

DNA damage induces downregulation of histone gene expression through the G₁ checkpoint pathway

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Activation of the G₁ checkpoint following DNA damage leads to inhibition of cyclin E-Cdk2 and subsequent G₁ arrest in higher eucaryotes. Little, however, is known about the molecular events downstream of cyclin E-Cdk2 inhibition. Here we show that, in addition to the inhibition of DNA synthesis, ionizing radiation induces downregulation of histone mRNA levels in mammalian cells. This downregulation occurs at the level of transcription and requires functional p53 and p21^{CIP1/WAF1} proteins. We demonstrate that DNA damage induced by ionizing radiation results in the suppression of phosphorylation of NPAT, an *in vivo* substrate of cyclin E-Cdk2 kinase and an essential regulator of histone gene transcription, and its dissociation from histone gene clusters in a p53/p21-dependent manner. Inhibition of Cdk2 activity by specific inhibitors in the absence of DNA damage similarly disperses NPAT from histone gene clusters and represses histone gene expression. Our results thus suggest that inhibition of Cdk2 activity following DNA damage results in the downregulation of histone gene transcription through dissociation of NPAT from histone gene clusters.

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Introduction

To preserve the integrity of the genome, mammalian cells respond to DNA damage by activating checkpoint pathways. These pathways are crucial for cellular survival, and their perturbation can cause severe diseases including cancer

(Zhou and Elledge, 2000; McGowan, 2002; Bartek and Lukas, 2003; Shiloh, 2003). Activation of the G₁ checkpoint triggered by ionizing radiation (IR) requires the participation of a number of signaling molecules. Following IR, the tumor suppressor protein p53 is stabilized and activated through events initiated by ATM, a protein kinase whose gene is mutated in the human disease ataxia-telangiectasia (Kastan *et al.*, 1992; Kuerbitz *et al.*, 1992; Zhou and Elledge, 2000; Bartek and Lukas, 2003; Shiloh, 2003). p53 then induces the expression of the cyclin-dependent kinase (CDK) inhibitor p21, which binds to and inhibits the activities of cyclin E/A-Cdk2 kinases (El-Deiry *et al.*, 1993; Gu *et al.*, 1993; Harper *et al.*, 1993; Xiong *et al.*, 1993; Dulic *et al.*, 1994). As cyclin E- and cyclin A-Cdk2 are required for S-phase entry (Fang and Newport, 1991; Girard *et al.*, 1991; Tsai *et al.*, 1993; van den Heuvel and Harlow, 1993; Jackson *et al.*, 1995; Ohtsubo *et al.*, 1995), DNA damage by IR thus induces a G₁ arrest. Although the signaling pathways that lead to the inhibition of Cdk2 kinase have been extensively studied, the critical targets of this kinase that mediate the DNA damage response remain to be determined.

Chromosome duplication during S phase of the eucaryotic cell cycle requires both replication of genomic DNA and the synthesis of histones, which are assembled with the newly synthesized DNA into chromatin fibers. In mammalian cells, the majority of histone synthesis occurs in S phase and is tightly coupled to the rate of DNA synthesis. The quantity of histones synthesized in mammalian cells is primarily determined by the abundance of histone mRNA, which in turn is controlled by multiple mechanisms including regulation of transcription, pre-mRNA processing and mRNA stability (Heintz, 1991; Osley, 1991; Stein *et al.*, 1992; Marzluff and Duronio, 2002).

We and others have previously shown that the cyclin E-Cdk2 substrate NPAT concentrates at a few nuclear foci that are associated with the histone gene clusters *in vivo* (Zhao *et al.*, 1998, 2000; Ma *et al.*, 2000). Ectopic expression of NPAT activates transcription of multiple histone promoter constructs as well as the endogenous histone genes ((Ma *et al.*, 2000; Zhao *et al.*, 2000; J Zhao *et al.*, unpublished observations). This NPAT-mediated transcriptional activation is regulated by cyclin E-Cdk2-mediated phosphorylation (Ma *et al.*, 2000; Zhao *et al.*, 2000). Inhibition of NPAT expression by siRNA (Gao *et al.*, 2003) impairs histone gene expression. Thus, NPAT is crucial for the activation of histone gene expression and links the cell cycle machinery to the regulation of histone synthesis.

As histone synthesis is coupled with DNA synthesis during S phase, and histone gene transcription is regulated by the cyclin E-Cdk2 complex through NPAT phosphorylation, it is possible that DNA damage may also trigger downregulation of histone gene expression. We therefore investigated the regulation of histone gene expression after DNA damage by IR. In this study, we show that histone gene expression

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is downregulated, in parallel with the inhibition of DNA synthesis, upon IR. A deficiency for p53 or p21 alleviates this downregulation. In an effort to explore the underlying mechanism, we found that phosphorylation of NPAT is inhibited and the NPAT protein becomes dispersed from histone gene clusters after IR. We suggest that DNA damage induces downregulation of histone gene expression through inhibition of cyclin E-Cdk2 activity, which in turn interferes with NPAT function. Consistently, inhibition of Cdk2 activity with specific inhibitors in the absence of DNA damage also causes dissociation of NPAT protein from histone clusters, repression of histone gene expression and inhibition of entry into S phase. Thus, our results indicate that the G₁ checkpoint regulates not only DNA synthesis but also histone gene expression. Moreover, these results identify the regulation of NPAT function as a downstream event of Cdk2 inhibition during the G₁ checkpoint activation.

Results

DNA damage induces downregulation of histone gene expression

To test directly whether DNA damage might cause decreased histone gene expression, we utilized HCT116 cells, a human colorectal cancer cell line that contains wild-type p53 and p21 genes and possesses an apparently normal G₁ checkpoint (Waldman *et al*, 1995; Bunz *et al*, 1998). The cells were treated with IR, and the protein levels of p53 and p21, cyclin E and Cdk2 associated kinase activity and the histone mRNA levels before and after irradiation were analyzed. As reported previously (Waldman *et al*, 1995; Bunz *et al*, 1998), DNA damage caused by IR resulted in stabilization of p53, increased synthesis of the CDK inhibitor p21, inhibition of cyclin E and Cdk2 associated kinase activity and cell cycle arrest (Figure 1A–C). Steady-state mRNA levels of the histone genes, encoding both the linker histone H1 and the four core histones, were significantly decreased after DNA damage as assayed by Northern blotting (Figure 1D). Moreover, the expression of all five subtypes of histone mRNA was reduced by a similar degree (Figure 1D). Decreases in histone mRNA levels were also observed in primary human fibroblast WI38 cells following IR (data not shown). Thus, IR-induced DNA damage results in coordinated downregulation of the expression of multiple histone genes in cultured human cells.

IR-induced repression of histone gene expression occurs in parallel with the inhibition of DNA synthesis

Repression of histone gene expression after DNA damage may result indirectly from the inhibition of DNA synthesis, as it has previously been shown that sustained histone gene expression requires continued DNA synthesis (Heintz *et al*, 1983; Sittman *et al*, 1983; Baumbach *et al*, 1987). Alternatively, the inhibition of histone gene expression may be caused by a more direct DNA-damage-generated signaling event that is independent of DNA synthesis. To distinguish between these two possibilities, we analyzed cell cycle distribution and histone mRNA levels at multiple time points after IR, and compared the change in S-phase population with levels of histone mRNA abundance. IR caused increases in p53 and p21 proteins over time. By 6 h after irradiation, p21 protein levels had increased significantly, cyclin E-Cdk2

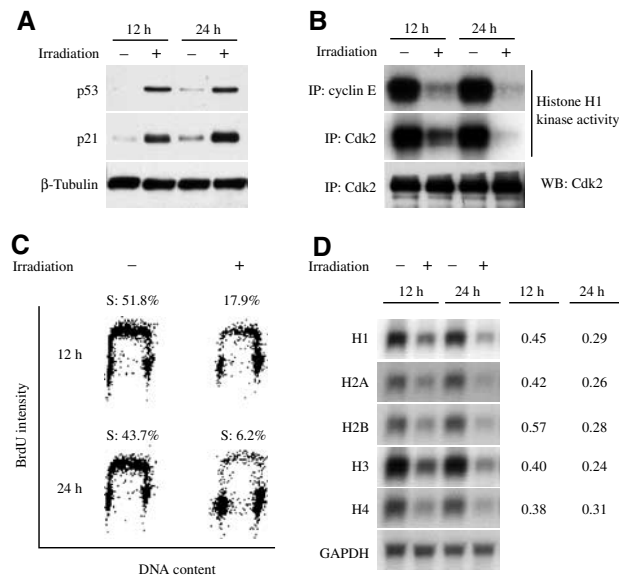


Figure 1 IR induces downregulation of histone gene expression. (A) Western blot analysis of p53 and p21 proteins. HCT 116 cells were untreated or irradiated with 6 Gy. At the indicated times after irradiation, the cells were harvested and the protein levels of p53 and p21 in the cell extract were analyzed. The analysis of β -tubulin was used as the loading control. (B) Analysis of cyclin E-Cdk2 kinase activity. HCT 116 cells were treated as described in panel A. The kinase activity associated with cyclin E or Cdk2 was assayed as described in Materials and methods. A Western blot of immunoprecipitated Cdk2 was used as the loading control. (C) Cell cycle profiles of cells treated with or without IR. HCT116 cells were treated as in panel A. BrdU was added to the culture medium 30 min before the cells were harvested for the FACS analysis as described in Materials and methods. (D) Analysis of histone mRNA levels. HCT116 cells were treated as described in panel A, and the mRNA levels of five subtypes of histones were analyzed on Northern blots. The signal of GAPDH mRNA was used as the loading control. The relative levels of histone mRNAs after IR, in comparison with histone mRNA levels in the nonirradiated cells, are shown on the right.

kinase activity had concomitantly decreased and a reduction of the S-phase population (BrdU-positive cells) was detectable at 8 h after irradiation (Figure 2A and B). The HCT116 cells carry a mutation in the mismatch repair system that is required for activation of the S-phase checkpoint (Brown *et al*, 2003). Therefore, there is no immediate decrease in DNA synthesis following irradiation in these cells. The decrease in S-phase population thus results from the activation of the G₁ checkpoint. As shown in Figure 2C, IR induced a gradual decrease of histone mRNA levels. Importantly, the decrease of histone mRNAs occurred no later than the decrease of S phase (Figure 2D). These results suggest that repression of histone gene expression is directly induced by DNA damage signals rather than as an indirect consequence of DNA synthesis inhibition.

p53 and p21 contribute to the downregulation of histone gene expression induced by ionizing radiation

It has previously been shown that activation of the G₁ checkpoint by IR requires functional p53 and p21 (Brugarolas *et al*, 1995; Deng *et al*, 1995; Waldman *et al*, 1995). To determine whether the inhibition of histone gene expression after IR also depends on these proteins, we

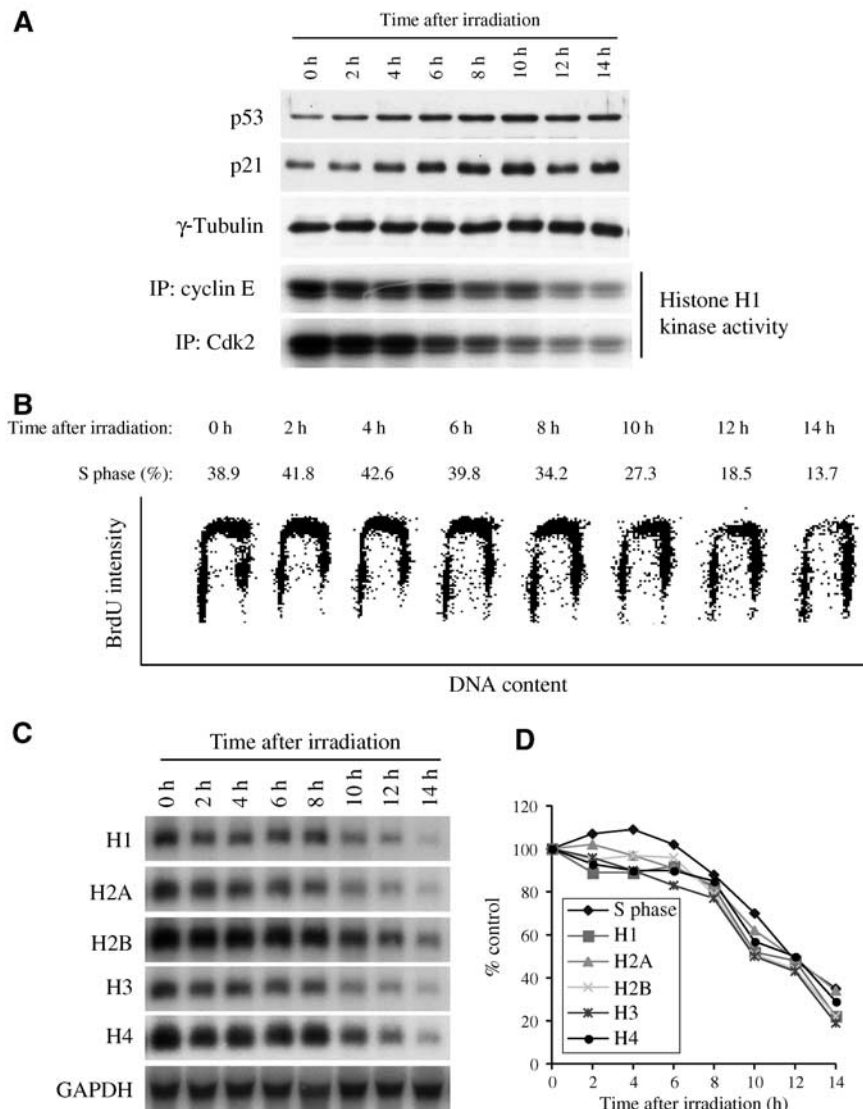


Figure 2 IR-induced downregulation of histone gene expression parallels inhibition of DNA synthesis. HCT116 were γ -irradiated (12 Gy) and harvested at the indicated times for the analysis of p53 and p21 protein levels and cyclin E–Cdk2 kinase activity (A), cell cycle distribution (B) and histone mRNA levels (C) as described in Figure 1. (D) Comparison of changes in S-phase population and histone mRNA levels after IR. The data from FACS analysis shown in panel B are expressed as percentage of control for S phase, and the Northern blot signals from panel C were quantitated using a phosphorimager and expressed as percentage of control for histone mRNAs. Data from nonirradiated samples (0 h) are set at 100%.

employed mutant derivatives of the HCT116 cell line in which either the p53 gene or the p21 gene had been disrupted by homologous recombination (Waldman *et al*, 1995; Bunz *et al*, 1998). We analyzed how histone mRNA levels change in these cells after IR. As reported previously (Waldman *et al*, 1995; Bunz *et al*, 1998), IR does not cause G₁-phase arrest in the p53- or p21-deficient HCT116 cells (data not shown, Figure 3B and D, and Supplementary Figure 1). Interestingly, the DNA damage-induced inhibition of histone gene expression, seen in the parental HCT116 cells (Figures 1 and 2), is largely alleviated in the p53- and p21-deficient cells, especially at early time points (4–10 h) following irradiation (Figure 3). Thus, similar to their roles in the inhibition of DNA synthesis at the G₁ checkpoint, the p53 and p21 proteins are critical for the downregulation of histone gene expression following DNA damage inflicted by IR.

DNA damage results in inhibition of histone gene transcription

Next, we asked whether IR downregulates histone mRNA abundance at the level of transcription. We thus carried out nuclear run-on assays (Ausubel *et al*, 1991; Carey and Smale, 2000) using nuclei from γ -irradiated cells as well as from nonirradiated control cells, and compared the rate of histone gene transcription in these samples. While IR resulted in the inhibition of histone gene transcription in the parental HCT116 cells at 10 h following the treatment, this effect was essentially relieved in the p21-deficient HCT116 cells at the same time point (Figure 4). These data demonstrate that the decrease of histone mRNA levels after DNA damage results, at least in part, from the inhibition of histone gene transcription, and that IR-induced inhibition of histone gene transcription depends on p21 protein.

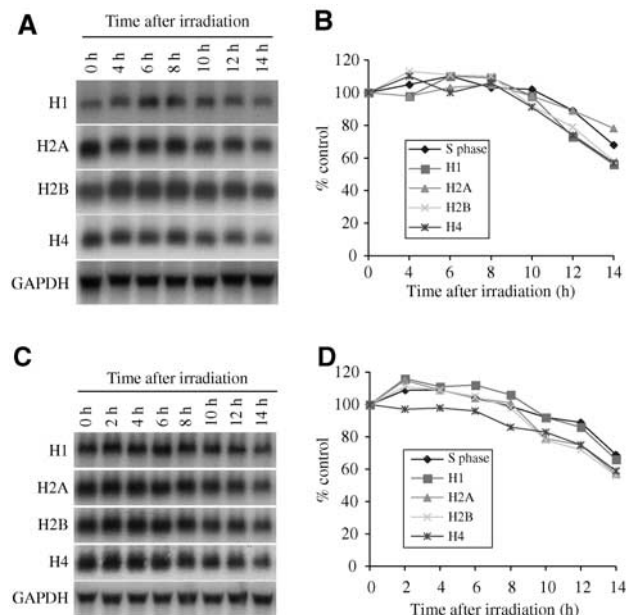


Figure 3 Effects of p21 and p53 deficiency on the IR-induced downregulation of histone gene expression. p21^{-/-} or p53^{-/-} HCT cells were treated with IR (12 Gy). At the indicated times after irradiation, histone RNA levels were analyzed on Northern blots (A, C), and the cell cycle distribution was analyzed by FACS. The percentage of control is calculated as described in Figure 2D (B, D). (A, B) Data from p21^{-/-} cells, and (C, D) data from p53^{-/-} cells.

DNA damage induced by ionizing radiation results in the suppression of NPAT phosphorylation

p21 protein interacts with CDKs and inhibits their activity (Gu *et al*, 1993; Harper *et al*, 1993; Xiong *et al*, 1993). The observation that inhibition of histone gene transcription following DNA damage requires functional p21 suggests that a CDK-controlled event is involved in this downregulation. The cyclin E-Cdk2 substrate NPAT associates with histone gene clusters *in vivo* and acts as a transcriptional regulator of histone genes (Zhao *et al*, 1998, 2000; Ma *et al*, 2000; Gao *et al*, 2003). It is thus possible that DNA damage induces downregulation of histone gene expression by modulating NPAT function. To investigate this possibility, we first examined the effect of IR on NPAT phosphorylation. It has previously been shown that NPAT is phosphorylated by cyclin E-Cdk2 both *in vitro* and *in vivo* (Ma *et al*, 2000; Zhao *et al*, 2000), and that phosphorylation of NPAT by cyclin E-Cdk2 at residues Ser 775 and Ser 779 causes a mobility shift of NPAT on SDS-PAGE gel (Ma *et al*, 2000). We employed a mobility shift assay to assess the phosphorylation state of NPAT after IR. While the majority of NPAT protein in growing HCT 116 cells was in the slower-migrating phosphorylated form, most (if not all) of the detectable NPAT protein converted to the unphosphorylated (faster-migrating form) after DNA damage (Figure 5, left panel). Treatment of NPAT from nonirradiated cells with lambda protein phosphatase also caused conversion of the slower-migrating form to the faster-migrating form (Figure 5), confirming that alteration in the gel mobility most likely resulted from a change in the NPAT phosphorylation state. Thus, IR leads to inhibition of NPAT phosphorylation.

In contrast to the mobility changes of NPAT in the parental HCT 116 cells after IR, no such effect was observed in either

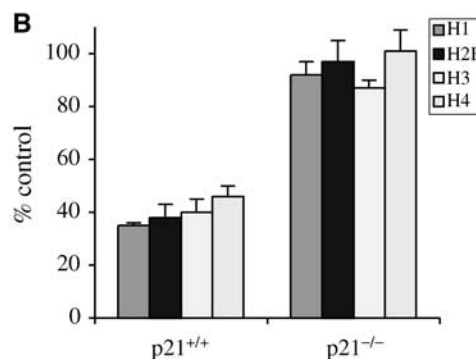
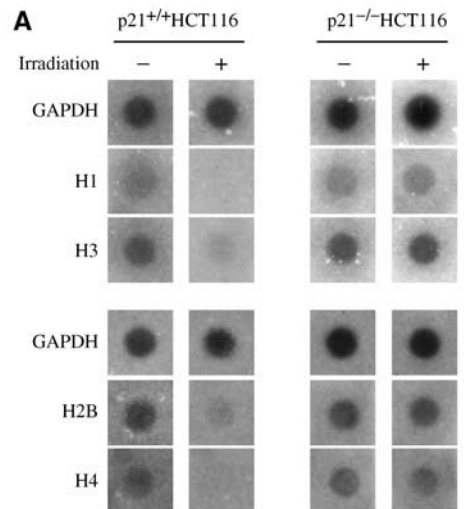


Figure 4 IR induces downregulation of histone gene transcription. (A) Parental HCT116 or p21^{-/-} HCT116 cells were treated with or without IR (12 Gy) as indicated. At 10 h after irradiation, the nuclei were harvested and nuclear run-on assays were carried out as described in Materials and methods. (B) The hybridization signals from the nuclear run-on assays, such as the one shown in panel A, were quantitated using a phosphorimager and expressed as the percentage of the nonirradiated controls.

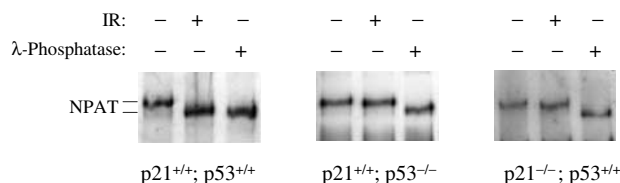


Figure 5 IR results in p53/p21-dependent inhibition of NPAT phosphorylation. The phosphorylation state of NPAT in the indicated cells before or 24 h after irradiation (12 Gy) was analyzed as described in Materials and methods. λ-PPase: Lambda protein phosphatase. Treatment of NPAT from nonirradiated cells with Lambda protein phosphatase was used as a control to locate the migration of unphosphorylated NPAT on SDS-PAGE gels.

p53^{-/-} or p21^{-/-} HCT116 cells (Figure 5, middle and right panels). Therefore, similar to the IR-induced downregulation of histone gene expression, IR-induced inhibition of NPAT phosphorylation requires functional p53 and p21 proteins. Given that IR induces inhibition of cyclin E-Cdk2 kinase activity in the parental HCT 116 cells (Figure 2), but not in the p53^{-/-} or p21^{-/-} HCT116 cells (see Supplementary Figure 1), and that NPAT is an *in vivo* substrate of cyclin

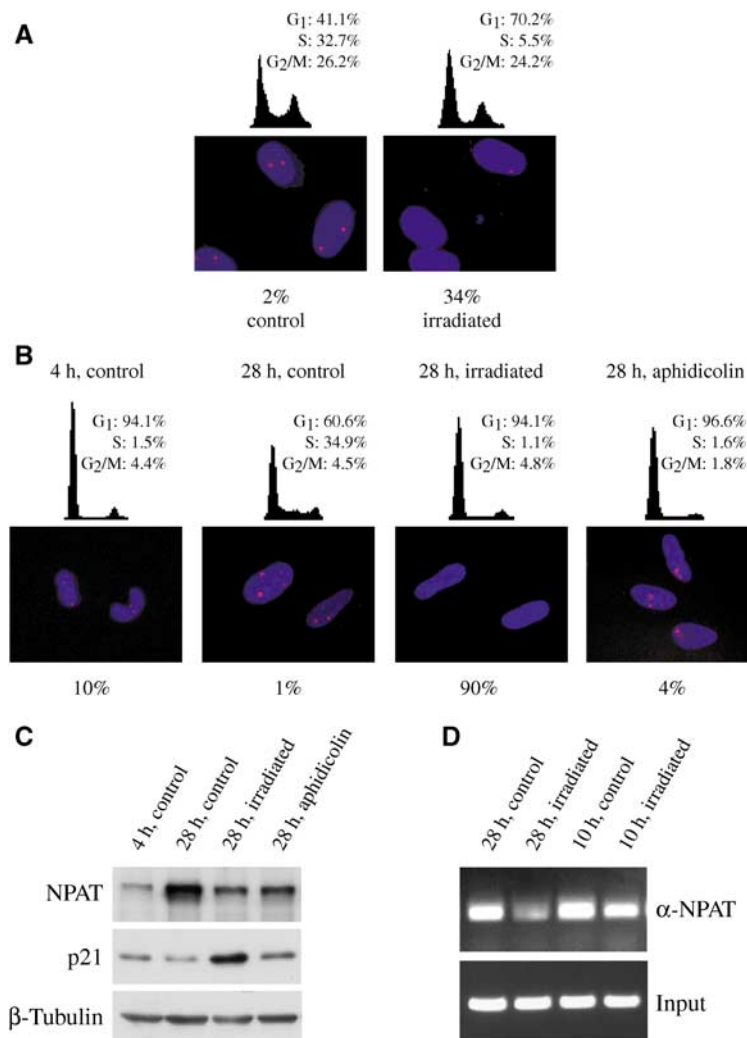


Figure 6 Effect of IR on NPAT localization. **(A)** WI38 cells were γ -irradiated (12 Gy) and the localization of NPAT protein was examined by immunofluorescence staining (IF) 24 h after the treatment. The NPAT staining is shown in red. The nuclei were stained with DAPI (blue). The cell cycle profiles of the irradiated cells and the control nonirradiated cells are depicted at the top. The percentage of cells that lost NPAT foci is shown at the bottom. **(B)** WI38 cells were first arrested at G₀ by serum starvation, and then stimulated to enter the cell cycle by serum addition. At 4 h after stimulation, the cells were fixed (4 h, control), left untreated (28 h, control), treated with IR (28 h, irradiated) or treated with aphidicolin (28 h, aphidicolin). After 24 h, the cells were fixed and analyzed for the localization of NPAT by IF. The cell cycle profiles of the cells are shown at the top. The percentage of cells that lost NPAT foci is shown at the bottom. **(C)** The NPAT and p21 protein levels in the cells treated as described in panel B were analyzed on Western blots. A Western blot of β -tubulin was used as a loading control. **(D)** MRC5 cells were first arrested at G₀ by serum starvation and then stimulated to enter the cell cycle with serum. At 10 and 28 h after stimulation, the association of NPAT protein with the histone H4/e promoter in the cells was determined by using ChIP assays as described previously (Zhao *et al*, 2000). PCR products from total DNA (0.5%) were used as the input controls.

E-Cdk2 kinase, inhibition of NPAT phosphorylation following DNA damage likely results from the inhibition of cyclin E-Cdk2 kinase activity.

DNA damage causes dissociation of NPAT protein from histone gene clusters

Having shown that the phosphorylation of NPAT is inhibited following DNA damage, we then asked whether IR has any effect on NPAT activity. NPAT protein concentrates at a few easily detectable nuclear foci that are associated with the histone gene clusters on chromosomes 1 and 6, and the association of NPAT with the histone gene clusters appears to be cell cycle dependent (Ma *et al*, 2000; Zhao *et al*, 2000; Zheng *et al*, 2003). We examined the localization of NPAT protein after exposing cells to IR. As shown in Figure 6A, the

NPAT foci disappeared in a fraction of irradiated cells. The percentage of cells that have lost NPAT foci appeared to correlate roughly with the fraction of G₁ cells at the time of irradiation, suggesting that the localization of NPAT in G₁ cells is most sensitive to DNA damage. Indeed, when WI38 cells were synchronized at early G₁ phase and γ -irradiated, greater than 90% of the cells either lost the NPAT foci completely or retained the NPAT foci but with dramatically decreased intensity (Figure 6B and data not shown). Loss of the NPAT foci in the irradiated G₁ cells is not simply a consequence of blocked S-phase entry as cells treated with aphidicolin, a specific inhibitor of DNA polymerases (Wright *et al*, 1994), also failed to enter S phase but retained the NPAT foci (Figure 6B). Thus, loss of the NPAT foci results from a specific signal generated by DNA damage.

Loss of the NPAT foci in γ -irradiated cells may result from a decrease of NPAT protein levels, as DNA damage induced cell cycle arrest and NPAT expression is cell cycle regulated (Zhao *et al*, 1998; Gao *et al*, 2003). Alternatively, NPAT protein may become dispersed after DNA damage. To distinguish between these two possibilities, we compared the NPAT protein levels in irradiated WI38 cells and control cells. IR caused induction of p21 protein, loss of NPAT foci and cell cycle arrest (Figure 6B and C). Yet, the NPAT protein levels were comparable in the irradiated cells and in cells treated with aphidicolin (Figure 6C). In addition, the level of NPAT protein in the irradiated cells was higher than that in the early G₁ cells (4 h, control) that contained detectable NPAT foci (Figure 6B and C). We also observed similar NPAT protein levels in irradiated and in untreated control cells when asynchronously growing WI38 or HCT116 cells were used (data not shown). These results demonstrate that the disappearance of NPAT foci following IR is due to dispersed localization, rather than loss of NPAT protein.

Using chromatin immunoprecipitation (ChIP) assays, we have previously shown that NPAT protein associates with the histone promoters *in vivo* (Zhao *et al*, 2000). Loss of the NPAT foci following IR suggests that DNA damage causes dissociation of NPAT protein from the histone gene promoters. To test this notion directly, we examined the association of NPAT protein with a histone H4 promoter after irradiation using ChIP assays. As shown in Figure 6D, the association of NPAT protein with the histone H4 promoter was greatly decreased following γ -radiation. Taken together, the above results show that DNA damage by IR causes dissociation of NPAT protein from the histone gene clusters. As decreased association of NPAT with the H4 promoter could be detected at 6 h after irradiation (10 h after serum stimulation, Figure 6D), a time point well before the cells reached S phase and initiated S-phase-dependent histone synthesis (data not shown), it is unlikely that the dissociation is the result of the inhibition of S-phase entry or the inhibition of histone synthesis. Given that NPAT plays a crucial role in histone gene transcription, the results instead suggest that dissociation of NPAT protein from the histone promoters following IR is responsible for the inhibition of histone gene transcription after DNA damage.

As p53 and p21 are required for IR-induced downregulation of histone gene expression and inhibition of NPAT phosphorylation (Figures 3–5), we examined whether they are also required for the dispersion of the NPAT protein following IR. As shown in Figure 7, while a fraction of parental HCT116 cells have lost NPAT foci after γ -radiation, virtually all interphase HCT116 cells deficient in either p53 or p21 retained the NPAT foci after irradiation. Thus, dissociation of NPAT protein from histone gene clusters after DNA damage also depends on functional p53 and p21 proteins.

Cdk2 activity is required for the formation of NPAT foci at histone gene clusters

The finding that IR-induced suppression of NPAT phosphorylation and dispersion of NPAT protein from the histone gene loci requires functional p21 suggests that a CDK activity, which is inhibited following DNA damage, is required for NPAT to localize at histone gene clusters. As NPAT is an *in vivo* substrate of cyclin E–Cdk2 (Zhao *et al*, 1998, 2000; Ma *et al*, 2000), it is possible that the cyclin E–Cdk2 activity is

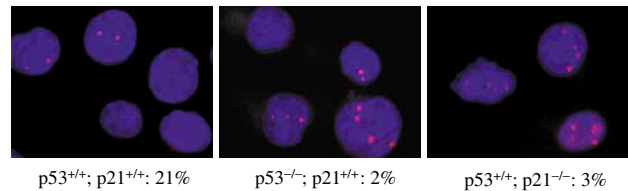


Figure 7 IR-induced dispersion of NPAT protein from histone gene loci depends on p53 and p21. HCT116 cells, p53^{-/-} or p21^{-/-} HCT116 cells were analyzed for the localization of NPAT protein by IF at 24 h after irradiation (12 Gy). The NPAT staining is shown in red and the nuclei are in blue. The percentage of cells that lost NPAT foci after irradiation is shown at the bottom. Without IR treatment, less than 4% of these cells lost NPAT foci (data not shown).

required for NPAT foci formation. To test this hypothesis, we examined the effect of inhibition of cyclin E–Cdk2 on NPAT localization in transiently transfected cells. As shown in Figure 8A, ectopic expression of CDK inhibitors p21 or p27, and of a dominant-negative Cdk2 mutant, all of which have been shown to inhibit Cdk2 activity (Gu *et al*, 1993; Harper *et al*, 1993; van den Heuvel and Harlow, 1993; Xiong *et al*, 1993; Polyak *et al*, 1994; Toyoshima and Hunter, 1994), resulted in the loss of NPAT foci in the transfected U2OS cells. In contrast, inhibition of Cdc2, a CDK involved in the G₂/M transition, by overexpression of a dominant-negative Cdc2 mutant (van den Heuvel and Harlow, 1993) had virtually no effect on NPAT foci formation. Ectopic expression of the Cdk2 inhibitors in HCT116 cells also caused dispersion of NPAT protein and inhibition of cell cycle progression (data not shown). Importantly, the effect of these inhibitors on NPAT localization could be alleviated by coexpression of cyclin E (Figure 8A), indicating that loss of NPAT foci is due to the specific inhibition of Cdk2 activity by the transfected inhibitors.

To provide additional evidence that Cdk2 activity is required for NPAT to form the foci at histone gene clusters, we treated cells with the chemical inhibitor roscovitine at a concentration that specifically blocks Cdk2 but not Cdk4 and Cdk6 activity (Meijer *et al*, 1997), and examined its effect on NPAT localization. Consistent with the idea that Cdk2 activity is required for the NPAT foci formation, cells treated with roscovitine lost their NPAT foci, while treatment of cells with DMSO, the solvent for roscovitine, had no effect on NPAT localization (Figure 8B). Taken together, our results indicate that the activity of Cdk2, likely in the form of the cyclin E–Cdk2 complex, is required for the formation of NPAT foci at the histone gene clusters.

Induction of p21 represses histone gene expression concomitantly with the dissociation of NPAT protein from histone gene clusters

The above results suggest that IR-induced downregulation of histone gene expression results from the suppression of NPAT phosphorylation and its dissociation from the histone gene promoters as a result of inhibition of cyclin E–Cdk2 by p21. If this suggestion is correct, one would predict that induction of p21 without γ -radiation should also inhibit histone gene expression. To test this idea directly, we generated a stable H1299 cell line that expresses p21 fused with the green fluorescent protein (GFP–p21) upon induction by doxycycline, and examined the effect of induction of the GFP–p21

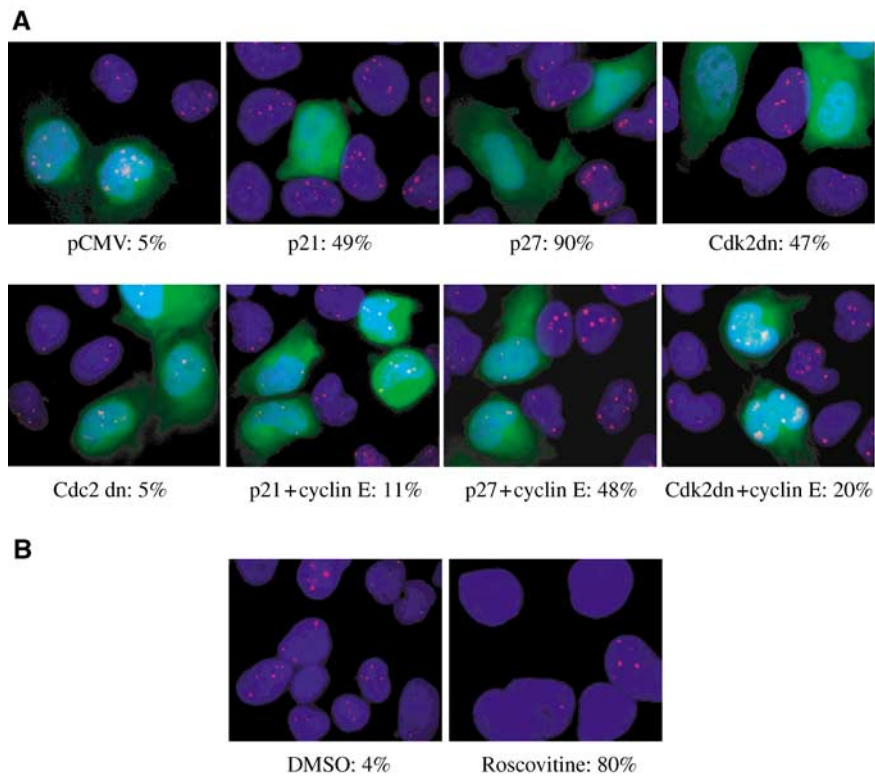


Figure 8 Inhibition of Cdk2 activity prevents NPAT foci formation. (A) Effect of ectopic expression of Cdk2 inhibitors on NPAT localization. U2OS cells were transfected with the indicated expression plasmids, together with a GFP-expressing plasmid to monitor the transfected cells. At 36 h after transfection, the cells were fixed and the localization of NPAT was analyzed by IF. The percentages of the transfected cells (green) that lost NPAT foci (red) are indicated. (B) Effect of Cdk2 kinase inhibitor roscovitine on NPAT localization. U2OS cells were treated with roscovitine (20 μ M) or DMSO for 24 h, and then fixed and examined for the localization of NPAT (red) by IF. The percentage of cells that lost NPAT foci after treatment is indicated.

on histone gene expression. These cells express very little GFP-p21 under noninducing conditions. Upon doxycycline induction, they express increasing amounts of GFP-p21 protein in a time-dependent manner (Figure 9A and B). Induction of the GFP-p21 in these cells had little effect on the levels of NPAT protein during the time period examined (Figure 9B). The induced GFP-p21 protein interacted with the endogenous cyclin-Cdk2 (data not shown) and inhibited cell cycle progression (Figure 9C). Importantly, induction of the GFP-p21 led to inhibition of NPAT phosphorylation (Supplementary Figure 2), loss of NPAT foci (Figure 9A), dissociation of NPAT protein from the histone promoters (Supplementary Figure 2) and downregulation of histone gene expression (Figure 9D). Moreover, loss of NPAT foci, inhibition of histone gene expression and decrease of S-phase population after GFP-p21 induction occurred in parallel (Figure 9E). Thus, inhibition of CDK activity, likely Cdk2 activity, is sufficient to cause dissociation of NPAT protein from histone gene clusters and inhibition of histone gene expression.

Discussion

In this study, we demonstrate that IR triggers p53/p21-dependent downregulation of histone gene expression at the level of transcription. Importantly, we show that IR, as well as inhibition of Cdk2 activity with specific inhibitors, results in the suppression of phosphorylation of the cyclin E-Cdk2

substrate NPAT and its dissociation from histone gene clusters. NPAT is crucial for histone gene transcription (Ma *et al*, 2000; Zhao *et al*, 2000; Gao *et al*, 2003), and association of NPAT protein with the histone gene clusters correlates with histone gene expression (this study). It is, therefore, likely that inhibition of histone gene transcription following IR results, at least in part, from the dissociation of NPAT protein from histone gene promoters. Thus, our studies have not only revealed that the G₁ checkpoint regulates histone gene expression as well as DNA synthesis following IR, but have also identified regulation of NPAT function as a critical event downstream of Cdk2 inhibition (Figure 10).

The results presented here support the idea that downregulation of histone gene expression following IR results directly from a DNA-damage-generated signal rather than indirectly from the inhibition of DNA synthesis. First, the decrease in histone mRNA levels following IR was observed slightly before a decrease in DNA synthesis (Figure 2D). Second, downregulation of histone mRNA levels triggered by inhibition of DNA synthesis is mediated by post-transcriptional processes (Heintz *et al*, 1983; Sittman *et al*, 1983; Baumbach *et al*, 1987; Marzluff and Duronio, 2002). In contrast, IR caused dissociation of NPAT from histone gene promoters (Figure 6) and the inhibition of histone gene transcription (Figure 4). Nevertheless, it is important to note that, in addition to the transcriptional regulation, post-transcriptional processes may also contribute to the downregulation of histone gene expression after IR.

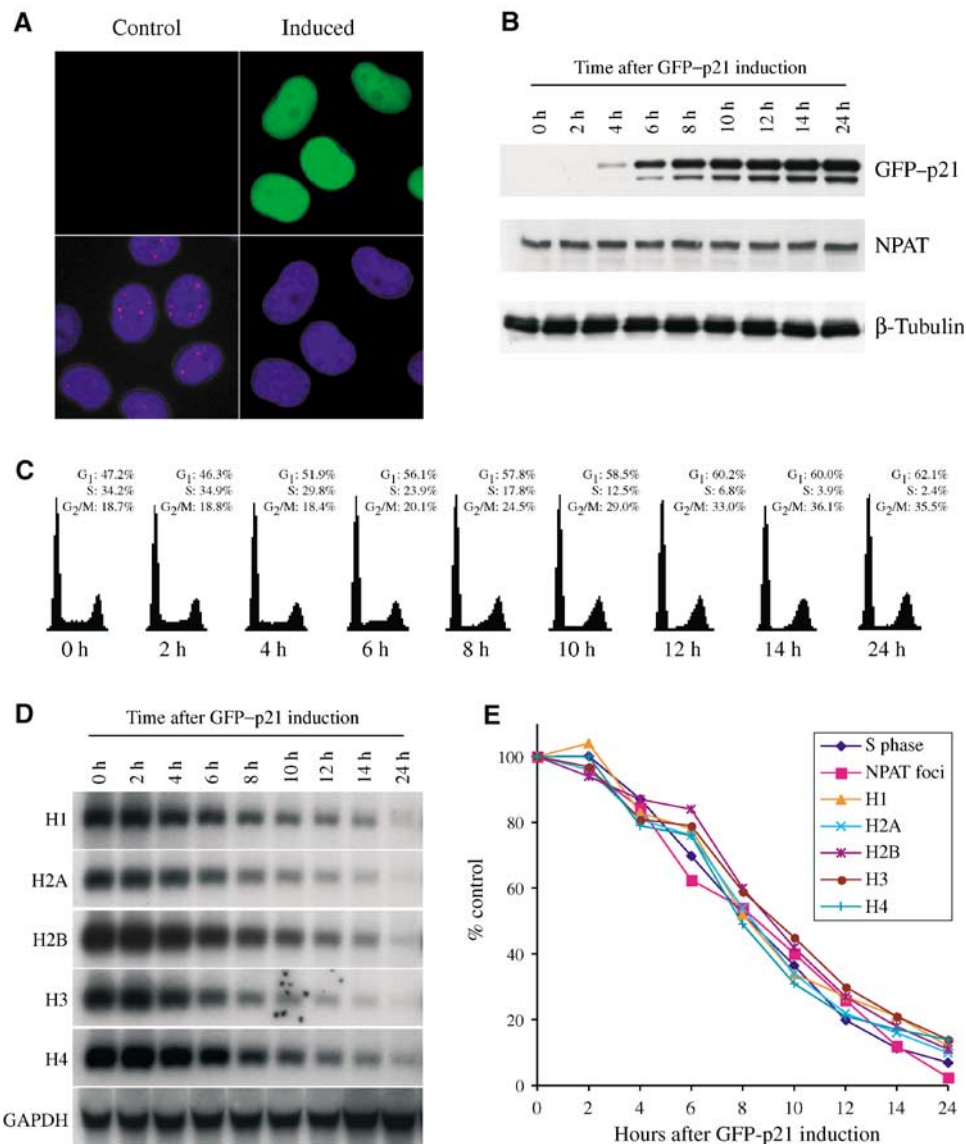


Figure 9 Induction of p21 disperses NPAT protein from histone gene loci and inhibits histone gene expression. (A) Effect of p21 induction on NPAT localization. EGFP-p21 H1299 cells were treated with or without doxycycline (2 μ g/ml) for 24 h. The expression of GFP-p21 was examined by the green fluorescence of the fusion protein (top panels) and the localization of NPAT was analyzed by IF (bottom panels). NPAT staining is shown in red and the nuclei are in blue. (B) Analysis of GFP-p21 and NPAT protein levels before and after induction. EGFP-p21 cells were treated with doxycycline for the indicated times, and the protein levels of GFP-p21 and NPAT were examined on Western blots using antibodies specific for GFP and NPAT, respectively. As a loading control, a Western blot of β -tubulin is also shown. (C) Cell cycle analysis following GFP-p21 induction. The cell cycle distribution of the EGFP-p21 cells at the indicated times following addition of doxycycline was analyzed by FACS. (D) Analysis of histone gene expression after GFP-p21 induction. The levels of histone mRNA in EGFP-p21 cells at the indicated times after GFP-p21 induction were examined on Northern blots. (E) Induction of p21 causes parallel loss of NPAT foci, downregulation of histone gene expression and the decrease of the S-phase population. EGFP-p21 cells were examined by IF at the indicated time following p21 induction to assess the presence or absence of the NPAT foci. The uninduced EGFP-p21 cells typically contain 6–9 NPAT foci, and the cells that contain more than three detectable NPAT foci were scored as NPAT-foci-positive cells. The percentage of cells that contain NPAT foci was compared with that of cells in S phase, as well as with that of histone mRNA levels, at the indicated time after p21 induction. The levels of uninduced cells were set at 100%. The FACS data from panel C and quantitation of hybridization signals from panel D by a phosphorimager were used for panel E.

We and others have previously shown that cyclin E-Cdk2 regulates the function of NPAT in histone gene transcription (Ma *et al*, 2000; Zhao *et al*, 2000). However, the underlying mechanism has remained unclear. Our observations that NPAT becomes dissociated from histone promoters in response to the inhibition of Cdk2 activity by DNA damage or by various Cdk2 inhibitors suggest that phosphorylation of NPAT by cyclin E-Cdk2 is required for its association with

histone gene clusters. Thus, one of the mechanisms by which cyclin E-Cdk2 controls NPAT function appears to be through regulating the interaction of NPAT with histone gene promoters. Consistently, it was recently reported that interaction of NPAT with both histone promoters and OCA-S, a coactivator essential for histone H2B transcription, occurs in an S-phase-dependent manner (Zheng *et al*, 2003). Our finding of a strong correlation between cyclin E-Cdk2 kinase activity,

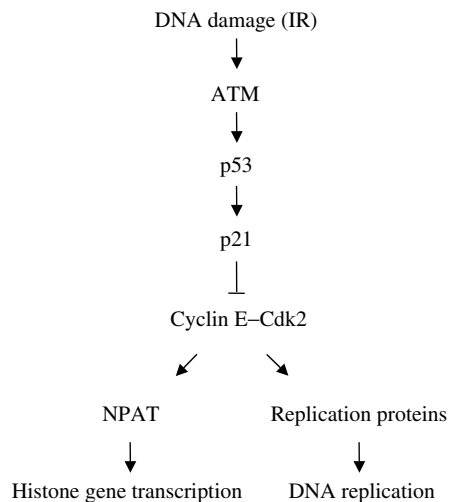


Figure 10 Model for the concurrent inhibition of DNA replication and histone gene expression by the G₁ checkpoint. See text for details.

NPAT phosphorylation, the association of NPAT with histone gene clusters and histone gene expression provides additional support for the conclusion that phosphorylation of NPAT by cyclin E-Cdk2 is critical for histone gene expression.

In this study, we have focused on investigating the effect of DNA damage inflicted by IR on histone gene expression. While treatment of cells with another DNA-damaging agent, adriamycin, also results in p53/p21-dependent downregulation of histone gene expression (Supplementary Figure 3), cisplatin apparently induces downregulation of histone gene expression via a p53/p21-independent pathway (data not shown). Thus, as for the regulation of DNA synthesis, multiple pathways exist in mammalian cells to downregulate histone gene expression in response to different forms of DNA lesions induced by various damaging agents.

DNA replication and bulk histone synthesis are tightly coupled and appear to be mutually dependent during normal cell cycle progression (Heintz *et al*, 1983; Sittman *et al*, 1983; Baumbach *et al*, 1987; Ye *et al*, 2003). Perturbation of this association has deleterious consequences (Meeks-Wagner and Hartwell, 1986; Marzluff and Duronio, 2002). Recent studies indicate that chromatin assembly is also crucial for S-phase progression (Ye *et al*, 2003) and may also be inhibited by a DNA damage checkpoint pathway (Groth *et al*, 2003). Thus, mammalian cells have evolved mechanisms that ensure the tight coupling of DNA replication, histone gene expression and chromatin assembly under both normal growth conditions and conditions where DNA damage may occur.

In addition to its role in histone gene transcription, expression of NPAT promotes G₁ progression (Zhao *et al*, 1998; Gao *et al*, 2003). Recent studies suggest that the ability of NPAT to promote S-phase entry is independent of histone gene activation (Wei *et al*, 2003; J Zhao *et al*, unpublished observation). Inhibition of NPAT expression impeded progression at multiple phases of the cell cycle (Gao *et al*, 2003). Thus, NPAT functions in multiple cellular processes during cell cycle progression. Given the observations reported here and the requirement of NPAT for S-phase entry (Gao *et al*, 2003), one may speculate that regulation of NPAT function

may also contribute to the inhibition of DNA synthesis following the activation of the G₁ checkpoint in mammalian cells.

Materials and methods

Cell culture and generation of GFP-p21-inducible cell lines

Primary human fibroblast WI38, MRC5 and human osteosarcoma U2OS cells were from American Type Culture Collection. Human colorectal cancer cell line HCT116 and p53- and p21-deficient HCT116 (Waldman *et al*, 1995; Bunz *et al*, 1998) were kindly provided by Dr Bert Vogelstein (The Johns Hopkins University School of Medicine). Human lung cancer cell line H1299 was from Dr Peter Keng (University of Rochester). WI38, MRC5, H1299 and U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), unless otherwise specified. HCT116 cells and their derivatives were cultured in McCoy's medium supplemented with 10% FBS. For synchronization of WI38 and MRC5, the cells were cultured in DMEM supplemented with 0.1% FBS for 3 days and then stimulated with 20% FBS (Zhao *et al*, 2000).

To generate stable cell lines that express tetracycline-inducible GFP-p21 fusion protein, the human p21 cDNA was cloned into the pBIG2i vector (Strathdee *et al*, 1999). An in-frame fusion of p21 and enhanced green fluorescence protein (EGFP) was then made by inserting the EGFP sequence from the pEGFP-C1 plasmid (Clontech) into the 5' of the p21 sequence. The resulting pBIG2i EGFPp21 plasmid was transfected into H1299 cells and stable clones were selected using 200 µg/ml hygromycin. Clones with inducible expression of GFP-p21 protein were initially selected by treating cells with 2 µg/ml doxycycline and screening for green fluorescence. Fluorescent colonies (EGFP-p21 H1299 cells) were picked and propagated in the absence of doxycycline.

Plasmids and antibodies

Plasmids pCMVneo, pCMVCdk2, pCMVCdk2dn, pCMVcyclinE, pCMVp21 and pCMVp27, the rabbit polyclonal antibody specific for NPAT and the mouse anti-cyclin E monoclonal antibodies were described previously (van den Heuvel and Harlow, 1993; LaBaer *et al*, 1997; Zhao *et al*, 2000). The rabbit polyclonal antibodies for p53, Cdk2 and β-tubulin were from Santa Cruz Biotechnology. The mouse anti-p21 antibody was from Upstate Biotechnology. The mouse monoclonal antibody specific for γ-tubulin was from Sigma. The mouse monoclonal antibody for GFP was from BD Biosciences Clontech.

Transient transfection and immunofluorescence staining

Transient transfections were carried out using FuGENE 6 (Roche) according to the protocol suggested by the manufacturer. U2OS cells were grown on glass coverslips placed in six-well plates and transfected with 500 ng expression plasmid plus 500 ng vector, or transfected with two expression plasmids, each 500 ng. To monitor the transfected cells, 50 ng pEGFP plasmid was also cotransfected.

Immunofluorescence staining (IF) was carried out as described previously (Zhao *et al*, 2000). For quantitative analyses, the experiments were carried out independently at least twice, and more than 200 cells were analyzed in each experiment.

Ionizing irradiation

Cells were seeded 18–24 h prior to treatment unless otherwise specified and were irradiated with a ¹³⁷Cs source. For irradiation of cells synchronized at G₁ phase, cells were first arrested at G₀ by serum starvation and then stimulated to enter the cell cycle as described above. At 4 h after serum addition, the cells were irradiated with a ¹³⁷Cs source.

Kinase assays

Extracts prepared from HCT 116 cells at the indicated times after IR were incubated with antibodies specific for Cdk2 (M2) or cyclin E (HE111 and HE172). The immunoprecipitation was carried out as described previously (Zhao *et al*, 1998). Following immunoprecipitation, the immunoprecipitates were washed twice with a kinase reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM DTT and 0.1 mg/ml BSA), and incubated with 5 µg of histone H1 and 2 µCi of [γ-³²P]ATP at 30°C for 20 min. The reaction was terminated

with SDS-PAGE loading buffer, and phosphorylation of histone H1 was analyzed by autoradiography following SDS-PAGE.

Lambda protein phosphatase treatment and NPAT mobility shift assays

Extracts were prepared from parent, p21^{-/-} or p53^{-/-} HCT 116 cells before and after IR. NPAT protein in the extracts was immunoprecipitated using a mouse monoclonal antibody specific for NPAT. The immunoprecipitates were divided into two equal parts, and one part was treated with Lambda protein phosphatase (New England Biolabs) using the protocol suggested by the manufacturer. Both treated and untreated samples were separated by SDS-PAGE. The gel electrophoresis was carried out at 200 V for 2.5 h using a Bio-Rad Mini-Protein 3 cell. In order to detect the mobility shift of NPAT protein after irradiation, electrophoresis was carried out with a longer running time than usually used (2.5 h versus 40 min). Phosphorylated and unphosphorylated forms of NPAT were analyzed on Western blots using a rabbit anti-NPAT antibody.

Northern blot analysis and nuclear run-on assays

Analysis of histone mRNA levels on Northern blots was carried out as described previously (Gao *et al*, 2003). The hybridization signals were quantitated using a phosphorimager (Bio-Rad) and normalized with the hybridization signal of GAPDH. Nuclear run-on assays were performed essentially as described (Ausubel *et al*, 1991), except that ³²P-labelled RNA transcripts were isolated using a Qiagen RNeasy kit and hybridization were carried out at 55°C. Histone DNA prepared by PCR (Gao *et al*, 2003) and full-length human GAPDH cDNA were spotted onto Hybond N+ membrane (Amersham) using a dot-blot apparatus. The hybridization signals

were quantitated using a phosphorimager (Bio-Rad) and normalized with the GAPDH signal.

FACS analysis

The cell cycle distribution before and after IR was determined using either propidium iodide (PI) staining or PI/BrdU double staining. FACS analysis using PI staining was carried out as described previously (Zhao *et al*, 1998). For the analysis using PI/BrdU double staining, BrdU (final concentration: 30 μM) was added to the culture medium 30 min before cells were harvested. The incorporation of BrdU was detected using a mouse anti-BrdU antibody (BD Biosciences) and an FITC-conjugated goat anti-mouse IgG antibody (Vector laboratories). Both DNA content and BrdU incorporation were analyzed by flow cytometry.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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