Association of p21 with NF-YA suppresses the expression of Polo-like kinase 1 and prevents mitotic death in response to DNA damage

Y-C Lin¹, Y-N Chen¹, K-F Lin¹, F-F Wang¹, T-Y Chou*¹,²,³ and M-Y Chen*¹,⁴

Polo-like kinase 1 (PLK1) is a Ser/Thr kinase that has important roles in multiple phases of mitosis; its function is involved in centrosome maturation, mitotic entry, anaphase progression and mitotic exit.¹,² PLK1 is tightly controlled in its abundance and activity during cell cycle progression.¹,³ Protein and mRNA levels of PLK1 remain low throughout G1 and S phases, start to increase in the G2 phase and peak during mitosis; the concordant changes in transcript and protein levels suggest that PLK1 expression is primarily controlled transcriptionally in these phases of cell cycle.⁴,⁵ The cell cycle-dependent element (CDE)/cell cycle gene homology region (CHR) in the PLK1 promoter represents a key transcriptional repression element; although the responsible cis-acting factors remain elusive, mutations in this bipartite cis element hinder the cell cycle-specific regulation of PLK1.⁴,⁵ Upon the mitotic exit, PLK1 is regulated at the protein level by anaphase-promoting complex/cyclosome-mediated ubiquitination, which destines PLK1 for proteosomal degradation.⁶ The expression of PLK1 is also regulated under genotoxic stress conditions.⁷,⁸ Both the expression and activity of PLK1 are downregulated upon the activation of the DNA damage-induced G2/M checkpoint, which prevents mitotic entry in p53-dependent and -independent manners.⁷,⁹ The transcriptional repression of PLK1 induced by DNA damage can occur through the activation of G2/M checkpoint kinases ATM and ATR, and is dependent on functional p53 and/or p21.¹⁰,¹¹ The repressive effect of p53 on PLK1 expression appears to be CDE/CHR independent and involves the binding of p53 to a response element (p53RE2) ~ 800-bp upstream of the CDE/CHR element and the recruitment of histone deacetylase to the vicinity of p53RE2.¹² Deletion studies suggest that the CDE/CHR element is important in p21-mediated repression of the PLK1,¹³ but the underlying mechanism has not been well elucidated. The PLK1 promoter also contains one single CCAAT box, which is essential for the promoter activity,¹⁴ the role of this element in stress-responsive PLK1 regulation has not been explored yet.

Nuclear factor Y (NF-Y) is a CCAAT box-binding transcription factor composed of three different subunits: YA, YB and YC. The association between NF-YB and NF-YC provides a docking site for NF-YA, and NF-YA is responsible for sequence-specific DNA binding.¹⁶,¹⁷ NF-Y-binding loci in the

Abbreviations: ADR, adriamycin; CDE, cell cycle-dependent element; CDK2, cyclin-dependent kinase 2; ChIP, chromatin immunoprecipitation; Co-IP, co-immunoprecipitation; CHR, cell cycle gene homology region; PCR, polymerase chain reaction; PLK1, Polo-like kinase 1; RT, reverse transcription

Received 04.5.13; revised 24.11.13; accepted 28.11.13; Edited by D Aberdam
p21 binds to NF-YA to repress PLK1 and prevent mitotic death
Y-C Lin et al

The CCAAT box in the PLK1 promoter is required for p53/p21-dependent transcriptional repression of PLK1 in response to DNA damage. We have previously shown in H1299 cells stably transfected with a temperature-sensitive p53 mutant (tp53) that the induction of functional p53 decreases PLK1 protein levels in a p21-dependent manner.11 In this study, we found that ADR treatment could increase p53 expression in HCT116 cells with or without p21 deletion, whereas levels of PLK1 protein and transcript were only decreased in the wild-type but not in the p21−/− cells (Figure 1a). These data confirmed the requirement of p21 in suppressing PLK1 expression in response to DNA-damaging reagent, and suggested a regulatory mechanism at the transcriptional level. We constructed a reporter plasmid pGL3-Luc-PLK1, which contains the −1717/+58-bp region of the PLK1 promoter, and tested its promoter activity in the isogenic HCT116 cell system. Results of luciferase assays showed that ADR treatment inhibited the PLK1 promoter activity in the wild-type but not in the p21−/− or p53−/− cells (Figure 1b), indicating that the DNA damage-induced transcriptional repression is dependent on the p53/p21 pathway. Introducing a p21-expressing plasmid into p21−/− or p53−/− cells restored the ADR-induced PLK1 promoter inhibition, demonstrating that p21 is sufficient for this transcriptional repression.

To investigate the importance of the CCAAT box in the PLK1 promoter for p53/p21-mediated repression, a pGL3-Luc-PLK1 variant carrying a mutated CCAAT box (mCCAAT; CCAAT to CAGCT) was prepared and compared with the wild-type reporter construct (wtCCAAT) in the previously established tsp53- and neo-H1299 (vector control) stable clones.11 Transfected cells were subjected to the temperature shift (for the induction of functional p53 in tsp53-H1299 cells) and treated by ADR to induce DNA damage, and their lysates were assayed for luciferase activities (Figure 1c). The activity of the wtCCAAT promoter in tsp53-H1299 cells was significantly lower than that in the neo-H1299 control clone, and this repressive effect of p53 could be alleviated by p21 knockdown; however, the mCCAAT promoter was unresponsive to p53 activation. Consistent with the above results, p21 overexpression in the p53-null H1299 background was sufficient to inhibit the activity of the wtCCAAT but not the mCCAAT promoter (Figure 1d). In H1299 and neo-H1299 cells, where p53 and p21 were not expressed, the mutation of CCAAT did not affect the PLK1 promoter activity (Figures 1c and d). Collectively, our results suggest that the CCAAT box is essential for the DNA damage-induced p53/p21-dependent PLK1 repression.

Results

The CCAAT box in the PLK1 promoter is required for p53/p21-dependent transcriptional repression of PLK1 in response to DNA damage. We have previously shown in H1299 cells stably transfected with a temperature-sensitive p53 mutant (tp53) that the induction of functional p53 decreases PLK1 protein levels in a p21-dependent manner.11 In this study, we found that ADR treatment could increase p53 expression in HCT116 cells with or without p21 deletion, whereas levels of PLK1 protein and transcript were only decreased in the wild-type but not in the p21−/− cells (Figure 1a). These data confirmed the requirement of p21 in suppressing PLK1 expression in response to DNA-damaging reagent, and suggested a regulatory mechanism at the transcriptional level. We constructed a reporter plasmid pGL3-Luc-PLK1, which contains the −1717/+58-bp region of the PLK1 promoter, and tested its promoter activity in the isogenic HCT116 cell system. Results of luciferase assays showed that ADR treatment inhibited the PLK1 promoter activity in the wild-type but not in the p21−/− or p53−/− cells (Figure 1b), indicating that the DNA damage-induced transcriptional repression is dependent on the p53/p21 pathway. Introducing a p21-expressing plasmid into p21−/− or p53−/− cells restored the ADR-induced PLK1 promoter inhibition, demonstrating that p21 is sufficient for this transcriptional repression.

To investigate the importance of the CCAAT box in the PLK1 promoter for p53/p21-mediated repression, a pGL3-Luc-PLK1 variant carrying a mutated CCAAT box (mCCAAT; CCAAT to CAGCT) was prepared and compared with the wild-type reporter construct (wtCCAAT) in the previously established tsp53- and neo-H1299 (vector control) stable clones.11 Transfected cells were subjected to the temperature shift (for the induction of functional p53 in tsp53-H1299 cells) and treated by ADR to induce DNA damage, and their lysates were assayed for luciferase activities (Figure 1c). The activity of the wtCCAAT promoter in tsp53-H1299 cells was significantly lower than that in the neo-H1299 control clone, and this repressive effect of p53 could be alleviated by p21 knockdown; however, the mCCAAT promoter was unresponsive to p53 activation. Consistent with the above results, p21 overexpression in the p53-null H1299 background was sufficient to inhibit the activity of the wtCCAAT but not the mCCAAT promoter (Figure 1d). In H1299 and neo-H1299 cells, where p53 and p21 were not expressed, the mutation of CCAAT did not affect the PLK1 promoter activity (Figures 1c and d). Collectively, our results suggest that the CCAAT box is essential for the DNA damage-induced p53/p21-dependent PLK1 repression.

p21 is recruited to a PLK1 promoter region containing the CCAAT box in response to DNA damage. To explore the mechanism by which p21 regulates PLK1 in cells with DNA damage, we tested if p21 can be recruited to the promoter following ADR treatment. Chromatin immunoprecipitation (ChIP) assays were performed using specific antibodies and primer sets to amplify regions of the PLK1 promoter from precipitated DNA (Figure 2a); region A was used as a control whereas region B is the region containing the CCAAT box (−39/−35 bp).14 ADR treatment induced the recruitment of p21 specifically to the CCAAT box-containing region in the PLK1 promoter in wild-type but not p21−/− or p53−/− HCT116 cells (Figure 2b). ChIP assays using an antibody against NF-YA, that is, the DNA-binding subunit of the CCAAT box-binding NF-Y, showed that NF-YA associated with the CCAAT box-containing PLK1 promoter region in wild-type, p21−/− or p53−/− HCT116 cells, either with or without ADR treatment (Figure 2b). CDK2, a kinase known to bind and phosphorylate NF-YA,22 also associated with this promoter region; intriguingly, the association of CDK2 with promoter was markedly reduced following ADR treatment (for the induction of functional p53 in tsp53-H1299 cells) and treated by ADR to induce DNA damage, and their lysates were assayed for luciferase activities (Figure 1c). The activity of the wtCCAAT promoter in tsp53-H1299 cells was significantly lower than that in the neo-H1299 control clone, and this repressive effect of p53 could be alleviated by p21 knockdown; however, the mCCAAT promoter was unresponsive to p53 activation. Consistent with the above results, p21 overexpression in the p53-null H1299 background was sufficient to inhibit the activity of the wtCCAAT but not the mCCAAT promoter (Figure 1d). In H1299 and neo-H1299 cells, where p53 and p21 were not expressed, the mutation of CCAAT did not affect the PLK1 promoter activity (Figures 1c and d). Collectively, our results suggest that the CCAAT box is essential for the DNA damage-induced p53/p21-dependent PLK1 repression.

p21 is recruited to a PLK1 promoter region containing the CCAAT box in response to DNA damage. To explore the mechanism by which p21 regulates PLK1 in cells with DNA damage, we tested if p21 can be recruited to the promoter following ADR treatment. Chromatin immunoprecipitation (ChIP) assays were performed using specific antibodies and primer sets to amplify regions of the PLK1 promoter from precipitated DNA (Figure 2a); region A was used as a control whereas region B is the region containing the CCAAT box (−39/−35 bp).14 ADR treatment induced the recruitment of p21 specifically to the CCAAT box-containing region in the PLK1 promoter in wild-type but not p21−/− or p53−/− HCT116 cells (Figure 2b). ChIP assays using an antibody against NF-YA, that is, the DNA-binding subunit of the CCAAT box-binding NF-Y, showed that NF-YA associated with the CCAAT box-containing PLK1 promoter region in wild-type, p21−/− or p53−/− HCT116 cells, either with or without ADR treatment (Figure 2b). CDK2, a kinase known to bind and phosphorylate NF-YA,22 also associated with this promoter region; intriguingly, the association of CDK2 with promoter was markedly reduced following ADR treatment (for the induction of functional p53 in tsp53-H1299 cells) and treated by ADR to induce DNA damage, and their lysates were assayed for luciferase activities (Figure 1c). The activity of the wtCCAAT promoter in tsp53-H1299 cells was significantly lower than that in the neo-H1299 control clone, and this repressive effect of p53 could be alleviated by p21 knockdown; however, the mCCAAT promoter was unresponsive to p53 activation. Consistent with the above results, p21 overexpression in the p53-null H1299 background was sufficient to inhibit the activity of the wtCCAAT but not the mCCAAT promoter (Figure 1d). In H1299 and neo-H1299 cells, where p53 and p21 were not expressed, the mutation of CCAAT did not affect the PLK1 promoter activity (Figures 1c and d). Collectively, our results suggest that the CCAAT box is essential for the DNA damage-induced p53/p21-dependent PLK1 repression.
The occupancy of the CDC25A promoter was also examined as a control; ADR treatment promoted the recruitment of p21 to the -222/-58-bp region that contains two CCAAT boxes, which was accompanied by a reduction in CDK2 association to the same region. The reciprocal changes in the amounts of promoter-associated p21 and CDK2 in response to DNA damage suggest that p21 may regulate transcription of those target genes with CCAAT box-containing promoters by replacing CDK2 in binding to CCAAT box-bound NF-Y.

Expression of p21 disrupts the interaction between CDK2 and NF-YA. It is known that CDK2 can form a complex with NF-YA. By expressing a fixed amount of NF-YA with increasing dosage of p21 in H1299 cells, we tested if p21 could affect the formation of the CDK2/NF-YA complex. Co-immunoprecipitation (co-IP) experiments showed that increasing p21 expression resulted in decreasing amounts of NF-YA detected in the CDK2 immunoprecipitates (Figure 3a).

To confirm this effect of p21, we used the tsp53-H1299 cell system; cells were induced to express functional p53 and treated with ADR to activate the p53/p21 pathway, and cell lysates were subjected to CDK2 immunoprecipitation combined with immunoblotting analysis to detect the association between NF-YA and CDK2. In vector-transfected tsp53-H1299 cells with induced p21 expression, the CDK2/NF-YA complex could not be detected. In the p53-null neo-H1299 control cells and the tsp53-H1299 cells with p21 knockdown (p21-KD), where p21 expression could not be induced, NF-YA was co-immunoprecipitated with CDK2 (Figure 3b). These results support a role of p21 in negatively regulating the association between CDK2 and NF-YA.

As p21 can bind to CDK2, we investigated if the CDK2-binding ability is required for the above-described p21 effect. A CDK2-binding-defective p21 mutant, p21-PRG, was tested. In co-IP experiments, unlike wild-type p21, p21-PRG was unable to reduce the association between CDK2 and NF-YA in the cellular context (Figure 3c). ChIP assays
p21 binds to NF-YA to repress PLK1 and prevent mitotic death

Y-C Lin et al

Figure 2 p21 is recruited to the CCAAT box-containing region of PLK1 promoter in response to ADR treatment. (a) A schematic representation of the PLK1 promoter. The regions (A and B) amplified in ChIP assays are indicated. Nucleotide positions are numbered relative to the translational start site (+1). The locations of wtCCAAT box, CDE and CHR are indicated and their exact sequences are underlined. (b) Recruitment of regulatory proteins to PLK1 promoter regions was assessed by ChIP assays using antibodies against p21, CDK2 or NF-YA. The -1569/-1217-bp region A as a control and the -301/+58-bp region B that contains the CCAAT box were amplified by PCR using specific primer sets. (b) Isogenic p21 +/- p53 +/- (WT), p21 +/- or p53 +/- HCT116 cells were treated with ADR for 12 h and subsequently cultured in the ADR-free medium for 48 h; –, no ADR treatment. (c) The tsp53-H1299 cells were cultured at 38 °C for 24 h, shifted to 32 °C and treated with ADR for 12 h, and transferred to drug-free medium at 32 °C for 48 h before samples were prepared for ChIP; –, no ADR treatment. The p21 response element (-222/-58 bp) in the CDC25A promoter was also amplified as a control.

showed that the amount of PLK1 promoter-associated CDK2 was significantly reduced when wild-type p21, but not p21-PRG, was overexpressed (Figure 3d). Compared with the wild-type p21, p21-PRG was less effective in suppressing the PLK1 promoter activity (Figure 3e) and in lowering the PLK1 mRNA levels (Figure 3f). Together, these results suggest that the CDK2-binding ability is required for p21 to interfere with the formation of the CDK2/NF-YA complex and to suppress PLK1 expression.

NF-YA binds to p21 directly and anchors p21 to the CCAAT box-containing promoter region for PLK1 repression. As both p21 and NF-YA were detected in the CCAAT box-containing region of the PLK1 promoter, the possibility that p21 binds to NF-YA was explored. Immunoprecipitation and immunoblotting on lysates from 293T cells co-expressing HA-tagged p21 and Flag-tagged NF-YA revealed that p21 could interact with NF-YA within cells (Figure 4a). Recombinant GST-tagged p21 purified from E. coli could associate with in vitro translated 35S-labeled NF-YA (Figure 4b). Further in vitro binding analysis using GST-tagged NF-YA and His-tagged p21 purified from E. coli demonstrated that NF-YA could directly interact with p21 (Figure 4c). We also tested p21-PRG and found it able to bind to NF-YA in the in vitro GST pull-down assay (Figure 4c). However, co-IP showed that, unlike the wild-type p21, p21-PRG failed to interact with NF-YA (Figure 4d), supporting that the CDK2-binding ability is required for p21 to interact with NF-YA in the cellular context.

The role of NF-YA in anchoring p21 to the PLK1 promoter was next examined. In Flag-p21-expressing H1299 cells, ChIP analysis demonstrated that NF-YA knockdown caused a significant reduction of the amount of p21 bound to the CCAAT box-containing region of PLK1 promoter (Figure 4e). In contrast, compared with the control, overexpression of wild-type NF-YA but not a mutant NF-YA (m29) impaired in DNA binding27 increased the amount of promoter-associated p21 (Figure 4f). Similarly, expression of NF-YA in tsp53-H1299 cells further increased the amount of promoter-associated p21 following p53 induction (Figure 4g). These results suggest that p21 associates with the PLK1 promoter through its interaction with the CCAAT box-bound NF-YA.

NF-YA has a pivotal role in p53/DNA damage-induced PLK1 repression. The role of NF-YA in p53-mediated PLK1 suppression during DNA damage response was also investigated. The tsp53-H1299 cells were co-transfected with the pGL3-Luc-PLK1 reporter construct together with a control vector (shVec) or a plasmid-expressing NF-YA-targeting shRNAs; lysates for the luciferase assay were prepared from transfected cells after temperature-induced expression of functional p53 and ADR treatment. Comparing vector-transfected p53-null neo-H1299 control and tsp53-H1299 cells, p53 induction/DNA damage resulted in suppression of the PLK1 promoter activity and this suppression was alleviated by the silencing of NF-YA expression (Figure 5a). Reverse transcription (RT)-PCR analysis demonstrated that NF-YA knockdown decreased the
suppressive effect of p53 induction/ADR treatment on PLK1 mRNA expression in tsp53-H1299 cells, indicating that NF-YA is important for the DNA damage-induced PLK1 repression (Figure 5b). To investigate whether the DNA-binding activity of NF-YA is required for the p21-mediated PLK1 inhibition, tsp53-H1299 cells were transfected to express wild-type or mutant NF-YA that are impaired in DNA binding (m28 or m29);27 transfected cells were induced by the temperature shift to activate the p53-p21 axis, and cell lysates were prepared for luciferase assays. The results showed that both NF-YA mutants did not suppress the PLK1 promoter activity but the wild-type NF-YA did (Figure 5c), indicating that the DNA-binding activity of NF-YA is required for repressing PLK1. Together, these results suggest that the promoter-bound NF-YA is essential for the p53/p21-dependent inhibition of PLK1 expression in the DNA damage response.

**NF-YA-mediated suppression of PLK1 expression is important for the p53 checkpoint function and prevention of mitotic death following DNA damage.** We have previously shown that p21-mediated suppression of PLK1 is necessary for the checkpoint function of p53.11 Given the above findings suggesting that NF-YA is required for p53-mediated repression of PLK1, we examined the functional significance of NF-YA in the p53-mediated stress response. In HCT116 cells transfected with a reporter construct containing the p53 response element, luciferase assays showed that NF-YA silencing, similar to p21 depletion, decreased the ADR-induced p53 transcriptional activity (Figure 6a). In tsp53-H1299 cells, RT-PCR analysis demonstrated that NF-YA silencing significantly reduced ADR-induced expression of p53 target genes p21 and MDM2 (Figure 6b). In NF-YA knocked-down tsp53-H1299 cells, PLK1 silencing abrogated the suppressive effect of NF-YA depletion on p53 transcriptional activity (Figure 6c), suggesting that the increased PLK1 expression resulting from NF-YA silencing may underlie the inhibition of p53 activity.

We have previously shown that p53 protects cells from ADR-induced mitotic death.28 By examining the formation of multinucleated cells and the increase in subG1 DNA content to assess mitotic death, we investigated the functional significance of NF-YA in the mitotic checkpoint function of p53. Compared with the control, HCT116 cells depleted of NF-YA and p21−/− cells similarly displayed a significant increase in the formation of multinucleated cells following exposure to ADR (Figure 6d), suggesting that both p21 and NF-YA participate in protecting cells from mitotic death. Analysis of the subG1 DNA content in ADR-treated tsp53-H1299 cells revealed that NF-YA silencing significantly increased the subG1 cell population; this effect was diminished by simultaneous knockdown of PLK1, suggesting that the effect of NF-YA depletion on mitotic death resulted from increased PLK1 expression (Figure 6e). Colony formation assays verified that depletion of NF-YA decreased the survival of ADR-treated cells. The clonogenic activity of NF-YA-depleted cells was substantially reduced relative to the vector control cells, and the depletion of PLK1 in these cells partially restored the colony formation activity (Figure 6f).

Collectively, our results suggest that by repressing PLK1 expression, NF-YA has an important role in the p53 checkpoint function that helps to prevent DNA damage-induced mitotic death.
Figure 4. NF-YA binds to p21 directly and anchors p21 to the CCAAT box-containing region in the PLK1 promoter. (a) Co-IP assays. Plasmids expressing Flag-NF-YA and HA-p21 were co-transfected into 293T cells. Lysates were immunoprecipitated with anti-Flag or anti-HA antibodies, and the amounts of NF-YA and p21 in the precipitates were determined by immunoblotting. (b and c) In vitro binding assays. Proteins bound to glutathione-sepharose beads were resolved by SDS-PAGE and detected by immunoblotting for the pulled-down proteins or Coomassie blue staining for GST-tagged proteins. (b) Recombinant GST-tagged p21 from E. coli was immobilized on glutathione-sepharose beads and incubated with 35S-labeled NF-YA prepared by immunoblotting for the pulled-down proteins or Coomassie blue staining for GST-tagged proteins. (c) Recombinant GST-tagged NF-YA from E. coli was immobilized on glutathione-sepharose beads and incubated with purified His-tagged p21 or p21-PRG. (d) Co-IP assays. Lysates were prepared from H1299 cells overexpressing NF-YA and Flag-tagged p21 or p21-PRG, and subjected to immunoprecipitation with anti-p21 antibodies. Proteins in the input lysates or precipitates were analyzed by immunoblotting using specific antibodies. (e–g) ChIP assays. Immunoprecipitation were performed using anti-Flag or anti-p21 antibodies. DNA in precipitates was amplified using primer sets for regions A and B shown in Figure 2a. Amounts of promoter-associated Flag-p21 or p21 were normalized to the individual inputs and compared with the value of the vector control sample (which was set as 1). Expression levels of NF-YA and p21 in samples were also analyzed by immunoblotting. (e) H1299 cells transfected with the control vector (Vec) or the Flag-p21-expressing plasmid in the absence or presence of NF-YA-specific shRNA expression were analyzed at 24 h after transfection. (f) H1299 cells transfected with a Flag-p21-expressing plasmid alone or in combination with wild-type or mutant NF-YA (m29) were analyzed at 24 h after transfection. (g) The tap53-H1299 cells transfected with a plasmid-expressing NF-YA were cultured at 32°C for 24 h (for the induction of functional p53) and analyzed.

Discussion

We have uncovered that beyond its well-known role as a p53 downstream checkpoint component that binds CDK2 and halts cell cycle progression, p21 can directly bind to NF-YA on the PLK1 promoter and actively suppress PLK1 transcription to inhibit mitotic entry and prevent mitotic catastrophe. We propose a model of NF-Y-mediated transcriptional regulation of PLK1 expression (Figure 7). In unstressed cells, NF-YA is associated with and phosphorylated by CDK2, which facilitates NF-Y binding to the CCAAT box and activates transcription22 presumably by recruiting coactivators with the histone acetyltransferase activity to the PLK1 promoter region to acquire an active chromatin environment,20 the cell cycle-dependent regulation of CDK2 may help to ensure the normal progression of cell cycle in this scenario. Upon DNA damage, the activation of p53 leads to increased p21 levels, which causes the replacement of CDK2 by p21 in interacting with the CCAAT box-bound NF-YA, turning the CCAAT box-associated regulatory complex into a repressive one; presumably through recruitment of corepressors with the histone deacetylase activity to remove the acetylation of histones,20 the p21/NF-Y complex causes transcriptional repression of PLK1 and thereby prevents mitotic catastrophe. Notably, we have also observed the replacement of CDK2 by p21 in association with the CCAAT box in the promoter of another p21-repressible gene, CDC25A, after ADR treatment.14,24 This finding suggests that the dynamic interplay among CDK2, p21 and NF-YA might be a commonly adopted mechanism in regulating CCAAT box-containing promoters in response to DNA damage.

Our results show that p21-PRG, a p21 mutant defective in CDK2 binding, retains the ability to directly interact with NF-YA in vitro, suggesting that the CDK-interacting and NF-YA-interacting regions of p21 are independent. In the cellular context, however, p21-PRG does not bind CDK2 or interact
NF-YA has an important role in p53 DNA damage-induced PLK1 repression. (a) NF-YA-depleted (shNF-YA-1, shNF-YA-2) and vector control (shVec) tsp53-neo cells were subjected to luciferase reporter assays after being co-transfected with pGL3-Luc-PLK1 and pRL-TK and treated with ADR as described in Figures 1c. (b) NF-YA-depleted (shNF-YA-1, shNF-YA-2) and vector control (shVec) tsp53-neo cells were treated with ADR and assayed for the expression of the indicated genes by RT-PCR. Expression of PLK1 at 48 h after ADR release was normalized to the GAPDH transcript level, and compared with the value obtained from the sample of the shVec-transfected cells. (c) The tsp53-neo cells were transfected with a plasmid expressing wild-type (WT) or mutant NF-YA (m28 or m29) together with pGL3-Luc-PLK1 and pRL-TK. Cells were shifted to 32°C for the induction of functional p53, and luciferase activities were assayed at 24 h after transfection. (a–c) Bars represent the mean ± S.D. from three independent experiments (*P < 0.05; **P < 0.01; ***P < 0.001, Student’s t-Test).
Materials and Methods

Cell culture. H1299 (p53-null human non-small cell lung carcinoma) cells were cultured at 37 °C in RPMI-1640 supplemented with 10% fetal bovine serum (FBS). The mutant tsp53Y143A-H1299 and the control neo-H1299 cell lines were maintained in RPMI-1640 supplemented with 10% FBS; these clones were cultured at 38 °C as p53-null cells, or transferred to 32 °C for the induction of functional p53 in tsp53Y143A-H1299 cells. Cells of p21-deficient tsp53-H1299 (p21-KD) and vector control clones were cultured at 38 °C in the presence of 400 μg/ml hygromycin and 0.2 mg/ml G418 (human embryonic kidney cells with wild-type p53 alleles and residual p53 activities despite the presence of SV40 large T antigen), and HCT116 (human colorectal carcinoma cells with wild-type p53) and its isogenic (p531/− or p21−/−) cell lines (provided by Dr. Tzu-Hao Cheng at the Institute of Biochemistry and Molecular Biology, National Yang-Ming University, Taipei, Taiwan) were cultured at 37 °C in DMEM supplemented with 10% FBS. All cells were maintained in a humidified atmosphere of 5% CO2/95% air.

Immunoblotting analysis. Cells were lysed in a lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM MgCl2, ethylenediaminetetraacetic acid (EDTA), 1 mM EGTA, 1% Triton X-100 and a protease inhibitor cocktail added immediately before use) on ice for 30 min and centrifuged at 12,000 g at 4 °C for 15 min. Proteins (10-μg samples) were subjected to 8-12% SDS-PAGE, transferred to the Hybond-C Extra membrane (Amersham Biosciences, Piscataway, NJ, USA), and incubated with antibodies against proteins of interest, including NF-YA (Santa Cruz Biotechnology, Santa Cruz, CA, USA), PLK1 (Zymed Laboratories, South San Francisco, CA, USA), p21 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CDK2 (Santa Cruz Biotechnology), PLK1 (Zymed Laboratories, South San Francisco, CA, USA), p21 (Santa Cruz Biotechnology), MDM2 (Proteintech, Chicago, IL, USA), p53 (Calbiochem, Merck KGaA, Darmstadt, Germany), GAPDH (Santa Cruz Biotechnology), β-actin (Sigma-Aldrich, St Louis, MO, USA) and tubulin (Sigma-Aldrich, St Louis, MO, USA).

Plasmid construction. The firefly luciferase reporter construct pGL3-Luc-PLK1 was generated by cloning the −1717 to +58 (numbered relative to the
p21 binds to NF-YA to repress PLK1 and prevent mitotic death

Y-C Lin et al

Cell Death and Disease

Figure 7 NF-YA participates in the transcriptional regulation of PLK1 expression. We propose a model in which the dynamic interplay among CDK2, p21 and NF-YA on the promoter fine tunes PLK1 expression. In untransformed CDK2, CDK2 associates with NF-YA bound on the CCAAT box in the PLK1 promoter to allow cell cycle progression. DNA damage activates p53 and induces p21 to replace CDK2 in binding to NF-YA, which causes PLK1 repression and prevents mitotic cell death. In cells depleted of p21 or NF-YA, DNA damage fails to repress PLK1 expression; as a result, PLK1 acts to attenuate functions of p53, thereby resulting in mitotic catastrophe. Ac, acetylation of histones; P, phosphorylation of NF-YA.

Translational start site) PLK1 promoter fragment through polymerase chain reactions (PCRs) amplifications and subsequent subcloning of the PCR product into KpnI/BglII sites of the pGL3-Basic plasmid (Promega). Primers used were PLK1 promoter-F (5’-GGGATCCACCTGGGAGCTTACACCTGTTTCTCTTCC-3’) and PLK1 promoter-R (5’-GAATTCCTGCTCCTCCTCTCAGATTCAAGCCTCG-3’). Mutagenesis was performed using a QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s protocols to generate variant reporter constructs with specific mutations in the PLK1 promoter, or a p21 mutant (p21-PRG)-expressing construct. Specific mutagenic primer pairs were PLK1 mCCAAT-F (5’-GGGATCCACCTGGGAGCTTACACCTGTTTCTCTTCC-3’) and PLK1 mCCAAT-R (5’-GAATTCCTGCTCCTCCTCTCAGATTCAAGCCTCG-3’) for the mCCAAT reporter construct, and p21-PRG-F (5’-GGGATCCACCTGGGAGCTTACACCTGTTTCTCTTCC-3’) and p21-PRG-R (5’-GGGATCCACCTGGGAGCTTACACCTGTTTCTCTTCC-3’) for the p21-PRG expression construct; underlined sequences shown in the primers are the mutated nucleotides. Multiple (12–16) PCR cycles were used to amplify the entire vector, and the PCR products were digested with DpnI for 1 h at 37 °C and subsequently transformed into E. coli. DNA was extracted from transformed and the target sequence was verified by DNA sequencing. For constructing GST fusion protein-expressing plasmids, NF-YA and p21 cDNA fragments were generated by PCR amplifications using specific primers. PCR fragments were ligated, in-frame, into pGEX-3X-5S (Amerham Biosciences) or pREST A (Invitrogen, Carlsbad, CA, USA) and transformed into BL21.

Luciferase reporter assay. Cells were co-transfected with a luciferase reporter plasmid, such as pGL3-Luc-PLK1, pGL3-Luc-mutPLK1 (with a mutated CCAAT box) or pGPU.PL-P53RE (a luciferase reporter construct containing four copies of p53RE), together with the Renilla luciferase control vector pRL-TK (Promega, Fitchburg, WI, USA). Lysates were prepared 24 h after transfection and assayed for the luciferase activity using a dual-luciferase reporter assay system (Promega). The activity of the firefly luciferase was normalized to that of the Renilla luciferase in the same assayed sample.

Lentiviral production and transduction. Plasmids for the expression of shRNAs targeting NF-YA (TRCN000014298 and TRCN000014530) or PLK1 (TRCN0000121073 and TRCN0000121325) were obtained from the National RNAi Core Facility Platform (located at the Institute of Molecular Biology/Genomic Research Center, Academia Sinica), which is supported by the National Core Facility Program for Biotechnology, Taiwan. Viral packaging was performed as described.13 For target cell transduction, cells were grown to 60% confluency, and the virus-containing media were added to cells with polybrene (8 μg/ml). At 24 h after transfection, the virus-containing media were removed and cells were collected for further experiments. As shNF-YA expression caused a rapid decrease in the number of HCT116 cells, it was not possible to select for infected cells; however, the efficacy of each batch of the NF-YA shRNA-expressing lentiviral particles was assessed by immunoblotting for NF-YA before conducting further experiments.

In vitro binding assay. E. coli transformants carrying plasmids for different fusion proteins were grown in the presence of 0.1 mM isopropyl-β-D-thiogalactoside for 3.5 h at 30 °C to induce the expression of fusion proteins. For purification of GST-tagged proteins, bacteria were lysed by sonication in buffer A (50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, 1% Triton X-100, 1 mM EDTA and 15% glycerol) and the lysates were filtered through a 0.45-μm filter membrane. GST-tagged proteins were purified using glutathione-sepharose beads in buffer A. For purifying His-tagged proteins, bacteria were lysed by sonication in the lysis buffer (50 mM Tris-HCl, pH 8.0, 300 mM NaCl, 20 mM imidazole and 0.05% Tween 20) and the lysates were filtered through a 0.45-μm filter membrane and subsequently incubated with the Ni-NTA resins (Qiagen, Hilden, Germany). His-tagged proteins bound on resins were eluted in a lysis buffer containing 200 mM imidazole. The eluted proteins were concentrated and exchanged in phosphate-buffered saline (PBS) by Microcon YM-3 (Millipore, Billerica, MA, USA) and were stored at −70 °C. The [35S] Met-labeled proteins were prepared using the TNT Quick Coupled Transcription/Translation system (Promega) according to the manufacturer’s instructions. For binding assays, the GST-tagged proteins were immobilized on glutathione-sepharose beads and incubated with purified His-tagged proteins at 4 °C overnight or with the [35S] Met-labeled proteins at 4 °C for 4 h. After washing with PBS, the beads-bound proteins were subjected to 10% SDS-PAGE and immunoblotting of autoradiography.

RNA extraction and RT-PCR. Total RNA was isolated with the Trizol reagent (Invitrogen). The RNA (5 μg) was reverse transcribed into cDNA using the oligo-dT(18) primer and reverse transcriptase in a 20-μl reaction mixture. Amplification was performed for CDKN1A (20 cycles) with primers p21-F (5’-AGTGGACACCGGCGTGGG-3’) and p21-R (5’-TTAGGCTCCTCCCTTGGAGA-3’), for MDM2 (20 cycles) using primers MDM2-F (5’-CAATCCACAAAGATAATAGCA-3’) and MDM2-R (5’-CAGACAACTTAAACGATAA-3’), and NF-YA (35 cycles) using primers NF-YA-F (5’-CTTGTGCTCTCTATCAAAAGAATTCC-3’) and NF-YA-R (5’-ACACTCGGATATGCTGTGCTTCC-3’), for PLK1 (30 cycles) using primers PLK1-F (5’-GCCAGATACAATTCCCTCAGGATC-3’) and PLK1-R (5’-GCAAAAAGCAGAAGAAGGACG-3’) and for GAPDH (25 cycles) using primers GAPDH-F (5’-AAGTATGACACAGCCTCAAGA-3’) and GAPDH-R (5’-CACACCCCTTCTGATCTCAATC-3’). The thermocycling program used was: 30 s at 95 °C, 30 s at 58 °C and 30 s at 72 °C in each cycle.

Co-immunoprecipitation. For immunoprecipitation, each 1-mg sample of cell lysates was pre-cleared by incubating with 20 μl of protein A-sepharose beads (Amershams Biosciences) for 1 h at 4 °C to reduce nonspecific binding, and subsequently allowed to react with 1 μg of an antibody against the protein of interest for 16 h at 4 °C. The antibody was incubated with protein A-sepharose beads at 4 °C overnight or with the [35S] Met-labeled proteins at 4 °C for 4 h. Before washing, proteins in the immunoprecipitated complexes were separated by 10% SDS-PAGE and detected by using a chemiluminescence detection system (Amershams Biosciences) and captured on the Super RX X-ray films (Fuji Film).

Chromatin immunoprecipitation. The assay was performed as described previously.11 The antibodies used include NF-YA (Santa Cruz Biotechnology), CDK2 (Santa Cruz Biotechnology), p21 (Santa Cruz Biotechnology) and Flag (Sigma-Aldrich). Promoter regions of PLK1 (Figure 2a) and CDC25A were amplified by PCR. Primer pairs used were PLK1 region A-F (5’-AGACTAGGAGGTGTGTTGGTGAGA-3’) and PLK1 region A-R (5’-TGAAAGACAAAGAAGAGAAGAGAAGGATTT-3’) and PLK1 region B-F (5’-CCCTGGCCGGGACTCTT-3’) and PLK1 region B-R (5’-AGGATCTCTCAGGAGAGAAGGATTT-3’) and PLK1 region C-F (5’-AAACCAGGGCGCTG-3’) and CDC25A-F (5’-AGAAGTGCGGATACCCCGAGCACAGG-3’) and CDC25A-R (5’-GCTTCTGCGTCTCCCAACCGCCT-3’).
Flow cytometry. Cells (1 x 10⁶) were fixed in ice-cold 70% ethanol at 4 °C for 16 h. To perform cell cycle analysis, cells were treated with RNase A (1 mg/ml), stained with propidium iodide (20 μg/ml), and subsequently subjected to flow cytometry using a FACSscan system (BD Biosciences, San Jose, CA, USA). Data were processed using the WinMDI software (http://facs.scripps.edu/software.html).

Clonogenic survival assay. Cells were exposed to ADR for 6 days, trypsinized, and subsequently re-seeded in a six-well plate (1.2 x 10⁴ cells per well) and cultured at 38 °C. After 10 days, the colonies were stained with crystal violet and scored.

Conflict of Interest
The authors declare no conflict of interest.

Acknowledgements. We thank Dr. Tzu-Hao Cheng for providing cell lines, and Min-Lun Li for critical reading of the manuscript. This work was supported by grants NSC99-2320-B-010-009-MY3 to F-FW and NSC99-2320-B-010-023-MY3 to T-YC from the National Science Council, DOH102-TD-C-111-007 to T-YC from the Department of Health, and 100AC-T506 to F-FW and 102AC-TC15 to M-YC from the Ministry of Education, Taiwan.

1. Bruinsma W, Raaijmakers JA, Medema RH. Switching Polo-like kinase-1 on and off in time and space. Trends Biochem Sci 2012; 37: 534–542.
2. Lens SM, Voest EE, Medema RH. Shared and separate functions of polo-like kinases and aurora kinases in cancer. Nat Rev Cancer 2010; 10: 825–841.
3. van Vught MA, Medema RH. Getting in and out of mitosis with Polo-like kinase-1. Oncogene 2005; 24: 2844–2859.
4. Martin BT, Strebehand K. Polo-like kinase 1: target and regulator of transcriptional control. Cell Cycle 2006; 5: 2881–2885.
5. Uchiumi T, Longo DL, Ferris DK. Cell cycle regulation of the human polo-like kinase (PLK) promoter. J Biol Chem 1997; 272: 9166–9174.
6. Lindon C, Pines J. Ordered proteolysis in anaphase inactivates Plk1 to contribute to proper mitotic exit in human cells. J Cell Biol 2004; 164: 233–241.
7. Smits VA, Klompmaker R, Arnaud L, Rijksen G, Nigg EA, Medema RH. Polo-like kinase-1 mediates the p53-dependent prevention of caspase-independent mitotic death. Mol Cancer Res 2002; 1: 321–330.
8. Takaki T, Trenz K, Costanzo V, Petronczki M. Polo-like kinase 1 reaches beyond mitosis-cytokinesis, DNA damage response, and development. Curr Opin Cell Biol 2008; 20: 650–660.
9. Yuan JH, Feng Y, Fisher RH, Maloid S, Longo DL, Ferris DK. Polo-like kinase 1 inactivation following mitotic DNA damaging treatments is independent of ataxia telangiectasia mutated kinase. Mol Cancer Res 2004; 2: 417–426.
10. van Vught MA, Smits VA, Klompmaker R, Medema RH. Inhibition of Polo-like kinase-1 by DNA damage occurs in an ATM- or ATR-dependent fashion. J Biol Chem 2001; 276: 41656–41660.
11. Lin YC, Sun SH, Wang FF. Suppression of Polo-like kinase 1 (PLK1) by p21(Waf1) mediates the p53-dependent prevention of caspase-independent mitotic death. Cell Signal 2011; 23: 1816–1823.
12. Jackson MW, Agnaral MK, Yang J, Bruss P, Uchiumi T, Agnaral ML et al. p130cip107/p15Sr-dependent transcriptional repression during DNA damage-induced cell-cycle exit at G2. J Cell Sci 2005; 118(Pt 9): 1821–1832.
13. McKenzie L, King S, Marcar L, Nicol S, Dias SS, Schumm K et al. p33-dependent repression of polo-like kinase-1 (PLK1). Cell Cycle 2010; 9: 4200–4212.
14. Zhu H, Chang BD, Uchiumi T, Robinson IB. Identification of promoter elements responsible for transcriptional inhibition of polo-like kinase 1 and topoisomerase IIalpha genes by p21(WAF1/CIP1/SDI1). Cell Cycle 2002; 1: 59–66.
15. Brauningr A, Strebehand K, Rubasimen-Waigmann H. Identification and functional characterization of the human and murine polo-like kinase (Pik) promoter. Oncogene 1995; 11: 1793–1800.
16. Maltz SN, de Crombrugghe B. Role of the CCAAT-binding protein CBF/NF-Y in transcription. Trends Biochem Sci 1998; 23: 174–178.

17. Mantovani R. The molecular biology of the CCAAT-binding factor NF-Y. Gene 1999; 239: 15–27.
18. Ceribelli M, Dolfini D, Merco D, Gatta R, Vigano AM, Pavesi G et al. The histone-like NF-Y is a bifunctional transcription factor. Mol Cell Biol 2006; 26: 4084–4088.
19. Peng Y, Jahroudi N. The NF-Y transcription factor functions as a repressor and activator of the von Willebrand factor promoter. Blood 2002; 99: 2408–2417.
20. Peng Y, Stewart D, Li W, Hawkins M, Kulak S, Ballermann B et al. Irradiation modulates association of NF-Y with histone-modifying cofactors PCAF and HDAC. Oncogene 2007; 26: 7576–7583.
21. Zhu X, Wang Y, Pi W, Liu H, Wickrema A, Tuan D. NF-Y recruits both transcription activator and repressor to modulate tissue- and development-stage specific expression of human gamma-globin gene. PloS ONE 2012; 7: e47715.
22. Chae HD, Yun J, Bang YJ, Shin DY. Cdk2-dependent phosphorylation of the NF-Y transcription factor is essential for the expression of the cell cycle-regulatory genes and cell cycle G1 and G2/M transitions. Oncogene 2004; 23: 4084–4088.
23. Yun J, Chae HD, Choi TS, Kim EH, Bang YJ, Chung J et al. Cdk2-dependent phosphorylation of the NF-Y transcription factor and its involvement in the p53–p21 signaling pathway. J Biol Chem 2003; 278: 36966–36972.
24. Vigneron A, Chetier J, Barre B, Gamelin E, Coqueret O. The cell cycle inhibitor p21wa1f1 binds to the myc and cdc25A promoters upon DNA damage and induces transcriptional repression. J Biol Chem 2006; 281: 34742–34750.
25. Xiong Y, Haunon GJ, Zhang H, Caso D, Kobayashi R, Beach D. p21 is a universal inhibitor of cyclin kinases. Nature 1993; 366: 701–704.
26. Garcia-Wilson E, Perkins ND. p21WAFl/CIP1 regulates the p300 sumoylation motif CRD1 through a C-terminal domain independently of cyclin/CDK binding. Cell Cycle 2005; 4: 1113–1119.
27. Mantovani R, Li XY, Pessara U, Hooft van Huijsduijnen R, Benoist C, Mathis D. Dominant negative analogs of NF-YA. J Biol Chem 1994; 269: 20340–20346.
28. Lin YC, Wang FF. Mechanisms underlying the pro-survival pathway of p53 in suppressing mitotic death induced by adriamycin. Cell Signal 2008; 20: 258–267.
29. Dalavi M, Mondesert O, Bourdon JC, Ducomman B, Dozer C. Cdc25B is negatively regulated by p53 through Sp1 and NF-Y transcription factors. Oncogene 2011; 30: 2035–2045.
30. St Clair S, Giono L, Varmeh-Ziaie S, Resnick-Silverman L, Liu WJ, Padi A et al. DNA damage-induced downregulation of Cdc25C is mediated by p53 via two independent mechanisms: one involves direct binding to the cdc25C promoter. Mol Cell 2004; 16: 725–736.
31. Innocente SA, Lee JM, p53 is a NF-Y- and p21-independent, Sp1-dependent repressor of cyclin B1 transcription. FEBS Lett 2005; 579: 1001–1007.
32. Yun J, Chae KD, Choy HE, Chung J, Yoo HS, Han MH et al. p33 negatively regulates cc2c transcription via the CCAAT-binding NF-Y transcription factor. J Biol Chem 1999; 274: 29677–29682.
33. Imbriano C, Gurtner A, Cociaciarella F, Di Agostino S, Basile V, Gostissa M et al. Direct p53 transcriptional repression: in vivo analysis of CCAAT-containing G2/M promoters. Mol Cell Biol 2005; 25: 3737–3751.
34. Imbriano C, Gresutta N, Mantovani R. The NF-Y/p33 liaison: well beyond repression. Biochim Biophys Acta 2012; 1825: 131–139.
35. Ando K, Otsuki T, Yamamoto H, Fuyuya K, Hosoda M, Hayashi S et al. Polo-like kinase 1 (Pik1) inhibits p53 function by physical interaction and phosphorylation. J Biol Chem 2004; 279: 25549–25561.
36. Gurtner A, Fuschi P, Martelli F, Manni I, Artuso S, Simonte G et al. Transcription factor NF-Y induces apoptosis in cells expressing wild-type p53 through E2F1 upregulation and p33 activation. Cancer Res 2010; 70: 9711–9720.
37. Schoffski P. Polo-like kinase (PLK) inhibitors in preclinical and early clinical development in oncology. Oncologit 2009; 14: 559–570.
38. Strebehand K, Ulrich A. Targeting polo-like kinase for cancer therapy. Nat Rev Cancer 2006; 6: 321–330.