H358 Small T–expressing cells (bottom) after treatment with DMSO vehicle control (blue) or 20 μM DT-061 (red) and collected at specified time points after release and drug addition. B, Western blot analysis of Chk1 and γH2AX in H358 control and Small T–expressing cells 4 h after release and the addition of 20 μM DT-061. Bar graphs are quantitative representation of the mean ± S.D. (error bars) from three biological replicates. A two-way ANOVA statistical analysis was performed. C, BrdU incorporation in SW620 cells expressing WT-Aα or mutant Aα 2 h after release from thymidine block and the addition of either DMSO (blue) or 20 μM DT-061 (red). Quantification of the percentage of BrdU-positive cells is shown on the right. The bar graph is a representation of the mean ± S.D. from three biological replicates. A two-way ANOVA statistical analysis was performed. D, Western blot analysis of Chk1 and γH2AX in SW620 cells from C. Bar graphs are quantitative representations of the mean ± S.D. from three biological replicates. A two-way ANOVA statistical analysis was performed.
6 h (data not shown). Consistent with previously published work, an H358 xenograft model treated with DMA vehicle control (blue) or 5 mg/kg DT-061 (red) twice daily for 38 days. Treatment groups were composed of 10 and 11 individual mice, respectively. Linear regression analysis was performed. B, tumor weights upon resection from treatment groups in A. A two-tailed Student’s t-test statistical analysis was performed. C, representative Western blot analysis of protein levels in tumors from A. D, bar graphs are quantitative representations of the mean ± S.D. (error bars) of all tumors in A. A two-tailed Student’s t-test statistical analysis was performed. E, representative Western blot analysis of protein levels in livers from A. F, bar graphs are quantitative representations of the mean ± S.D. A two-tailed Student’s t test statistical analysis was performed.

Mouse body and liver weight were measured at the end of the study showing no difference between SMAP-treated and control animals (Fig. 6, C and D). Western blot analysis of treated H358 tumors showed significantly increased levels of phosphor-
PP2A controls DNA replication through regulation of CDC45

ylated Chk1 and γ-H2AX compared with control tumors (Fig. 6, E and F). Livers were collected and analyzed by Western blotting for the induction of replication stress response pathway with the DT-061–treated mice showing no significant induction in either phosphorylated Chk1 or γ-H2AX as compared with control mice (Fig. 6, G and H). These data suggest that DT-061 treatment induces replication stress in vivo and preferentially targets cancer cells, leaving normal cells unaffected.

Active PP2A induces replisome destabilization

Given the evidence of replication fork collapse, we were interested in identifying the effects of PP2A on the replisome complex in response to PP2A activity. To study replisome composition, we isolated the chromatin-bound fraction of synchronized H358, SW620, and U2OS cells after 2 h of treatment with SMAPs, as this was the earliest time point where there was evidence of DNA fork collapse. Western blot analyses of the S1 (cytosolic) fraction and the P2 (chromatin-bound) fraction of cells were performed, and the specificity of the chromatin isolation was validated by using β-tubulin and GAPDH as markers of cytosolic and soluble cell fractions and histone 3 as a marker for chromatin (Fig. 7A). Initially, we analyzed the levels of chromatin-bound helicase proteins upon PP2A activation. PP2A activation with DT-061 treatment resulted in no differences in the levels of DNA bound MCM2–4 in all three cell lines tested (Fig. 7B). Next, samples were analyzed for changes in the replisome tether protein CDC45, responsible for connecting the replisome components to the main DNA helicase (28). All three cell lines had significantly less chromatin-bound CDC45 in the presence of activated PP2A (Fig. 7C). Consistent with previous literature, this loss of CDC45 binding corresponded with significantly decreased levels of CDC45 binding partner polymerase α with PP2A activation (Fig. 7C). Additionally, synchronized SW620 cells expressing only WT-Aα or R183W mutant Aα PP2A scaffolding subunits showed significantly more chromatin-bound CDC45 in the presence of R183W mutant Aα versus WT-Aα 2 h after release from thymidine (Fig. 7, D and E). Using the SW620 cells expressing V5-tagged WT-Aα, co-immunoprecipitation assays were performed in double-thymidine–synchronized cells 1.5 h after release and treatment with DT-061. During replication, Aα-PP2A was found in complex with CDC45 and PP2A C subunit in both control and DT-061–treated cells (Fig. 7F). However, upon PP2A activation by DT-061, the levels of CDC45 binding with Aα-PP2A significantly increased with no change in the total level of PP2A C subunit binding, suggesting that acute activation of PP2A stimulates PP2A Aα-CDC45 binding (Fig. 7, F and G). Together, these findings present evidence that PP2A activity directly regulates CDC45 during ongoing replication, disrupting the replisome and arresting DNA replication.

Impaired PP2A in patients is associated with increased replication errors

Our previous data have shown that PP2A is capable of regulating ongoing DNA replication, initiate homologous recombination, and activate a replication stress response, all of which are essential processes for a proper intra-S-phase checkpoint response to allow for repair of inappropriately replicated DNA. To study PP2A’s role as a surveillance mechanism of DNA replication in humans, we leveraged available clinical data from cancer patients through the Cancer Genome Atlas (TCGA) project. Using these data, we identified all patients harboring loss-of-function Arg-183 or Pro-179 Aα mutations that we have characterized throughout this present study. We identified that 78% of tumors harboring one of these loss-of-function mutations were characterized as uterine cancer, so we chose to focus on uterine cancer patients for our subsequent analysis (Fig. 8A). Comparing the tumor genomes of patients with a loss-of-function Arg-183 or Pro-179 Aα mutation or unaltered Aα, we found no differences in the patients’ total mutational burden levels, suggesting that dysfunctional PP2A Aα has no effect on the cells’ proofreading or mismatch repair processes (Fig. 8B). Alternatively, a comparison of the total fraction of altered genome demonstrated significantly lower levels of genomic alterations in Aα WT tumors compared with those harboring a loss-of-function PP2A mutation (Fig. 8C and Fig. 5S). These data suggest that in cancer patients, PP2A’s regulation of ongoing replication could serve as a surveillance mechanism by inhibiting ongoing replication in the event of inaccurate DNA replication and by facilitating the activation of homologous recombination and a replication stress response. Therefore, in the presence of an incompetent PP2A heloenzyme, the cell loses this critical surveillance mechanism, resulting in impaired DNA replication and chromosomal aberrations.

Discussion

The ability to inhibit ongoing replication is a critical step in an intra-S-phase checkpoint response. Here, we describe how PP2A plays an important role in the regulation of DNA replication and how activated PP2A can lead to stalled and collapsed replication forks through a destabilization of the replisome. PP2A-mediated replication fork stalling and replication stress was identified in multiple different cancer types, including in both cellular and in vivo models, suggesting that PP2A’s regulation of replication is conserved across tissue types.

This study presents a mechanism for PP2A’s control of ongoing replication through its regulation of CDC45 binding. CDC45 dissociation from the replisome has previously been reported as a mechanism for the intra-S-phase checkpoint (29). Specifically, in response to DNA-damaging agents, CDC45 lost its association with the MCM complex to facilitate the arrest of DNA replication. This work is consistent with our findings that CDC45 dissociation from the replisome correlated with arrested DNA replication upon higher PP2A activity. Additionally, work done by Chou et al. (30) has shown that PP2A is capable of regulating CDC45 binding to chromatin during the formation of the pre-replication complex during G1 (31). Here, we show PP2A in complex with CDC45 during replication. Furthermore, we show using both chemical activation and genetic inhibition to alter PP2A activity, that active PP2A during replication is associated with decreased chromatin-bound CDC45. Consistent with previous literature, CDC45 dissociation from the replisome had no effect on MCM protein binding but resulted in decreased chromatin binding to polymerase α (32).
Destabilization of the replisome complex through CDC45 would result in the impaired DNA replication and DNA fork collapse that is consistent with the phenotypes identified in this paper. Moreover, reports from Chi-Wu Chiang’s laboratory (33, 34) have described PP2A-B56 γ activity and nuclear localization during S phase correlating with the critical CDK inhibitor p27Kip1, further supporting a role for PP2A signaling as an intra-S-phase checkpoint with the ability to shut down ongoing CDK activity and cell cycle progression. Together, these studies support the claims that PP2A directly regulates DNA replication and characterize this regulation as an alternative mechanism for the induction of replication stress.
PP2A controls DNA replication through regulation of CDC45

![Diagram of mutations by cancer type](image)

**Figure 7.** Active PP2A induces replisome destabilization and uncouples CDC45 complex from the chromatin. Chromatin isolation was performed in cells 2 h after release from thymidine block and the addition of either DMSO or 20 μM DT-061. A, Western blot analysis confirming the specificity of the chromatin isolation in H358, SW620, and U2OS cells. S1 (cytosolic) and P2 (chromatin) cellular fractions were analyzed for β-tubulin (cytosol), GAPDH (cytosol and nucleus), and histone 3 (chromatin). B, Western blot analysis of MCM protein levels in P2 cellular fractions. The bar graph is a representation of the mean ± S.D. from three biological replicates. C, Western blot analysis of replisome complex proteins CDC45 and polymerase α in the P2 cellular fraction. The bar graph is a representation of the mean ± S.D. from three biological replicates. D, Western blot analysis of S1 and P2 cellular fractions validating the specificity of the chromatin isolation in SW620 cells expressing WT-A or mutant A 2 h after release from thymidine block. E, Western blot analysis of replisome complex protein CDC45 in P2 cellular fractions. Bar graph is a representation of the mean ± S.D. from three biological replicates. F, Western blot analysis of a co-immunoprecipitation assay performed in SW620 cells expressing WT-A or mutant A 1.5 h after release from thymidine block and the addition of DMSO or 20 μM DT-061. G, the bar graph is a quantification of F, representing the mean ± S.D. from three biological replicates. A two-tailed Student’s t test statistical analysis was performed for all statistical analysis.

Important to this work, we show that the effects seen with the small-molecule activator of PP2A, DT-061, are dependent on PP2A activity. Specifically, expressing the PP2A inhibitor small T antigen or a loss-of function mutation in the Aα scaffold subunit of PP2A both abrogated SMAP-induced replication stalling and subsequent DDR pathway activation, suggesting that these effects are PP2A-dependent. Consistent with this finding, we have shown previously that the expression of Small T abrogated the SMAP-induced tumor growth inhibition in vivo, further supporting the notion that PP2A regulation of replication stress may be essential for SMAP-induced tumor inhibition and cell death (15, 16).

Multiple therapeutic strategies for inducing replication fork stalling are currently used in the clinic. Topoisomerase inhibitors, ribonucleotide reductase inhibitors, DNA-intercalating agents, and inhibitors of the replication stress response all result in toxic levels of replication stress (1–3, 36). In patients, these replication-targeting agents are consistently associated with high levels of toxicity to normal tissues due to their effects on all replicating cells (2). Our studies present the potential for PP2A activation to be a replication-targeting therapeutic strategy specifically targeted to cancer cells. In preclinical toxicity studies in both rodent and canine species, dose escalation studies with SMAPs showed no visible signs of toxicity at more than 40 times the therapeutic dose (data not shown). In the current studies, mice treated with SMAPs showed no signs of toxicity or lethargy, while maintaining normal body weights throughout the 38 days of twice-daily treatment (Fig. 6). Additionally, livers from DT-061–treated mice showed no signs of hepatotoxicity based on liver weights after 38 days of treatment, and PP2A activation resulted in tumor-specific DNA replication stress and cell death with no changes in these same markers in treated mouse livers (Fig. 6, G and H). We believe that this tolerability is due to a regulated inhibition of PP2A activity that has been described in transformed cells, which may sensitize them to acute PP2A activation compared with normal cells that have higher PP2A activity (9, 37, 38). Furthermore, the replication-dependent nuclear co-localization of PP2A-B56γ and the

**Figure 8.** Impaired PP2A in patients is associated with increased replication errors. A, Arg-183 and Pro-179 mutations in patient tumors analyzed by cancer type. B, analysis of the mutational burden in patients with uterine cancer harboring either a mutant (Arg-183 or Pro-179) or WT Aα. Analysis was performed on 80 total mutant samples and 476 WT samples. C, analysis of the fraction of altered genome in patients with uterine cancer harboring either a mutant (Arg-183 or Pro-179) or WT Aα. Analysis was performed on 43 mutant samples and 468 WT samples. Samples were collected off of TCGA pan-cancer atlas data sets. A two-tailed Student’s t test statistical analysis was performed for all statistical analysis. Graphs represent mean ± S.E. (error bars).
cell cycle regulator p27Kip1 has been shown to be disrupted in cancer, suggesting that PP2A-mediated regulation of DNA replication is dysregulated in cancer cells that have adapted to survive without replication-dependent PP2A signaling (33). These data suggest that the PP2A-mediated replication stress induced by SMAPs preferentially affects cancer cells and could provide valuable insight into the development of pharmacologically tractable drug strategies that modulate cancer cell DNA replication to drive greater therapeutic efficacy across a wide range of human cancers.

Previous studies have relied on chemical inhibitors of PP2A or genetic silencing approaches to identify PP2A-dependent pathways during replication stress (12, 39–41). PP2A inhibition after the induction of DNA double-strand breaks results in increased phosphorylation of γ-H2AX as well as RPA and impaired double-strand break repair (39, 40). These studies have focused on PP2A in the context of external DNA-damaging agents and described a role for PP2A in the negative regulation of many members of the replication stress response pathway (12). Our findings show that acute chemical activation of PP2A in replicating cells induces a PP2A-dependent DNA damage response, resulting in increased phosphorylation of these same phosphosites. Furthermore, we show that replicating cells expressing competent WT PP2A have increased levels of active ATR and Chk1 when compared with cells expressing impaired mutant forms PP2A, suggesting a basal role for PP2A in regulating replication dynamics in the absence of chemical activators. These data indicate that the aggregate effect of PP2A on regulating replication is not merely through the negative regulation of replication stress response elements. Additionally, studies utilizing PP2A inhibition strategies have relied heavily on immortalized cell models. However, cellular transformation studies have characterized the inhibition of PP2A as an essential, early event in cellular transformation, suggesting that the immortalized cell lines used to characterize much of our knowledge of PP2A may have partially inhibited PP2A activity and therefore may be unable to detect the complete range of PP2A signaling (26, 27, 37, 38, 42). As this study shows, the ability to use direct PP2A activators to identify PP2A-regulated signaling pathways allows us to gain new insights into PP2A signaling that has remained undetected by previous strategies.

Materials and methods

Cell lines

SW620 (ATCC, Manassas, VA) and H358 (ATCC) were grown in RPMI 1640 (Corning Mediatech, Inc.), and U2OS (ATCC) cells were grown in Dulbecco’s modified Eagle’s medium (Corning Mediatech, Inc., Manassas, VA). HEC50B cells were grown in Eagle’s minimum essential medium (ATCC). H358 Small T cells were previously characterized by Sangodkar et al. (43). All cell lines were grown according to ATCC recommendations in medium supplemented with 10% FBS, with the exception of HEC50B, which was grown in 15% FBS (VWR International, Avantor Performance Materials) and 50 units/ml penicillin-streptomycin (GE Healthcare, Little Chalfont, UK). All cell lines were maintained at 37 °C in 5% CO2 atmosphere.

In vivo xenograft models and drug treatment

Female nu/nu mice (Nu/J) mice aged 6–8 weeks at the start of the study were used. 10 × 10^6 H358 cells were injected subcutaneously in 50% Matrigel into the right flank of 6–8-week-old female nu/nu mice. Tumor volumes were measured every 3 days by caliper measurements. When tumor volume reached an average of 150 mm³, mice were randomized into treatment groups. Mice were kept in original mixed housing between groups with 5 mice per cage. DT-061 was prepared in a 500 µg/ml solution of 10% N,N-dimethylacetamide (DMA) (Sigma-Aldrich) and 10% Solutom HS15 (Kolliphor HS 15, “Solutom”) (Sigma-Aldrich) in distilled H2O. Solution is prepared by first reconstituting DT-061 in DMA, followed by the dropwise addition of prewarmed Solutom (55 °C) and mix by vortex. Pre-warmed distilled H2O (55 °C) is then added dropwise for a final concentration of 500 µg/ml. Mice are dosed by oral gavage twice daily at 1% of their total body weight for a final dose of 5 mg/kg.

Mouse body weights were recorded weekly, and percentages of mouse body weights during treatment were calculated as follows: weight at each time point/initial weight. Animals were observed for signs of toxicity (mucous diarrhea, abdominal stiffness, and weight loss). Tissues were harvested 2 h after the final dose of the treatment study. Upon resection, tumor and liver tissues were flash-frozen in liquid nitrogen. All mouse work was performed under the guidelines of the Case Western Reserve University institutional animal care and use committee.

Study approval

Animal studies were performed under protocols approved by the institutional animal care and use committee of Case Western Reserve University (protocol 2013-0132).

Cell culture drug treatment

DT-061 (generously provided by the laboratory of Michael Ohlmeyer) was reconstituted in DMSO at a stock concentration of 80 mM. Treatment medium was prepared to a final concentration of 20 μM or equal volume of DMSO. Upon drug treatment, treatment medium was exchanged with cell medium and incubated for the designated time. After the incubation period, the treatment medium and cells were collected by trypsinization followed by centrifugation at 300 × g for 5 min. Cell pellets were processed for further analysis by propidium iodide staining or protein analysis.

Propidium iodide staining

After cell collection, cells were resuspended in cold PBS. Ice-cold 100% ethanol was added to samples dropwise for a final concentration of 80% ethanol. Samples were stored at −20 °C overnight or until final analysis. On the day of flow cytometry analysis, DNA was harvested by centrifugation at 1200 × g for 7 min at 4 °C. Samples were washed twice in cold PBS and resuspended in 400 µl of PBS with 50 µg/ml propidium iodide (Sigma-Aldrich) and 100 µg/ml RNase A (Sigma-Aldrich).
Cells were incubated in the dark for 20 min before being transferred into filter tip cytometry tubes (Thermo Fisher Scientific).

Double thymidine synchronization

24 h after cell plating, filter-sterilized thymidine (Agros Organics) was added to cell medium to a final concentration of 2 mM and incubated overnight at 37 °C. Postincubation, cells were washed with PBS, and fresh medium was added and incubated at 37 °C. After 8 h, sterile thymidine was added to a final concentration of 2 mM and incubated overnight at 37 °C. Postincubation, cells were washed with PBS and fresh medium containing treatment as indicated in the experiment.

BrdU labeling

At the start of the experiment, cells were seeded on top of sterilized glass coverslips (Thermo Fisher Scientific). After treatment as described in the experiment, BrdU (EMD Millipore) was added to medium to a final concentration of 10 μM, and cells were incubated at 37 °C for 30 min. Cells were fixed with 70% ice-cold ethanol in PBS overnight at −20 °C. Ethanol solution was removed, and cells were incubated in 3 ml of 0.08% pepsin in 0.1 N HCl at 37 °C for 20 min. Pepsin was removed, and nuclei were incubated in 1.5 ml of 2 N HCl at 37 °C for 20 min. Solution was neutralized with 3 ml of 0.1 M sodium borate and washed with 2 ml of IFA buffer (10 mM HEPES, pH 7.4, 150 mM NaCl, 4% FBS, and 0.1% sodium azide with 0.5% Tween 20. Nuclei were then incubated overnight at 4 °C with anti-BrdU clone MoBU-1 conjugated to Alexa Fluor 488 (Thermo Fisher Scientific, catalog no. B35130) in IFA buffer. Nuclei were incubated for 30 min in 50 μg/ml PI (BioLegend, catalog no. 421301) and 5 μg/ml RNase A (Roche Applied Science, catalog no. 10109169001) in IFA buffer. Cell cycle analysis was analyzed using FlowJo software. BrdU positivity was calculated using a y axis cutoff of Alexa Fluor 488 intensity. Early and late S phases were calculated by separating BrdU-positive cells into two groups based on their PI concentration to identify DNA concentrations of G1 and G2 cells.

DNA fiber combing

Cells were synchronized with double-thymidine block as described above. After a 4-h incubation in conditioned medium, CldU (MP Biomedicals) was added to medium to a final concentration of 100 μM, and cells were incubated at 37 °C for 30 min. Postincubation, cells were washed with PBS and incubated with new conditioned medium including 25 μM IdU (MP Biomedicals) for 30 min. After incubation, cells were collected in 1 ml of PBS and stored at −80 °C for further analysis. To analyze the DNA fibers, cells are diluted in PBS, and cells were seeded on a glass slide and allowed to dry. Dried cell residues were lysed, and gravity flow pulled the mixture down slide and left to dry for 4 h. Slides were fixed in a 3:1 mixture of methanol and acetic acid for 15 min at room temperature. Postfixing, slides were washed and incubated overnight at −20 °C. Fixed slides were treated with 2.5 M HCl for 1 h. Slides were washed with PBS-Tween, followed by three 5-min washes with PBS. Slides were blocked with 5% BSA-PBS-Tween for 20 min and rinsed three times with PBS for 5 min. Then slides were incubated in a humidified chamber at room temperature for 6 h with 1:250 mouse-anti-BrdU/IdU (catalog no. 347580) (BD Biosciences) followed by three PBS rinses and incubation in a humidified chamber at 4 °C overnight with 1:100 rat anti-BrdU/CldU (ab6326) (Abcam). Postincubation, slides were rinsed five times with PBS for 5 min and incubated in a humidified chamber at room temperature for 1 h with both 1:500 anti-mouse Alexa Fluor 488 and 1:500 anti-rat Alexa Fluor 594 secondary antibodies (Thermo Fisher Scientific). Finally, slides were rinsed five times with PBS for 5 min, mounting medium and coverslips were added, and slides were stored at −20 °C.

Western blotting

Proteins from whole cells were lysed in radioimmune precipitation buffer (Thermo Fisher Scientific) supplemented with 1 phosphoSTOP (Roche Pharmaceuticals, Basel, Switzerland) and 1 cComplete Ultra tablet (Roche Pharmaceuticals) per 10 ml of buffer. Proteins from tissue were lysed in tPER buffer supplemented with 1 phosphoSTOP (Roche Pharmaceuticals) and 1 cComplete Ultra tablet (Roche Pharmaceuticals) per 10 ml of buffer. Protein concentrations of cell extracts were determined by a Pierce BCA protein assay kit (Thermo Fisher Scientific), and equal quantities of protein were separated by SDS-PAGE 4–12% polyacrylamide gels (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). Primary antibodies were incubated overnight at 4 °C as follows. ATR (E1S3S) at 1:1000 (13934S), phospho-histone H2A.X (Ser-139) (20E3) incubated at 1:1000 (9718S), phospho-ATR (Ser-428) antibody (2853S), phospho-Chk1 (Ser-345) (133D3) incubated at 1:500, Chk1 (2G1D5) incubated at 1:1000, phospho-ATM (Ser-1981) (D6H9) incubated at 1:500, ATM (D2E2) incubated at 1:1000, and the PARP antibody incubated at 1:1000 were all purchased from Cell Signaling Technologies. Anti-SV40 small T antigen clone PAb280 was incubated at 1:500 and purchased from EMD Millipore. Primary antibodies were detected with goat anti-mouse or donkey anti-rabbit conjugated to horseradish peroxidase (VWR) using the ChemiDoc XRS chemiluminescence imager (Bio-Rad). Densitometry analysis was performed using Image Lab software (Bio-Rad).

Immunofluorescence analysis

Rad51 foci—Cells grown on coverslips were fixed with ice-cold 3.7% paraformaldehyde, 2% sucrose for 20 min and washed three times with PBS. Cells were incubated with 0.2% Triton X-100 for 10 min, washed three times with PBS, and blocked with 0.5% BSA, 0.05% Tween 20 in PBS for 1 h. Slides were next incubated in 0.5% BSA, 0.05% Tween 20 with anti-Rad51 (GeneTex, catalog no. GTX70230) at 1:300 dilution overnight at 4 °C. Slides were washed three times with PBS and incubated with anti-mouse IgG Alexa Fluor 594 diluted 1:500 in 0.5% BSA, 0.05% Tween 20 for 1 h at room temperature. Cells were washed three times with PBS, and coverslips were mounted on slides using mounting medium with DPAI (Novus Biologics, catalog no. H-1200-NB).

RPA32 and γ-H2AX foci—Cells growing on slides were extracted for 5 min on ice with 0.5% Triton X-100 in cytoskeletal buffer (10 mmol/liter PIPES, 300 mmol/liter sucrose, 100 mmol/liter NaCl, 3 mmol/liter MgCl2, pH 6.8) supplemented with 1 mmol/liter phenylmethylsulfonyl fluoride, 0.5 mmol/
PP2A controls DNA replication through regulation of CDC45

liter sodium vanadate, and proteasome inhibitor. Then extracted cells were fixed with 3–4% paraformaldehyde. The cells were permeabilized with PBS containing 0.5% Triton X-100 for 15 min at room temperature, followed by blocking with 1% BSA, and then incubated with primary antibodies (mouse anti-γ-H2AX (Ser-139, clone JBW301, Millipore) was used at 1:500 dilution; rabbit anti-RPA32 (Ser-4/Ser-8) (A300-245A; BETHYL) were used at 1:500 dilution). The bound secondary antibodies were revealed with goat anti-mouse IgG Alexa Fluor 594 and chicken anti-rabbit IgG Alexa Fluor 488. Slides were viewed at ×60 magnification with a Zeiss Axio Observer inverted fluorescence microscope (X-Cite 120LED).

**Comet assay**

The neutral comet assay was performed using the comet assay kit from Trevigen (Gaithersburg, MD) following the manufacturer’s instructions. The lyses occurred at 4 °C for 30 min. Comets were analyzed using CometScore software version 2.0.0.38 (TriTek, Sumerduck, VA).

**Generation of CRISPR/Cas9-mediated knockout cell lines**

The double-nicking strategy utilizing mutant Cas9 was used for the generation of CRISPR knockout cells to increase specificity (41). sgRNAs targeting PP2A α (PPP2R1A) and PP2A β (PPP2R1B) were designed using the MIT CRISPR tool. Within the CRISPR sgRNA design tool, a 200-bp sequence within exon 5 was utilized for PPP2R1A, and a 200-bp sequence within exon 1 was utilized for PPP2R1B. For both designed pairs, there were zero predicted off targets. The top and bottom strands of the sgRNAs were purchased from IDT and cloned into the PX-461 pSpCas9n(BB)-2A-GFP (Addgene, catalog no. 48410), following the previously described protocol (42). The guide RNA sequences used were as follows: for PPP2R1A, Guide 1a (GCTGCCGCCCAGCCACCATGGG) and Guide 1b (CAAGCTGGGGGAGTTTGCCAAGG); for PPP2R1B, Guide 1a (GCTGCCGCCCAGCCACCATGGG) and Guide 1b (CAAGCTGGGGGAGTTTGCCAAGG); for PPP2R1B, Guide 1a (ACTTCTTTAATACTGTTGAGTCCG) and Guide 1b (TCAACAATTGCCCCTAGCACCAGTGG); for PPP2R1B, Guide 1a (ACTTCTTTAATACTGTTGAGTCCG) and Guide 1b (TCAACAATTGCCCCTAGCACCAGTGG). The double PPP2R1A/PPP2R1B knockout cells were generated in a stepwise fashion, first knocking out PPP2R1A and then re-expressing exogenous V5-tagged PP2A-Aα, described below, and finally knocking out PPP2R1B. The PPP2R1A knockout cells were generated as described previously (43). For CRISPR-mediated knockout, the appropriate PX-461 plasmids were transfected into the target cell line, SW620, using Lipofectamine 3000 (Invitrogen, catalog no. L3000-015). After 72 h, GFP-positive cells were sorted into 96-well plates at 1 cell/well and allowed to grow for 3 weeks. After this time, surviving clones were expanded and split, creating one master plate and one replica plate. The replica plate was used to isolate genomic DNA using QuickExtract DNA reagent (Lucigen, catalog no. QEO9050). Genomic DNA was amplified using Promega master mix (Promega, catalog no. M7502). PCR primers used were purchased from Invitrogen and were as follows: PPP2R1A, TACTTCCGAAACCTTGTCCT (forward) and CCAGGAGACGA-AACTCACCT (reverse); PPP2R1B, GGGACTCCACAGCCT-TGT (forward) and GGCCCTCGTCCCTACTGC (reverse). PCR products isolated from clones with identified deletions were analyzed for the presence of knockouts by immunoblotting.

**Plasmids and viral infection**

Gateway V5-tagged lentiviral expression vector pLX304-PP2A-Aα (WT Aα) was obtained from the DNASU plasmid repository (HsCD00444402) deposited by the ORFeome Collaboration (44). The pLX304-PP2A-Aα plasmid was confirmed by Sanger sequencing to be WT. pLX304-PP2A-A-R183W (mutant Aα) was generated by site directed mutagenesis (Agilent, catalog no. 210513). After mutagenesis, the mutant plasmid was sequence-confirmed by Sanger sequencing. Lentiviruses were packaged using 293T cells using X-tremeGENE transfection reagent (Sigma, catalog no. 63666244001) and the second-generation packaging constructs pMD2.G (Addgene, catalog no. 12259) and psPAX2 (Addgene, catalog no. 12260). Supernatant medium containing the virus was collected 24–48 h post-transfection, filtered using 0.45-μm filters, and supplemented with 4 μg/ml Polybrene (Santa Cruz Biotechnology, Inc., catalog no. sc-134220). Target cells were transduced for 24 h and cultured for 72 h before being selected in medium containing 16 μg/ml blasticidin (Invitrogen, catalog no. ant-bl-5b).

**Chromatin isolation**

Chromatin isolation was performed according to the methods published by Méndez and Stillman (35). In brief, cells were fixed by adding 10 ml of 1% formaldehyde in PBS and incubating at room temperature for 20 min. Cross-linking was quenched through the addition of 1 ml of 1.25 M glycine and resuspension at 4 × 10⁷ cells/ml in buffer A (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl₂, 0.34 M sucrose, 10% glycerol, 1 mM DTT, 1 tablet of PhosStop (Sigma-Aldrich, catalog no. 04906837001), and 1 tablet of Complete ULTRA (Sigma-Aldrich, catalog no. 05892791001)) with 0.1% of Triton X-100 and incubated on ice for 5 min. Cells were spun at 1300 g for 5 min at 4 °C. The supernatant (S1) was removed and clarified by centrifugation at 20,000 × g for 15 min at 4 °C. Nuclear pellet was washed in buffer A and then lysed in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 1 tablet of PhosStop, and 1 tablet of Complete ULTRA) and centrifuged at 1700 × g for 5 min at 4 °C. The pelleted chromatin (P2) was washed once in buffer B and resuspended in 2X Laemmli buffer (Sigma-Aldrich, catalog no. S3401). Resuspended chromatin was pulse-sonicated at 4 °C for 20 s with 40 s in between for a total of three pulses.

**Co-immunoprecipitation assay**

The co-immunoprecipitation assays were performed according to the Dynabeads co-immunoprecipitation kit (Thermo Fisher Scientific, catalog no. 14321D). Before harvest, cells were fixed by adding 10 ml of 1% formaldehyde in PBS, followed by incubation at room temperature for 20 min. Cross-linking was quenched through the addition of 1 ml of 1.25 M glycine. Normal mouse IgG (Santa Cruz Biotechnology, catalog no. sc-2025) and anti-V5 antibody (Thermo Fisher Scientific, catalog no. NB10062264) were conjugated to beads for the immunoprecipitations. Protein elution was performed by resuspending protein-bound beads in 2X Laemmli buffer (Sigma-Aldrich, catalog no. S3401) and incubation of samples at 100 °C for 10 min. Conformation-specific mouse (Abcam, catalog no. ab131368) and rabbit (Cell Signaling, catalog no. 5127S) sec-
PP2A controls DNA replication through regulation of CDC45

ordinary antibodies were used for Western blot analysis of co-immunoprecipitation samples to minimize off-target signals.

Quantification and statistical analysis

Densitometry quantification of Western blots was performed within the Bio–Rad Image Lab software. Statistical analyses were performed with GraphPad Prism version 7. Statistical analyses between treatment and control groups used an unpaired t test. For cell cycle analyses, the FCS Express Multicycle Analysis tool was used, and multiple t tests were run for statistical significance. For DNA fiber analysis, at least 100 fibers were used. For the xenograft efficacy study, a linear regression analysis was used. Each experiment was repeated at least three times, and representative data are shown.

*p < 0.05; **, p < 0.01; ***, p < 0.001; ****, p < 0.0001.


References


PP2A controls DNA replication through regulation of CDC45


