Loss of PPP2R2A inhibits homologous recombination DNA repair and predicts tumor sensitivity to PARP inhibition

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Abstract

Reversible phosphorylation plays a critical role in DNA repair. Here we report the results of a loss-of-function screen that identifies the PP2A heterotrimeric serine/threonine phosphatases PPP2R2A, PPP2R2D, PPP2R5A and PPP2R3C in double-strand break (DSB) repair. In particular, we found that PPP2R2A-containing complexes directly dephosphorylated ATM at S367, S1893, and S1981 to regulate its retention at DSB sites. Increased ATM phosphorylation triggered by PPP2R2A attenuation dramatically upregulated the activity of the downstream effector kinase CHK2, resulting in G1/S phase cell cycle arrest and downregulation of BRCA1 and RAD51. In tumor cells, blocking PPP2R2A thereby impaired the high-fidelity homologous recombination repair pathway and sensitized cells to small molecule inhibitors of poly(ADP-ribose) polymerase (PARP). We found that that PPP2R2A was commonly downregulated in non-small cell lung carcinomas, suggesting that PPP2R2A status may serve as a marker to predict therapeutic efficacy to PARP inhibition. In summary, our results deepen understanding of the role of PP2A family phosphatases in DNA repair and suggest PPP2R2A as a marker for PARP inhibitor responses in clinic.
Introduction

Genome stability is essential for the prevention of undue cellular death and cancer development. To maintain genome integrity, cells have evolved multiple DNA repair pathways (1). One of the most powerful activators of the DNA repair response is double-strand breaks (DSB).

Initiation of DSB repair is controlled by the phosphatidylinositol 3 kinase–like kinase (PIKK) family. A wave of phosphorylation events radiating from PIKKs is amplified to convey the signals to a large number of substrates. Although this cascade has been studied in great detail, the biological relevance of many of these phosphorylation events and the mechanisms that control their down-regulation remain unknown (2). It is conceivable that Ser/Thr protein phosphatases could be responsible for keeping proteins involved in DNA repair response in an inactive state under normal conditions or for inactivating the signaling once DNA has been repaired. However, phosphorylation of a number of PIKKs, including ataxia telangiectasia mutated (ATM), ATR, and CHK2, oscillates during the DNA repair process (3, 4), suggesting that phosphatases may serve not only as negative regulators but also as active modulators of DNA damage response.

Protein phosphatase 2A (PP2A) refers to a large family of heterotrimeric Ser/Thr phosphatases. The PP2A core enzyme consists of a catalytic C subunit and a structural A subunit. In mammals, two distinct genes encode closely related versions of both the PP2A A (Aα/PPP2R1A and Aβ/PPP2R1B) and C (Cα/PPP2CA and Cβ/PPP2CB) subunits. The AC dimer recruits a third, regulatory B subunit, which is responsible for the substrate specificity and function of the PP2A heterotrimeric complex. Four unrelated families of B subunits have been identified to date:
B/B55/PPP2R2A, B'/B56/PR61/PPP2R5, B''/PR72/PPP2R3, and Striatin/STRN. Approximately 100 distinct complexes can be formed through combinatorial association of these subunits, and it is believed that specific PP2A complexes mediate particular physiological functions (5, 6).

PP2A has been directly implicated in the negative regulation of DSB DNA repair proteins, including γH2AX, ATM, CHK1, and CHK2 (2). However, consistent with the idea that protein phosphatases are not just negative regulators of DNA repair signaling, selective inhibition of PP2A activity impairs DNA repair (7-9). PP2A function is also essential for activation of cell cycle checkpoints in response to IR (10, 11). One potential explanation for this apparent discrepancy is that several distinct PP2A complexes may modulate different steps of the DNA repair process.

Here we assessed the role of specific PP2A complexes in control of DSB repair and identify PPP2R2A as a critical effector of the homologous recombination (HR) repair through modulation of ATM phosphorylation. Critically, defects of HR DNA repair in PPP2R2A-depleted cells dramatically increased their sensitivity to poly(ADP-ribose) polymerase (PARP) enzyme inhibition. Finally, we show that PPP2R2A is commonly down-regulated in non–small cell lung carcinomas (NSCLCs) and therefore propose PPP2R2A status as a predictive marker for sensitivity to PARP inhibition.
Material and Methods

Plasmids and cell lines.

Lentiviral pLA CMV N-Flag vector was used to generate Flag-tagged PPP2R2A. The pLKO.1-puro shGFP, pLKO.1-puro shLuciferase (shLuc), and pLKO.1-puro vectors containing shRNAs targeting specific PP2A subunits were provided by the RNAi Consortium (12). The RNAi experiments were performed using ON-TARGETplus SMARTpools against PPP2R2A and PPP2CA (Thermo Scientific).

Cells were cultured in DMEM or RPMI (GIBCO) medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. Lentiviral infections were carried out as described (12). To induce DSBs, cells were treated with bleomycin (Sigma-Aldrich) or irradiated using the linear accelerator (6 MV photons, Varian Medical Systems).

Cell cycle analysis

Cells were washed with phosphate-buffered saline (PBS), fixed with 70% ethanol, treated with 100 μg/ml RNase A, and stained with 50 μg/mL propidium iodide (PI). 5-Bromo-2'-deoxyuridine (BrdU) incorporation was analyzed using the In Situ Cell Proliferation Kit FLUOS (Roche) according to the manufacturer’s protocol. Cell cycle distribution and BrdU immunostaining were assessed by FACSCanto (Becton-Dickinson).

Immunoblotting and immunoprecipitation

Cells were resuspended in 0.5% Triton X-100, 100 mM NaCl, 3 mM MgCl₂, 300 mM sucrose, 1 mM EGTA, 10 mM PIPES pH 6.8 containing protease inhibitor
and phosphatase inhibitor cocktails (Roche). The cell lysates were incubated on ice for 10 minutes and then centrifuged at 500 x g for 5 minutes at 4°C. The supernatant and pellet were designated as cytoplasmic and nuclear fractions, respectively. The nuclear fraction was resuspended in 10 mM NaCl, 5 mM MgCl₂, 250 mM sucrose, 1 mM EGTA, 10 mM Tris-HCl pH 7.6 containing protease and phosphatase inhibitor cocktails and treated with RNase-free DNase I (80 μg/mL; Roche) for 30 minutes at 37°C.

Immunoprecipitations were performed 24 hours after transfection in lysis buffer containing 50 mM Tris pH 7.4, 1% NP-40, 250 mM NaCl, and proteinase inhibitor cocktail (Roche). The protein lysates were pre-cleaned by incubation with sepharose-A beads and incubated with anti-Flag M2 beads (Sigma-Aldrich) or with anti-ATM antibody (Novus Biologicals) and sepharose-protein A beads (GE Healthcare) overnight at 4°C. Flag complexes were eluted using 3xFlag peptide (Sigma-Aldrich).

The following antibodies were used: goat polyclonal anti-ATM, (Novus Biologicals), mouse monoclonal anti-PP2AC (BD Biosciences, clone 46), mouse monoclonal anti-BRCA1 (Santa Cruz, clone D-9). The following antibodies were purchased from Cell Signaling: rabbit polyclonal anti-Flag; rabbit polyclonal anti-phospho-CHK1 (S296); rabbit polyclonal anti-phospho-CHK2 (T68); rabbit polyclonal anti-phospho BRCA1 (S1524); rabbit polyclonal anti-CDC25A; rabbit polyclonal anti-PPP2R2A; mouse monoclonal anti-phospho-ATM (S981) (clone 10H11.E12), mouse monoclonal anti-CHK2 (clone 1C12); mouse monoclonal anti-PP2A A (clone 4G7), and; rabbit polyclonal anti-phospho-CHK1 (S317); rabbit monoclonal anti-phospho-CHK1 (S345) (clone 133D3).

Neutral comet assay
Microscope slides were pre-coated with a thin layer of normal melting point agarose. Cell suspensions were placed into a tube containing low melting point agarose and transferred to a glass microscope slide. Slides were placed in 2 M NaCl, 30 mM Na₂EDTA, 10 mM Tris-HCl, 1% Triton X-100 and 10% DMSO, pH 8.0 for 1 hour at 4°C. After lysis, the slides were rinsed for 20 minutes in solution 0.3 M NaOH, 1 mM EDTA and were subjected to electrophoresis at 0.7 V/cm and 50 mA for 20 minutes. The slides were then rinsed with 1 M Tris-HCl, pH 7.5 and dried with pre-chilled absolute ethanol and stained with PI (2 μg/ml). Comet images were examined at 200× magnification with Nikon ECLIPSE Ti microscope. The tail moment values were quantified by CometScore software v1.5.

**Immunofluorescent microscopy**

Cells were plated on cover slips or μClear-96 well plates (Greiner Bio-One) and fixed with 4% paraformaldehyde for 10 minutes at RT and permeabilized with ice-cold methanol for 5 minutes at -20°C. The cells were incubated in blocking buffer 3% bovine serum albumin (BSA) in PBS for 30 minutes at RT and then with primary antibodies. After three washes with 0.3% BSA in PBS, the secondary antibody was added. The following antibodies were used: mouse monoclonal anti-γH2AX (S139) (Millipore, clone JBW301), mouse monoclonal anti-RAD51 (Abcam, clone 14B4).

Automated Image analysis was performed using IN Cell Analyzer 2000 (GE Healthcare) (Suppl. Figure 1,2).

**Quantitative real-time reverse-transcription PCR (qRT-PCR)**

Total RNA was isolated using RNeasy (Qiagen), and cDNA was synthesized using the Advantage RT-PCR kit (Clontech). A list of primers used for real-time qPCR
is presented in Suppl. Figure 3A. Real-time PCRs were conducted in a Roche LightCycler-480-96 (Roche), using SYBR Green PCR Master Mix (Roche).

qRT-PCR analysis of The Lung Cancer cDNA and Cancer Survey cDNA arrays (OriGene) was performed using an ABI 7500 (Applied Biosystems).

NHEJ and HR DNA repair assays

Non-homologous end joining (NHEJ) repair efficiency was analyzed using H1299dA3-1 cells, which contain a chromosomally integrated copy of the pIRES-TK-EGFP DNA construct (13) (a generous gift of Dr. Kohno, National Cancer Center Research Institute, Tokyo, Japan). To assess HR efficiency we utilized 293 DR-GFP cells, which contain a single integrated copy of the transgenic reporter DR-GFP. H1299dA3-1 and 293 DR-GFP cells were transfected with shRNAs against luciferase or PPP2R2A. Following puromycin selection, cells were transfected with I-SceI expression vector. The number of GFP-positive cells was measured 48 hours later by flow cytometry analysis using a FACSCanto (Becton-Dickinson).

In vitro phosphatase assay

293T cells were transfected with Flag-tagged PPP2R2A, Wip1, and a kinase-dead Wip1-D314A mutant. FLAG-tagged proteins were immunoprecipitated in anti-Flag (M2) coated 96 well plates (Sigma-Aldrich). Immunoprecipitated complexes were incubated with phosphopeptides for 30 minutes at 37°C. Release of free phosphate was measured 20 minutes after addition of Malachite Green solution using a VictortmX3 Multilabel plate reader (Perkin Elmer).

Clonogenic survival assay and cell viability
10^6 cells per 10 cm dish were plated in triplicate and then irradiated or treated with ABT-888 (Enzo Life Sciences). After 12 days colonies were stained with crystal violet (Sigma-Aldrich) and counted using ImageJ software. Cell viability was analyzed with a Vi-Cell XR counter.

**Human xenograft assays**

Xenografts were established in female NMRI-nu (nu/nu)/6 week mice (Janvier) with A549 cell line by s.c. injection (2.0×10^6 cells per 0.2 mL/mouse). Tumors were staged to a preselected size using the following formula: volume = (tumor length× tumor width^2)/2. ABT-888 (25 mg/kg) was administered orally (twice daily for 9 days) in a vehicle containing 0.85% NaCl adjusted to pH 4.0.

Tumors were fixed in 4% paraformaldehyde, paraffin-embedded, sectioned, and immunostained with rat anti-mouse Ki-67 antibody (DAKO). Immunodetection was performed with R.T.U. VECTASTAIN kit (VECTOR laboratories). Sections were counterstained with hematoxylin. For apoptosis detection In Situ Death detection kit, Fluorescein (Roche) staining was performed according to the manufacturer’s protocol.
Results

Identification of PP2A specific complexes involved in DSB repair

Although previous studies demonstrated that PP2A negatively regulates PIKK-induced signaling, more recent reports have shown that PP2A inhibition impairs DNA repair (7, 8). To further investigate this apparent conundrum, we silenced the more abundant PP2A catalytic subunit, PPP2CA. Because cells expressing very low levels of PPP2CA proliferate poorly (14, 15), we used shRNAs that only partially suppress PPP2CA expression (Figure 1A). Consistent with a previous report (7), both immunoblot analysis of γH2AX and automatic image analysis of γH2AX immunostaining revealed that partial depletion of PPP2CA resulted in inefficient DNA repair (Figure 1A,B).

To systematically identify PP2A complexes that mediate DSB repair, we performed a loss-of-function screen using shRNA library that targets each of the known PP2A B regulatory subunits (16). qRT-PCR analysis confirmed the knockdown (KD) in each of the shRNA-expressing cell lines (Figure 1C, Suppl. Figure 3B). Whenever specific antibodies were available, we also confirmed silencing of the PP2A subunits by immunoblotting (Suppl. Figure 3C).

In addition to PPP2CA, we found that suppression of four PP2A regulatory subunits, PPP2R2A, PPP2R2D, PPP2R5A, and PPP2R3C, resulted in a statistically significant (p<0.05 by Student's t test) increase of γH2AX compared to cells expressing shGFP (Figure 1C). For PPP2R5B and STRN3 subunits, only expression of one out of two shRNAs resulted in γH2AX increase, most probably due to off-target effects (Figure 1C). These results indicate that PP2A B regulatory subunits,
PPP2R2A, PPP2R2D, PPP2R5A, and PPP2R3C, could be involved in control of DSB repair.

**PPP2R2A is commonly down-regulated in NSCLCs**

Inefficient DNA repair mediated by suppression of specific PP2A B subunits suggests the contribution of these PP2A subunits to tumor suppression. In consonance with this idea, recent analysis of outlying expression coincident with extreme copy number alterations has identified PPP2R2A, one of our hits, as a putative tumor suppressor gene in breast cancer (17). Moreover, high-resolution analyses of somatic copy-number alterations of large-scale datasets revealed statistically significant loss-of-heterozygosity (LOH) of the PPP2R2A-containing region in human cancers (18, 19). Reduced PPP2R2A mRNA expression was also found in prostate adenocarcinoma samples (20, 21).

Our qRT-PCR analysis of PPP2R2A mRNA expression in 245 samples covering 14 cancer types confirmed that PPP2R2A was downregulated in lung and thyroid carcinomas (p<0.05) (Figure 2A; Suppl. Figure 4A). Further analysis of PPP2R2A mRNA expression in a panel of 21 NSCLC samples with matched normal tissues revealed at least a 2-fold decrease in PPP2R2A mRNA in 8 out of 21 NSCLC samples (Figure 2B).

A significant correlation (p=0.0683) between the frequency of PPP2R2A LOH and the percentage of samples with down-regulated PPP2R2A expression across different cancer types (Suppl. Figure 4B) suggests that decreased PPP2R2A expression in human cancers is due to LOH of the PPP2R2A-containing region. To test this idea, we analyzed PPP2R2A expression in a set of lung carcinoma cell lines.
with known status of PPP2R2A LOH (Suppl. Figure 4C). In fact, immunoblot analysis of PPP2R2A expression revealed lower levels of PPP2R2A in cell lines harboring PPP2R2A LOH (Figure 2C). A common down-regulation of PPP2R2A in NSCLCs due to LOH of PPP2R2A-containing region further support the idea that PPP2R2A functions as a tumor suppressor. Thus, our further studies were focused on PPP2R2A.

**PPP2R2A inhibits the HR repair by affecting cell cycle progression in response to DNA damage**

We validated the results of the loss-of-function screen by analyzing γH2AX after PPP2R2A suppression at different time points following IR or bleomycin treatment (Figure 3A,B). Moreover, overexpression of shRNA resistant form of PPP2R2A in PPP2R2A KD cells restored γH2AX levels (Figure 3C), indicating that inefficient DSB repair is triggered specifically by loss of PPP2R2A. Single-cell gel electrophoresis also revealed that unresolved DNA damage induced by camptothecin was significantly higher in PPP2R2A KD cells (Figure 3D). Taken together, our observations strongly support the idea that PPP2R2A plays a key role in DSB DNA repair.

To assess a possible mechanism by which PPP2R2A modulate DSB repair, we elucidated the effect of PPP2R2A suppression on two major pathways of DSB repair, NHEJ and HR (22, 23). We found that although suppression of PPP2R2A facilitated the NHEJ repair, the HR pathway was dramatically inhibited in PPP2R2A KD cells (Figure 4A,B). Consistently, we found a decreased Rad51 foci formation in cells expressing shPPP2R2A (Figure 4C; Suppl. Figure 2). Moreover, 8 hours after IR, expression of both BRCA1 and RAD51 was significantly decreased in PPP2R2A
KD cells (Figure 4D). Together, these results indicate that loss of PPP2R2A impairs the HR mechanism.

Because the balance between NHEJ and HR repair depends on the phase of cell cycle (22, 24), we examined the effect of PPP2R2A on IR-induced cell cycle checkpoints. We found that 8 hours after IR, HeLa cells expressing shGFP accumulated in G2/S phase, whereas cells with suppressed PPP2R2A were mostly in the G1 phase of the cell cycle (Figure 4E,F). In addition, BrdU incorporation after IR was dramatically decreased in PPP2R2A KD cells (Figure 4G). We also observed that I-SceI-induced DSBs resulted in accumulation of 293DR-GFP-shPPP2R2A cells in the G1 phase of the cell cycle (Figure 4H). In concordance with these results, we found that in response to IR expression of the cell cycle–promoting phosphatase CDC25A (25) was significantly decreased in PPP2R2A-depleted cells (Figure 4I). Taken together, these data indicate that PPP2R2A modulates the HR repair through regulation of G1/S cell cycle checkpoint.

Accelerated Cdc25A turnover after DNA damage is regulated by the CHK1 and CHK2 kinases (26, 27). Therefore, we analyzed the effect of PPP2R2A on CHK1 or CHK2 activation. Although inhibition of PPP2R2A did not affect CHK1 phosphorylation at S296, S217, and S345, we observed a significant accumulation of CHK2 phosphorylated at T68 in PPP2R2A KD cells (Figure 4I), suggesting that G1/S checkpoint induced by loss of PPP2R2A is mediated by prolonged activation of the CHK2 kinase.
PPP2R2A directly regulates ATM phosphorylation

CHK2 is a direct downstream target of ATM, while ATM has been shown to be directly regulated by PP2A. In particular, a previous report (28) reveals that a specific PP2A inhibitor, okadaic acid, induces ATM autophosphorylation at S1981. Moreover, the authors demonstrated interaction between ATM and PP2A A and C subunits. However, specific PP2A regulatory subunit(s) involved in ATM dephosphorylation has not been identified, whereas our data suggest that PPP2R2A may affect CHK2 activity by regulating ATM dephosphorylation.

Indeed, we found that suppression of PPP2R2A resulted in a significant increase of ATM phosphorylated at S1981 (Figure 5A), while restoration of PPP2R2A expression by overexpression of shRNA-resistant silent mutant of PPP2R2A rescued this increase (Figure 4B). Although ATM phosphorylation is considered as a sign of ATM activation, there are contradictory data how it affects ATM functions. Studies of ATM-S1987A (mouse homologue of human S1981) mice demonstrated normal ATM-dependent phosphorylation of ATM substrates, activation of cell cycle checkpoints, and localization of ATM to DSBs (29, 30). In vitro studies using human recombinant ATM proteins revealed that phosphorylation does not affect its kinase activity (31-33). In human cells ATM phosphorylation at S1981 is also dispensable for the ability of ATM to localize to DSBs, but it is required for ATM retention at DSBs (34). Consistently with this report, immunofluorescent analysis of ATM revealed an increase of the size of ATM foci in PPP2R2A KD cells (Figure 5C), further confirming the idea that phosphorylation regulates ATM retention at the sites of DSBs (34).

These findings suggest that PPP2R2A controls DNA repair through regulation of the ATM signaling. To test this hypothesis, we analyzed whether PPP2R2A affects
DNA repair in HeLa ATM SilenciX (ATM KD) cells. Indeed, analysis of γH2AX levels in ATM-deficient cells revealed that suppression of PPP2R2A in these cells did not affect the kinetics of DNA repair (Figure 5D).

To determine whether PPP2R2A directly regulates ATM dephosphorylation, we performed a set of reciprocal immunoprecipitations. We found PPP2R2A together with PP2A A and C subunits in complex with ATM (Figure 6A). Using in vitro phosphatase assays, we also found that phosphopeptides corresponding to human ATM pS367, pS1893, and pS1981, but not pT1885 and pS2996, were efficiently dephosphorylated by PPP2R2A-specific complexes (Figure 6B). Given that we observed an interaction between PPP2R2A heterotrimeric complexes and ATM, increased ATM phosphorylation in PPP2R2A-depleted cells, and in vitro dephosphorylation of three ATM autophosphorylation sites (S367, S1893, and S1981) by PPP2R2A-specific complexes, it seems likely that ATM is a direct target for PPP2R2A-specific PP2A phosphatase in vivo.

Wip1 phosphatase has been also demonstrated to dephosphorylate ATM at S1981 and S367, but not at S1893 (35, 36) (Figure 6B), indicating that PPP2R2A and Wip1 may differentially regulate ATM dephosphorylation at different sites. Moreover, in contrast to PP2A subunit PPP2R2A, Wip1 expression is extremely low in undamaged cells (Figure 6C), and depletion of Wip1 does not affect the level of phosphorylated ATM under normal conditions (35). Taken together, these data suggest that PPP2R2A-containing PP2A holoenzyme is the major ATM phosphatase in the absence of DNA damage.

IR triggered transient dissociation of the ATM–PPP2R2A complex, allowing accumulation of phosphorylated ATM (Figure 6D). This observation is consistent with
a previous report (28) that revealed a significant reduction in the amount of ATM associated with PP2A A and C subunits after IR. Interestingly, ATM mediates the IR-induced dissociation of the PPP2R2A subunit from the PP2A AC core dimer (37), implicating ATM in the disruption of functional PPP2R2A-containing PP2A complexes. Consistently with this report, 15 minutes after IR PPP2R2A associated phosphatase activity was decreased. 30 minutes after IR PPP2R2A was found again in complex with ATM, which is also correlated with an increase of PPP2R2A-specific phosphatase activity towards ATM phosphopeptides (Figure 6D,E). This kinetics is consistent with the kinetics of ATM recruitment/dissociation to the sites of DSBs (34). These data also suggest the existence of a negative-feedback loop between PPP2R2A and ATM.

**PPP2R2A down-regulation increases sensitivity to PARP inhibitors**

Inhibition of the HR repair in PPP2R2A-depleted cells suggest that these cells are highly sensitive to DNA damage. Indeed, colony formation assay revealed that inhibition of PPP2R2A sensitized cells to IR (Figure 7A). Moreover, HR deficiency predicts hypersensitivity to inhibition of poly(ADP-ribose) polymerase (PARP) (38, 39). In fact, analysis of cell survival after treatment with ABT-888 (veliparib), a potent inhibitor of PARP1 and PARP2, revealed that PPP2R2A KD cells are more sensitive to ABT-888 treatment (Figure 7B). To validate this observation, we examined the sensitivity to PARP inhibition of lung carcinoma cell lines with different levels of PPP2R2A expression. Consistently, we found that cell lines with higher PPP2R2A expression had lower sensitivity to ABT-888 compared to cell lines with lower PPP2R2A expression (Figure 2C, 7C).
ABT-888 treatment also inhibited growth of PPP2R2A KD A549 xenografts (Figure 7D). Moreover, we observed significantly reduced proliferation rate and increased apoptosis in PPP2R2A KD tumors compared to shGFP expressing tumors (Figure 7E,F). These results strongly indicate that both PPP2R2A expression levels and LOH in the PPP2R2A-containing region can be used to predict tumor sensitivity to treatment with PARP inhibitors.
Discussion

PP2A is a ubiquitously expressed family of Ser/Thr protein phosphatases, and the diversity of PP2A functions suggests that particular PP2A complexes may contribute independently to complex phenotypes, such as the DNA damage response (5, 6). Indeed, we found that suppression of four different PP2A regulatory B subunits (PPP2R2A, PPP2R2D, PPP2R5A, and PPP2R3C) impairs the efficiency of DNA repair, suggesting that these specific PP2A complexes are involved in control of DNA repair. In our prior study, we performed a loss-of-function screen using a similar shPP2A library to identify PP2A subunits involved in human cell transformation (16). This screen implicates three PP2A regulatory subunits, PPP2R5A, PPP2R5C, PPP2R3A, in control of cell transformation. Our present results suggest that in addition to modulating c-Myc and Wnt activity (16), PPP2R5A could also contribute to the tumorigenic phenotype by impairing DNA repair efficiency. Consistent with this idea, it has been shown that PPP2R5A could directly dephosphorylate both c-Myc (40) and CHK2 kinase (41). However, the exact mechanism(s) by which PPP2R2D, PPP2R5A, and PPP2R3C contribute to DNA response needs to be further investigated.

In summary, our study revealed that dephosphorylation of ATM at S367, S1893, and S1981 by PPP2R2A-specific complexes regulates retention of ATM foci. Dysregulation of ATM triggered by loss of PPP2R2A induces G1/S checkpoint and inhibits HR repair. These observations further challenge the view that protein phosphatases serve only as negative regulators of DNA repair. Instead, our data suggest that tight regulation of phosphorylation events during DNA repair by specific protein phosphatases is required for timely DSB DNA repair.
Analysis of PPP2R2A expression in human cancers revealed that PPP2R2A mRNA expression is commonly down-regulated in NSCLCs. PPP2R2A gene is located at 8p21 chromosome region, which is frequently deleted in a wide range of epithelial cancers (42). However, a homozygous deletion of PPP2R2A-containing locus is more rare event in human cancers. As an example, a recent analysis of 141 clinical prostate tumors revealed PPP2R2A deletions in 61.7% of tumor samples with only three recurrent homozygous deletions (2.1%) (43). These results are consistent with our observation that PPP2R2A expression is down-regulated in NSCLC cell lines but never completely lost. As far as no potentially important somatic mutations of PPP2R2A have been reported, these data implicates PPP2R2A as a haploinsufficient tumor suppressor gene in epithelial cancers.

PARP inhibitors are emerging as a promising class of anticancer agents particularly effective against tumors, which loss of functional BRCA1 and BRCA2 (38). However, BRCA1 and BRCA2 mutation carriers comprise less than 5% of breast cancer cases and in lung cancer BRCA mutations are even much less common. Therefore, there is a pressing need to identify novel biomarkers that would predict responsiveness to PARP inhibitors. Here we demonstrate that PARP inhibition suppresses growth of tumors with decreased PPP2R2A expression. Together with the finding that LOH of PPP2R2A-containing region or decreased PPP2R2A expression is observed in about 40% of lung carcinomas, these data implicate PPP2R2A as a novel predictive marker in BRCA-proficient NSCLCs for the efficiency of treatment with PARP inhibitors.
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Figure Legends

Figure 1. shRNA screen to identify PP2A subunits involved in control of DNA damage response. A, Immunoblot analysis of PP2A C and γH2AX in nuclear (N) and cytoplasmic (C) fractions in HeLa cells expressing shGFP or shPPP2CA 8 hours after IR with 2 Gy. B, Automated image analysis of γH2AX immunostaining of HeLa cells expressing shGFP or shPPP2CA at different time points after 2 Gy of IR. C, Automated image analysis of γH2AX immunostaining of HeLa cells expressing shRNAs targeting the indicated PP2A B subunits 8 hours after 2 Gy of IR. Expression levels of PP2A subunits was assessed by qRT-PCR.

Figure 2. PPP2R2A expression is commonly down-regulated in NSCLCs due to LOH of the PPP2R2A-containing region. A, qRT-PCR analysis of PPP2R2A expression in a set of 245 tumor samples across 14 cancer types. B, qRT-PCR analysis of PPP2R2A expression in a panel of 21 lung NSCLC samples with matched normal tissues. PPP2R2A expression was normalized to GAPDH expression. C, Immunoblot analysis of PPP2R2A expression in NSCLC cell lines with and without LOH in the PPP2R2A-containing region.

Figure 3. Loss of PPP2R2A impairs DSB repair. A, Automated image analysis of γH2AX immunostaining of HeLa cells expressing shGFP or shPPP2R2A at different time points after 2 Gy of IR. Suppression of PPP2R2A was confirmed by immunoblotting. B, Analysis of γH2AX immunostaining in HeLa cells transfected with ON-TARGETplus SMART pools targeting GFP, PPP2CA, or PPP2R2A at different time points after treatment with bleomycin (10μM) for 1 hour. Suppression of PP2A C and PPP2R2A subunits was confirmed by immunoblotting. C, The effect of
PPP2R2A expression on γH2AX as detected by automated image analysis. shPPP2R2A-resistant (rPPP2R2A) form of PPP2R2A was overexpressed in HeLa-shPPP2R2A-2 cells; V, refers to an empty vector. D, Comet assay of HEK TER cells expressing shGFP or shPPP2R2A after treatment with 2 μM of camptothecin (CPT) for 1 h. The comet tail moment from 75 cells was quantified with CometScore software. *p=0.05 and **p=0.01, as determined by Student’s t-test.

Figure 4. Loss of PPP2R2A impairs the HR repair by triggering G1/S cell cycle arrest. A, The efficiency of I-SceI–induced NHEJ repair in H1299dA3-1 cells expressing shRNAs specific to luciferase or PPP2R2A. B, The efficiency of I-SceI–induced HR repair in 293 DR-GFP cells expressing the indicated shRNAs. C, Automated image analysis of Rad51 foci formation of HeLa cells expressing shRNAs targeting GFP or PPP2R2A after 2 Gy of IR. *p=0.0033 and **p=0.0054, as determined by Student’s t-test. D, Immunoblot analysis of the indicated proteins in HeLa cells expressing shGFP or shPPP2R2A after 2 Gy of IR. E, F, Cell cycle distribution of PI-stained HeLa cells expressing the indicated shRNAs 8 hours after 5 Gy of IR. G, Analysis of BrdU incorporation by HeLa cells expressing shRNAs specific to GFP or PPP2R2A 8 hours after 5 Gy of IR. H, The proportion of 293 DR-GFP cells expressing the indicated shRNAs cells in G1 phase after transfection with YFP or I-SceI-YFP. I, Immunoblot analysis of the indicated proteins in HeLa cells expressing shRNAs against GFP or PPP2R2A at different time points after 2 Gy of IR.

Figure 5. PPP2R2A negatively regulates ATM phosphorylation. A, Immunoblot analysis of phospho-ATM (S1981) and ATM in HeLa cells expressing shGFP or shPPP2R2A at different time points after 2 Gy of IR. B, Immunoblot analysis of
phospho-ATM (S1981) and ATM in HeLa-shPPP2R2A-2 cells and HeLa-shPPP2R2A-2/rPPP2R2A cells at different time points after 2 Gy of IR. C, Representative images and automated image analysis of ATM immunostaining in HeLa cells expressing shRNAs targeting GFP or PPP2R2A after 2 Gy of IR. *p=0.0021 and **p=0.0034, as determined by Student’s t-test. D, Automated image analysis of γH2AX immunostaining of HeLa and HeLa ATM SilenciX (ATM KD) cells expressing shGFP or shPPP2R2A at different time points after 2 Gy of IR.

**Figure 6.** PPP2R2A-containing complexes directly dephosphorylates ATM at S367, S1893, and S1981. A, Reciprocal immunoprecipitations of ATM or Flag-tagged PPP2R2A expressed in HEK TE cells followed by immunoblotting using antibodies specific for Flag, ATM, PP2A C, and PP2A A. NS refers to a non-specific band. B, *In vitro* phosphatase activity of PPP2R2A-specific complexes and Wip1 toward ATM phosphopeptides. ND refers to not detected. C, Immunoblot analysis of the indicated proteins at different time points after 2 Gy of IR. D, Immunoprecipitations of PPP2R2A-containing complexes at different time points after 2 Gy of IR followed by immunoblotting with antibodies specific for Flag, ATM, and PP2A C. E, PPP2R2A-associated phosphatase activity of at different time points after 2 Gy of IR toward the indicated ATM phosphopeptides.

**Figure 7.** Decreased expression of PPP2R2A increases tumor sensitivity to PARP inhibition. A, Colony formation assay of HeLa cells expressing shRNAs specific to GFP and PPP2R2A after exposure to increasing doses of IR. B, Cell survival of HeLa cells expressing shGFP or shPPP2R2A-1 after ABT-888 treatment. C, Cell survival of lung carcinoma cell lines treated with increasing concentrations of ABT-888. D, Growth of A549 xenograft tumors expressing the indicated shRNAs in
response to ABT-888 treatment. *p=0.0011, as determined by ANOVA test. E, Ki67 immunohistochemistry of A549 xenografts expressing shGFP or shPPP2R2A-1 after treatment with ABT-888. F, TUNEL analysis of A549 xenografts expressing the indicated shRNAs after 7 days of ABT-888 treatment.
Figure 1

A

B

C

Time after IR (hours)

Suppression level

0 18% 36% 75% 85% 73% 62% 60% 53% 70% 85% 60% 56% 70% 59% 63% 72% 60% 53% 70% 97% 75% 92% 91% 70% 60%

Mean+2SEM

Relative γH2AX intensity

PP2A C

shGFP

shPPP2CA-1

shPPP2CA-2

PP2A C

γH2AX

Vinculin

Short Exposure

Long Exposure

Figure 2

A

B

C

Relative expression of PPP2R2A mRNA (%)
Figure 3

(A) Western blot analysis showing the expression levels of PPP2R2A and GAPDH in cells transfected with shGFP or shPPP2R2A-1/2. The blots are presented with different treatments: untreated, 2 hours, and 12 hours after bleomycin treatment.

(B) Graph illustrating the time course of γH2AX intensity after X-irradiation. The data show a significant increase in γH2AX intensity over time for both shGFP and shPPP2R2A-2 conditions.

(C) Western blot analysis of vinculin expression in cells treated with different combinations of shGFP, siPPP2CA, and siPPP2R2A. The blots demonstrate the effect of these treatments on vinculin expression.

(D) Comet assay results showing the effect of CPT (2 μM) on γH2AX positive cells. The figure includes untreated and treated conditions for shGFP and shPPP2R2A-1, with quantification of Comet Tail Moment for 2 and 12 hours after CPT treatment.

* p < 0.05, ** p < 0.01

Figure 4

A. NHEJ repair (%)

B. HR repair (%)

C. Rad51 foci per nucleus

D. Untreated 2h after IR 8h after IR

E. Untreated 8h after IR

F. Cell Cycle phase (%)

G. BrdU-positive cells (%)

H. % of YFP-positive cells in G1 phase

V. I-SceI

I. CDC25A p-CHK2 (T68) 

p-CHK1 (S317) p-CHK1 (S345) CHK1 CHK2 GAPDH
Figure 5

A

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C

D

- shGFP
- shPPP2R2A-2
- ATM KD shGFP
- ATM KD shPPP2R2A-2

Number of ATM foci per nuclei

Relative size of ATM foci

Total γH2AX intensity

Time after IR, hours
Figure 6

A) Western blot analysis showing the expression of various proteins under different conditions.

B) Table showing the phosphatase activity, pmol PO_4^+/min, for different substrates under various conditions.

C) Time course analysis of protein expression after IR exposure.

D) Western blot analysis showing the expression of proteins at different time points.

E) Graph showing the phosphatase activity over time for different substrates.

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Figure 7

A. Cell Survival (%)

- shGFP
- shPPP2R2A-1
- shPPP2R2A-2

B. Concentration of ABT-888 (μM)

- shGFP
- shPPP2R2A-1
- shPPP2R2A-2

C. Concentration of ABT-888 (μM)

- shGFP
- shPPP2R2A-1
- shPPP2R2A-2

D. Tumor volume, mm

- A549-shGFP vehicle (n=6)
- A549-shPPP2R2A-1 vehicle (n=6)
- A549-shGFP ABT-888 (n=12)
- A549-shPPP2R2A-1 ABT-888 (n=9)

E. Ki67

- shGFP
- shPPP2R2A-1

F. TUNNEL

- shGFP
- shPPP2R2A-1
Loss of PPP2R2A inhibits homologous recombination DNA repair and predicts tumor sensitivity to PARP inhibition

Peter Kalev, Michal Simicek, Iria Vazquez, et al.

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