Oncogene (2006) 25, 2829–2838

www.nature.com/onc

ORIGINAL ARTICLE

P21$^{Cip1/WAF1}$ downregulation is required for efficient PCNA ubiquitination after UV irradiation

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p21$^{Cip1/WAF1}$ is a known inhibitor of the short-gap filling activity of proliferating cell nuclear antigen (PCNA) during DNA repair. In agreement, p21 degradation after UV irradiation promotes PCNA-dependent repair. Recent reports have identified ubiquitination of PCNA as a relevant feature for PCNA-dependent DNA repair. Here, we show that PCNA ubiquitination in human cells is notably augmented after UV irradiation and other genotoxic treatments such as hydroxyurea, aphidicolin and methylmethane sulfonate. Intriguingly, those DNA damaging agents also promoted downregulation of p21. While ubiquitination of PCNA was not affected by deficient nucleotide excision repair (NER) and was observed in both proliferating and arrested cells, stable p21 expression caused a significant reduction in UV-induced ubiquitinated PCNA. Surprisingly, the negative regulation of PCNA ubiquitination by p21 does not depend on the direct interaction with PCNA but requires the cyclin dependent kinase binding domain of p21. Taken together, our data suggest that p21 downregulation plays a role in efficient PCNA ubiquitination after UV irradiation.
Oncogene (2006) **25,** 2829–2838. doi:10.1038/sj.onc.1209315; published online 9 January 2006

Keywords: p21; PCNA; ubiquitination; proteolysis; DNA repair

Introduction

Proliferating cell nuclear antigen (PCNA) is a ring-shaped trimeric complex highly conserved through evolution that has essential roles in DNA replication and repair (Warbrick, 2000). PCNA forms a sliding platform required both for the processivity of the DNA polymerase complex and the correct coordination of leading and lagging strand DNA synthesis (Prelich and Stillman, 1988). PCNA also plays a pivotal role in several forms of DNA repair (including nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR)) and various aspects of postreplicative processing (Jonsson and Hubscher, 1997; Kelman, 1997). The p21$^{Cip1/waf1}$ protein, a member of a family of cyclin dependent kinase (CDK) inhibitors (CKIs), is capable of blocking PCNA functions through multiple mechanisms. *In vitro*, p21 blocks PCNA-dependent DNA replication by interfering with its interaction with RFC (replication factor C) (Oku *et al*., 1998; Waga and Stillman, 1998), DNA polymerase δ (Podust *et al*., 1995), and FEN1 (Chen *et al*., 1996). P21 also interferes with PCNA interaction with DNA repair factors required for NER (Gary *et al*., 1997). In those scenarios, p21 interaction with PCNA inhibits the resynthesis step of the repair process (Pan *et al*., 1995; Shivji *et al*., 1998). *In vivo*, however, whereas some groups have found an inhibitory role of the C terminus of p21 on unscheduled DNA synthesis (UDS) (Cooper *et al*., 1999), others have reported a positive or null role of p21 in NER (McDonald *et al*., 1996; Sheikh *et al*., 1997). While the biological significance of the PCNA/p21 interaction is not yet clear, a recent report shows that p21 downregulation is required for efficient PCNA dependent-UDS after UV irradiation, (Bendjennat *et al*., 2003) implying that, in cultured cells, p21 accumulation might be sufficient to inhibit PCNA-dependent DNA repair.

Recently, PCNA ubiquitination has been identified as a repair relevant modification of the sliding clamp in *Saccharomyces cerevisiae*, as yeast expressing, from the original gene locus, a PCNA mutant unable to undergo ubiquitination are very sensitive to UV light and methyl methane sulfonate (MMS) treatment (Hoege *et al*., 2002; Stelter and Ulrich, 2003). This is particularly interesting as ubiquitin conjugation systems such as Rad6/Rad18 and Mms2/Ubc13/Rad5 have long been known to participate in error-free post replicative repair but their repair-related substrates were unknown (Pickart, 2002). Importantly, Hoege *et al*. (2002) not only showed that PCNA is a substrate for those enzymes after MMS and UV treatments but they also demonstrated that PCNA functions are exquisitely regulated by sumoylation, mono- and multiubiquitination at lysine 164. While the role of PCNA multiubiquitination in mammals is less well explored, the PCNA ubiquitination site is conserved (Hoege *et al*., 2002), and in human cells,

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Received 19 July 2005; revised 24 October 2005; accepted 9 November 2005; published online 9 January 2006

PCNA monoubiquitination is strongly impaired when Rad18 levels are downregulated, thus suggesting that the enzymatic pathway responsible for ubiquitin conjugation to PCNA is conserved as well (Kannouche *et al*., 2004). In agreement with those findings, photobleaching experiments in mammalian cells have shown that ubiquitination of PCNA is required for the accumulation and stability of PCNA at the sites of damage (Solomon *et al*., 2004). Moreover, ubiquitinated PCNA has a much higher affinity than unmodified PCNA for DNA Polymerase η, an enzyme that can replicate past CPDs (cyclobutane pyrimidine dimers) with similar efficiency to undamaged templates (Kannouche *et al*., 2004). Importantly, Rad18 not only triggers PCNA monoubiquitination but also constitutively interacts with Pol η therefore facilitating PCNA/Pol η colocalization at the sites of UV-induced DNA lesions (Watanabe *et al*., 2004). Thus, a polymerase switch triggered by changes in the PCNA ubiquitination status might be promoted to translesion DNA synthesis (TLS) *in vivo*.

Different lines of evidence reported herein suggested that p21 could also interfere with PCNA ubiquitination after DNA damage. Accordingly, p21 proteolysis increases after a number of genotoxic stresses that promote PCNA ubiquitination and sustained expression of p21 after UV irradiation negatively modulates PCNA ubiquitination. Therefore, increased p21 turnover might represent a significant aspect of the cellular response to UV irradiation by promoting increased PCNA ubiquitination. The identification of the CDK binding domain of p21 as the central motif required for p21-dependent inhibition of PCNA ubiquitination sheds light on the potential pathways connecting these ubiquitin-regulated processes when damage to DNA takes place.

Results

PCNA ubiquitination correlates with inefficient accumulation of p21

It has been demonstrated that p21 can interfere with PCNA-dependent UDS after UV irradiation (Bendjennat *et al*., 2003). PCNA is modified by ubiquitination after UV irradiation or treatment of cells with hydroxyurea (HU) and MMS but not after γ irradiation (Kannouche *et al*., 2004). As the current model of p21 inhibition of UV triggered PCNA-dependent DNA repair does not take PCNA ubiquitination into account, we decided to explore the relationship between p21 and PCNA ubiquitination. To do so, PCNA ubiquitination at lysine 164 was confirmed after UV irradiation (Supplementary Figure 1). In agreement with previous reports (Hoege *et al*., 2002; Kannouche *et al*., 2004; Watanabe *et al*., 2004), UV irradiation and MMS treatments resulted in the accumulation of a Triton-resistant, slowly migrating form of PCNA whose molecular weight was increased by approximately 10 kDa which corresponds to a single ubiquitin bound to PCNA (Figure 1a). It has also been suggested that a signal relevant for PCNA ubiquitination is the accumulation of stall replication forks (Kannouche and Lehmann, 2004). In agreement, PCNA ubiquitination was observed after the inhibition of ribonucleotide reductase by HU (as previously reported by Kannouche *et al*. (2004)) and by the DNA polymerase α inhibitor, aphidicolin (APH). Other treatments, such as actinomycin D (ActD) and daunorubicin (Dauno), which arrest cells in the G_1 and G_2 phases, respectively, did not promote the accumulation of ubiquitinated PCNA (Figure 1a). Also, neocarcinostatin (NCS), a γ-irradiation mimicking treatment, results in no detectable PCNA ubiquitination. It is interesting to note that ubiquitinated PCNA accumulated at a slower rate after HU treatment when compared to UV irradiation and MMS (Figure 1a and Supplementary Figure 2), suggesting that while the stalling of replication forks might be necessary for the ubiquitination of PCNA, the accumulation of DNA damage on the stalled forks might also be required to activate PCNA ubiquitination. In line with this speculation are the results obtained with hypoxia (Hammond and Giaccia, 2004) and the hypoxia-mimicking drug, deferoxamine mesylate (DFX) (Ashcroft *et al*., 2000; Gottifredi *et al*., 2004), which result in the accumulation of HCT116 and other cell lines at the G1/S boundary without detectable damage to DNA (Hammond *et al*., 2002, 2003). Our experiments show that whereas HU and APH increased PCNA ubiquitination, hypoxia and DFX failed to do so (Figure 1a). We, therefore, speculate that the cell cycle arrest beyond origin firing is not sufficient for the ubiquitination of PCNA and the accumulation of longer patches of ssDNA or broken DNA might be the trigger for PCNA ubiquitination.

To explore the relationship between p21 and PCNA ubiquitination, total extracts of cells treated in parallel were also prepared. p21 proteolysis increases after UV (Bendjennat *et al*., 2003), MMS (our unpublished results) and HU and APH (Gottifredi *et al*., 2004). As a consequence, p21 levels are reduced with respect to unstressed cells in UV- and MMS-treated cells, whereas in HU- and APH-treated cells, levels of p21 do not increase over unstressed control even when p53 levels are high (Figure 1b). In those cases, PCNA ubiquitination is efficiently induced. On the contrary, treatments such as ActD, Dauno and NCS, well-characterized inducers of p21 (Figure 1b), did not upregulate PCNA ubiquitination (Figure 1a). Our results thus show a striking inverse correlation between the upregulation of p21 and the accumulation of PCNA ubiquitination. Additionaly, although PCNA ubiquitination and p21 proteolysis might be coupled, increased p21 proteolysis is not a trigger for PCNA ubiquitination since treatments that upregulate p21 degradation such as DFX (Gottifredi *et al*., 2004) do not promote PCNA ubiquitination.

The effect of UV irradiation on PCNA ubiquitination and p21 proteolysis is dominant over other genotoxic agents

The results described in Figure 1 suggested that stalled replication forks might not be the only signal that

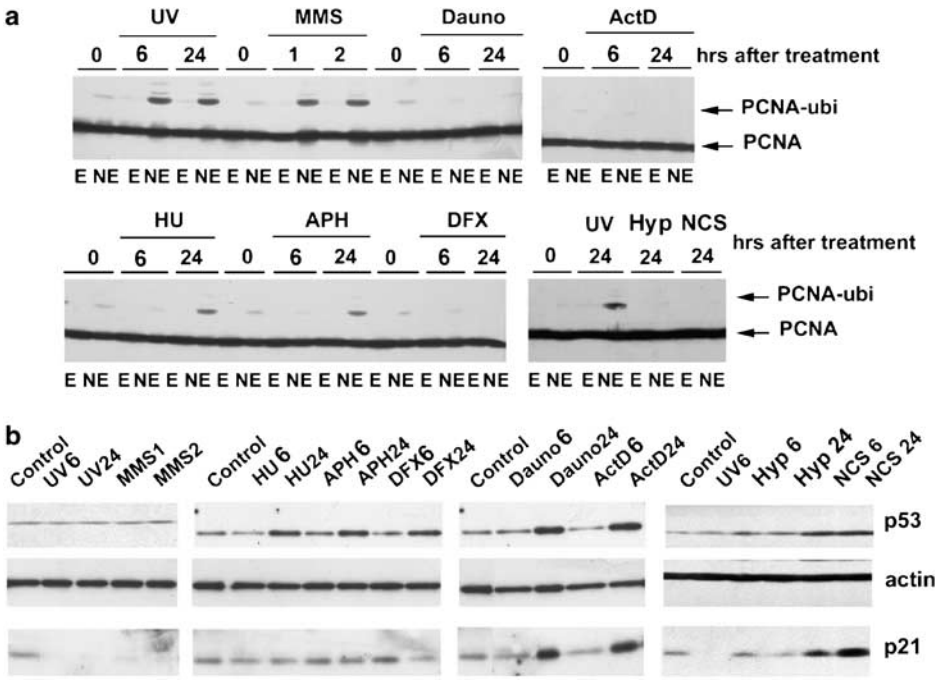

Figure 1 Defective p21 accumulation correlates with PCNA ubiquitination. (**a**) Exponentially growing HCT116 cells were exposed to the indicated treatments as described in Materials and Methods. At the indicated times, cells were subjected to Triton extractions followed by Western blot analyses with PCNA specific antibodies. E (Extractable fraction); NE (Nonextractable fraction). (**b**) Exponentially growing HCT116 cells were exposed to the indicated treatments as described in Materials and Methods. At the indicated times, total cell extracts were prepared and samples were subjected to Western blot analyses with p21 and p53 specific antibodies. Actin was used as the loading control. Numbers next to each treatment corresponds to the time (in hours) of exposure to the indicated genotoxic stress.

triggers PCNA ubiquitination. To test this, HCT116 cells arrested in G_1 or G_2 phases of the cell cycle were UV irradiated. HCT116 cells were serum starved (0.5% FBS) or treated with ActD to accumulate them in G_1 (see cell cycle profiles – Figure 2a) or with Dauno (D) and nocodazole (Noc) to accumulate them in G_2 (see cell cycle profiles – Figure 2a). UV irradiation was performed 6 h before lysis and the genotoxic agents were maintained for the whole length of the experiment. UV irradiation did not dramatically change the cell cycle distribution after 6 h. However, a slight increase in S-phase was observed in asynchronous control cells after UV irradiation and cell death became detectable when ActD, Dauno, Noc and starved cells were UV irradiated. Interestingly, we observed that PCNA ubiquitination might occur in cells arrested by different means and at different stages of the cell cycle (Figure 2a). In all cases, a strong dominant effect of UV irradiation was observed on p21 levels that were strongly reduced even when cells were treated with p53 inducers, such as ActD and Dauno.

To avoid the complexity of stress-induced alterations in p21 mRNA levels in cells with wild-type p53, we performed similar experiments in engineered H1299 cells in which p21 can be induced by tetracycline removal (Baptiste *et al*., 2002). Here, we observed that the levels of PCNA ubiquitination after UV irradiation are similar when tetracycline has or has not been removed (Figure 2b). Also, UV irradiation of this cell line alone or in combination with other treatments does not affect the cell cycle distribution (see FACS analysis – Figure 2b). Interestingly, in this case PCNA ubiquitination also correlates with efficient p21 downregulation by UV thus suggesting that the same or related signals to the ones that trigger PCNA ubiquitination could also upregulate p21 proteolysis after UV. Moreover, similar results were observed when cells were treated with ActD or Dauno in the presence or absence of tetracycline (Figure 2b). Taken together, these data indicate that PCNA ubiquitination is cell cycle independent and that other signals, perhaps arising from the DNA repair or the checkpoint machinery, are determinants of PCNA ubiquitination.

Accumulation of p21 after UV treatment impairs PCNA ubiquitination

The above-mentioned results are particularly interesting in the context of a recent report that demonstrates p21 downregulation is required for efficient PCNA dependent DNA repair after UV irradiation (Bendjennat *et al*., 2003). In fact, the results in Figures 1 and 2 suggest that while PCNA ubiquitination and p21 proteolysis can take place independently, PCNA ubiquitination is not detected when p21 levels are high. To explore the relevance of p21 proteolysis on PCNA ubiquitination, it was imperative to create an artificial scenario in which p21 levels are upregulated during UV

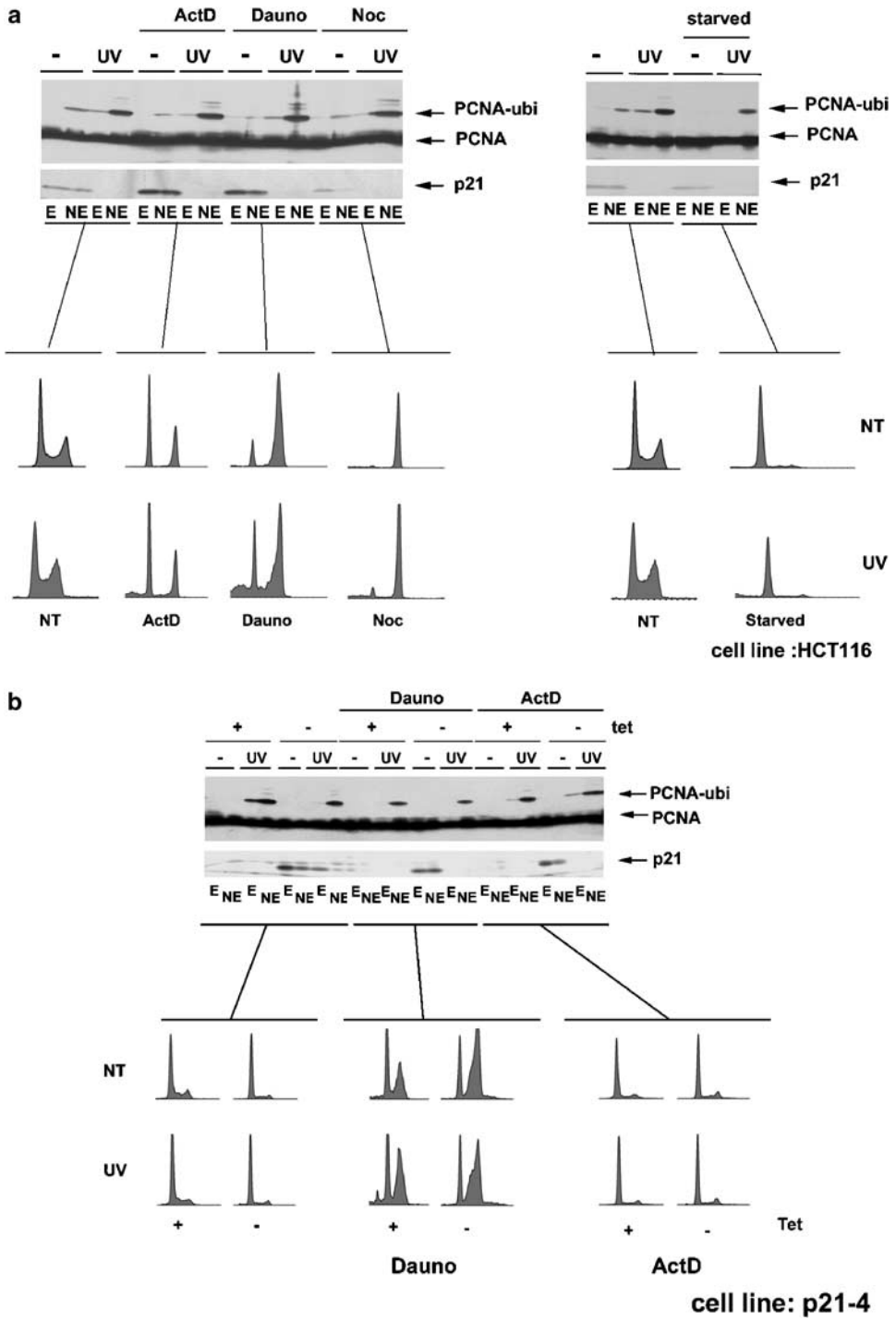

Figure 2 PCNA ubiquitination is not dependent on the cell cycle. (**a**) G1 and G2 arrest do not prevent induction of PCNA ubiquitination by UV. HCT116 cells were treated with the indicated compounds for 24 h or serum starved (0.5% FBS) for 48 h. At 6 h before lysis, the cell culture medium was removed and cells were UV irradiated. Cell culture medium containing the indicated compounds was immediately readded to cells after UV irradiation. At the indicated times, cells were subjected to Triton extraction and Western blot analyses were performed with PCNA and p21 specific antibodies. FACS analysis was performed to monitor the effective accumulation of cells in the expected phase of the cell cycle. E (Extractable fraction); NE (Nonextractable fraction). (**b**) UV promotes both p21 downregulation and PCNA ubiquitination. H1299 cells expressing a tetracycline-regulated p21 were washed extensively to induce p21 when indicated. Tetracycline removal was combined with ActD and Dauno treatments when indicated. Cells were UV irradiated 6 h before Triton extraction and PCNA and p21 protein levels were determined by Western blot. In all cases, tetracycline, ActD and Dauno treatment were maintained for the whole length of the experiment. FACS analysis was performed to monitor the effective accumulation of cells in the expected phase of the cell cycle. E (Extractable fraction); NE (Nonextractable fraction).

treatment. Although it is clear that p21 degradation is regulated by the proteasome, the mechanism behind p21 degradation has been a subject of debate (Blagosklonny *et al*., 1996; Sheaff *et al*., 2000; Touitou *et al*., 2001; Bendjennat *et al*., 2003; Bloom *et al*., 2003; Bornstein *et al*., 2003). Initially, it was reported that the degradation of p21 is ubiquitin independent (Sheaff *et al*., 2000), but more recent work demonstrated that p21 degradation switches from ubiquitination-independent to ubiquitination-dependent proteolysis after UV irradiation (Bendjennat *et al*., 2003). Finally, it was shown that ubiquitination of the N-terminus is required for p21 degradation in unstressed cells, and that p21 proteolysis can be blocked by fusing six tandem Myc epitopes to the N-terminus (Bloom *et al*., 2003). Thus, we tested the effect of UV irradiation on different p21 mutants. A p21 construct in which all internal lysines were mutated, p21 K6R, was as unstable as wild-type p21 after different doses of UV irradiation (Figure 3a) and was degraded with similar kinetics (not shown). On the contrary, 6 Myc p21 was stable after UV irradiation irrespective of the presence of internal lysines (Figure 3a). This result is in agreement with N-terminal ubiquitination being the trigger for p21 degradation both in unstressed (Bloom *et al*., 2003) and stressed cells (this work). We cannot fully understand the difference of stability in p21K6R between our experiments and those reported by Fotedar and co-workers (Bendjennat *et al*., 2003) but they might depend on the lack of N-terminal tag in our p21 K6R construct or the different cell lines employed. Importantly, the 6 Myc p21 mutant has unaltered capacity to interact with PCNA and CDK2 and effectively localizes to the nucleus (see Supplementary Figure 3a and Figure 4b). We thus tested the effect of this stable p21 mutant on PCNA ubiquitination. Wt p21 and 6 Myc p21 were cotransfected with V5-PCNA into H1299 cells, and Triton extractable and insoluble fractions were collected before and after UV irradiation. Importantly, impaired accumulation of PCNA ubiquitination was observed when 6 Myc p21 transfected cells were UV irradiated (Figure 3b), therefore suggesting that p21 proteolysis increases after UV irradiation to allow efficient PCNA ubiquitination. Moreover, sustained expression of p21 after UV irradiation impaired the accumulation of ubiquitinated PCNA both at low and high doses of UV irradiation (Figure 3d).

The interaction between p21 and PCNA has been extensively analysed in the past. In fact, both the inhibition of PCNA dependent replication and PCNA-dependent repair by p21 depend on this interaction (Dotto, 2000). A second important function of p21 relies

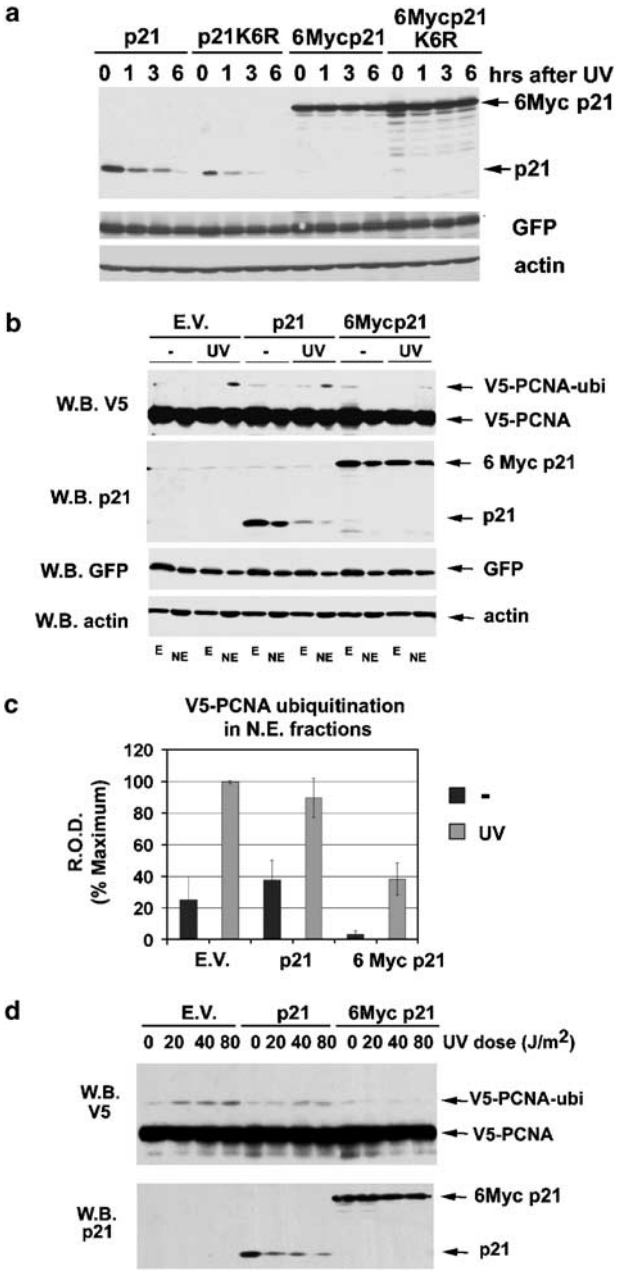

Figure 3 Stable p21 impairs PCNA ubiquitination by UV irradiation. (**a**) N terminal tagged but not lysine-less p21 is stable after UV treatment. Wt p21, p21-K6R, 6 Myc p21 and 6 Myc p21K6R were transfected into H1299 cells. Cells were UV irradiated at the indicated hours before lysis. GFP was cotransfected to monitor efficiency of transfection. Actin was used as a loading control. (**b**) 6 Myc p21 downregulates PCNA ubiquitination. Empty vector (E.V.), wt p21 and 6 Myc-21 were transfected with V5-PCNA as indicated. At 48 h after transfection, the indicated samples were exposed to UV light as indicated. At 6 h after UV irradiation, samples were Triton extracted and Western blots were performed with the indicated specific antibodies. (**c**) Levels of V5-PCNA ubiquitination in NE fractions obtained from densitometric analysis performed on V5 and PCNA Western blots from three independent experiments. Values are expressed as percentage with respect to the signal obtained for the V5-PCNA ubiquitinated band corresponding to the UV irradiated sample transfected with V5- PCNA and empty vector (lane 4 in Figure 3b). ROD (%) = relative optical density (as % of maximal value). E (Extractable fraction); NE (Nonextractable fraction). (**d**) The effect of 6 Myc p21 on PCNA ubiquitination is independent of the dose of UV irradiation. Empty vector (E.V.), p21 wt and 6 Myc21 wt were co-transfected with V5-PCNA. At 48 h after transfection, samples were exposed to the indicated doses of UV irradiation. After 6 h, samples were Triton extracted and the NE fraction was resolved in SDS-PAGE, and Western blots were performed with the indicated specific antibodies.

on its interaction with CDKs and their cyclin partners. The repression of the activity of CDKs by p21 is central for its inhibition of DNA replication (Dotto, 2000). However, p21 dependent inhibition of CDKs has not been linked to any aspect of PCNA dependent DNA repair. We therefore created two 6 Myc p21 mutants encoding previously described mutations that disrupt the CDK and the PCNA binding motif of p21 (Gulbis *et al*., 1996). As expected, 6 Myc p21(PCNA-) did not interact with PCNA (Supplementary Figure 3a), and 6 Myc p21(CDK-) did not interact with CDK2 (Supplementary Figure 3a), and both mutants localized in the nuclear compartment (Figure 4b). Additionally, these mutants were as stable as 6 Myc p21 after UV irradiation (Figure 4a). The effect of 6 Myc p21 wt, 6 Myc p21(PCNA-) and 6 Myc p21(CDK-) mutants on the cell cycle was also determined. whereas transient transfection of 6 Myc p21 wt and 6 Myc p21(PCNA-) resulted in cell cycle arrest similar to the ones obtained for wt p21, the cell cycle profile that resulted from 6 Myc p21(CDK-) transfection was very similar to the one obtained after transfection of empty vector (Figure 4c). We also performed a 5-bromodeoxyuridine (BrdU) incorporation experiment that revealed that transfection with p21 wt, 6 Myc p21 wt, 6 Myc p21(PCNA-) resulted in about a 50% inhibition of BrdU incorporation. Opposite results were obtained with the 6 Myc p21(CDK-), mutant which was not able to reduce the incorporation of BrdU when compared to controls transfected with empty vector (Supplementary Figure 3b). When the effect of these p21 mutants on PCNA ubiquitination was tested, we observed that 6 Myc p21(PCNA-) was almost as capable as 6 Myc p21 wt of inhibiting V5-PCNA ubiquitination after UV (Figure 4d), therefore demonstrating that modulation of PCNA ubiquitination by high levels of p21 does not require interaction between these two proteins. On the contrary, the 6 Myc p21(CDK-) mutant, in spite of its unaltered capacity to bind PCNA after UV irradiation, was very inefficient in down regulating PCNA ubiquitination (Figure 4d). Similar results were obtained in three cell lines (Supplementary Figure 4) therefore suggesting that CDKs or a related factor might promote PCNA ubiquitination. Thus, the CDK binding capacity might be a central feature of p21 that requires negative modulation in cells after UV irradiation to allow efficient PCNA ubiquitination.

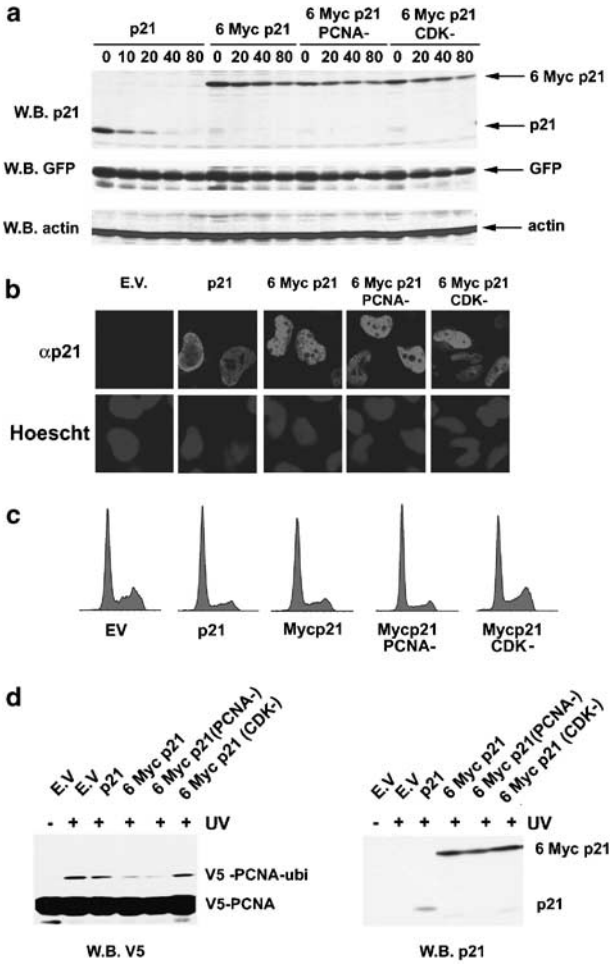

Figure 4 The CDK binding domain of p21 is required for p21 to reduce PCNA ubiquitination. (**a**) 6 Myc p21 mutants defective in PCNA and CDK interaction are stable after UV irradiation. HCT116 cells were transfected with empty vector (E.V.), p21, 6 Myc p21 and 6 Myc p21(PCNA-) and 6 Myc p21 (CDK-) as indicated. After 48 h, the indicated samples were irradiated with the indicated doses of UV irradiation. After 6 h, samples were lysed and levels of p21 determined by Western blot analysis with specific antibodies to p21. GFP was cotransfected to monitor efficiency of transfection. Actin was used as a loading control. (**b**) All p21 mutants localize to the nucleus. H1229 cells plated on coverslips were transfected with empty vector, wt p21, 6 Myc p21 and 6 Myc p21 (PCNA-) and 6 Myc p21 (CDK-). After 24 h, cells were fixed and p21 localization was determined by incubation of coverslips with a p21 specific antibody. Hoescht staining was used to identify the cellular nuclei. (**c**) 6 Myc p21 (PCNA-) and 6 Myc p21 (CDK-) have differential effects on cell cycle distribution. H1299 cells were transfected with the indicated vectors and GFP. The cell cycle distribution of GFP positive cells was determined by FACS. (d) 6 Myc p21(CDK-) cannot impair PCNA ubiquitination after UV irradiation. H1299 cells were transfected with the indicated vectors. After 48 h, the indicated samples were UV irradiated. After 6 h, cells were triton extracted and fractions were collected. NE fractions were resolved by SDS PAGE, and Western blot analysis with V5 and p21 specific antibodies revealed PCNA and p21 levels.

Discussion

An increasing amount of evidence supports an essential role of PCNA ubiquitination in DNA repair both in yeast and mammals. Whereas both multi- and mono-ubiquitination of PCNA were reported to have differential roles in yeast (Hoege *et al*., 2002; Pickart, 2002) monoubiquitination of PCNA in mammals after DNA damage might prevent fatal arrest of replication forks by allowing lesion bypass (Kannouche *et al*., 2004; Watanabe *et al*., 2004). Here, we show that ubiquitination of PCNA is independent of the cell cycle but is highly dependent on genotoxic stimuli. The integrity of the repair machinery does not alter PCNA ubiquitination after UV (Kannouche *et al*., 2004) HU or APH

(Supplementary Figure 5), thus suggesting that the signals regulating PCNA ubiquitination might result from the exposure of ssDNA, its coating protein RPA, or checkpoint activation. In an effort to identify regulators of PCNA monoubiquitination, we explored the effect of p21 on this process and found a striking correlation between inefficient p21 accumulation and efficient PCNA ubiquitination. Moreover, the levels of ubiquitinated PCNA are negatively modulated upon transient expression of stable p21 showing that, indeed, p21 is a negative regulator of PCNA ubiquitination after UV irradiation. Therefore, the increased p21 turnover after UV irradiation might not only promote DNA repair by NER (Bendjennat *et al*., 2003) but it might also be relevant for TLS. The implications of our findings are discussed below.

P21 as a negative regulator of PCNA ubiquitination

The observations reported herein reveal a novel aspect of the regulation of PCNA functions. whereas many groups have shown that the p21/PCNA interaction competitively impairs the interaction of PCNA with essential replicative and repairative factors, the regulation of PCNA-dependent DNA synthesis by p21 *in vivo* remains controversial. Here, we show that p21 impairs PCNA ubiquitination, a repair-relevant modification of PCNA, through its CDK binding motif. The main mechanism by which PCNA ubiquitination promotes DNA repair is the bypass of DNA lesions that hamper the progression of DNA synthesis. In fact, ubiquitinated PCNA preferentially binds to DNA Pol η, a polymerase capable of replicating DNA across various lesions (Kannouche *et al*., 2004). Notably, p21 degradation after UV irradiation is essential for the repair-related synthesis of DNA as measured in UDS (unscheduled DNA synthesis) assays (Bendjennat *et al*., 2003). Our work might shed light on new aspects of p21-dependent inhibition of DNA repair. Although the cellular pool of ubiquitinated PCNA that accumulates after UV might specifically interact with permissive polymerases and favor TLS, excess of p21 would downregulate ubiquitinated PCNA levels, inhibiting the formation of PCNA/Pol η complexes and thus hampering TLS. At this point it is interesting to mention that the activities of ubiquitinated PCNA might not be restricted to TLS. In fact, after UV irradiation of cells arrested in the G1 and G2 phases of the cell cycle, PCNA is efficiently ubiquitinated and p21 is also degraded, therefore, suggesting that both ubiquitin-dependent processes might be coupled to DNA repair in all phases of the cell cycle. Some of the effectors of p21 and PCNA ubiquitination have been identified. In fact, the monoubiquitination of PCNA depends on the E2 ubiquitin conjugating enzyme, Rad6, and the E3 ubiquitin ligase and DNA binding protein Rad18. The nondegradative multiubiquitination of the sliding clamp is dependent on another E2-E3 system, the Ubc13/Mms2 dimer. As for p21, a clear link with SKP2 has been established both *in vitro* and *in vivo* (Bendjennat *et al*., 2003; Bloom *et al*., 2003; Bornstein *et al*., 2003). However, p21 is also unstable in the G_0 phase of the cell cycle when SKP2 is not expressed, which implies that another E3 ligase could participate in p21 degradation (Bendjennat *et al*., 2003). Furthermore, the ligase responsible for the N-terminal ubiquitination of p21 has not been identified yet, but the fact that *Xenopus* p21 (Xic1) is recruited by PCNA to DNA where its proteolysis is coupled to the initiation of DNA synthesis (Furstenthal *et al*., 2001; Chuang and Yew, 2005) may provide a hint for its discovery. In that respect, it is interesting to point out that the ubiquitin ligases for PCNA are DNA binding proteins, suggesting that PCNA ubiquitination and p21 degradation could occur in the same vicinity. In fact, it could be speculated that ubiquitination of both p21 and PCNA might be coordinately triggered by DNA damage that requires and/or mimics replicative DNA synthesis. In line, p21 downregulation is required for the efficient restart of DNA synthesis after S-phase block, which suggests that p21 could hamper a broad range of PCNA activities in cells (Gottifredi *et al*., 2004).

Although the binding of p21 to PCNA is required for p21 to interfere with DNA replication and NER in both *in vitro* and *in vivo* models, our study suggests that their interaction does not directly affect PCNA ubiquitination. Using a mutant version of p21 that cannot bind to PCNA, we were able to show that interaction of p21 with PCNA is not required for p21 to negatively modulate PCNA monoubiquitination. Moreover, the result obtained with 6 Myc p21 (CDK-) suggests that the kinase activity of CDKs might have a role in PCNA ubiquitination. It is therefore possible to hypothesize that p21 could interfere with UDS through direct p21/PCNA interaction, and with TLS through indirect modulation of PCNA ubiquitination by CDK or another related kinase. This is particularly interesting as it has been recently reported that CDK2 phosphorylates an abasic sites translesion polymerase (DNA Pol λ) (Frouin *et al*., 2005). In addition, CDK2 has been implicated in double strand break (DSB) repair (Muller-Tidow *et al*., 2004). Although this DNA repair process is not directly relevant to this work, this observation allows speculation that CDK2 activity might be relevant to other DNA repair processes. Moreover, protein phosphorylation generally precedes protein ubiquitination, therefore suggesting that CDK kinase activity could promote PCNA ubiquitination. In that respect, it is important to highlight that PCNA has no consensus site for CDKs. Also, CDK2 might already be inactive during UV treatment in a p21-independent manner that correlates with increased and sustained inhibitory phosphorylation of CDK2 at Y15 (Bendjennat *et al*., 2003); therefore, its activity might not directly affect PCNA ubiquitination. Also, we observed PCNA ubiquitination in G1 arrested cells where no CDK kinase activity is expected. On the other hand, the requirement of low, residual activity of CDKs for PCNA ubiquitination cannot be discarded. Also, other kinases such as JUNK and p38, which are also inactivated by p21 (Dotto, 2000), are suitable candidates for regulators of PCNA ubiquitination after UV. A better characterization of the functions of CDKs and other kinases on

PCNA ubiquitination may shed light on the potential kinase requirement for PCNA ubiquitination. Finally, it is also important to point out that while the 6 Myc p21 (PCNA-) mutant is as efficient as p21 wt in blocking cell cycle progression, the 6 Myc p21 (CDK-) mutant is not capable of altering cell cycle distribution with respect to control populations. Whereas the differential effect of p21 mutants on PCNA ubiquitination could be interpreted as an indirect effect of p21 mutants on the cell cycle, the data in Figure 2 showing that PCNA ubiquitination is not impaired in any phase of the cell cycle argue against that possibility.

Signals that upregulate PCNA ubiquitination

Whereas the enzymes involved in PCNA ubiquitination have been identified (Hoege *et al*., 2002; Stelter and Ulrich, 2003; Kannouche *et al*., 2004), the signals that trigger their activity towards PCNA are not fully understood. Our studies performed with different drugs indicate that a certain type of DNA damage is required but that the NER-repairing machinery itself does not modulate PCNA ubiquitination. Further, although stalled replication promotes PCNA ubiquitination, the damage incurred on the replicating forks by prolonged tension may be the real trigger. Moreover, although PCNA ubiquitination is maximally triggered by low doses of UV irradiation (Supplementary Figure 2), damage itself might still be essential to promote increased PCNA modification by ubiquitin binding. In fact, whereas HU and APH induce accumulation of DNA breaks in comet assays, hypoxia does not (Hammond *et al*., 2003), thus suggesting that deficient PCNA ubiquitination after hypoxia and its mimicking factor, DFX, may be related to the lack of DNA damage to replicating forks. In fact, signals that trigger PCNA ubiquitination may be as sensitive as some checkpoint signals that require small amounts of damaged DNA for activation (Kuhne *et al*., 2003; Shroff *et al*., 2004). Our data also suggests that only damage to DNA involving PCNA-depending synthesis of DNA triggers PCNA ubiquitination in a cell cycle independent manner. Interestingly, a common intermediate in stalled replication and NER is the formation of single stranded DNA that gets immediately coated by the ssDNA binding protein RPA (Carr, 2003; Binz *et al*., 2004). Along this line, Rad18 localization at stalled forks during MMS treatment of mammalian cells is dependent on phosphorylation by checkpoint kinases (Nikiforov *et al*., 2004). Thus, checkpoint proteins may contribute to the ubiquitination of PCNA.

Materials and methods

Cell cultures and regulation of p21 expression

Parental H1299 human lung epithelial carcinoma cells (p53 null), HCT116 and RKO human colorectal cancer cell lines expressing wild-type p53 and WI38 human fibroblasts were cultured in DMEM medium supplemented with 10% FCS. H1299 derivatives expressing tetracycline regulated wild-type p21 were previously described (Baptiste *et al*., 2002) and were maintained in DMEM medium supplemented with 10% FCS, 2 μg/ml puromycin (SIGMA), 300 μg/ml G418 (GIBCO) and 4.5 μg/ml tetracycline. The human fibroblasts used in this study were GM00500 (normal human fibroblasts) and GM00544 and GM02991 (XP-A deficient human fibroblasts), and they were purchased from Coriell Institute for Medical Research and were maintained in MEM medium supplemented with 15% serum and essential and nonessential amino acids.

Genotoxic agents used include Dauno, 0.22 μM (Oncogene Research Products) (Gewirtz, 1999); ActD, 5 nM (Calbiochem) (Sobell, 1985); HU, 1.5 mM (SIGMA) (Timson, 1975); APH, 5 μg/μl (Calbiochem) (Ikegami *et al*., 1978); DFX 250 μM (SIGMA)(Ashcroft *et al*., 2000); methyl methanesulfonate, 0.02% (SIGMA) (Lakin and Jackson, 1999), neocarzinostatin 500 ng/ml and Noc, 100 ng/ml (SIGMA) (Thyberg and Moskalewski, 1999). When p21-4 cells were used, genotoxic agents were added at the same time as tetracycline was removed. Cells were irradiated with UVC (254 nM) following the protocol described by Bendjennat *et al*. (2003). Cells were irradiated at the indicated times before lysis. In the case of serum-starved HCT116 cells, exponentially growing cells were kept in 10% serum for 24 h after they reached confluence and then they were serum starved (0.5%) for 48 h. In the case of the oxygen deprivation experiments, cells were placed into a modular incubator chamber (Billups-Rothenberg) for the indicated times at oxygen concentrations of 0.1% and were immediately lysed at the times indicated in the figure legend.

Transfections and construction of expression vectors

Plasmids were transfected into cells using Lipofectamine 2000 according to the manufacturer's instructions. CS2p21 and CS2p21-K6R were previously described (Sheaff *et al*., 2000) and kindly provided by Dr Clurman. V5/his-PCNA (V5-PCNA) and V5/his-PCNAK164R (V5-PCNA-KR) were described elsewhere (Hoege *et al*., 2002) and were kindly provided by S Jentsch. HA-ubiquitin was originally described in (Treier *et al*., 1994) and was provided by Xavier Jacq. To generate the CS2MT-p21 (6 × Myc tagged p21) and CS2MT-p21K6R (6xMyc tagged p21-K6R) expression vectors, PCR fragments encoding the complete p21 sequence flanked by an *Eco*RI and *Xba*I site were obtained from CS2p21 and CS2p21-K6R. The *Eco*RI and *Xba*I sites were used to insert the PCR fragments into a CS2MT vector with the N-terminal methionine of p21 directly fused to the last amino acid of the 6 × -Myc tag in the vector. The CS2MT vector containing the 6 × -Myc tag was generously provided by Dave Turner (University of Michigan). To generate the p21 mutant defective in PCNA binding (CS2MT-p21 (PCNA-)), the CS2MT-p21 was subjected to quick-change site-directed mutagenesis (Stratagene) using the following primers,

Forward:	cggcggcagaccagcgcgacagctgcctaccactccaaac gccggctg;
Reverse:	cagccggcgtttggagtggtaggcagctgtcgcgctggtctg ccgccg,

that change amino acids M147 to A, D149 to A and F150 to A. These mutations have been characterized previously (Gulbis *et al*., 1996).

To generate the p21 mutant defective in CDK binding (CS2MT-p21 (CDK-)) the CS2MT-p21 was subjected to quick-change site directed mutagenesis (Stratagene) using the following primers

Forward:	ctgcatccaggaggcccgtgagcgaaggaactccgcctttgt caccga;
Reverse:	tcggtgacaaaggcggagttccttcgctcacgggcctcctgga tgcag,

that change amino acids W49 to R, F51 to S and D52 to A. These mutations have been characterized previously (Gulbis *et al*., 1996).

Protein analysis

Total extracts were prepared by incubating approximately 2×10^5 cells in 100 μl of protein extraction buffer containing 10 mM Tris (pH7.5), 1 mM EDTA, 400 mM NaCl, 10% glycerol, 0.5% NP40, and 5 mM NaF, 0.5 mM sodium orthovanadate, 1 mm dithiothreitol (DTT), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and protease inhibitors for 20 min at 4°C. For Triton extractability experiments, cells were incubated for 60 s in PBS containing 1% Triton. The Triton soluble fraction was collected and the remaining insoluble fraction was solubilized by direct resuspension in an equal volume of sample buffer.

In the case of lysates used for immunoprecipitations, either low- or high-stringency buffers were used as indicated. The low-stringency buffer contains HEPES 25 mM, 100 mM NaCl, 10% glycerol, 2.5 mM NaF, 1 mM EDTA, Triton 0.5%, 0.5 mM sodium orthovanadate, 1 mM DTT 0.1 mM PMSF and protease inhibitors. The high-stringency buffer (RIPA) has been described before (Sheaff *et al*., 2000) and contains 10 mM Tris, 25 mM NaF, 300 mM NaCl, 1% NP40, 1% sodium deoxycholate NaDOC, 0.1% SDS, 0.5 mM sodium orthovanadate, 1 mM DTT, 0.1 mM PMSF and protease inhibitors. In the case of RIPA extraction, cell extracts were also subjected to sonication before centrifugation. After separation of the soluble fraction from the insoluble pellet, protein extracts were subjected to immunoprecipitations. Antibodies used for immunoblotting were C19 for human p21, monoclonal PC10 (Santa Cruz) and polyclonal FL261 (Santa Cruz) for PCNA, HA.11 for the hemagglutinin epitope (Covance), anti-V5 (Invitrogen) for the V5 epitope of PCNA, anti-GFP (Santa Cruz), DO-1 for p53 (hybridoma supernatant) and antiactin (SIGMA). For immunoprecipitations, AB-1 and C19 were used for p21, whereas PC10 anti-PCNA and anti-his (Santa Cruz) were used for transfected PCNA. In experiments with sequential immunoprecipitations, the first incubation was with anti-p21 monoclonal antibody (AB-1; Oncogene Science) for 3 h at 4°C, followed by a second incubation with anti-p21 polyclonal antibody (C19; Santa Cruz) overnight at 4°C. Densitometric analysis was performed with the Image Master 2D software version 3.10. In order to maximize the sensitivity and accuracy of the densitometry, exposures were carefully chosen to be in the linear range of the film and to be similar to one another when more than one experiment was analysed.

Cell cycle analysis

Cells were fixed with ice-cold methanol. When farnesylated-GFP was used cells were fixed in ethanol. The samples were resuspended in 0.9 ml of phosphate-buffered saline containing RNase I (50 μg/ml) and propidium iodide (PI) (25 μg/ml, Sigma). The stained samples were analysed in a fluorescence-activated cell sorter (FACSCalibur, Becton Dickinson) using the ModFit *LT* program.

Immunofluorescence

H1299 cells plated on coverslips were washed and then fixed in 4% paraformaldehyde-sucrose 4% and for 15 min at room temperature. After blocking with PBS 1% donkey serum (SIGMA), the coverslips were first incubated with AB1 or Myc primary antibodies and then with anti-mouse-conjugated Cy2 antibody (1:100, Jackson ImmunoResearch). Antibodies were all diluted in PBS 1% donkey serum. Nuclei were stained with bisbenzimide–Hoescht No33258 (SIGMA). Images were obtained with a Zeiss Axioplan confocal microscope. For BrdU incorporation assays, cells were incubated with 10 μM BrdU for 60 min before fixation with methanol. DNA was denatured with HCl 1.5 M for 30 min and fluorescence staining was performed with monoclonal anti-BrdU antibody (Amersham) according to the manufacturer's instructions.

Abbreviations

ActD, actinomycin D; APH, aphidicolin; CDK, cyclin dependent kinase; Dauno, daunorubicin; DFX, deferoxamine mesylate; HU, hydroxyurea; MMS, methylmethane sulfonate; NER, Nucleotide excision repair; NCS, neocarzinostatin; Noc, nocodazole; PCNA, proliferating cell nuclear antigen; TLS, translesion DNA synthesis; BrdU, 5-bromodeoxyuridine.

Acknowledgements

We thank Dr Kristine McKinney and Dr Fernanda Ceriani for helpful suggestions and careful reading of the manuscript. We also thank Marcos Barboza for excellent technical assistance. We are very grateful to Dr S Jentsch, Dr B Clurman and Dr D Turner for advice and plasmids. This work has been supported by grants from Leukemia and Lymphoma Society, Fundación Antorchas, Agencia Nacional de Promoción Cientifica y Tecnológica and CONICET to VG; Fundación Barón to OP and NIH (CA058316) to CP.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)