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Distinct phosphatases antagonize the p53 response in different phases of the cell cycle

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The basic machinery that detects DNA damage is the same throughout the cell cycle. Here, we show, in contrast, that reversal of DNA damage responses (DDRs) and recovery are fundamentally different in G1 and G2 phases of the cell cycle. We find that distinct phosphatases are required to counteract the checkpoint response in G1 vs. G2. Whereas WT p53-induced phosphatase 1 (Wip1) promotes recovery in G2-arrested cells by antagonizing p53, it is dispensable for recovery from a G1 arrest. Instead, we identify phosphoprotein phosphatase 4 catalytic subunit (PP4) to be specifically required for cell cycle restart after DNA damage in G1. PP4 dephosphorylates Krüppel-associated box domain-associated protein 1-S473 to repress p53-dependent transcriptional activation of p21 when the DDR is silenced. Taken together, our results show that PP4 and Wip1 are differentially required to counteract the p53-dependent cell cycle arrest in G1 and G2, by antagonizing early or late p53-mediated responses, respectively.

A cell's genomic integrity is constantly challenged by endogenous and exogenous sources of DNA damage. Double-strand breaks (DSBs) are particularly threatening to the genomic stability of proliferative cells and provoke a checkpoint response that coordinates repair processes with further cell cycle progression to prevent the replication and segregation of broken DNA. This DNA damage response (DDR) is orchestrated by multiple kinases that sense the DNA damage and relay this signal (1). Cellular recovery from a DNA damage insult ultimately requires the termination of the DDR once repair of the DNA is complete.

PI3-kinase–related kinases (PIKKs), ataxia telangiectasia mutated (ATM), and ATM- and Rad3-related (ATR) are activated by distinct structures of damaged DNA and phosphorylate histone H2AX in the vicinity of the damaged site to recruit repair proteins (2). In addition to such local events, ATM and ATR activate a subsequent layer of checkpoint kinase 2 (Chk2) and Chk1, respectively, that disseminates from the damaged site (3, 4). ATM also activates p38 mitogen-activated protein kinase (MAPK), which coordinates the DDR outside the nucleus (5, 6). Combined, these checkpoint kinases ensure that cell cycle progression is prevented at the G1/S or G2/M boundary (7).

PIKKs and checkpoint kinases commonly converge on the transcription factor p53, a key regulator of stress responses. Phosphorylation of p53 prevents its degradation by mouse double minute 2 (Mdm2)-mediated polyubiquitination, allowing p53 to accumulate and induce its target genes, including p21 (1). Both p53 and its transcriptional target p21 are sufficient to impose an arrest in both G1 and G2, and they are absolutely required for a bona fide checkpoint arrest in G1 (8–11).

Recovery from a checkpoint-induced arrest requires silencing of the checkpoint machinery and coincides with the removal of phosphorylations deposited by PIKKs and other checkpoint kinases. We have previously shown that WT p53-induced phosphatase 1 (Wip1) is essential for checkpoint recovery from a DNA damage-induced arrest in G2, by preventing p53-dependent repression of several mitotic regulators (12). Wip1 is also known to act as a homeostatic antagonist of p53 by removal of ATM-dependent S15 phosphorylation on p53 (13–16). In addition, Wip1 dephosphorylates other ATM substrates, including ATM itself, phosphorylated H2AX pS139 (γ-H2AX), Chk2, p38 MAPK, and Mdm2 (14, 17–19). Given this role of Wip1 in the silencing of p53 as well as other components of the DDR, we expected Wip1 to be essential for recovery from a G1 arrest. Here, we show, instead, that Wip1 is not required for recovery from a G1 arrest caused by γ-irradiation. This finding prompted us to screen for other phosphatases that are essential for the reversal of a checkpoint-dependent arrest in G1.

Results

Wip1 Is Required for Spontaneous Recovery After Low-Dose Irradiation in G2, but Not G1. We previously uncovered the Wip1 phosphatase as a critical regulator of recovery from a DNA damage-induced G2 arrest (12). How recovery from a DNA damage-induced G1 arrest is regulated is not known. To study this process, we used non-transformed retinal pigment epithelial (RPE) cells immortalized with human telomerase reverse transcriptase (hTert) and expressed fluorescent ubiquitination-based cell cycle indicators (FUCCIs) (20).

G1 cells were identified by exclusive expression of Cdt1 (amino acids 30–120) fused to an orange fluorescent protein (mKO2-Cdt1) at the start of the experiment and followed over time to the moment of S-phase entry, marked by the coexpression of Geminin (amino acids 1–110) fused to a green fluorescent protein (mAG1-Geminin) (Fig. 1*A*). To determine the fate of S/G2 cells after irradiation, we followed cells exclusively expressing fluorescent mAG1-Geminin into mitosis.

Significance

Cell cycle checkpoints coordinate repair of DNA damage with progression through the cell cycle to prevent propagation of DNA mutations and tumor formation. Here, we show that two phosphatases, phosphoprotein phosphatase 4 catalytic subunit (PP4) and WT p53-induced phosphatase 1 (Wip1), are required to promote cell cycle reentry after DNA damage. PP4 is essential for cell cycle reentry in G1, whereas Wip1 is required for reentry in G2, but both act to revert the p53 response. These findings help us understand how overexpression of PP4 and Wip1, frequently observed in human cancers, may translate to a poor response to genotoxic therapies.

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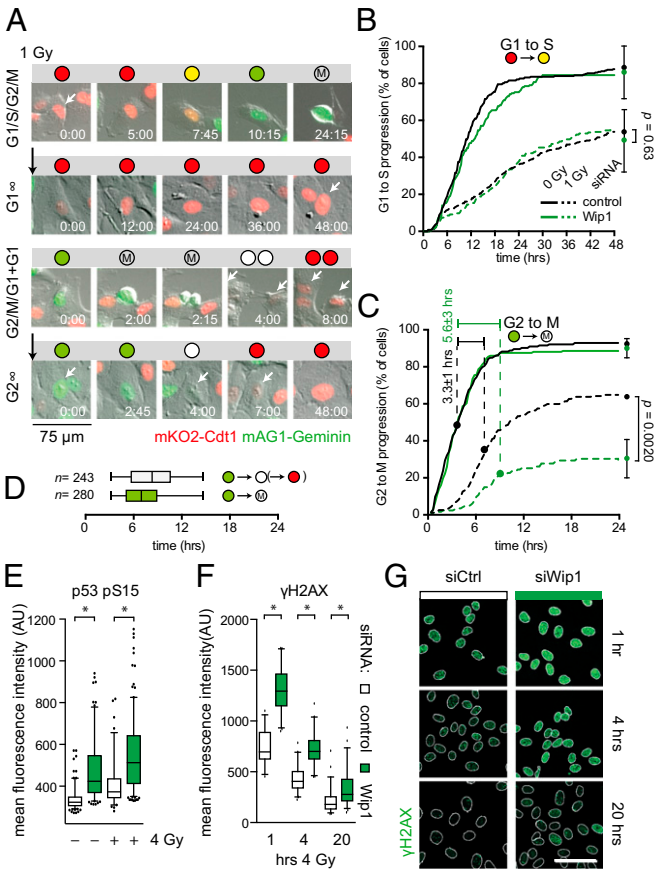

Fig. 1. Wip1 is differentially required for recovery from a DNA damage-induced arrest in G1 and G2 of the cell cycle. (*A*) Example still views of observed cell fates in RPE-FUCCI cells. (*B* and *C*) Asynchronously proliferating RPE-FUCCI cells were transfected with indicated siRNAs for 48 h, irradiated, and filmed immediately thereafter. (*B*) Cells in G1 (mKO2-Cdt1$^+$, mAG1-Geminin$^-$) at the start of the experiment were followed to mAG1-Geminin coexpression (S-phase entry). (*C*) Cells in G2 (mKO2-Cdt1$^-$, mAG1-Geminin$^+$) at the start of the experiment were followed to mitosis. Black and green dots mark means and SD of Wip1-depleted cells normalized to control transfections of the same experiments. (*D*) Timing of mitotic entry or loss of mAG1-Geminin expression in 1-Gy irradiated RPE-FUCCI cells. (*E*) Phosphorylation of p53 on S15 was determined by immunofluorescence of asynchronous cultures depleted of Wip1 after irradiation. Individual 2n cells were identified by DAPI fluorescence (Fig. S2), and the mean nuclear fluorescence intensity of p53-pS15 was quantified. Whiskers represent 5–95% of data points. (*F* and *G*) RPE cells were treated with siRNAs targeting Wip1 during G1 synchronization, and the clearance of γ-H2AX after irradiation was determined by immunofluorescence. Nuclear mean fluorescence intensity was quantified. Whiskers represent 5–95% of data points. Shown are representatives of three independent experiments. (Scale bar: 75 µm.) AU, arbitrary units; siCtrl, small interfering control; siWip1, small interfering Wip1. *$P < 0.0001$.

In the absence of DNA damage, S-phase entry of G1 cells occurred within 20 h, although after exposure to 1 Gy of γ-irradiation, the rate of S-phase entry was much reduced with ~53 ± 13% of cells progressing within 48 h (Fig. 1*B* and Movie S1). The RPE-FUCCI cells thus enable sensitive detection of recovery from irradiation in G1.

Exposure in G2 resulted in a rapid checkpoint arrest of a defined duration of 3.3 ± 1 h (measured as the time difference between reaching half of the maximal mitotic entry). Approximately 60% of irradiated G2 cells ultimately entered mitosis within 9 h, contrasting with the fourfold slower cell cycle progression of cells irradiated in G1 (Fig. 1*C*). RPE-FUCCI cells that were irradiated in G2 but failed to enter mitosis returned to a G1-like state expressing mKO2-Cdt1 3–15 h after irradiation within the same time window as mitotic entry in the recovering population (Fig. 1 *C* and *D*).

When we compared the performance of 1 Gy-irradiated RPE-FUCCI cells after siRNA-mediated depletion of Wip1, the mitotic entry of cells irradiated in G2 was reduced twofold compared with control siRNA-transfected cells, and the overall delay in mitotic entry after irradiation was longer (5.6 ± 3 h) (Fig. 1*C*). In contrast, we did not detect any effect on the spontaneous recovery of cells irradiated in G1, either on the rate of S-phase entry or on the percentage of cells that ultimately entered S-phase (Fig. 1*B*). We obtained similar results with hTert-immortalized BJ human foreskin fibroblasts expressing the FUCCI system (Fig. S1). These results suggest that Wip1 is differentially required to promote recovery in G1 vs. G2.

Wip1 is a transcriptional target of p53 (13) and a negative regulator of DDR signaling by removing S15 phosphorylation on p53 and S139 phosphorylation of H2AX (12, 15, 18). Induction of Wip1 protein levels after irradiation is intact in RPE cells synchronized in G1 by serum starvation and restimulation (Fig. S2). siRNA-mediated depletion of Wip1 resulted in increased (both basal and after irradiation) levels of S15 phosphorylation of p53 as detected by immunofluorescence of asynchronous cultures, irrespective of cell cycle phase (Fig. 1*E* and Fig. S2). Furthermore, cells depleted of Wip1 during G1 synchronization displayed markedly elevated levels of γ-H2AX after irradiation (Fig. 1 *F* and *G*), confirming that Wip1 is functional as a phosphatase following DNA damage in both the G1 and G2 phases of the cell cycle.

DNA Damage Checkpoint in G1 Is Maintained by Chk2 and p38. Redundancy of Wip1 for recovery from DNA damage in G1 suggests recovery is differentially regulated during the different phases of the cell cycle. To study how recovery from an irradiation-induced arrest in G1 is controlled, we first tested which checkpoint kinases maintain the G1 arrest. Exposure to 4 Gy of irradiation arrested G1-synchronized RPE cells in G1 for at least 40 h, as measured by BrdU incorporation (Fig. 2*A*). Addition of an ATM inhibitor (KU55933) before irradiation prevented the cells from arresting in G1 (Fig. 2*B*), confirming that the observed cell cycle arrest is a result of an ATM-dependent DSB response. However, addition of ATM inhibitor at later time points failed to abrogate the checkpoint arrest (Fig. 2*B*). ATM is the most upstream kinase to initiate signaling from a DSB, and it controls the activation of several downstream checkpoint kinases, including Chk1, Chk2, and p38 MAP kinase (7). We applied inhibitors of these kinases to cells arrested in G1 for 16 h and determined S-phase entry of the cells in the following 24 h. Combinations of a Chk2 inhibitor and one of three different p38 MAPK inhibitors caused a full reversal of the established G1 arrest (Fig. 2 *A* and *B* and Fig. S2). A similar setup with BJ fibroblasts confirmed a critical role for Chk2- and p38 MAPK-dependent signaling to maintain a DNA damage-induced arrest in G1 (Fig. S1).

As expected, the observed arrest requires p53 and p21, and it is prevented by siRNA-mediated depletion of either p53 or p21 (Fig. S2). Inhibition of Chk2 and p38 MAPK requires the presence of Mdm2, but not Wip1, to promote S-phase entry in G1-arrested cells, confirming that Wip1 is not necessary to revert these p53-mediated responses (Fig. S2). Irradiation of G1 cells results in increased protein levels of p53 and p21, and reduced cyclin-Cdk activity, as evidenced by a lack of phosphorylation on the pocket proteins retinoblastoma (Rb) and p130 (Fig. 2 *C*–*E*). Indeed, treatment of cells with p38 MAPK and Chk2 inhibitors results in loss of protein levels of p53 and p21 over a period of 8 h with concomitant rephosphorylation of pocket proteins, coinciding with the cell cycle restart (Fig. 2*C*). This phosphorylation of Rb occurs without the resolution of remaining γ-H2AX foci (Fig. 2 *D* and *E*). Together, these experiments show that p38 MAPK

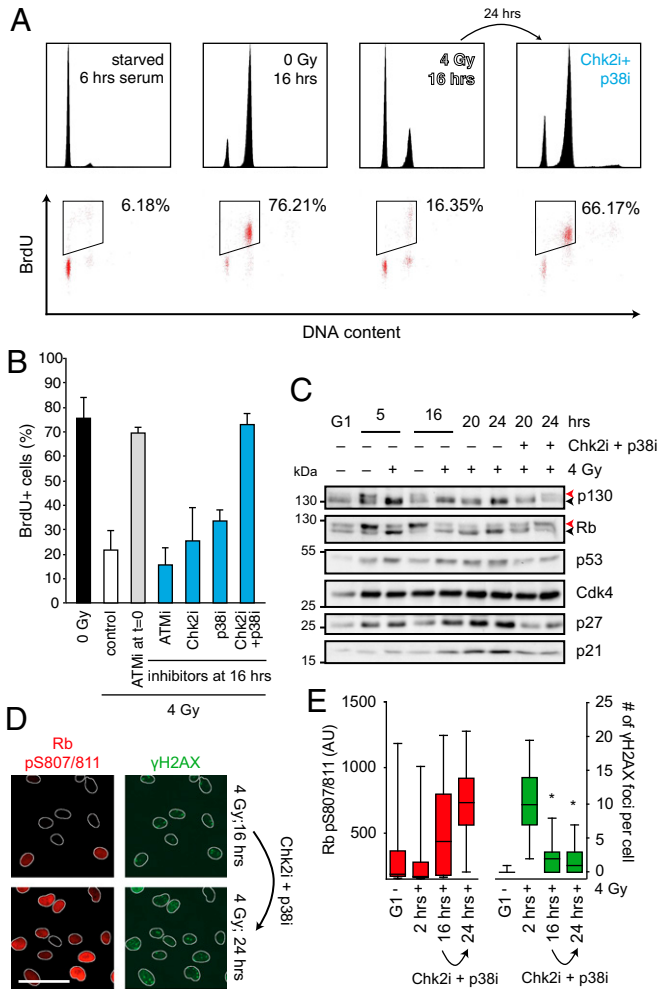

Fig. 2. DNA damage-induced checkpoint arrest in G1 is maintained by p38 MAPK and Chk2. RPE cells were synchronized and arrested in G1 by 4 Gy of γ-irradiation as schematically represented (*A*) and analyzed by flow cytometry (*B*). Mitotic drug S-trityl-L-cysteine and BrdU were added to prevent the accumulation of daughter cells and to monitor S-phase entry, respectively. Sixteen hours after irradiation, various checkpoint kinases were inhibited with small-molecule inhibitors to promote cell cycle reentry of these G1-arrested cells. (*C*) Phosphorylation status of pocket proteins and protein levels of cyclin-dependent kinase inhibitors after DNA damage and during recovery as determined by Western blot. Red and black arrowheads indicate hyper- and hypophosphorylated forms, respectively. (*D*) Immunofluorescence images of RPE cells synchronized and irradiated as in *A* probed with antibodies directed against γ-H2AX and phosphorylated Rb pS807/811. (*E*) Mean nuclear fluorescence intensity of Rb pS807/811 and the number of γ-H2AX foci were quantified. Error bars indicate SD ($n = 3$). Whiskers indicate 5–95% of data points. (Scale bar: 75 μm.) *$P < 0.0001$.

and Chk2 cooperate to sustain activation of p53 and maintain expression of p21 during the G1 arrest and that their inhibition promotes checkpoint recovery even when DNA damage persists.

Phosphatases Required for Recovery from a G1 Arrest. Because p38 MAPK and Chk2 kinases are essential for the maintenance of the checkpoint arrest in G1 (Fig. 2*B*), we expected checkpoint recovery to require dephosphorylation or degradation of their substrates. We therefore tested a siRNA library of 224 phosphatases and regulatory proteins to identify the phosphatase(s) required for recovery of DNA-damaged G1 cells. We monitored 5′-ethynyl-2′-deoxyuridine (EdU) incorporation in RPE cells irradiated (4 Gy) in G1 and stimulated to reenter the cell cycle by inhibition of p38 MAPK and Chk2 (Fig. 3*A* and Dataset S1). The majority of siRNAs did not prevent EdU incorporation after serum stimulation or after inhibition of Chk2 and p38 MAPK in cells arrested in G1 for 16 h, including siRNAs targeting Wip1. Importantly, phophoprotein phosphatase 4 catalytic subunit (PP4), phosphatases PSPH, PTPRN2, PP5, PTPN6, DUSP2, and the B56delta-subunit of the PP2A phosphatase (PP2R5D) were identified as potential regulators of G1 checkpoint recovery that compromised EdU incorporation after DNA damage and subsequent checkpoint silencing (recovery < mean − 2 SD of mock-transfected) but allowed normal cell cycle progression in the absence of damage (unperturbed/recovery >1.67, unperturbed entry >67% of mock-transfected in more than two of three

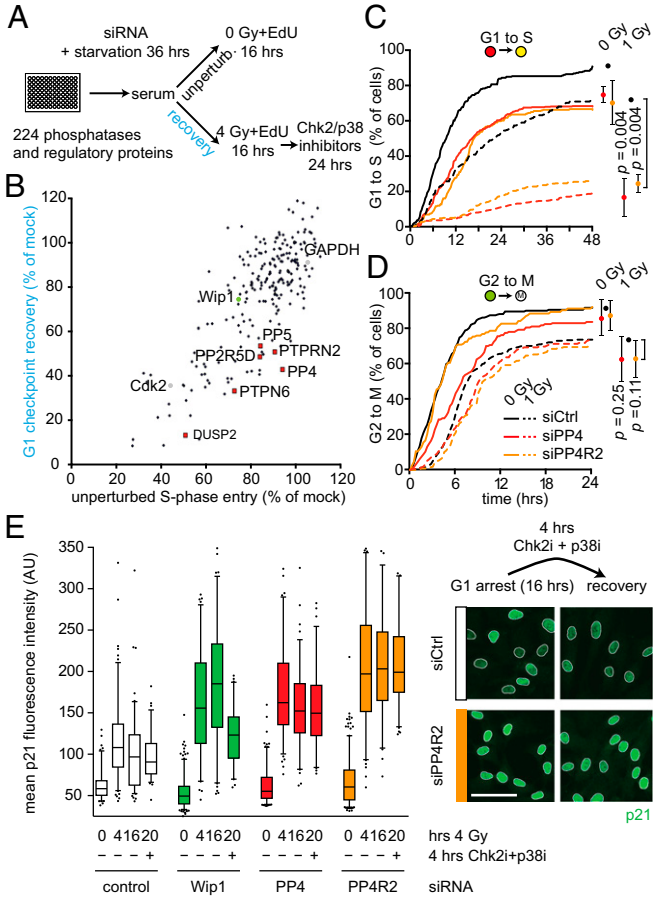

Fig. 3. PP4 is required for recovery from an arrest in G1, but not in G2. (*A*) RPE cells were transfected with siRNA pools during serum starvation and treated further as in Fig. 2*A* to identify phosphatases required for S-phase entry after a DNA damage-induced arrest, but not normal cell cycle progression. EdU incorporation was determined by automated microscopy (Fig. S3). (*B*) Scatterplot of recovery/unperturbed S-phase entry is shown as determined by the percentage of EdU-positive cells normalized to mock-transfected control wells. Shown are means of three independent experiments. siRNA pools targeting GAPDH and Cdk2 were included in each plate as controls for normal and impaired S-phase entry. Time-lapse microscopy was performed for cell cycle progression of asynchronously proliferating RPE-FUCCI cells irradiated in G1 (*C*) or G2 (*D*) after depletion of PP4 or PP4R2 by siRNA. Shown is the average cumulative progression of three independent experiments. Dots represent cell cycle progression normalized to control siRNA-transfected RPE-FUCCI cells of the same experiment. Error bars represent SD ($n = 3$). (*E*) Immunofluorescence detection of p21 in RPE cells arrested 16 h in G1 (4 Gy) and incubated for an additional 4 h with Chk2 and p38 inhibitors. Mean nuclear intensity was quantified. Whiskers represent 5–95% of data points. (Scale bar: 75 μm.)

experiments). Phenotypes were confirmed with multiple single siRNAs for PP4, PTPRN2, PTPN6, and DUSP2 (Fig. S3).

PP4 Is Required for Recovery After Irradiation in G1, but Not G2. To look further into the reversal of Chk2 and p38 phosphorylations, we proceeded with analysis of the only phosphoserine/phosphothreonine phosphatase, PP4. PP4 is a multimeric type 2A phosphatase that functions in dimeric or trimeric complexes to balance a wide range of signaling pathways (21–25). In particular, PP4 in complex with its regulatory subunit 2 (PP4R2) has well-documented roles in the reversal of DDR phosphorylations (21, 26–29). Indeed, we find that PP4R2, but not other regulatory subunits, is required for recovery from a G1 checkpoint arrest after inhibition of p38 MAPK and Chk2 (Fig. S3).

When we depleted PP4 or PP4R2 in RPE-FUCCI cells, effects on cell cycle progression of unperturbed cells were limited (Fig. 3 *B* and *C*). In contrast, with depletion of Wip1, which did not affect the spontaneous recovery of cells irradiated (1 Gy) in G1, depletion of either PP4 or PP4R2 reduced recovery by 75 ± 15% and 64 ± 7%, respectively (Fig. 3 *B* and *C* and Movie S2). Conversely, the PP4 phosphatase complex did not contribute significantly to mitotic progression of cells irradiated in G2 (mAG1-Geminin^{+}, mKO2-Cdt1^{-}), whereas mitotic progression was reduced by 46 ± 7% in the absence of Wip1 (Figs. 1*C* and 3*C*). BJ-hTert FUCCI cells showed a similar G1-specific defect after 2 Gy of γ-irradiation when PP4 or PP4R2 was depleted (Fig. S1).

These results confirm a critical role for the PP4 phosphatase complex in G1, but more importantly, they reveal differential and complementary requirements for checkpoint recovery in different phases of the cell cycle.

Inhibition of p38 and Chk2 promotes recovery in the presence of persistent foci of γ-H2AX (Fig. 2 *D* and *E*) but not after depletion of PP4 or PP4R2 (Fig. 3*A* and Fig. S3), indicating that PP4 acts independent of DNA repair. Indeed, foci of γ-H2AX disappeared with normal kinetics in PP4-depleted RPE cells irradiated in G1 (Fig. S4). The DNA damage-induced checkpoint in G1 absolutely requires a functional p53 pathway (11), and whenever we codepleted PP4 and p53 or PP4R2 and p53 in G1-synchronized RPE cells or RPE-FUCCI cells, we observed a complete checkpoint override (Fig. S4). PP4 must therefore act downstream of p38 and Chk2 in the reversal of p53-dependent responses.

We had observed previously that p53's principal transcriptional target, p21, is eliminated within several hours after inhibition of p38 and Chk2 kinases (Fig. 2*C*). Although p53 protein levels dropped normally after checkpoint silencing in PP4-depleted cells (Fig. S4), we found elevated levels of p21 throughout the arrest in G1 that persisted after checkpoint silencing (Fig. 3*D*), suggesting a role to antagonize p21, particularly once the DDR is terminated.

PP4-PP4R2 Regulates Krüppel-Associated Box Domain-Associated Protein 1 S473 Phosphorylation to Eliminate p21 in Recovery. Transcription of p21 is dynamically regulated to enable rapid expression in response to stress, as well as to ensure timely shutdown of transcription when the stressor is under control (30). The Krüppel-associated box domain-associated protein 1 (KAP1) is a transcriptional corepressor protein for the p21 gene *CDKN1A* and prevents transcription initiation of the poised promoter (31, 32). Its activity is impeded by DNA damage through phosphorylation by ATM on S824 and by Chk1/Chk2 on S473 (31, 33–35). Dephosphorylation of these sites, in turn, is regulated by PP4 (36). Consistently, we find that PP4-depleted cells display increased and prolonged phosphorylation of KAP1 in response to damage (Fig. 4*A*). ATM-dependent phosphorylation on S824 is rapid and transient, even after depletion of PP4, and it disappears within the first hours after irradiation. Checkpoint kinase-dependent phosphorylation of S473, on the other hand, is delayed in onset, is pan-nuclear, and persists well up to 16 h after irradiation (Fig. 4 *A* and *B*), as described before (34, 36). These observations suggested to us that PP4 might be required to reactivate KAP1 during recovery by removing the Chk2-dependent phosphorylation on S473 and repressing p21 transcription.

Upon inhibition of p38 MAPK and Chk2, phosphorylation on S473 of KAP1 was lost within 2 h (Fig. 4*B*), correlating with decreasing levels of p21 protein (Fig. 3*D*) as observed by immunofluorescence. In PP4 or PP4R2-depleted cells, however, phosphorylation on S473 was sustained.

Because persistent phosphorylation is inhibitory to KAP1 function, we tested whether KAP1 depletion would mimic depletion of PP4 or PP4R2. Indeed, cells depleted of KAP1 had elevated levels of p21 in response to DNA damage in G1, failed to eliminate p21 protein and mRNA after pharmacological checkpoint silencing, and did not progress to S-phase (Fig. 4*C*

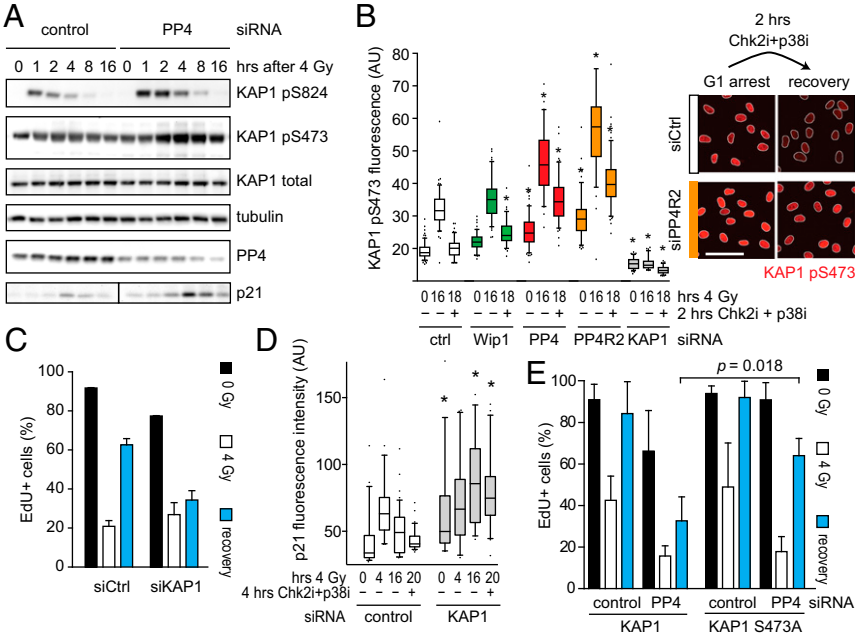

Fig. 4. PP4 dephosphorylates KAP1 S473 to eliminate p21 during recovery from a G1 arrest. (*A*) Inhibitory phosphorylations of KAP1 on S824 and S473 detected by Western blot in response to irradiation 48 h after siRNA transfection in G1-synchronized RPE cells. (*B*) KAP1 S473-phosphorylation 16 h after irradiation determined by immunofluorescence and after inhibition of p38 and Chk2 in G1-synchronized RPE cells. Nuclei are outlined in white based on DAPI signal, and the nuclear mean fluorescence of pS473 was quantified. (Scale bar: 75 μm.) (*C*) RPE cells treated as in Fig. 3*A*. S-phase entry was detected by EdU incorporation 40 h after irradiation. (*D*) Mean nuclear fluorescence intensity of p21 by immunofluorescence of cells treated as in *B*. (*E*) Stable RPE clones expressing FLAG-KAP1 or S473A mutant were transfected with siRNA targeting endogenous KAP1 alone (control) or in combination with PP4 during serum starvation and treated further as in *C*. Protein levels of the same experiments were detected by Western blot (Fig. S4). Immunofluorescence experiments are representative of three independent experiments. Whiskers represent 5–95% of data points. *P < 0.0001. Error bars represent SD (n = 3).

and Fig. S4). To pinpoint KAP1 further as the relevant substrate of PP4/PP4R2, we replaced endogenous KAP1 with a non-phosphorylatable S473A mutant and assessed its ability to circumvent the requirement for PP4 in recovery from a G1 arrest. When we thus prevented initial phosphorylation of KAP1 S473 after DNA damage, cells progressed to S-phase (Fig. 4*E* and Fig. S4) upon checkpoint silencing without PP4, confirming phosphorylated S473 on KAP1 as the critical substrate for PP4 in recovery from a G1 arrest. Taken together, these data show that PP4 is required for recovery in G1 by promoting the dephosphorylation of S473 on KAP1, allowing it to repress p21.

Wip1, but Not PP4, Prevents p53-Dependent Repression of Cyclin B1. The p21 and p53 are sufficient to induce a cell cycle arrest in both G1 and G2 (8, 9), yet we find that PP4 is only required in G1 to recover from the checkpoint-induced cell cycle arrest. Inversely, Wip1 is required to moderate p53 activity during a G2 arrest (12), but we show here that its activity is not essential to recover from a checkpoint-induced arrest established in G1. Because depletion of Wip1 was shown to lead to p53-dependent repression of cyclin B1, we examined if we could confirm a differential requirement for Wip1 and PP4 in G2 by analysis of cyclin B1 expression during an ongoing DDR. To this end, we made use of RPE cells in which the endogenous locus of cyclin B1 was C-terminally fused to YFP, allowing sensitive and quantitative detection of endogenous cyclin B1 levels under its normal transcriptional and posttranscriptional regulation (Fig. S5).

As expected, codepletion of p53 and Wip1 relieved the G2 recovery defect in RPE cells observed by Wip1 depletion alone (Fig. S5). In addition, the rate of cyclin B1-YFP accumulation decreased in a p53-dependent manner starting 2 h after irradiation (Fig. 5 *A* and *B* and Fig. S5). When Wip1 was depleted, cyclin B1-YFP failed to accumulate beyond 2 h after damage and started to decline to nondetectable levels after 4 to 8 h, corresponding in time to the loss of the Geminin probe in irradiated RPE-FUCCI cells (Fig. 1*D*). Depletion of PP4R2 or KAP1 had no effect on cyclin B1-YFP levels during this checkpoint arrest, indicating that PP4-dependent dephosphorylation of KAP1 has no role in p53-mediated regulation of cyclin B1 immediately after DNA damage. Consistent with this notion, we find that KAP1 S473 dephosphorylation occurs well after the onset of cyclin B1 repression (Fig. 4*A* and Fig. S4).

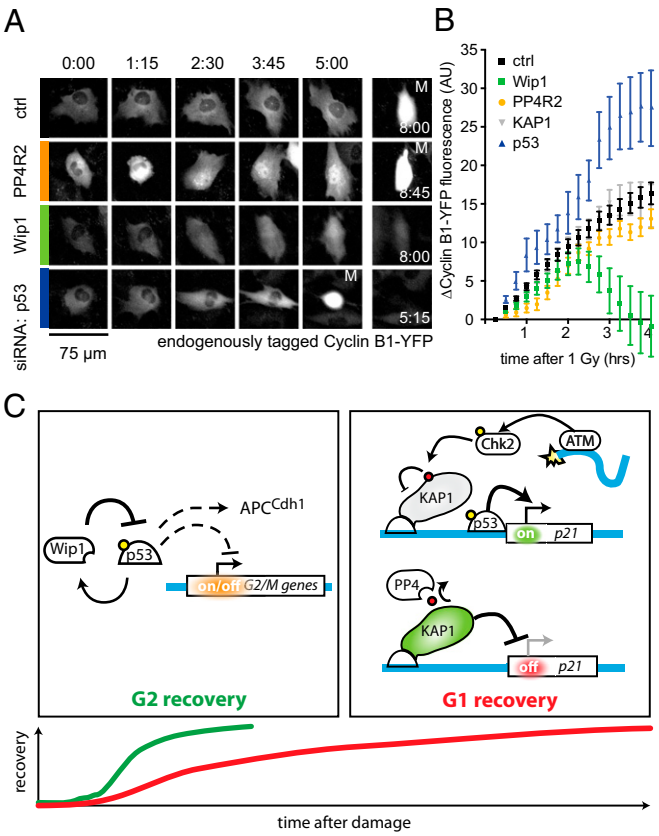

Fig. 5. Wip1 prevents p53-dependent repression of cyclin B1. (*A*) RPE cells expressing endogenously tagged cyclin B1-YFP (Fig. S5). Still views of single RPE-cyclin B1-YFP cells followed in time after 1 Gy of γ-irradiation 48 h after transfection. M, mitosis. (*B*) Quantification of the change in mean fluorescence intensity after irradiation in RPE-cyclin B1-YFP cells. Error bars represent SEM ($n > 20$). Results are representative of three independent experiments. (*C*) Model of mechanisms of action of Wip1 and PP4, which are separated in time corresponding to the timing of recovery in G2 and G1, respectively. Red (inhibitory) and yellow (activating) dots represent phosphorylation.

Discussion

At any given time, the vast majority of cells with proliferative capacity in the human body are in G0/G1. However, we know little about the particular molecular pathways that these cells use to recover from a DNA damage insult and resume proliferation. We do know that deregulation of the p53 and Rb pathways is common in cancer and results in the loss of a functional G1 checkpoint (11, 37, 38). Not only are G1-specific mechanisms in dealing with DNA damage undeniably significant for normal cellular behavior, but their frequent inactivation in cancer also highlights a pivotal role in preventing the accumulation of mutations during carcinogenesis.

We find that recovery from irradiation in G1 or G2 of the cell cycle is fundamentally different, in terms of their kinetics, in the checkpoint kinases that maintain the arrest and the phosphatases that promote recovery. In two nontransformed hTert-immortalized cell lines, we show that mitotic entry after exposure to irradiation is restricted in time, whereas S-phase entry after exposure to the same dose is permitted 72 h after the insult (Fig. 1). Within several hours after irradiation in G2, p53-dependent responses compromise the competence for further cell cycle progression, whereas a G1 arrest remains fully reversible in the first 16 h (Fig. 2).

Our experiments show that these differences are paralleled by a differential requirement for phosphatases in the reversal of the DDR in a G1-arrested cell vs. a G2-arrested cell (Fig. 5*C*). PP4 facilitates turning off engaged p21 transcription, and thereby antagonizes the p53 pathway to allow recovery from a G1 arrest hours after irradiation. Even though Wip1 acts on DDR substrates in G1, it is redundant for the elimination of p21 after checkpoint silencing, and Wip1 is therefore dispensable for recovery from irradiation in G1. In G2, however, Wip1 is essential to prevent premature p53-dependent loss of cyclin B1, and possibly other mitotic regulators, immediately after irradiation to permit subsequent recovery. Thus, both phosphatases antagonize p53-dependent responses, but each does so in a distinct time frame, required for recovery in distinct phases of the cell cycle.

Contrary to our expectations, depletion of Wip1 did not affect cell cycle restart of G1-arrested cells. Even in mouse embryonic fibroblasts from Wip1 KO animals, S-phase entry after irradiation is only decreased by 10% (39). This limited role of Wip1 in recovery from a G1 arrest after irradiation is striking, given the overt effects on p53 S15 and H2AX phosphorylation levels and its requirement for recovery from a G2 arrest. Nonetheless, even though γ-H2AX is cleared inefficiently in the absence of Wip1, most foci are resolved within 16 h, allowing ample time for the relatively slow recovery from a G1 arrest to occur. Our results predict that the proposed clinical application of compounds that inhibit Wip1 will prove ineffective for tumors that retain a G1 checkpoint arrest after DNA damage and most effective for p53-proficient tumors that arrest primarily in G2 (40).

Overexpression of Wip1 and PP4 occurs frequently in cancer and is associated with poor disease outcome (41–43). Because carcinogenesis requires multiple mutation events, overexpressed Wip1 or PP4 may help overcome the anticancer barrier posed by the DDR, promoting cell cycle progression after damage to the DNA and acquisition of additional mutations. In established tumors, overexpressed Wip1 and PP4 may promote recovery at the expense of the envisioned cell killing, conferring resistance to conventional genotoxic chemotherapy and radiotherapy.

Reversibility of a DNA damage-induced checkpoint arrest is an inherent prerequisite for cellular recovery after repair of the damaged DNA. Although the machinery that detects DNA damage is shared throughout the cell cycle, available repair pathways and the cell cycle machinery that must be called to a halt differ significantly in G1 and G2. Reversal of DNA damage-induced checkpoints in G1 and G2 is correspondingly regulated in distinct manners.

Materials and Methods

Cell Culture. The hTert-immortalized BJ fibroblasts, hTert-immortalized retinal pigment epithelium, and derived cell lines were maintained in DMEM/F12 (Gibco) supplemented with ultraglutamine, penicillin/streptomycin, and 6% (vol/vol) FBS.

G1 Synchronization, Checkpoint Silencing, and Flow Cytometry. We seeded RPE or BJ-hTert cells grown to confluency at 15,000 cm^{-2} and withdrew serum for 36 h after cell attachment. Six hours after serum stimulation, cells were exposed to γ-irradiation from a caesium-137 source (1 $Gy \cdot min^{-1}$). Inhibitors and BrdU (10 μM; Sigma) or EdU (10 μM; Invitrogen) were added as indicated, and at various time points, cells were collected by trypsinization or fixed in wells for automated fluorescence microscopy.

Immunofluorescence. At indicated time points after irradiation and inhibitor treatment, we fixed cells in PBS-buffered 3.7% formaldehyde permeabilized with −20 °C methanol and blocked aspecific binding with Tris-buffered saline containing 4% BSA and 0.1% Tween-20 before antibody incubation for immunofluorescence.

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