DNA Damage Triggers p21^{WAF1}-dependent Emi1 Down-Regulation That Maintains G2 Arrest

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Several regulatory proteins control cell cycle progression. These include Emi1, an anaphase-promoting complex (APC) inhibitor whose destruction controls progression through mitosis to G1, and p21^{WAF1}, a cyclin-dependent kinase (CDK) inhibitor activated by DNA damage. We have analyzed the role of p21^{WAF1} in G2-M phase checkpoint control and in prevention of polyploidy after DNA damage. After DNA damage, p21^{+/+} cells stably arrest in G2, whereas p21^{-/-} cells ultimately progress into mitosis. We report that p21 down-regulates Emi1 in cells arrested in G2 by DNA damage. This down-regulation contributes to APC activation and results in the degradation of key mitotic proteins including cyclins A2 and B1 in p21^{+/+} cells. Inactivation of APC in irradiated p21^{+/+} cells can overcome the G2 arrest. siRNA-mediated Emi1 down-regulation prevents irradiated p21^{-/-} cells from entering mitosis, whereas concomitant down-regulation of APC activity counteracts this effect. Our results demonstrate that Emi1 down-regulation and APC activation leads to stable p21-dependent G2 arrest after DNA damage. This is the first demonstration that Emi1 regulation plays a role in the G2 DNA damage checkpoint. Further, our work identifies a new p21-dependent mechanism to maintain G2 arrest after DNA damage.

INTRODUCTION

Cell cycle checkpoints safeguard genome integrity. After DNA damage, cells arrest at the G1 phase with 2N DNA content or at the G2 phase with 4N DNA content (Kastan *et al.*, 1991). The p53 tumor suppressor plays an important role in checkpoint response to DNA damage by activating the transcription of several genes including p21^{WAF1}, an inhibitor of cyclin-dependent kinases (CDKs; reviewed in Boulaire *et al.*, 2000). A role of p21^{WAF1} in G2 arrest was suggested by studies in which the ectopic expression of p53 or p21^{WAF1} (hereafter referred to as p21) led to an arrest in both G1 and G2 phases of the cell cycle (Agarwal *et al.*, 1995; Bates *et al.*, 1998; Medema *et al.*, 1998; Niculescu *et al.*, 1998). The importance of p53 and p21 in the G2 DNA damage response became evident when the human colorectal cell line HCT116 lacking p21 (p21^{-/-} HCT116) failed to sustain G2 arrest after γ -irradiation and attained a DNA content of 8N (Waldman *et al.*, 1996; Bunz *et al.*, 1998). Although a transient G2 arrest after DNA damage can be initiated in the absence of p53 or p21 at least in part by inhibition of CDC25 phosphatase, it is generally accepted that the maintenance of the G2 arrest after DNA damage involves p53 and p21 (Bartek and Lukas, 2001; Taylor and Stark, 2001).

The anaphase-promoting complex (APC) is a multiprotein complex with E3-ubiquitin ligase activity (King *et al.*, 1995; Sudakin *et al.*, 1995). Ubiquitination of specific substrates by APC targets them for degradation by the proteasome. APC is present throughout the cell cycle; however, in synchro-

nized cells its activity is high only from mitosis through G1 (Fang *et al.*, 1998; Kramer *et al.*, 1998). APC activity regulates progression through mitosis to G1 and its substrates in mammalian cells include the inhibitor of anaphase onset (securin), cyclins (A2 and B1), other mitotic kinases (polo-like kinase-1, aurora kinases), and regulators of pre-replication complex formation (Cdc6, Geminin; Reed, 2003 and references therein). Cdc20 (Fang *et al.*, 1998; Kramer *et al.*, 1998) and Cdh1 (Kramer *et al.*, 1998) associate with APC and activate it. Cdh1-deficient chicken cells show delayed mitotic entry after DNA damage, indicating a requirement for APC (Sudo *et al.*, 2001), but the physiological relevance of this role for APC has not been addressed. In mammalian cells, the binding of Emi1 (early mitotic inhibitor 1) to Cdc20 and Cdh1 inhibits the ubiquitination activity of APC^{Cdc20} and APC^{Cdh1} (Reimann *et al.*, 2001b). In human cells, the Emi1 protein level is uniform throughout the cell cycle except for its absence from mitotic entry to G1. Emi1 restrains the activation of APC in interphase (Hsu *et al.*, 2002), and its degradation in prophase by F-box protein β -Trcp1 leads to the activation of APC (Guardavaccaro *et al.*, 2003; Margottin-Goguet *et al.*, 2003). Overexpression of Emi1 leads to mitotic defects (Guardavaccaro *et al.*, 2003; Margottin-Goguet *et al.*, 2003; Lehman *et al.*, 2006), and Emi1 expression is frequently up-regulated in human tumors (Hsu *et al.*, 2002; Margottin-Goguet *et al.*, 2003).

After γ -irradiation, asynchronous p21^{+/+} cells arrest in G2, whereas p21^{-/-} cells ultimately progress into mitosis (Bunz *et al.*, 1998; Andreassen *et al.*, 2001). Previous work showed p21 suppresses CDK activity after γ -irradiation in G2 (Bunz *et al.*, 1998; Andreassen *et al.*, 2001). In this study, we demonstrate a role for p21 in the maintenance of G2 arrest after DNA damage, through degradation of cyclins and key mitotic proteins. We show that p21 down-regulates Emi1 in cells that have initiated transient G2 arrest through the ATM/ATR pathway (Sarkaria *et al.*, 1999; Busby *et al.*,

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2000; Liu *et al.*, 2000; Zhao *et al.*, 2002; Brown and Baltimore, 2003). This Emi1 down-regulation activates APC, which in turn degrades key substrates that prevent the G2-arrested cells from entering mitosis. Our results offer an explanation for the prolonged maintenance of G2 arrest after DNA damage by p21.

MATERIALS AND METHODS

Cell Culture and Synchronization

HCT116 (parental and isogenic p21^{-/-}; parental and isogenic p53^{-/-}) are near diploid colorectal carcinoma cells and were kindly provided by Bert Vogelstein (Johns Hopkins University, Baltimore, MD) (Waldman *et al.*, 1996; Bunz *et al.*, 1998). Irradiation was delivered by a ¹³⁷Cs γ -irradiator. HCT116 cells released from S phase synchronization were irradiated with 12 Gy (Bunz *et al.*, 1998) because we found that at doses lower than 12 Gy, p21^{+/+} HCT116 cells do not completely arrest in G2, but continue to cycle. U2OS, MCF-7, MDA-MB231, MDA-MB435, and IMR90 cell lines were purchased from ATCC (Manassas, VA). For synchronization with hydroxyurea (HU), cells were treated for 20 h with 2 mM HU. For synchronization by double thymidine block, cells were treated with 2 mM thymidine for 18 h, released for 6 h from the block, and then treated with thymidine for an additional 18 h. To release cells from double thymidine or HU block, cells were washed twice with PBS and replated in drug-free medium.

Flow Cytometric Analysis

Cells were analyzed by two-dimensional (2D) flow cytometry using MPM-2 as a mitotic marker and propidium iodide (PI) as a marker of DNA content as described previously (Jascur *et al.*, 2005). Cells with less than 2N DNA content have not been gated out.

Cell Extracts and Antibodies

Cells were trypsinized, pooled with nonattached cells, washed, and lysed with buffer containing 50 mM Tris, 0.5% NP-40, 150 mM NaCl, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μ g/ml aprotinin, and 5 μ g/ml leupeptin. After incubation for 30 min on ice, the extracts were centrifuged at 14,000 rpm in a microfuge for 20 min at 4°C, and the supernatant was collected. The following antibodies were used: cyclin B1 (GNS1, sc-245), Cdc20 (p55CDC, sc-1906), vinculin (sc-5573), lamin B (M-20, sc-6217) and HRP-conjugated anti-goat IgG antibody (sc-2020) all from Santa Cruz Biotechnology (Santa Cruz, CA); Rb (554136) and p21 (556430) from PharMingen (San Diego, CA); securin (34-1500), polo-like kinase 1 (Plk1; 33-1700) and Emi1 (37-6600) from Zymed (South San Francisco, CA); polyclonal antibodies recognizing securin (J. A. Pintor-Toro, IRNA, Sevilla, Spain) and Emi1 (P. Jackson, Stanford University, Stanford, CA) were gifts. Other antibodies used were phospho-histone H2AX (4411-PC, Trevigen, Gaithersburg, MD), Cdh1 (MS-1116-PABX, Neomarkers, Fremont, CA), actin (A2066, Sigma, St. Louis, MO), phospho-histone-H3 (Upstate Biotechnology, Lake Placid, NY), HRP-conjugated anti-mouse (1050-05, Kirkegaard & Perry Laboratories, Gaithersburg, MD), Cy3 (705-166-147, Jackson ImmunoResearch, West Grove, PA) and HRP-conjugated anti-rabbit (AL13404, Biosource Technologies, Vacaville, CA). Polyclonal Cdk2-, Cdk1-, and cyclin A2-specific rabbit antibodies were as described (Fotadar *et al.*, 1996).

Kinase Assays

Histone H1 kinase assays were performed using 100 μ g cell extract as described previously (Fotadar *et al.*, 1996). For Plk1 kinase assays, 300 μ g cell extract was incubated with 10 μ l of packed protein A-agarose (Sigma) that had been preincubated with anti-Plk1 antibody. After 1 h at 4°C, the beads were washed four times with Plk1 immunoprecipitation buffer (50 mM Tris, 0.01% NP-40, 150 mM NaCl, 10 mM sodium fluoride, 1 mM sodium orthovanadate, 1 mM PMSF, 10 μ g/ml aprotinin, and 5 μ g/ml leupeptin) and once with Plk1 kinase buffer (10 mM HEPES, pH 7.5, 150 mM KCl, 10 mM MgCl₂, 2 mM DTT, and 1 mM EGTA). The kinase assays were performed in 16 μ l of kinase reaction mixture containing 166 μ M ATP, 5 μ Ci [γ -³²P]ATP, 1 μ g dephosphorylated casein (C8032, Sigma), and 10 μ l packed protein A-agarose for 20 min at 37°C.

Chromosome Spreads

Cells were trypsinized, washed twice with 75 mM KCl, and resuspended in 75 mM KCl. The cell suspensions were dropped onto coverslips and incubated at 37°C for 30 min. Coverslips were centrifuged at 230 $\times$ g for 1 min. Cells were fixed with 25% acetic acid, 75% methanol for 30 min at room temperature. After fixation, cells were dried and chromosomes stained with PI (10 μ g/ml) for 10 min.

Immunofluorescence Staining

Cells were processed for immunofluorescence as described previously (Jascur *et al.*, 2005). For lamin B staining, cells were fixed with methanol, permeabilized with 0.2% Triton X-100, incubated with lamin B-specific antibody followed by goat anti-mouse IgG (H+L)-Cy3 fluorochrome (Jackson ImmunoResearch). The DNA was stained with Hoechst for 10 min. High-resolution images were collected with a DeltaVision imaging system (Applied Precision, Issaquah, WA) built on an inverted microscope (IX70, Olympus, Melville, NY) using a 60 $\times$ NA 0.4 oil objective. Images were acquired at 0.2- μ m intervals in the z dimension and were deconvolved. Projections of multiple sections were created using SoftWoRx software (Applied Precision). Figures were processed in Photoshop CS2 (Adobe, San Jose, CA).

RNA Interference

Two Emi1 small interfering RNA (siRNA) duplexes AAA CUU GCU GCC AGU UCU UCA (Emi1 A) and AAG CAC UAG AGA CCA GUA GAC (Emi1 B; Hsu *et al.*, 2002) were synthesized (DharmaconResearch, Boulder, CO). Both Emi1 siRNA duplexes efficiently reduced the levels of Emi1. Emi1 B was used for the experiments shown in the article. Luciferase GL3 siRNA (CUU ACG CUG AGU ACU UCG A) was used as a negative control. p21 siRNA was as described (Jascur *et al.*, 2005). Cdh1 siRNA duplex UGA GAA GUC UCC CAG UCA G was synthesized (Dharmacon; Brummelkamp *et al.*, 2002). Four Rb siRNA duplexes AAA CAG AAG AAC CUG AUU UUA (Rb-1), AAG AUA CCA GAU CAU GUC AGA (Rb-2), AAG UUG AUA AUG CUA UGU CAA (Rb-3), and AAC CCA GCA GUU CGA UAU CUA (Rb-4) were synthesized (Dharmacon). All four Rb siRNA duplexes efficiently reduced the levels of Rb. Rb-3 was used for the experiments shown in the article. Cdk2 (45-1464) and Cdk1 (45-1454) siRNA were from Invitrogen (Carlsbad, CA). RNA interference (RNAi) experiments were performed as described (Jascur *et al.*, 2005).

Quantitative Real-Time PCR

mRNA expression levels were quantified by real-time quantitative PCR (ABI7900; Advanced Biotechnologies, Columbia, MD). Total RNA, 0.5 μ g, from each time point was reverse-transcribed into cDNA using the Superscript II RNase H-Reverse Transcriptase Kit from Invitrogen. Validated Taqman primers (Applied Biosystems, Foster City, CA) for Emi1 were used for the PCR reactions and the quantitative measurements. The results were normalized to the value observed for glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

RESULTS

DNA Damage Activates APC in a p21-dependent Manner

To study the role of p21 in the cellular response to DNA damage, we used parental HCT116 containing wild-type p21 (p21^{+/+}) and HCT116 cells in which the p21 gene was disrupted by homologous recombination (p21^{-/-}; Waldman *et al.*, 1996; Bunz *et al.*, 1998). The effect of p21 on γ -irradiation-induced G2 arrest was examined in cells synchronized by treatment with HU. At 2.5 h after release into fresh medium, 60-80% of the cells were bromodeoxyuridine positive, and there was no MPM2 signal (Supplemental Figure S1). Cells released from HU block continue to cycle normally, indicating the absence of DNA damage as a result of synchronization protocol (Supplemental Figure S1). To address the effect of irradiation on G2-to-M progression, cells released from HU synchronization for 2.5 h were irradiated. We noticed that although cyclin B1 protein disappeared in γ -irradiated p21^{+/+} cells, its levels remained relatively unchanged after irradiation of p21^{-/-} cells (Figure 1A). Cyclin B1 is degraded after APC-dependent ubiquitination (King *et al.*, 1995; Sudakin *et al.*, 1995; Kramer *et al.*, 1998). We therefore tested the stability of other known substrates of APC, such as cyclin A2 and Cdc20, after irradiation. Protein levels of both Cdc20 and cyclin A2 decreased in p21^{+/+} cells after irradiation, whereas their levels remained high in irradiated p21^{-/-} cells (Figure 1A). As expected, irradiation of p21^{+/+} cells resulted in an increase in p21 protein (Figure 1A). Histone H2AX phosphorylation, indicative of double-strand breaks, was observed after DNA damage (Figure 1A). Notably, APC substrates are seen in both irradiated p21^{+/+} and p21^{-/-} cells at early time points, and substrate degradation

Figure 1. DNA damage-induced activation of APC is p21-dependent. (A) Degradation of APC substrates in HU-synchronized γ -irradiated p21^{+/+} cells. p21^{+/+} and p21^{-/-} HCT116 cells were synchronized with HU, released for 2.5 h (indicated as 0 h), and then γ -irradiated (12 Gy). Cell extracts were examined for the stability of APC substrates (cyclin B1, Cdc20, and cyclin A2) and the levels of other proteins by immunoblotting with specific antibodies. Immunoblotting for vinculin is shown as a loading control. (B) Degradation of APC substrates in thymidine-synchronized γ -irradiated p21^{+/+} cells. p21^{+/+} and p21^{-/-} HCT116 cells were synchronized by double thymidine block, released for 2 h (indicated as 0 h), and then γ -irradiated (12 Gy). Stability of APC substrates and the levels of other proteins were examined by immunoblotting cell extracts with specific antibodies. (C) Inhibition of APC substrate degradation in irradiated p21^{+/+} cells by proteasome inhibitor MG132. p21^{+/+} HCT116 cells were synchronized with HU, released for 2.5 h (indicated as 0 h), and then γ -irradiated (12 Gy). Cells were treated with MG132 (10 μ M) at 9 h after irradiation. Stability of APC substrates and the levels of other proteins were examined by immunoblotting cell extracts with specific antibodies. (D) p21 down-regulation in p21^{+/+} cells leads to stabilization of APC substrates after DNA damage. p21^{+/+} HCT116 cells transfected with p21 siRNA were synchronized with HU, released for 2.5 h (indicated as 0 h), and then γ -irradiated (12 Gy). Stability of APC substrates and the levels of p21 protein were examined by immunoblotting cell extracts with specific antibodies.

in p21^{+/+} cells begins after 15 h. Protein levels of APC substrates were similarly reduced in p21^{+/+} but not p21^{-/-} cells irradiated after release from double thymidine block (Figure 1B). Importantly, the presence of proteasome inhibitor MG132 prevented the degradation of APC substrates (cyclin A2, cyclin B1, securin, and Cdc20) after γ -irradiation of HU-synchronized p21^{+/+} cells, consistent with the activation of APC after DNA damage (Figure 1C).

We likewise observed a failure of p21^{-/-} cells released from HU synchrony to degrade APC substrates after treatment of cells with adriamycin, a drug that intercalates with DNA and generates double-strand breaks (Supplemental Figure S2).

p21^{-/-} HCT116 cells are an isogenic line generated from p21^{+/+} HCT116 cells in which p21 was deleted by homol-

ogous recombination (Waldman *et al.*, 1996). To rule out the possibility that p21^{-/-} cells had acquired additional mutations that account for the effects observed here, we down-regulated p21 in p21^{+/+} HCT116 cells using specific siRNA. As expected, APC substrates were degraded in γ -irradiated control siRNA-transfected cells but not in p21 siRNA-transfected cells (Figure 1D).

p21-dependent APC Substrate Degradation Occurs in Cells Arrested in G2

The degradation of APC substrates commences after 15 h of irradiation. The low levels of Ser-10 phosphorylated histone H3 in irradiated p21^{+/+} cells at all time points up to 48 h suggest that the cells are arrested in G2 (Figure 1A). Phosphorylation of histone H3 at Ser-10 by Aurora kinase begins

in G2 and increases coincident with mitotic chromosome condensation (Crosio *et al.*, 2002). Therefore to test whether APC activation in irradiated p21^{+/+} cells is detected in cells arrested in G2, we monitored cells by 2D flow cytometry by staining for both DNA content and MPM2, a well-established mitosis-specific phospho-epitope and a mitotic marker (Davis *et al.*, 1983) to distinguish 4N cells that were in G2 from those that were in mitosis. Release from S phase synchrony without irradiation shows that both p21^{+/+} and p21^{-/-} HCT116 cells commence mitosis by 6–8 h, as evidenced by MPM2 staining of 4N cells (Figure 2A). In contrast to unirradiated cells, p21^{+/+} HCT116 cells irradiated after release from HU synchrony appear to be arrested in G2 and do not enter mitosis even at longer times after DNA damage (Figure 2B). Unlike p21^{+/+} cells, p21^{-/-} cells enter mitosis as evidenced by MPM2 staining of 4N cells. The percentage of p21^{-/-} cells in mitosis after irradiation is consistent with that reported for irradiated p21^{-/-} HCT116 in the absence of nocodazole (Bunz *et al.*, 1998).

To further confirm that p21^{+/+} HCT116 cells released from synchrony and irradiated are arrested in G2, we analyzed lamin breakdown, a marker of prometaphase entry (Georgatos *et al.*, 1997). Analysis of lamin staining every 2 h after release from synchrony and irradiation showed that p21^{+/+} cells mostly maintain circular lamin rings around single nuclei and show no deformed nuclei or micronucleation (Figure 2C). Lamin breakdown occurs after chromosome condensation (Georgatos *et al.*, 1997). Therefore, we ruled out the possibility that the irradiated p21^{+/+} cells were arrested in prophase by demonstrating the near absence of condensed chromosomes using chromosome spread technique and by staining DNA in fixed whole cells (Figure 2D; Beaudouin *et al.*, 2002). These results confirm that irradiated p21^{+/+} cells are predominantly arrested in G2 and that APC activation occurs in G2-arrested cells.

In contrast to irradiated p21^{+/+} cells, a substantial number of irradiated p21^{-/-} cells show condensed chromosomes (Figure 2D). Irradiated p21^{-/-} cells show a decrease in normal lamin rings around circular nuclei and a concomitant increase in micronucleation consistent with aberrant progression of cells through mitosis to interphase (Figure 2C). Micronucleation gives evidence that the cells have exited an abortive mitosis (Heddle and Carrano, 1977). γ -irradiated p21^{-/-} cells that exited mitosis and entered interphase with 4N DNA content as a result of improper mitosis (Bunz *et al.*, 1998; Andreassen *et al.*, 2001) underwent DNA synthesis and ultimately a subpopulation exhibited a DNA content of 8N (Figure 2B), as reported earlier (Bunz *et al.*, 1998).

These data show that both irradiated p21^{+/+} and p21^{-/-} cells are in G2 by 6–9 h. The irradiated p21^{+/+} cells are blocked in G2 and are competent to enter mitosis because the addition of caffeine, an inhibitor of ATR and ATM kinases (Sarkaria *et al.*, 1999) or UCN-01 that inhibits Chk1 (Busby *et al.*, 2000) and Chk2 kinases (Yu *et al.*, 2002), drives them into mitosis (Supplemental Figure S3). After a transient p21- and p53-independent G2 delay most likely mediated by Cdc25 phosphatase (Bartek and Lukas, 2001), the p21^{-/-} cells enter mitosis by 15 h, whereas the p21^{+/+} cells remain in G2 as a result of p21-dependent CDK inhibition.

Cdh1 Down-Regulation Overcomes p21-dependent DNA Damage-induced G2 Arrest

Our results suggest that p21-dependent APC activation may be an important contributor to prolonged G2 arrest after DNA damage by driving the degradation of APC substrates such as cyclins and other key mitotic components. To dem-

onstrate that APC is indeed involved in maintaining G2 arrest in DNA-damaged cells, p21^{+/+} cells were transfected with Cdh1 siRNA or control siRNA, synchronized with HU, released, and irradiated. Cells were monitored by 2D flow cytometry, staining for both DNA content and the mitotic marker MPM2. In comparison to irradiated control siRNA-transfected p21^{+/+} cells, which failed to progress to mitosis, irradiated Cdh1 siRNA-treated cells entered mitosis, as verified by MPM2 staining (Figure 3A). siRNA-mediated suppression of Cdh1 protein at the time of irradiation and 15 h after irradiation is shown in Figure 3B. This result shows that p21-dependent DNA damage induced G2 arrest can be attributed to APC activation.

p21-dependent Emi1 Down-Regulation Links APC Activation to DNA Damage

Emi1 is a negative regulator of APC activity (Reimann *et al.*, 2001a,b), and its presence maintains the stability of cyclins and other proteins required for entry into mitosis (Hsu *et al.*, 2002; Di Fiore and Pines, 2007). Given our results, we tested whether Emi1 protein levels were regulated after DNA damage in a p21-dependent manner. HU-synchronized p21^{+/+} and p21^{-/-} cells were γ -irradiated or treated with adriamycin after release, and the levels of Emi1 protein were tested at the times shown (Figure 4A). Interestingly, Emi1 protein was dramatically reduced in p21^{+/+} cells by 15 h after DNA damage, the time when irradiated cells were arrested in G2 (see Figure 2B). The decrease in Emi1 protein precedes the activation of APC and destruction of APC substrates in irradiated p21^{+/+} cells (Figures 1, A and B). In contrast, Emi1 protein levels remained relatively high even at longer times after DNA damage in p21^{-/-} cells (Figure 4A) and in p21 siRNA-treated p21^{+/+} cells (Figure 4C). We observed a similar failure of irradiated p21^{-/-} cells to down-regulate Emi1 after release from double thymidine synchrony (Figure 4B). A decrease in Emi1 and in APC substrates was observed upon irradiation of synchronized human breast adenocarcinoma MCF-7 cells, which contain wild-type p53 (Supplemental Figure S4A). Similarly, a decrease in Emi1 and in APC substrates was also observed upon irradiation of IMR90, a nontransformed human fibroblast cell line (Supplemental Figure S4B). Emi1 protein levels remained relatively high after DNA damage of p53^{-/-} HCT116 cells and two breast cell lines with mutant p53 (Runnebaum *et al.*, 1991), MDA-MB231, and MDA-MB435, as expected (Supplemental Figure S4, C and D). The one exception that we have found was U2OS cells, which show delayed decrease of Emi1 and retention of APC substrates after DNA damage (Supplemental Figure S4E).

p21^{+/+} HCT116 cells arrested in G2 after γ -irradiation have low Cdk2 activity, whereas p21^{-/-} HCT116 cells have high Cdk2 activity (Figure 4D), as reported previously (Bunz *et al.*, 1998; Andreassen *et al.*, 2001). Consistent with the low kinase activity in p21^{+/+} cells, we found an increased association of p21 with Cdk2 after γ -irradiation (Figure 4D). We considered the possibility that the high CDK activity in irradiated p21^{-/-} HCT116 cells may be linked to the failure to activate APC and arrest in G2. HU-synchronized p21^{-/-} cells were γ -irradiated after release, and the cells were treated with roscovitine, an inhibitor of Cdk1- and Cdk2-associated kinase activity (Knockaert *et al.*, 2002), at 4 h after irradiation. Addition of roscovitine to γ -irradiated p21^{-/-} cells resulted in down-regulation of Emi1 and the degradation of APC substrates (Figure 4E, left panel). Similar results were obtained when roscovitine was added to p21^{-/-} cells at 10 h after γ -irradiation (Supplemental Figure S5). The down-regulation of Emi1 and the degradation of APC sub-

Figure 2. APC substrate degradation after DNA damage occurs in G2-arrested cells. (A) Cell cycle profile of $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells synchronized with HU and released. Cells were not γ -irradiated. Cells were stained with propidium iodide (PI) and MPM2 antibody

strates by roscovitine were both prevented by the addition of the proteasome inhibitor LLnL (Supplemental Figure S5). The cell cycle profile of γ -irradiated $p21^{-/-}$ cells treated with roscovitine showed that the cells arrested in G2 and failed to enter mitosis at the time when control $p21^{-/-}$ cells were in mitosis (Figure 4E, right panel). Thus, APC substrate degradation occurs in G2. The effect is unlikely to be due to Erk1/Erk2 inhibition by roscovitine, as the IC_{50} of roscovitine for Cdk1-cyclin B and Cdk2-cyclin A is 0.45 and 0.7 μ M, respectively, and 14–35 μ M for Erk1/Erk2 (Knockaert *et al.*, 2002). Nevertheless, we directly confirmed that APC substrate degradation in roscovitine-treated $p21^{-/-}$ cells was not due to Erk1 or Erk2 inhibition. Treatment of γ -irradiated $p21^{-/-}$ cells with U0126, an inhibitor of Erk1/Erk2 activation, resulted in the inhibition of Erk1/Erk2 phosphorylation, but it did not lead to the degradation of APC substrates in irradiated cells (data not shown).

To corroborate the roscovitine results, we show that siRNA-mediated down-regulation of Cdk2 or Cdk1 in γ -irradiated $p21^{-/-}$ cells led to the down-regulation of Emi1 and degradation of APC substrates (Figure 4, F and G). Our results are thus consistent with a model in which continued inhibition of CDK activity after DNA damage in $p21^{+/+}$ cells leads to Emi1 down-regulation and APC activation during G2 arrest, causing the destruction of proteins required for mitotic entry.

p21-dependent Down-Regulation of Emi1 mRNA in G2-arrested Cells after DNA Damage

Emi1 exhibits a short half-life at the time of irradiation of cells (Figure 5A). To test whether Emi1 down-regulation after irradiation is linked to transcription control, γ -irradiated $p21^{-/-}$ cells in which Emi1 is stable, were treated with transcription inhibitor actinomycin D (ActD) 4 h after irradiation. ActD treatment resulted in rapid loss of Emi1 pro-

Figure 2 (cont). at the indicated times and examined by flow cytometry. Cells progress to 4N and become MPM2-positive by 6 h after release from HU synchrony. Numbers in MPM2 dot plots are percent of 4N population in mitosis. (B) Cell cycle analysis of $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells after γ -irradiation. Cells synchronized with HU were released for 2.5 h (indicated as 0 h) and then γ -irradiated (12 Gy). Cells were stained with PI and MPM2 antibody at the indicated times and examined by flow cytometry. $p21^{+/+}$ cells arrest in G2, whereas $p21^{-/-}$ cells continue to cycle and generate a subpopulation with 8N DNA content. Numbers in MPM2 dot plots are percent of 4N population in mitosis. (C) Absence of lamin breakdown in irradiated $p21^{+/+}$ cells. Synchronized and irradiated $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells obtained as in B were stained for lamin and DNA. The percent of cells ($n = 250$ /time point) with intact circular lamin rings around single nuclei, with no abnormalities, were scored (triangles). The percent of cells ($n = 250$ /time point) showing micronucleation were scored by both lamin and DNA staining (circles). The graph shows an average of two experiments. Representative fields of cells showing lamin staining, and micronucleation when present, after irradiation, is shown in the right panels. (D) Absence of chromosome condensation in irradiated $p21^{+/+}$ cells. $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells were synchronized and irradiated as in B. At the time of irradiation, nocodazole (200 ng/ml) was added to accumulate cells in mitosis. Chromosome spreads were prepared, and the DNA was stained with PI. Cells ($n = 250$ /experiment) with condensed chromosomes were scored. The table shows an average of two experiments. Two representative fields of chromosome spreads after irradiation are shown in the left panel. In addition, chromatin condensation was analyzed by staining methanol-fixed whole cells for DNA with Hoechst. Cells ($n = 250$ /experiment) with condensed chromatin were scored. The table shows an average of two experiments.

Figure 3. Down-regulation of Cdh1 overcomes $p21$ -dependent G2 arrest after DNA damage. (A) $p21^{+/+}$ HCT116 cells were transfected with control or Cdh1 siRNA for 24 h. Transfected cells were synchronized with nocodazole (0.05 μ g/ml) for 6 h, and mitotic cells collected by shake-off were released for 3 h and then synchronized with HU for 20 h. Cells released for 2.5 h (indicated as 0 h) were γ -irradiated (12 Gy). Cells were stained with PI and MPM2 antibody and examined by flow cytometry. Irradiated Cdh1 siRNA $p21^{+/+}$ cells enter mitosis as verified by MPM2 staining. The percent of cells in mitosis (indicated by the numbers in MPM2 dot plots) in the absence of Cdh1 is similar to the percent of unirradiated $p21^{+/+}$ cells in mitosis at 6 or 8 after release from G1/S synchronization (Figure 2A). (B) Cell extracts from A were examined for down-regulation of Cdh1 protein by immunoblotting with specific antibodies. Immunoblotting for actin is shown as loading control.

tein, suggesting that the sustained Emi1 levels in $p21^{-/-}$ cells after DNA damage may be linked to continuous transcription and protein turnover (Figure 5B). The effect of ActD on Emi1 down-regulation after DNA damage was dose dependent. To test whether Emi1 RNA levels were down-regulated in $p21^{+/+}$ cells when irradiation resulted in the loss of Emi1 expression, real-time PCR analysis was performed. The analysis revealed a striking reduction in Emi1 mRNA levels in HU-synchronized γ -irradiated $p21^{+/+}$ cells in comparison to $p21^{-/-}$ cells in which the Emi1 mRNA levels remain relatively unchanged after irradiation (Figure 5C). A reduction in Emi1 mRNA levels was also seen in γ -irradiated $p21^{+/+}$ cells released from double thymidine synchronization in comparison to similarly irradiated $p21^{-/-}$ cells in which the Emi1 mRNA levels remain relatively unchanged (Supplemental Figure S6).

$p21$ -dependent CDK inhibition after γ -irradiation is known to lead to the accumulation of hypophosphorylated Rb (Brugarolas *et al.*, 1999). Hypophosphorylation of Rb promotes Rb-E2F interaction, resulting in suppression of transcription (Frolov and Dyson, 2004). As Rb-E2F has been proposed to regulate Emi1 transcription (Hsu *et al.*, 2002), we analyzed the Emi1 mRNA levels in γ -irradiated Rb siRNA-transfected $p21^{+/+}$ HCT116 cells. Real-time PCR analysis revealed that Emi1 mRNA levels were maintained at significant levels in $p21^{+/+}$ cells transfected with Rb siRNA, as would be expected if Rb mediated suppression played a role in the decrease of Emi1 mRNA after irradiation

(Figure 5D). Further, Emi1 protein levels were substantially stabilized in Rb siRNA-transfected irradiated p21^{+/+} cells compared with control p21^{+/+} cells after irradiation (Figure 5E).

G2 Arrest Imposed by Emi1 siRNA after Irradiation Is Abrogated by Cdh1 siRNA

Results with p21^{+/+} HCT116 cells show that p21-mediated down-regulation of Emi1 in irradiated cells strongly correlates with activation of APC in G2-arrested cells and prevention of cells from entry into mitosis. To directly establish the role of Emi1 in G2 arrest after DNA damage, we assayed the effect of Emi1 down-regulation. Emi1 siRNA-transfected p21^{-/-} cells were synchronized with HU, released, and irradiated. The irradiated cells progressed to G2 but unlike control siRNA-treated cells, Emi1 siRNA-transfected p21^{-/-} cells were significantly delayed in entry into mitosis as evidenced by the lack of MPM2 staining at 15 h (Figure 6A). In parallel, we tested the effect of Emi1 siRNA on progression of cells from G2 through mitosis without irradiation. For this, Emi1 siRNA-transfected p21^{-/-} cells were synchronized with HU and released. Because HU-released cells enter mitosis by 6–8 h, peaking at 10 h (Figure 2A), we examined cells at 0, 10, and 12 h after release. Emi1 siRNA-transfected p21^{-/-} cells failed to progress to mitosis as evidenced by the lack of MPM2 staining (Figure 6B). This is consistent with previous work in which Emi1 siRNA was demonstrated to delay HeLa cells in G2 (Di Fiore and Pines, 2007). Examination of cells at 24 h after release confirms that cells fail to enter mitosis (Figure 6B). Emi1 siRNA-transfected p21^{+/+} show a similar failure to progress to mitosis without irradiation (Supplemental Figure S7).

We reasoned that if Emi1 down-regulation in G2 after DNA damage leads to a failure of cells to enter mitosis through its effect on APC, then down-regulation of both Emi1 and Cdh1 should allow entry of irradiated cells into mitosis. Indeed, down-regulation of both Cdh1 and Emi1 in p21^{-/-} cells, released from HU block and irradiated, re-

sulted in progression of irradiated cells to mitosis (Figure 6C). Quantitation of MPM2 staining shows that Emi1 depletion decreases the percent of MPM2 staining in irradiated p21^{-/-} cells, and Emi1 and Cdh1 double depletion brings the percent of MPM2 staining to that of control irradiated p21^{-/-} cells. Down-regulation of Emi1 in irradiated p21^{-/-} cells results in loss of APC mitotic substrates, thus corroborating failure of mitotic entry in p21^{-/-} cells (Figure 6D). Further, double depletion of Emi1 and Cdh1 in irradiated p21^{-/-} cells results in substantial stabilization of APC substrates (Figure 6D). These results show that G2 arrest observed upon Emi1 down-regulation is at least partly mediated through APC^{cdh1} activity.

At longer times after irradiation, the failure of Emi1 siRNA-transfected p21^{-/-} cells to progress to mitosis prevented the generation of cells with 8N DNA content in comparison to control siRNA-transfected and irradiated p21^{-/-} cells (Figure 6E, left panels). Consistent with these results, Emi1 siRNA-mediated down-regulation of Emi1 in irradiated p21^{-/-} cells resulted in APC activation and degradation of APC substrates, including cyclins (Figure 6E, right panels). Together, these results show that down-regulation of Emi1 after DNA damage prevents progression into mitosis by APC activation and degradation of proteins necessary for mitotic entry.

DISCUSSION

After DNA damage, p21^{+/+} cells stably arrest in G2, whereas p21^{-/-} cells ultimately progress into mitosis. We show here that DNA damage induces stable G2 arrest through down-regulation of Emi1 in p21^{+/+} cells but not in p21^{-/-} cells. Down-regulation of Emi1 activates APC, which in turn leads to the degradation of APC substrates, including cyclins, in γ -irradiated p21^{+/+} cells. The loss of critical mitotic proteins blocks cells from proceeding into mitosis. By contrast, Emi1 protein levels are unchanged in γ -irradiated p21^{-/-} cells, and APC remains inactive, causing the retention of APC substrates. Thus, irradiated p21^{-/-} cells fail to sustain G2 arrest and progress into mitosis. We conclude that stable p21-dependent G2 arrest after DNA damage requires Emi1 down-regulation.

We confirmed the involvement of Emi1 down-regulation and APC activation in G2 arrest after DNA damage in two ways. First, we demonstrated that down-regulation of Cdh1 in irradiated p21^{+/+} cells overcame the G2 arrest and led to the progression of cells into mitosis. Second, we showed that Emi1 siRNA inhibits the entry of irradiated p21^{-/-} cells into mitosis and that this inhibition can be overcome if Cdh1 is also down-regulated in the cells. Treatment of γ -irradiated p21^{-/-} cells with the CDK inhibitor roscovitine, as well as down-regulation of CDKs with siRNA, leads to a decrease in Emi1 levels, activates the APC, and arrests cells in G2 phase.

We have examined whether APC-dependent substrate degradation in irradiated p21^{+/+} cells occurs in G2-arrested cells. Our criteria for G2 arrest were the near absence of the following: nuclear envelope breakdown, MPM2 staining, histone H3 phosphorylation, and condensed chromosomes. Although it is possible that a small fraction of irradiated p21^{+/+} cells proceed to mitosis, this fraction cannot account for the nearly complete degradation of APC substrates observed in irradiated p21^{+/+} cells. We therefore favor the conclusion that APC substrate degradation in irradiated p21^{+/+} cells occurs predominantly in G2-arrested cells. The entry of irradiated p21^{-/-} cells into and out of mitosis has been demonstrated convincingly by Bunz *et al.* (1998), and we have confirmed this finding.

Figure 4 (cont). were released for 2.5 h (indicated as 0 h) and then γ -irradiated (12 Gy). Cdk2-associated kinase activity was measured in Cdk2 immunoprecipitates (IP) of cell extracts prepared at different times after γ -irradiation (top row). Histone H1 was used as a substrate. Cdk2 immunoprecipitates of cell extracts were immunoblotted with p21-specific antibody (middle row). p21 protein levels in the cell extracts are shown in the bottom row. (E) Roscovitine leads to the degradation of APC substrates in γ -irradiated p21^{-/-} cells. p21^{-/-} cells synchronized and γ -irradiated as in A were treated with 50 μ M roscovitine (Rosco) 4 h after γ -irradiation, and roscovitine was present for the duration of the experiment. Cell extracts were examined for the degradation of APC substrates by immunoblotting with specific antibodies (left panels). Cell cycle profile of roscovitine-treated p21^{-/-} cells stained with PI and MPM2 antibody is shown in right panels. Roscovitine-treated p21^{-/-} cells fail to enter mitosis, as evidenced by the lack of MPM2 staining. Numbers in MPM2 dot plots are percent of 4N population in mitosis. (F) Emi1 down-regulation in irradiated p21^{-/-} cells upon down-regulation of Cdk2 or Cdk1. p21^{-/-} HCT116 cells transfected with control, Cdk2, or Cdk1 siRNA were synchronized with HU, released for 2.5 h (indicated as 0 h), and then γ -irradiated (12 Gy). Cell extracts were examined for Emi1 down-regulation by immunoblotting with specific antibody. Down-regulation of Cdk1 and Cdk2 proteins by the respective siRNAs in cell extracts obtained at 0 h is shown in the bottom panels. (G) APC substrate degradation in irradiated p21^{-/-} cells upon down-regulation of Cdk2 or Cdk1. Cell extracts obtained as in F were examined for the degradation of APC substrates cyclin A2 and B1 by immunoblotting with specific antibodies.

Figure 5. p21-dependent Emi1 down-regulation of Emi1 mRNA in G2-arrested cells after DNA damage. (A) Emi1 protein has a short half-life. p21^{+/+} and p21^{-/-} cells synchronized with HU were released for 2.5 h (indicated as 0 h) and then γ -irradiated. Cycloheximide (CHX) was added immediately after irradiation. Emi1 protein levels in cell extracts prepared at different times after addition of CHX were analyzed by immunoblotting. (B) Inhibition of transcription leads to loss of Emi1 protein in irradiated p21^{-/-} cells. p21^{-/-} cells synchronized with HU were released for 2.5 h (indicated as 0 h) and then γ -irradiated (12 Gy). The cells were treated with actinomycin D (1 μ M) 4 h after irradiation, and the samples collected at different times after irradiation were analyzed for Emi1 protein. (C) mRNA levels of Emi1 are reduced in irradiated p21^{+/+} cells. p21^{+/+} and p21^{-/-} cells synchronized with HU were released for 2.5 h (indicated as 0 h) and then γ -irradiated (12 Gy). mRNA was isolated from synchronized and γ -irradiated p21^{+/+} and p21^{-/-} cells, and the relative Emi1 mRNA levels were determined by quantitative real-time PCR. GAPDH was used as an internal control. Mean $\pm$ SD from four different experiments is shown. Emi1 transcript levels in p21^{+/+} cells at 0 time was given an arbitrary value of 100%. (D) Emi1 mRNA is stabilized in irradiated Rb siRNA-treated p21^{+/+} cells. p21^{+/+} HCT116 cells were transfected with Rb siRNA (Rb) or control siRNA (Ctrl) 6 h before synchronizing cells with HU. The cells were released from HU block for 2.5 h (0 h) and then γ -irradiated (12 Gy). Emi1 mRNA levels were determined by quantitative real time PCR as in C. Mean $\pm$ SD from four different experiments is shown. Cell extracts obtained at the time of release (0 h) were examined for Rb protein (bottom panel). (E) Emi1 is stabilized in irradiated p21^{+/+} cells with down-regulated Rb. p21^{+/+} HCT116 cells were transfected with Rb siRNA or control siRNA (Ctrl) as in D. The stability of Emi1 after irradiation was examined by immunoblotting cell extracts with the specific antibodies.

There is a delay between Emi1 down-regulation and the degradation of APC substrates. It is possible that this delay in degradation of substrates may originate from the competing effects of deubiquitinating enzymes (DUBs) that rapidly remove ubiquitin from substrates and delay degradation (Amerik and Hochstrasser, 2004) and activation of degradation through APC activity. For example, USP28 is a DUB that controls the abundance of several checkpoint proteins that otherwise become unstable in response to irradiation (Zhang *et al.*, 2006). DUBs might balance opposing APC E3 activity in DNA-damaged cells for a period of time until the balance of these opposing pathways changes in favor of APC. It is possible that this shift of balance does not occur in some cells (see Supplemental Figure S4E).

We have shown that Emi1 protein has a short half-life in cells released from S phase synchrony. This prompted us to test whether CDK inhibition in G2 might act to repress Emi1 mRNA after DNA damage. Indeed, Emi1 mRNA levels are dramatically reduced in γ -irradiated p21^{+/+} cells, consistent with the reduction of Emi1 protein after DNA damage. Although Emi1 mRNA levels remain relatively unchanged after irradiation of p21^{-/-} cells, CDK inhibition by roscovitine leads to the reduction of Emi1 mRNA to a level <0.2% of control irradiated p21^{-/-} cells. A role for Rb in regulating Emi1 transcript levels has been suggested from work in Rb, p107, p130 triple knockout mouse embryonic fibroblasts (Balciunaite *et al.*, 2005). Exogenous E2F1 or E2F3 were shown to induce Emi1 gene expression, whereas transfection with constitutively active Rb represses Emi1 gene expression

(Hsu *et al.*, 2002). We observe significant stabilization of Emi1 mRNA after down-regulation of Rb in irradiated p21^{+/+} cells, thereby suggesting that DNA damage induced transcriptional repression of Emi1 in p21^{+/+} cells may be mediated in part through Rb. Further investigation using chromatin immunoprecipitation- and Emi1-specific PCR is in progress to address how the Emi1 promoter is regulated after DNA damage.

It is interesting that Emi1 decrease over the long term in nonirradiated and in irradiated cells has different consequences. Emi1 decrease in nonirradiated cells leads to rereplication (Di Fiore and Pines, 2007), whereas we show that decrease in Emi1 in G2-irradiated cells results in long-term G2 arrest. Di Fiore and Pines show that although cyclins A2 and B are reduced, cyclin E is increased in Emi1 down-regulated cells, and this may be sufficient to drive rereplication. Our results show that cells respond differently to DNA damage. We have shown that Cdk2-associated kinase activity is decreased in irradiated p21^{+/+} cells. We also have found that cyclin E-associated kinase activity is inhibited after DNA damage in p21^{+/+} cells (data not shown). Most importantly, p21^{+/+} cells do not progress toward rereplication because the degradation of critical mitotic proteins prevents their progression to mitosis.

Normal cell division is critical for maintaining genome ploidy. p21^{-/-} HCT116 cells become polyploid after DNA damage because the cells enter mitosis, but fail to execute mitosis properly (Bunz *et al.*, 1998; Andreassen *et al.*, 2001). As a consequence, the cells exit mitosis and enter interphase

Figure 6. G2 arrest imposed by Emi1 siRNA after irradiation is abrogated by Cdh1 siRNA. (A) Down-regulation of Emi1 arrests irradiated p21^{-/-} cells in G2. HCT116 cells were transfected with Emi1 siRNA (Emi1) or control siRNA (Ctrl) 6 h before synchronization with HU.

with 4N DNA content. Our finding that APC substrates, such as securin and cyclin B, are not degraded in irradiated p21^{-/-} cells even after mitotic exit further explains the failure of the cells that enter M phase to execute mitosis properly, as degradation of APC substrates is necessary for separation of sister chromatids in anaphase and for correct mitotic exit to form two daughter cells (reviewed in Peters, 2002).

The role of APC in the DNA damage checkpoint has not been extensively studied in mammalian cells. APC activity is not high in G2 phase (Fang *et al.*, 1998; Kramer *et al.*, 1998). Here we show that APC activation in G2 phase leads to stable arrest in irradiated p21^{+/+} cells. We show that down-regulation of Emi1 after DNA damage leads to APC activation. Emi1 can competitively inhibit the binding of substrates to Cdc20 and Cdh1 (Reimann *et al.*, 2001b). In addition, low CDK activity after DNA damage may facilitate activation of APC^{Cdh1}, as CDK phosphorylated Cdh1 does not activate APC (Zachariae *et al.*, 1998; Jaspersen *et al.*, 1999; Lukas *et al.*, 1999; Kramer *et al.*, 2000). The ability of Cdh1-depleted cells to enter mitosis after DNA damage as reported here and by Sudo *et al.* (2001) is intriguing given that CDK activity is inhibited after irradiation.

We further show that APC activation, which leads to degradation of APC substrates including cyclins, is p21-dependent. Sudo *et al.* (2001) have shown APC^{Cdh1} activation after DNA damage, but neither the mechanism of APC activation nor its physiological relevance were addressed. In their study, APC activation did not alter the levels of APC substrates examined. While this manuscript was in preparation,

Bassermann *et al.* (2008) reported that APC^{Cdh1} targets Plk1 after DNA damage but not 13 other APC substrates tested, including cyclins. They proposed that there are two pools of APC^{Cdh1} in G2 cells. One pool that targets substrates other than Plk1 is inactive both in the presence and absence of DNA damage. The other pool of APC^{Cdh1}, which targets Plk1, is activated after DNA damage and controls the G2 checkpoint. In contrast to Sudo *et al.* (2001) and Bassermann *et al.* (2008), we find that APC activation after DNA damage leads to the degradation of APC substrates, including cyclin A2 and B1. Our results further show that APC activation is observed late, between 15 and 24 h after irradiation, in contrast to the activation of APC^{Cdh1} within hours of DNA damage, as reported in HeLa cells, and the early activation of a Plk1-specific APC^{Cdh1} pool in U2OS cells. Although we also find that APC activation after DNA damage leads to Plk1 degradation (Figure 1C, Supplemental Figure S8), this degradation occurs late, in keeping with the timing of APC activation in our study. In contrast to Bassermann *et al.*, we observe that the Plk1 protein levels and Plk1 activity are maintained up to 24 h after irradiation (Supplemental Figure S8B). A decrease in Emi1 and in APC substrates upon DNA damage is not cell type-specific, as we observed these effects in several human cell lines, depending on their p53 or p21 status. Additional work is required before issues, such as whether there are two pools of active APC^{Cdh1} after DNA damage and whether Plk1 activity is a target of the early active APC, are resolved.

In summary, p21-dependent down-regulation of Emi1 in G2 phase after DNA damage plays an essential role in sustained G2 arrest and our work thus offers a new insight into the mechanism of p21-dependent cell cycle arrest after DNA damage.

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Figure 6 (cont). Synchronized cells were released from HU for 2.5 h (indicated as 0 h) and then γ -irradiated (12 Gy). Cells were stained with PI and MPM2 antibody and examined by flow cytometry. Numbers in MPM2 dot plots are percent of 4N population in mitosis. Cell extracts obtained at 0 h were examined for Emi1 protein (bottom panel). (B) Down-regulation of Emi1 in p21^{-/-} cells blocks mitotic entry. Cells were transfected with Emi1 siRNA (Emi1) or control siRNA (Ctrl) 6 h before synchronization with HU. The cells were released from HU and examined by flow cytometry after staining with PI and MPM2. Cells were not γ -irradiated. Numbers in MPM2 dot plots are percent of 4N population in mitosis. Cell extracts obtained at the time of release (indicated as 0 h) were examined for Emi1 protein (bottom panel). (C) DNA damage induced G2 arrest by down-regulation of Emi1 in p21^{-/-} cells depends on Cdh1. Cells were transfected with Emi1 siRNA (Emi1), control siRNA (Ctrl), or both Emi1 and Cdh1 siRNAs 6 h before synchronization with HU. Cells released from HU for 2.5 h (indicated as 0 h) were γ -irradiated (12 Gy) and were examined by flow cytometry after staining with PI and MPM2. Cell extracts obtained at 0 h were examined for Emi1 and Cdh1 protein (right panels). Numbers in MPM2 dot plots are percent of 4N population in mitosis. (D) Emi1 down-regulation in irradiated p21^{-/-} cells results in loss of APC substrates, and double depletion of Emi1 and Cdh1 overcomes this effect. Cells were transfected with Emi1 siRNA (Emi1), control siRNA (Ctrl), or both Emi1 and Cdh1 siRNAs (Emi1/Cdh1) 6 h before synchronization with HU as in C. Cells were released from HU for 2.5 h (indicated as 0 h) and then γ -irradiated (12 Gy). The stability of APC substrates after irradiation was examined by immunoblotting cell extracts with the indicated antibodies. Down-regulation of Cdh1 and Emi1 protein after siRNA transfection is shown in the bottom panels. (E) DNA damage-induced generation of cells with 8N DNA content fails to occur in p21^{-/-} cells with down-regulated Emi1. Cells were transfected with siRNA, synchronized, and irradiated as in A. Flow cytometric analysis of cells transfected with Emi1 siRNA or control siRNA is shown in the left panels. Numbers in the DNA plots indicate the percent of 8N population. Cell extracts were examined for Emi1 protein and the stability of APC substrates (right panels).

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