JCS ePress online publication date 9 September 2008

p21 differentially regulates DNA replication and DNA-repair-associated processes after UV irradiation

Gaston Soria[1], Juliana Speroni[1], Osvaldo L. Podhajcer[2], Carol Prives[3] and Vanesa Gottifredi[1,*]

[1]Cell Cycle and Genomic Stability Laboratory and [2]Laboratory of Molecular and Cellular Therapy, Fundación Instituto Leloir-CONICET, Universidad de Buenos Aires, Buenos Aires, Argentina
[3]Department of Biological Sciences, Columbia University, New York, NY 10027, USA
*Author for correspondence (e-mail: vgottifredi@leloir.org.ar)

Accepted 3 July 2008
Journal of Cell Science 121, 3271-3282 Published by The Company of Biologists 2008
doi:10.1242/jcs.027730

Summary

Although p21 upregulation is required to block cell-cycle progression following many types of genotoxic insult, UV irradiation triggers p21 proteolysis. The significance of the increased p21 turnover is unclear and might be associated with DNA repair. While the role of p21 in nucleotide excision repair (NER) remains controversial, recent reports have explored its effect on translesion DNA synthesis (TLS), a process that avoids replication blockage during S phase. Herein, we analyze the effect of p21 on different PCNA-driven processes including DNA replication, NER and TLS. Whereas only the CDK-binding domain of p21 is required for cell-cycle arrest in unstressed cells, neither the CDK-binding nor the PCNA-binding domain of p21 is able to block early and late steps of NER. Intriguingly, through its PCNA-binding domain, p21 inhibits the interaction of the TLS polymerase, pol η (pol eta), with PCNA and impairs the assembly of pol η foci after UV. Moreover, this obstruction correlates with accumulation of phosphorylated H2AX and increased apoptosis. By showing that p21 is a negative regulator of PCNA-pol η interaction, our data unveil a link between efficient TLS and UV-induced degradation of p21.

Supplementary material available online at
http://jcs.biologists.org/cgi/content/full/121/19/3271/DC1

Key words: Cell death, p21 (CDKN1A), PCNA, pol η (pol eta), UV irradiation

Introduction

PCNA (proliferating cell nuclear antigen) is a ring-shaped trimeric complex that is highly conserved through evolution and which has essential roles in DNA replication and repair (Maga and Hubscher, 2003; Moldovan et al., 2007; Warbrick, 2000). PCNA forms a sliding platform required for the processivity of DNA polymerases δ and ε during DNA replication (Burgers, 1991). PCNA also participates in several forms of DNA repair [including nucleotide excision repair (NER), base excision repair (BER) and mismatch repair (MMR)] and in various aspects of post-replicative processing (Moldovan et al., 2007). Recently, a role of PCNA in the activation of translesion DNA synthesis (TLS), a process that avoids replication blockage during S phase, was revealed. TLS was initially linked to PCNA ubiquitylation, a modification of PCNA that is essential for post-UV cell survival in *S. cerevisiae* (Hoege et al., 2002; Stelter and Ulrich, 2003). In mammals, ubiquitylated PCNA has been reported to have a much higher affinity than unmodified PCNA for the TLS-specific DNA polymerase η (pol η) (Bienko et al., 2005; Kannouche et al., 2004; Parker et al., 2007; Plosky et al., 2006), an enzyme that replicates past cyclobutane pyrimidine dimers (CPDs). More recently, others have proposed that PCNA ubiquitylation promotes the disassembly of factors that prevent pol η recruitment to replication foci (Haracska et al., 2006). Together, these results suggest a central role of PCNA ubiquitylation in the switch from replicative to TLS polymerases at sites of stalled replication.

Although PCNA is clearly a master regulator of DNA synthesis-associated processes, much less is known about potential modulators of its functions. The p21 protein (also known as CDKN1A and $p21^{Cip1/Waf1}$), a member of the family of cyclin-dependent kinase (CDK) inhibitors (CKIs), has been shown to interact with PCNA and to inhibit PCNA functions (Bruning and Shamoo, 2004; Warbrick, 1998). In vitro, p21 interferes with the interaction of PCNA with replication factor C (RFC) (Oku et al., 1998), DNA polymerase δ (Podust et al., 1995; Waga et al., 1994) and FEN1 (Chen et al., 1996). p21 also obstructs the interaction of PCNA with DNA-repair factors required for NER (Gary et al., 1997). Taken together, these data argue that, in vitro, p21 inhibits the resynthesis step of the repair process (Pan et al., 1995; Shivji et al., 1998). In vivo, however, although some groups have found an inhibitory role of p21 in NER-related unscheduled DNA synthesis (UDS) outside of S phase (Bendjennat, 2003; Cooper et al., 1999), others have reported a positive or null role of p21 in NER (McDonald et al., 1996; Perucca et al., 2006; Sheikh et al., 1997; Smith et al., 2000). The role of p21 in TLS is also under investigation and a recent report has demonstrated that p21 reduces TLS efficiency and TLS-associated mutagenic load (Avkin et al., 2006). In all cases, the effect of p21 on a given UV-associated process is difficult to assess because p21 is promptly degraded after UV irradiation (Kaur et al., 2007; Lee et al., 2006; Lee et al., 2007; Soria et al., 2006). To overcome such a limitation, it was imperative to utilize a non-degradable p21 protein. Using this approach, we have recently shown that PCNA ubiquitylation is impaired when p21 is stabilized (Soria et al., 2006), which suggests a negative effect of p21 on some UV-stimulated process(es).

Using various non-degradable mutants of p21 we addressed, in parallel, the role of p21 in DNA replication, NER and TLS. Our data indicate that only the CDK-binding domain of p21 is essential for the inhibition of DNA replication. In agreement, the p21-PCNA interaction was not sufficient to displace replicative polymerases such as pol δ. This was also in line with the inability of p21 to inhibit the NER-dependent DNA synthesis attributed to replicative

polymerases. The PCNA-p21 interaction efficiently impairs pol η association with PCNA and inhibits pol η foci assembly. This correlates with increased histone H2AX phosphorylation and cell death. Thus, in contrast to the current proposed model that links the negative effect of p21 on TLS with a positive effect of p21 on the ubiquitylated PCNA-pol η axis (Livneh, 2006), our data identify p21 as a selective negative regulator of PCNA partners in TLS. Moreover, the increased levels of PCNA-pol η interaction and pol η foci formation in unstressed *p21*$^{-/-}$ cells suggest that during unstressed DNA replication, p21 might prevent the mutagenesis that results from uncontrolled activity of permissive polymerases. In turn, after UV irradiation, the progressive reduction in p21 levels might allow gradual loading of TLS polymerases onto damaged DNA.

Results

The CDK-binding but not PCNA-binding motif of p21 inhibits DNA replication

Although the majority of DNA-damaging agents promote p53 activation, UV irradiation does not always result in p53 stimulation (supplementary material Fig. S1A) (Soria et al., 2006). Independently of p53 status, p21 is promptly degraded after UV exposure (Kaur et al., 2007; Lee et al., 2006; Lee et al., 2007; Soria et al., 2006) and its impact on DNA replication and repair remains obscure. To study the involvement of p21 in such processes after UV irradiation we used p21 mutants that resist UV-induced proteolysis (Fig. 2) (Soria et al., 2006). First, we established the amount of exogenous p21 expression that resembles physiological p21 upregulation. A comparison between the upregulation of p21 observed after well-characterized genotoxic treatments such as daunorubicin and actinomycin D (Gottifredi et al., 2004; Soria et al., 2006) and after transfection of various amounts of p21 expression vector is shown in supplementary material Fig. S1B. In addition to wild-type p21 (p21wt), we used a non-degradable p21 (6Mycp21) and mutants lacking the binding domains for PCNA [6Mycp21 (PCNA–)] and CDK [6Mycp21 (CDK–)]. The point mutations in the CDK and PCNA binding sites were sufficient to disrupt the targeted interactions (Soria et al., 2006). Additionally, the mutation in 6Mycp21 (PCNA–) also impaired the interaction of p21 with exogenously expressed GFP-PCNA (supplementary material Fig. S1C). Cell-cycle analysis revealed that both the p21wt and the stable mutant, 6Mycp21, efficiently block cell-cycle progression (Fig. 1A). Interestingly, the 6Mycp21 (PCNA–) construct, which retains the capacity to interact with CDK, was as efficient as the wild-type protein in promoting the accumulation of cells at G1/G2. However, the 6Mycp21 (CDK–) mutant, despite its ability to interact with PCNA, was clearly unable to modify the cell-cycle distribution (Fig. 1A). This is not dependent on GFP-PCNA expression, as similar results were obtained using only GFP as a transfection marker (Soria et al., 2006). In agreement, short

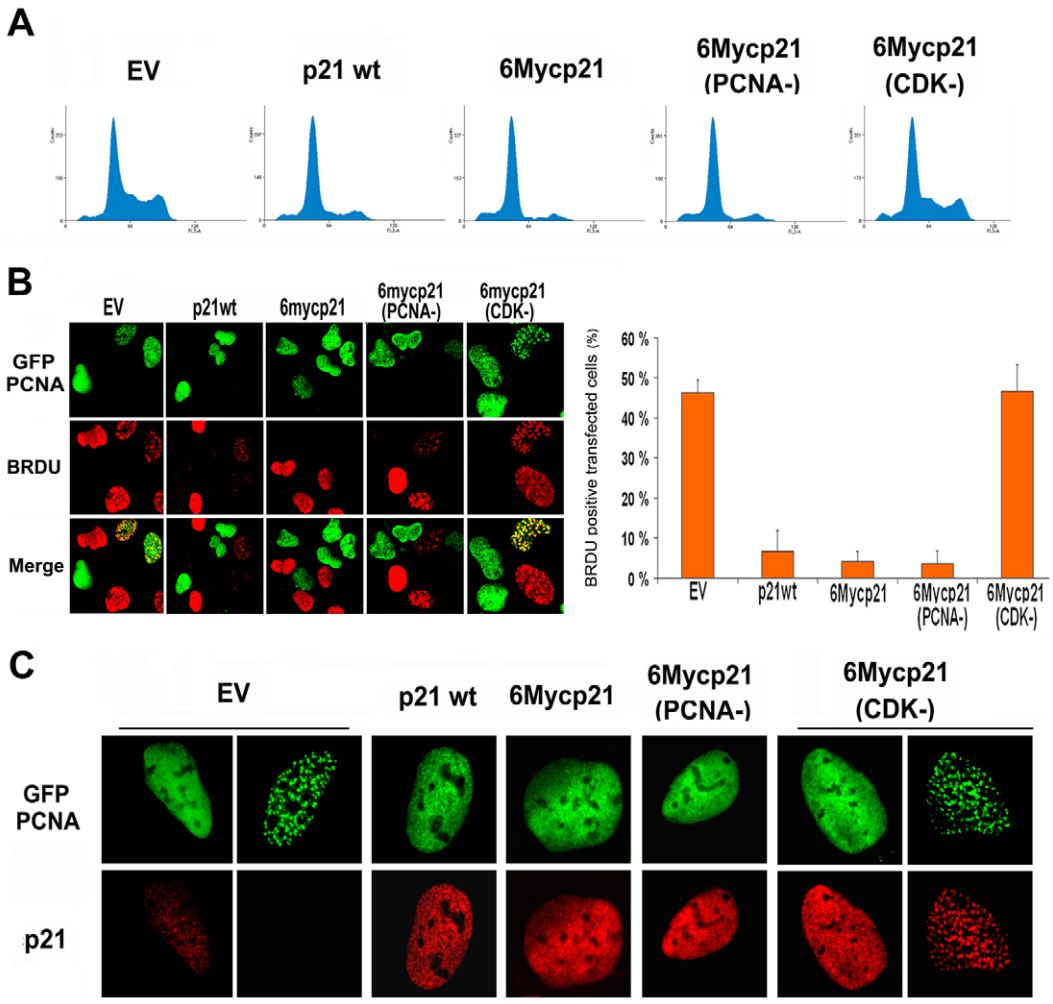

Fig. 1. The PCNA-binding domain of p21 is not essential to block cell-cycle progression. (A) U2OS cells were transfected with GFP-PCNA and the indicated p21 plasmids and the cell-cycle profile of the transfected population was determined. (B) U2OS cells were transfected as in A. BrdU (10 μM) was added 30 minutes before fixation and was detected with specific antibodies. Representative images are shown. The percentage of GFP-PCNA-positive cells that incorporated BrdU was determined (bar chart). At least 200 transfected nuclei/sample were counted. Values are the average and error bars are the standard deviation between equivalent samples in two independent experiments. (C) U2OS cells were transfected as in A, and the sub-nuclear distribution of GFP-PCNA was determined. In the case of the empty vector (EV) and 6Mycp21 (CDK–), ~60% of the cells showed a diffuse distribution, whereas the remainder showed focal PCNA. The two images shown for EV and 6Mycp21 (CDK–) are representative of each situation (merged panels in supplementary material Fig. S5). The quantification of three independent experiments is reported in Fig. 3B.

pulses of BrdU incorporation that identify S-phase cells showed that replicative DNA synthesis was completely abolished in almost every cell transfected with p21wt, 6Mycp21 and 6Mycp21 (PCNA–), but not with 6Mycp21 (CDK–) (Fig. 1B). Finally, because a correlation between PCNA foci formation and S phase has been clearly established (Essers et al., 2005; Leonhardt et al., 2000; Sporbert et al., 2002), we tested whether the different p21 constructs impaired GFP-PCNA foci formation (Fig. 1C). A significant percentage (~40%) of control cells (empty vector, EV) showed a focal distribution of GFP-PCNA. The remaining ~60% of the cells showed pan-nuclear GFP-PCNA localization, which corresponds to cells outside S phase (Essers et al., 2005; Leonhardt et al., 2000). As expected, when p21wt was cotransfected the number of cells with GFP-PCNA foci was drastically reduced (to less than 5%). Similar results were obtained when 6Mycp21 or 6Mycp21 (PCNA–) was expressed. However, the number of cells with GFP-PCNA foci was similar to that of control cells when 6Mycp21 (CDK–) was transfected (see Fig. 1C and Fig. 3B). Moreover, a clear colocalization of 6Mycp21 (CDK–) and PCNA (Fig. 1C and supplementary material Fig. S2A) and between 6Mycp21 (CDK–) and BrdU-positive foci (supplementary material Fig. S2A) was observed. Taken together, these data demonstrate that 6Mycp21 (CDK–) is unable to block cell cycle progression and, in agreement with previous findings (Chen et al., 1995), suggest that p21 binding to PCNA does not prevent the correct function of replicative polymerases.

The CDK-binding and PCNA-binding motifs of p21 do not inhibit DNA synthesis associated with NER

Since the p21-PCNA interaction is insufficient to halt DNA replication in cells, we wondered whether it could affect the participation of PCNA in NER. This is of interest because we and others have reported a strong p21 downregulation induced by UV irradiation (Fig. 2A) (Kaur et al., 2007; Lee et al., 2006; Lee et al., 2007; Soria et al., 2006) that might be linked to DNA-repair-associated processes. NER is activated within minutes after UV exposure and is characterized by pan-nuclear relocalization of NER factors, including PCNA, to damaged DNA (Volker et al., 2001). This fast reorganization of NER factors neither requires nor affects PCNA foci assembly at these early time points. In fact, no changes in the number of cells with PCNA foci were detected at this time, or even at later times such as 1 hour (see Fig. 3A). To check whether

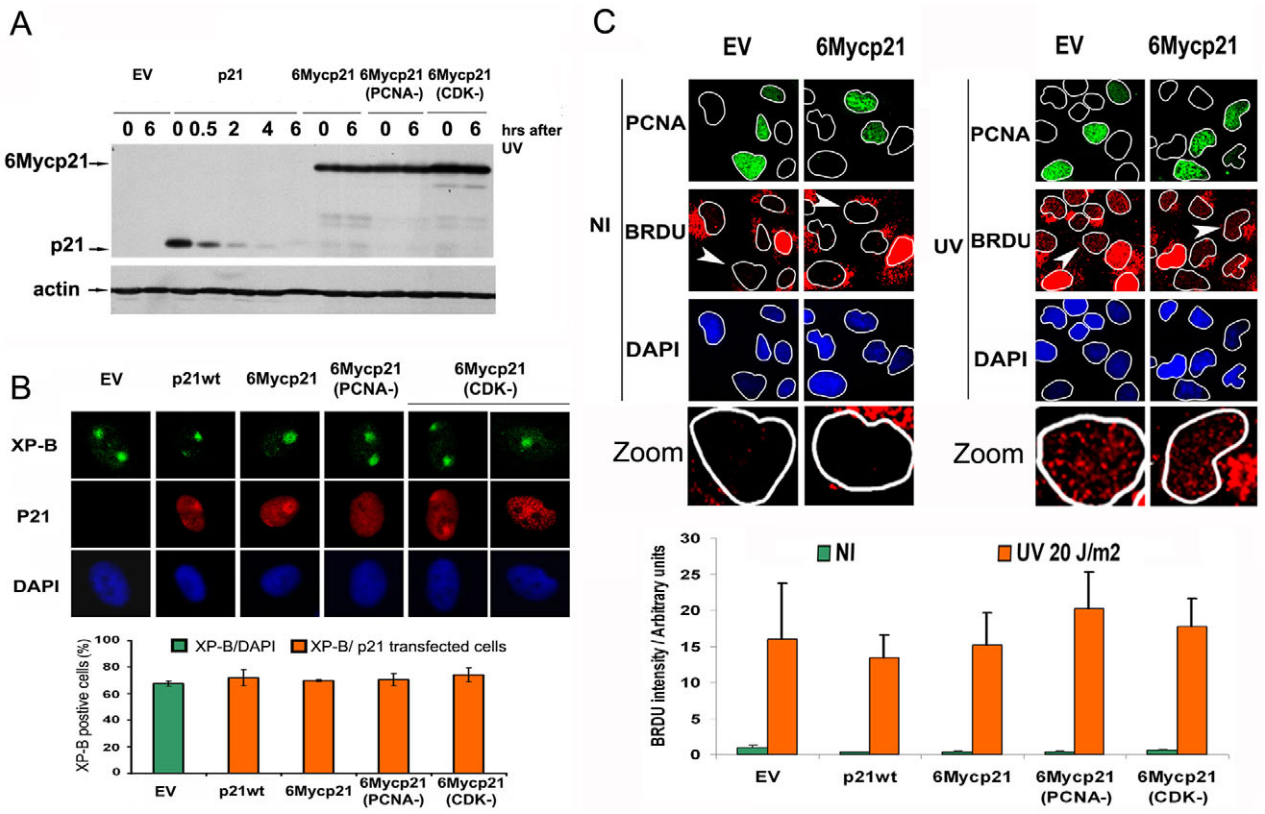

Fig. 2. Neither the PCNA-binding nor the CDK-binding domain of p21 inhibits early or late steps of NER. (A) U2OS cells transfected with the indicated plasmids were subjected to UV irradiation. Samples were collected at different time points and p21 protein levels were determined using specific antibodies. Actin was used as a loading control. (B) U2OS cells transfected with the indicated plasmids were UV irradiated (80 J/m^2) using polycarbonate filters. Thirty minutes later, cells were fixed and immunostained with XPB- and p21-specific antibodies. DAPI staining was used to visualize the nucleus. Quantification is reported in the bar chart. The first column (EV) indicates the percentage of total cells that showed XPB recruitment to the irradiated spots. The other columns represent the percentage of p21-positive cells with XPB relocalization to irradiated spots. In all cases, at least 200 transfected nuclei/sample were counted. Values are the average and error bars are the standard deviation between equivalent samples in two independent experiments. (C) U2OS cells were transfected with the indicated plasmids and GFP-PCNA as a transfection marker. Samples were UV irradiated (20 J/m^2) or not (non-irradiated, NI) and BrdU incorporation (100 μM) was performed for 4 hours. Cells were fixed and stained with BrdU-specific antibodies and DAPI. The white outline, generated using confocal software, was used to distinguish nuclear BrdU signal from mitochondrial DNA synthesis. The magnified (Zoom) images show the nuclei indicated by arrowheads. Quantification of the results obtained with all p21 mutants is reported in the bar chart (10 nuclei/sample were quantified). A complete panel showing all the p21 mutants is shown in supplementary material Fig. S3B.

the recruitment of NER-specific factors was affected by p21, human U2OS cells were UV irradiated through polycarbonate filters, which are porous and expose only discrete areas of the nucleus. This technique allows visualization of the sub-nuclear recruitment of NER factors to the irradiated spots (Essers et al., 2005; Green and Almouzni, 2003; Volker et al., 2001). As previously reported, spots with elevated levels of the helicase XPB (also known as ERCC3) were detected 30 minutes after UV irradiation in a high percentage of control cells (see Fig. 2B). None of the p21 constructs was able to alter this XPB accumulation into irradiated spots (Fig. 2B), suggesting that p21 does not affect early steps of NER. In line with this, XPB^+ spots were observed in all phases of the cell cycle and PCNA relocalization to XPB^+ spots was evident in cells outside S phase (diffused PCNA) in the presence of all the p21 mutants (supplementary material Fig. S3A). Also, all p21 constructs relocalized to XPB^+ spots, with the exception of 6Mycp21 (PCNA–) (Fig. 2A). This suggests that p21 is recruited to NER sites by its interaction with PCNA.

The effect of the different p21 constructs on UV-induced DNA synthesis was then tested on U2OS (Fig. 2C) and human WI38 VA (data not shown) cells, with GFP-PCNA as a marker for transfected cells. After UV irradiation, the cells were incubated in high concentrations of BrdU for 4 hours. Non-irradiated (NI) cells transiting through S phase exhibited intense BrdU incorporation, whereas cells in G1/G2 presented no detectable BrdU incorporation (Fig. 2C). Perinuclear cytoplasmic BrdU was attributed to mitochondrial DNA synthesis as previously described (Davis and Clayton, 1996). By contrast, detectable accumulation of nuclear BrdU was observed in all cells outside of S phase after UV irradiation (see Fig. 2B). As expected, this UDS outside of S phase, previously associated with NER (Li et al., 1994; Perucca et al., 2006), was not observed in cells with deficient expression of the NER essential factor, XPA (supplementary material Fig. S3C). Regardless of the capacity of p21, 6Mycp21 and 6Mycp21 (CDK–) to relocalize into irradiated spots, no significant effect of p21 on UDS was observed (Fig. 2C and supplementary material Fig. S3B). Thus, in spite of its ability to form a complex with PCNA, p21 is incapable of blocking NER in vivo. Importantly, taken together, the data shown in Figs 1 and 2 suggest that p21-PCNA interaction does not affect the activity of replicative polymerases, neither during DNA replication nor NER.

PCNA, but not CDK, binding by p21 impairs assembly of new PCNA foci after UV irradiation

GFP-PCNA reorganizes into well-defined sub-nuclear foci after treatment with cis-diamminedichloroplatinum or UV irradiation, which suggests the involvement of these structures in DNA repair-associated activities (Solomon et al., 2004). We tested the effect of the different p21 constructs on GFP-PCNA foci formation after UV irradiation. In line with previous observations (Solomon et al., 2004), in control samples, cells with GFP-PCNA foci increased from 40% to almost 80% in 6 hours following UV exposure (EV, Fig. 3B). Organic solvent extraction is necessary to immunodetect endogenous PCNA (Kannouche et al., 2001); by contrast, GFP-PCNA detection is not limited by such a procedure (Essers et al., 2005; Leonhardt et al., 2000). In fact, we have obtained similar results using three different extraction procedures (pre-extraction with detergents before PFA fixation; PFA fixation followed by Triton extraction; or methanol/acetone fixation) or direct counts on living cells (not shown). The increase in the number of cells with detectable PCNA foci was much slower than the activation of pan-nuclear NER (2 hours versus 15-30 minutes) and it is unlikely to be directly linked to classic NER. However, it is worth mentioning that differences were observed in the architecture of GFP-PCNA foci before and after UV irradiation. Although the distribution of GFP-PCNA foci in some irradiated cells was indistinguishable from that of replication foci in unstressed cells, others displayed a greater number of smaller GFP-PCNA foci (compare the two cells shown for EV in Fig. 3A).

When p21wt was expressed we observed delayed, but not blocked, GFP-PCNA redistribution into foci after UV irradiation (Fig. 3B,C) that correlated with p21 degradation (see Fig. 2A). This also suggested that PCNA foci formation occurs outside S phase when cells are exposed to UV light. In line with this, the UV irradiation of cells accumulated in G1/G2 by the ectopic expression of non-degradable 6Mycp21 (PCNA–) also resulted in delayed, but yet efficient, reorganization of GFP-PCNA into foci structures (Fig. 3B,C). Significantly, these GFP-PCNA foci were also greater in number and smaller in size [Fig. 3A, p21wt and 6Mycp21 (PCNA–)], which suggests a different composition to PCNA foci outside of S phase. By contrast, 6Mycp21 strongly impaired GFP-PCNA foci formation at all times (Fig. 3A,C). Since both 6Mycp21 and 6Mycp21 (PCNA–) provoke the accumulation of cells in G1 phase (even after UV irradiation), these observations indicate that the PCNA-binding domain of p21 impairs GFP-PCNA foci formation outside of S phase. Taken together, these data also demonstrate that the p21 interaction with CDK blocks replication-associated, but not UV-induced, GFP-PCNA foci formation.

A completely different scenario was observed when cells expressing 6Mycp21 (CDK–) were subjected to UV irradiation. In this case, the number of cells with GFP-PCNA foci was unaffected at all times after UV irradiation (Fig. 3B,C; Table 1). Thus, PCNA binding by p21 might prevent PCNA foci formation in G1/G2, but fails to disrupt replication-associated PCNA foci in S phase. Interestingly, some cells were characterized by larger and fewer GFP-PCNA foci [6Mycp21 (CDK–), Fig. 3A]. Intriguingly, three-dimensional reconstructions of these structures indicate that they might derive from fusion/collapse of smaller, single foci (supplementary material Fig. S2B).

Taken together, these observations indicate that different domains of p21 regulate PCNA foci formation before and after UV irradiation. Whereas the CDK-p21 interaction inhibits replication-associated PCNA foci formation in non-irradiated cells [Fig. 1, see DNA-replication inhibition by the 6Mycp21 (PCNA–) mutant], it does not impair PCNA foci formation after UV irradiation [Fig. 3B,C, delayed but efficient PCNA foci formation with 6Mycp21 (PCNA–)]. By contrast, the PCNA-p21 interaction does not affect PCNA recruitment to replication foci [Fig. 1, 6Mycp21 (CDK–)], but impairs PCNA foci formation outside of S phase after UV irradiation [Fig. 3B,C, no increase in PCNA foci formation after UV with 6Mycp21 (CDK–)].

We also performed a FLIP/FRAP analysis (fluorescence lost in photobleaching/fluorescence recovery after photobleaching) to establish the effect of p21 mutants on the intranuclear mobility of PCNA (supplementary material Fig. S4A). Cells with a pan-nuclear distribution of GPF-PCNA were characterized by high PCNA dynamics, independent of p21 status (supplementary material Fig. S4B, left panel). As expected (Essers et al., 2005), cells with PCNA foci were characterized by a major decrease in PCNA mobility after UV irradiation (see EV in supplementary material Fig. S4B, right panel). However, the mobility of GFP-PCNA was not strongly

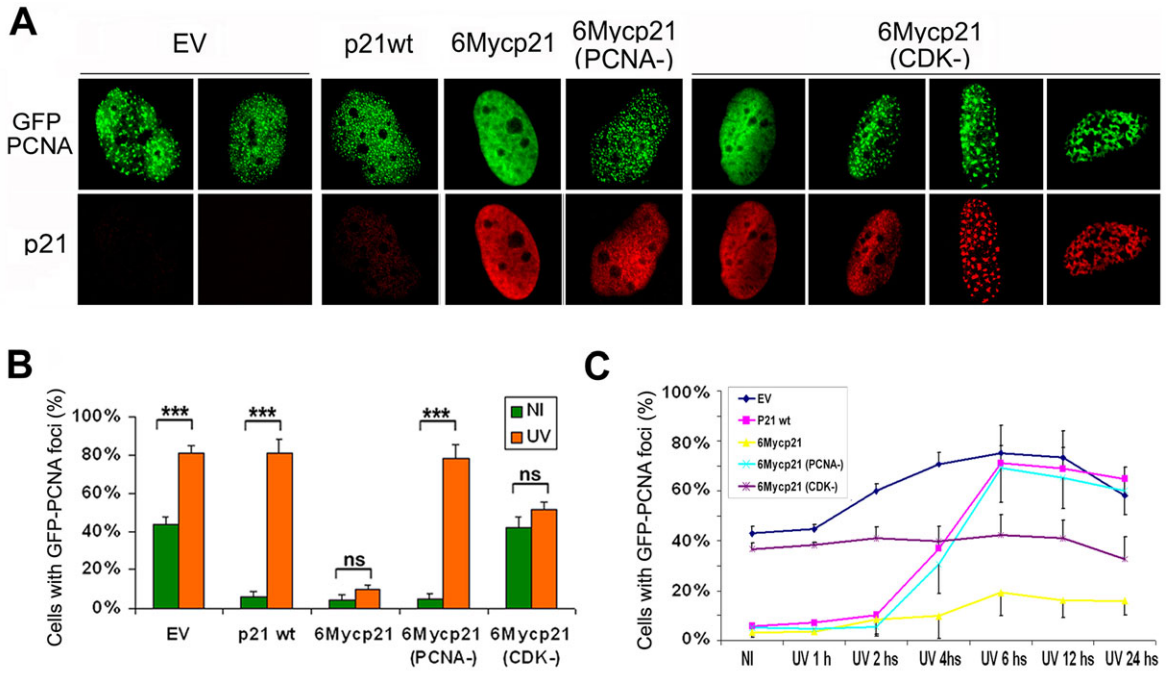

Fig. 3. The p21-PCNA interaction impairs the assembly of new GFP-PCNA foci after UV irradiation. (A) U2OS cells transfected with GFP-PCNA and the indicated p21 plasmids were UV irradiated (20 J/m^2). Six hours later, cells were fixed and the sub-nuclear distribution of PCNA and p21 determined by confocal microscopy. Non-irradiated (NI) controls obtained in parallel are shown in Fig. 1C. After UV irradiation, PCNA foci were similar to replication PCNA foci (EV, left-hand panel) or much smaller (EV, right-hand panel). When the 6Mycp21 (CDK–) mutant was transfected, the PCNA distribution ranged from diffuse to increasingly collapsed nuclear foci as shown in the set of panels to the right. Merged panels are shown in supplementary material Fig. S5. (B) The percentage of cells with GFP-PCNA foci before and after 6 hours of UV irradiation (20 J/m^2) was determined. In all cases, at least 200 transfected nuclei were counted. Values are the average and error bars are the standard deviation between equivalent samples in three independent experiments. The significance of the differences between the NI and UV samples was assessed by Student's *t*-test for each p21 variant (***P<0.001; ns=P>0.05, not significant). Further statistical comparison was performed by one-way ANOVA with Tukey-Kramer post-test (Table 1). (C) The percentage of cells with GFP-PCNA foci was determined at different time points after UV irradiation (20 J/m^2). In all cases, 200 transfected nuclei/sample were counted. Values are the average and error bars are the standard deviation between equivalent samples in two independent experiments.

affected by 6Mycp21 (CDK–). Interestingly, only subtle changes in PCNA mobility were revealed in those few cells in which 6Mycp21 allowed GFP-PCNA to reorganize into foci (see supplementary material Fig. S4B, right-hand panel). These data suggest that other factors that modulate the consolidation of PCNA foci are regulated by the p21-PCNA interaction.

The PCNA-binding but not CDK-binding domain of p21 inhibits pol η association with PCNA and its assembly into nuclear foci

Previous work from our group and others had suggested a role for p21 as a regulator of TLS (Avkin et al., 2006; Soria et al., 2006). Since pol η recruitment to stalled replication forks has been linked to the accumulation of pol η in nuclear foci that colocalize with PCNA (Kannouche et al., 2004; Plosky et al., 2006; Watanabe et al., 2004), we decided to test the effect of the various p21 constructs on pol η foci formation using a previously described GFP-tagged construct of pol η (Kannouche et al., 2001). As expected, a low percentage of unstressed cells showed GFP-pol η foci (Fig. 4A, upper panel and Fig. 4B, NI), and this number increased steeply after UV irradiation in control (EV) cells. Similar results were obtained when cells with pol η foci were quantified after detergent-extraction and PFA fixation (not shown). When p21wt and 6Mycp21 (PCNA–) were transfected, a delayed but otherwise unimpaired induction of pol η foci formation was observed. This correlates with the retardation in PCNA foci formation observed when these constructs were

Table 1. One-way ANOVA with Tukey-Kramer post-test for the experiment reported in Fig. 3B

Comparison	Significance
One-way ANOVA for untreated samples (P=0.0001)	
EV versus p21wt	P<0.001
EV versus 6Mycp21	P<0.001
EV versus 6Mycp21 (PCNA–)	P<0.001
EV versus 6Mycp21 (CDK–)	P>0.05
p21wt versus 6Mycp21	P>0.05
p21wt versus 6Mycp21(PCNA–)	P>0.05
p21wt versus 6Mycp21(CDK–)	P<0.001
6Mycp21 versus 6Mycp21(PCNA–)	P>0.05
6Mycp21 versus 6Mycp21(CDK–)	P<0.001
6Mycp21 (PCNA–) versus 6Mycp21(CDK–)	P<0.001
One-way ANOVA for irradiated samples (P=0.0001)	
EV versus p21wt	P>0.05
EV versus 6Mycp21	P<0.001
EV versus 6Mycp21 (PCNA–)	P>0.05
EV versus 6Mycp21 (CDK–)	P<0.001
p21wt versus 6Mycp21	P<0.001
p21wt versus 6Mycp21(PCNA–)	P>0.05
p21wt versus 6Mycp21(CDK–)	P<0.001
6Mycp21 versus 6Mycp21(PCNA–)	P<0.001
6Mycp21 versus 6Mycp21(CDK–)	P<0.001
6Mycp21 (PCNA–) versus 6Mycp21(CDK–)	P<0.001

The test was applied to the data reported in Fig. 3B, performing a full comparison among all non-irradiated and UV-treated samples separately. P>0.05, not significant.

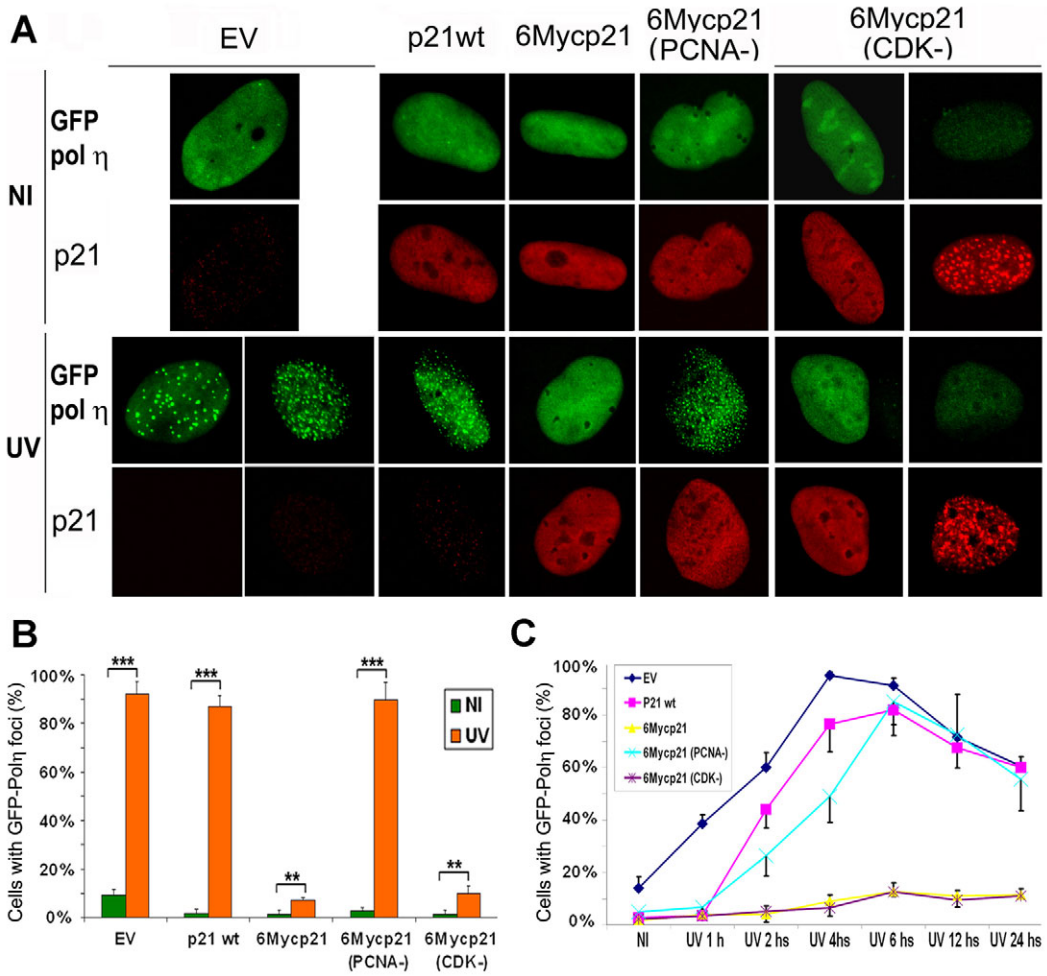

Fig. 4. The PCNA-binding domain of p21 inhibits GFP-pol η foci formation after UV irradiation. (A) U2OS cells transfected with GFP-pol η and the indicated p21 plasmids were UV irradiated (20 J/m^2) when indicated. Six hours later, cells were fixed and the sub-nuclear distribution of pol η and p21 determined by confocal microscopy. In control samples, pol η foci detected after UV irradiation were of two types: larger and fewer (EV, left-hand panel), or much smaller and greater in number (EV, right-hand panel). When 6Mycp21 (CDK–) was present, pol η did not reorganize into foci structures after UV irradiation, neither in cells with pan-nuclear [6Mycp21 (CDK–) left-hand panel] or focal [6Mycp21 (CDK–) right-hand panel] p21 distribution. See merged panels in supplementary material Fig. S5. (B) The percentage of cells with GFP-pol η foci before and after 6 hours of UV irradiation (20 J/m^2) was determined. In all cases, at least 200 transfected nuclei were counted. Values are the average and error bars are the standard deviation between equivalent samples in three independent experiments. The significance of the differences between the non-irradiated (NI) and UV samples was assessed by Student's *t*-test for each p21 variant (***P<0.001; **P<0.01). Further statistical analysis was performed using one-way ANOVA with Tukey-Kramer post-test (Table 1). (C) The percentage of cells with GFP-pol η foci was determined at different time points after UV irradiation (20 J/m^2). In all cases at least 200 transfected nuclei/sample were counted. Values are the average and error bars are the standard deviation between equivalent samples in two independent experiments.

expressed (Fig. 3C; Table 2). In agreement with GFP-PCNA foci organization (Fig. 3), a percentage of mock-transfected cells (EV) equivalent to the proportion of cells outside S phase and almost all cells transfected with p21wt or 6Mycp21 (PCNA–) were characterized by a smaller GFP-pol η foci size. 6Mycp21 impaired GFP-pol η foci formation after UV irradiation, which correlates with its effect on GFP-PCNA foci. Intriguingly, however, 6Mycp21 (CDK–) strongly impaired pol η recruitment to foci at all times, despite the constant proportion of cells (40%) with PCNA foci [Fig. 4B,C, 6Mycp21 (CDK–)]. In fact, the strong colocalization of PCNA and 6Mycp21 (CDK–) indicates that p21-PCNA interaction prevents GFP-pol η foci formation and chromatin association (see merged panels in supplementary material Fig. S5). Thus, whereas p21 recruitment to replication foci depends on PCNA foci formation, after UV irradiation the persistence of p21 at the replication sites by means of its interaction with PCNA interferes with pol η recruitment to PCNA foci.

To determine the role of endogenous p21 in GFP-pol η foci formation after UV irradiation, we used isogenic human HCT116 $p21^{+/+}$ and $p21^{-/-}$ cells as previously described by B. Vogelstein and colleagues (Bunz et al., 1998). A significant number of $p21^{-/-}$ cells with detectable pol η focal organization were observed even before UV irradiation (Fig. 5). These pol η foci were not as abundant as in UV-treated cells (see $p21^{-/-}$ samples in Fig. 5C). After UV irradiation, pol η foci increased in both cells lines, but the number of cells with pol η foci was higher in $p21^{-/-}$ up to 8 hours after UV (Fig. 5A). This correlated with the reduction in the levels of p21 in the $p21^{+/+}$ cells (Fig. 5B). Similar results were obtained when p21wt was transiently transfected into the $p21^{-/-}$ cells (Fig. 5D,E), indicating that p21wt expression was indeed associated with the retardation in pol η foci formation in $p21^{+/+}$ cells.

Table 2. One-way ANOVA test for the experiment reported in Fig. 4

Comparison	Significance
One-way ANOVA for untreated samples (*P*=0.015)	
EV versus p21wt	*P*<0.01
EV versus 6Mycp21	*P*<0.01
EV versus 6Mycp21 (PCNA–)	*P*<0.05
EV versus 6Mycp21 (CDK–)	*P*<0.01
p21wt versus 6Mycp21	*P*>0.05
p21wt versus 6Mycp21(PCNA–)	*P*>0.05
p21wt versus 6Mycp21(CDK–)	*P*>0.05
6Mycp21 versus 6Mycp21(PCNA–)	*P*>0.05
6Mycp21 versus 6Mycp21(CDK–)	*P*>0.05
6Mycp21 (PCNA–) versus 6Mycp21(CDK–)	*P*>0.05
One-way ANOVA for irradiated samples (*P*=0.0001)	
EV versus p21wt	*P*>0.05
EV versus 6Mycp21	*P*<0.001
EV versus 6Mycp21 (PCNA–)	*P*>0.05
EV versus 6Mycp21 (CDK–)	*P*<0.001
p21wt versus 6Mycp21	*P*<0.001
p21wt versus 6Mycp21(PCNA–)	*P*>0.05
p21wt versus 6Mycp21(CDK–)	*P*<0.001
6Mycp21 versus 6Mycp21(PCNA–)	*P*<0.001
6Mycp21 versus 6Mycp21(CDK–)	*P*>0.05
6Mycp21 (PCNA–) versus 6Mycp21(CDK–)	*P*<0.001

The test was applied to the data reported in Fig. 4, performing a full comparison among all non-irradiated and UV-treated samples separately. *P*>0.05, not significant.

It is well established that the interaction between PCNA and pol η increases after UV irradiation (Kannouche et al., 2004). We therefore determined the effect of p21 on pol η extractability and its interaction with PCNA in the chromatin-bound fraction. Whereas p21 expression did not alter the amount of Triton-insoluble GFP-pol η, either before or after UV (Fig. 6A), we observed a clear inhibition of GFP-pol η-PCNA interaction after UV, but only when stable p21 with an intact PCNA binding site was expressed (Fig. 6B). Pol δ-PCNA interaction was not affected by p21, before or after UV. Importantly, endogenous p21 also modulated endogenous pol η-PCNA interaction without affecting pol δ-PCNA (Fig. 6C). These data are completely in line with results described in Figs 1, 2 and 4, and demonstrate that under equivalent experimental conditions, the PCNA-permissive polymerases interaction might be more efficiently impaired by p21 than is the PCNA-replicative polymerases interaction.

The PCNA-binding motif of p21 increases cell death after UV irradiation

Together, our data suggest that p21 downregulation after UV irradiation might promote efficient TLS. To establish whether the deficient recruitment of pol η to chromatin was associated with defective processing of DNA lesions and/or decreased cell viability, we first determined the levels of histone H2AX phosphorylation (γH2AX). This marker tightly associates with DNA damage, including that resulting from UV irradiation (Marti et al., 2006). In control cells, a substantial increase in γH2AX at 4 hours after UV was followed by a return to basal levels at 24 hours (Fig. 7A, EV). None of the p21 constructs significantly altered the number of $\gamma H2AX^{+}$ cells at 4 hours. However, 6Mycp21 (CDK–) promoted the accumulation of pan-nuclear γH2AX to elevated levels, an event previously associated with S phase (Marti et al., 2006), that remained high even after 24 hours [Fig. 7A, 6Mycp21 (CDK–) and Fig. 7B]. Such increased levels of γH2AX were only observed with the 6Mycp21 (CDK–) mutant (supplementary material Fig. S6). Moreover, by performing local irradiation experiments, we observed that pol η failed to be recruited to damaged $\gamma H2AX^{+}$ spots when 6Mycp21 (CDK–) was present, emphasizing the link between impaired pol η recruitment and defects in DNA damage processing (Fig. 7C). Finally, a marked increase in cell death was observed when 6Mycp21 (CDK–) was transfected (Fig. 7D). Interestingly, 6Mycp21, which also inhibited pol η foci formation and pol η-PCNA interaction, promoted the maintenance of higher levels of γH2AX and upregulated cell death. We believe that its effect on cell viability might be less evident than that of 6Mycp21 (CDK–) because 6Mycp21-expressing cells accumulate in G1 and pol η function might be less crucial for survival outside of S phase. Taken

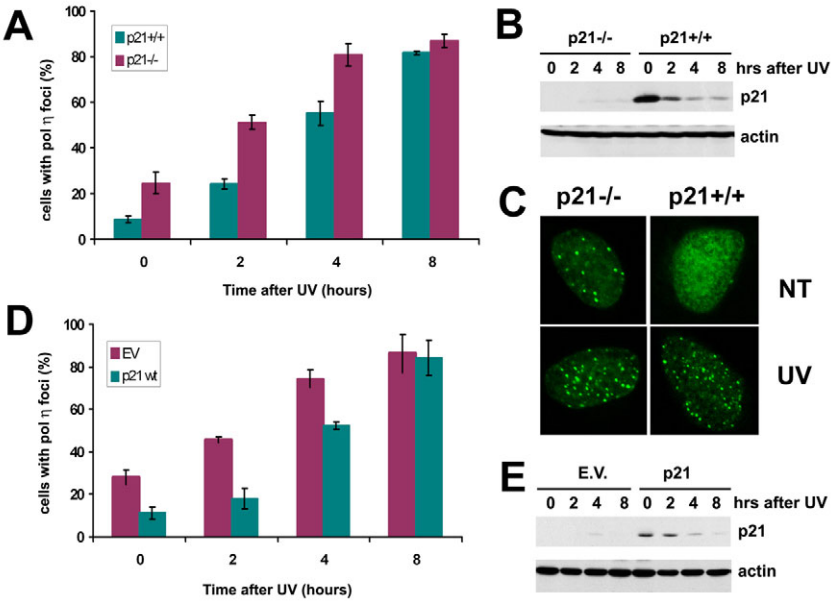

Fig. 5. Endogenous p21 modulates pol η foci assembly. (A) HCT116 $p21^{+/+}$ and HCT116 $p21^{-/-}$ cells transfected with GFP-pol η were UV irradiated (20 J/m^2) when indicated. The sub-nuclear distribution of pol η was determined by confocal microscopy. The percentage of cells with GFP-pol η foci before and after UV irradiation was determined. In all cases, 150 transfected nuclei were counted. Values are the average and error bars are the standard deviation between equivalent samples in three independent experiments. (B) $p21^{+/+}$ and $p21^{-/-}$ cells were subjected to UV irradiation and p21 protein levels determined using specific antibodies. Actin was used as a loading control. (C) Confocal analysis of pol η organization in $p21^{+/+}$ and $p21^{-/-}$ cells before (NT) and after UV irradiation. (D) $p21^{-/-}$ cells were transfected with GFP-pol η and p21 or EV when indicated and UV irradiated (20 J/m^2). At different times, cells were fixed and the sub-nuclear distribution of pol η determined by confocal microscopy. The percentage of cells with GFP-pol η foci was determined. In all cases, 150 transfected nuclei were counted. Values are the average and error bars are the standard deviation between equivalent samples in three independent experiments. (E) $p21^{-/-}$ cells transfected with p21 or EV were UV irradiated and p21 protein levels determined using specific antibodies. Actin was used as a loading control.

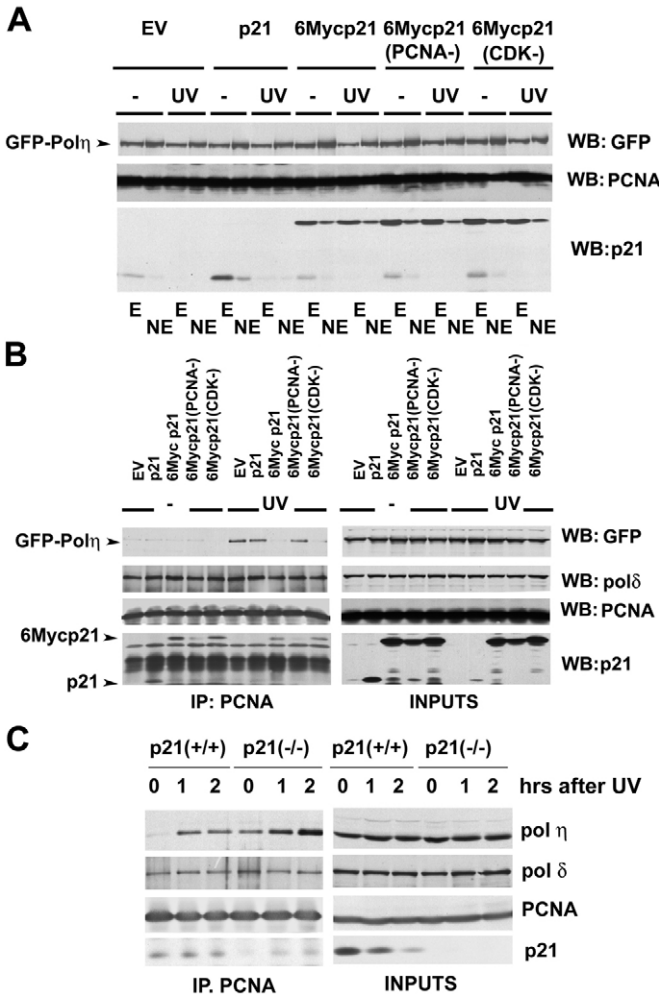

Fig. 6. The PCNA-binding domain of p21 inhibits the PCNA-pol η interaction after UV irradiation. (A) U2OS cells transfected with GFP-pol η and the indicated plasmids were UV irradiated. Four hours later, Triton-soluble (extractable, E) and Triton-insoluble (non-extractable, NE) fractions were collected and p21, PCNA and pol η distribution in both fractions was determined using specific antibodies. (B) U2OS cells transfected with GFP-pol η and the indicated plasmids were UV irradiated. Four hours later, the chromatin-bound fraction was cross-linked, sonicated and PCNA was immunoprecipitated (IP) as described in Materials and Methods. PCNA, GFP-pol η, pol δ and p21 were detected utilizing specific antibodies. The left-hand set of panels shows PCNA IP, whereas that on the right shows an aliquot of the chromatin-bound fraction used for the PCNA IPs (INPUTS). (C) HCT116 $p21^{+/+}$ and $p21^{-/-}$ cells (1×10^6) were UV irradiated and treated as in B at the indicated time points. After PCNA immunoprecipitation, endogenous pol η, PCNA, pol δ and p21 were detected utilizing specific antibodies. Note that high exposures of the blot to film were necessary to detect p21 in the IPs shown in C.

together, these data suggest that the disruption of p21-PCNA interaction might be crucial to allow efficient TLS, which is necessary to prevent cell death associated with stalled forks.

Discussion

Differential effects of p21 domains on DNA synthesis

The contribution of the various domains of p21 to the inhibition of DNA synthesis has been intensely evaluated over the last decade. Although the ability of its CDK-interacting domain to block cell-cycle progression is broadly accepted, there is still much controversy regarding the function of its PCNA-interacting domain. In vitro, high p21:PCNA ratios (up to 100:1) block DNA synthesis (Flores-Rozas et al., 1994; Podust et al., 1995; Waga et al., 1994) and NER (Cooper et al., 1999; Luo et al., 1995; Pan et al., 1995; Shivji et al., 1998). In vivo, however, although some studies suggest that the PCNA-interacting domain of p21 blocks DNA replication (Cayrol et al., 1998; Cazzalini et al., 2003) and NER (Bendjennat, 2003; Cooper et al., 1999), many others report little or no effect on DNA replication (Chen et al., 1995; Lin et al., 1996; Luo et al., 1995; Medema et al., 1998; Nakanishi et al., 1995; Ogryzko et al., 1997) and NER (Li et al., 1994; Perucca et al., 2006). The landmark consideration that arose is that the p21:PCNA ratio is critical for the inhibition of DNA synthesis. PCNA is a highly abundant protein, especially during S phase, and even the highest physiological levels of p21 upregulation might be insufficient to titrate PCNA as the p21:PCNA ratio might never exceed 1:1 in vivo (Gottifredi et al., 2004; Luo et al., 1995). Conversely, in vitro, the inhibitory effect of the PCNA-interacting domain of p21 on DNA synthesis requires p21:PCNA ratios of 10:1 or higher (Cooper et al., 1999; Gottifredi et al., 2004; Shivji et al., 1998). In addition, the amount of p21 available could also depend on other events, such as p21 sequestration by CDK/cyclins and modifications to chromatin accessibility.

In this work, by integrating various single-cell analysis approaches we demonstrate that the CDK-p21 interaction is pivotal for p21-dependent cell-cycle arrest. In fact, disruption of the CDK-binding domain of p21 is sufficient to allow cell-cycle progression, which also suggests that the p21-PCNA interaction does not efficiently contribute to cell-cycle arrest (Fig. 1). In line with this, the p21-PCNA interaction is also incapable of blocking the resynthesis step of NER, an event that also depends on replicative polymerases (Fig. 2). Therefore, we suggest that the effect of p21 on PCNA function might not relate to the inhibition of the loading/processivity of replicative polymerases, as discussed below.

p21 effects on PCNA, pol η and replicative polymerases recruitment

The DNA polymerases of the B family (α, δ and ε) function in DNA replication. The function of pol α is independent of PCNA and is associated with the priming of DNA replication. Subsequently, DNA polymerases δ and ε, assisted by PCNA, take over DNA synthesis. Pol ε is responsible for the synthesis of the leading strand and pol δ associates with the synthesis of the lagging strand (Garg and Burgers, 2005). Pol η belongs to a second group of polymerases (the Y family) that is involved in DNA damage tolerance and which is indispensable for translesion synthesis (Lehmann, 2006). Pol η, δ and ε, interact with PCNA and all contain conserved PCNA-interacting protein motifs (PIP boxes) that allows binding to the interdomain connecting loop (IDCL) of the PCNA monomer (Moldovan et al., 2007; Warbrick, 1998). Importantly, p21 also interacts with the IDCL, and it does so with much higher affinity than any of the other known PCNA-interacting proteins (Bruning and Shamoo, 2004). This has led to the general belief that p21 blocks DNA polymerase recruitment by competing for the same PCNA binding site. This might represent an oversimplification for the multi-subunit replicative polymerases (pol ε and δ) because their interactions with PCNA involve different PCNA-interacting motifs and occur at multiple sites (Eissenberg et al., 1997; Johansson et al., 2004; Maga et al., 1999; Xu et al., 2001; Zhang et al., 1999). For detailed reviews on multi-domain interactions between PCNA

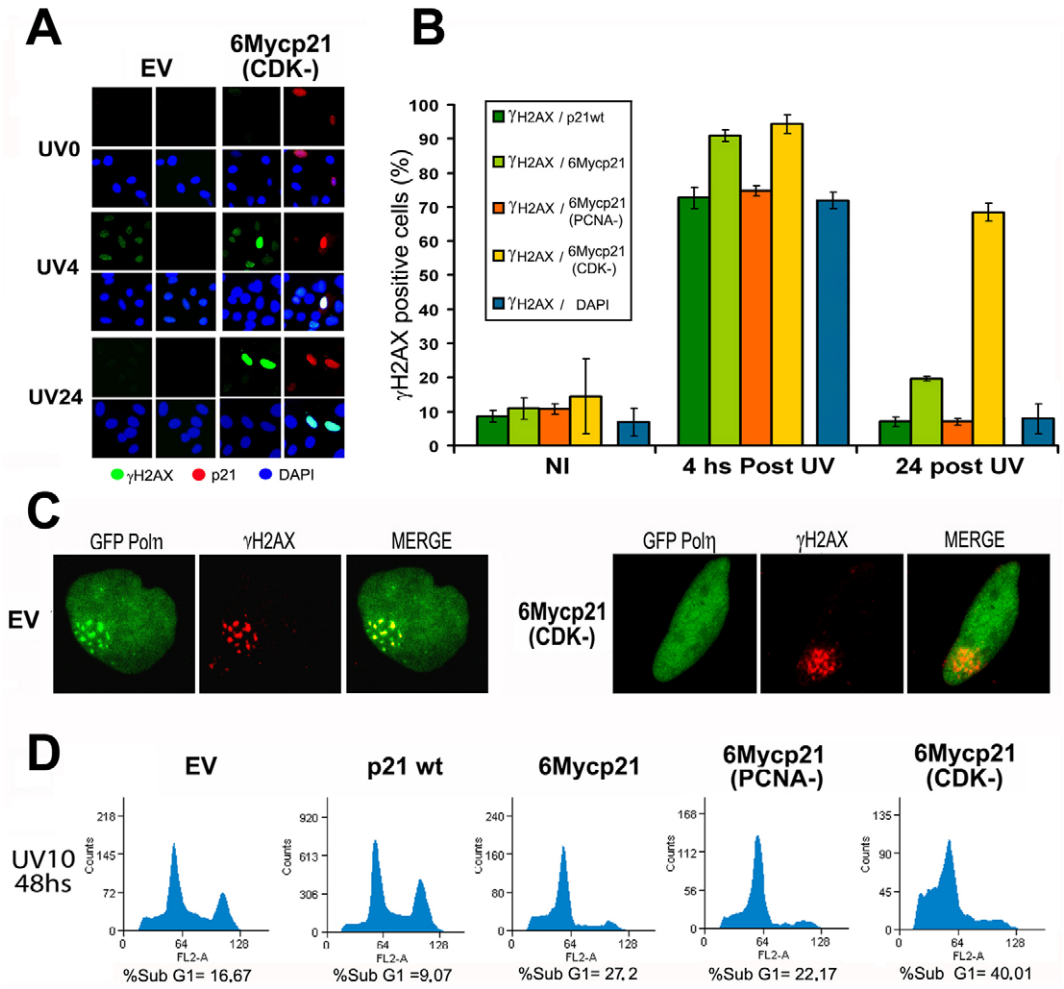

Fig. 7. p21-PCNA interaction is detrimental for cell survival after UV irradiation. (A) U2OS cells transfected with empty vector (EV) or 6Mycp21 (CDK–) were UV irradiated (10J/m^2). After fixation, immunostaining with phosphorylated H2AX (γH2AX)- and p21-specific antibodies was performed. DAPI staining was used to visualize the nucleus. A complete panel showing all p21 mutants is shown in supplementary material Fig. S5. (B) U2OS cells transfected with the indicated plasmids were irradiated with 10J/m^2. The number of cells with γH2AX accumulation was quantified for each time point. The data shown represent the percentage of p21-positive cells with detectable accumulation of γH2AX. In all cases, 100 transfected nuclei were counted. The last column in each group corresponds to the percentage of total cells with detectable accumulation of γH2AX. Values are the average and error bars are the standard deviation between equivalent samples in two independent experiments. (C) U2OS cells transfected with EV or 6Mycp21 (CDK–) and GFP-pol η were UV irradiated utilizing polycarbonate filters and γH2AX staining used to detect the irradiated areas. (D) U2OS cells transfected with GFP-PCNA and the indicated p21 plasmids were UV irradiated (10J/m^2). The cell-cycle profile of the transfected population was determined 48 hours later.

and its partners see Moldovan et al. (Moldovan et al., 2007) and Prosperi (Prosperi, 2006). The single-domain interaction model does however appear to be applicable to the structurally simpler single-subunit TLS polymerases. In fact, a single PIP box motif on human pol ι and pol η is largely responsible for their interaction with PCNA (Haracska et al., 2005; Haracska et al., 2001).

Our data are consistent with the observations mentioned immediately above. p21 does not directly block the recruitment of PCNA to S-phase replication foci, PCNA-associated DNA synthesis (Fig. 1 and supplementary material Fig. S2) or the PCNA-pol δ interaction (Fig. 6B). Conversely, p21 obstructs pol η recruitment to the replication foci after UV irradiation and, remarkably, also in unstressed cells (Fig. 4B; Fig. 5; Table 2). Moreover, p21 binding to PCNA is also a crucial modulator of pol η-PCNA interaction after UV exposure (Fig. 6B,C). In agreement with previous reports (Li et al., 1994; Medema et al., 1998; Perucca et al., 2006), our data reinforce the inability of p21 to displace replicative polymerases from DNA synthesis factories. Nevertheless, the ability of p21 to block pol η recruitment to stalled replication sites may impair and/or delay polymerase switching during TLS.

p21 effects on TLS

In a previous report (Soria et al., 2006), we showed that p21 downregulation is required for efficient PCNA ubiquitylation after UV irradiation. Non-degradable p21 (6Mycp21) impairs PCNA ubiquitylation and, surprisingly, this effect depends on the CDK-binding domain of p21. PCNA ubiquitylation modulates the function of TLS polymerases (Hoege et al., 2002; Kannouche et al., 2004; Plosky et al., 2006; Stelter and Ulrich, 2003; Watanabe et al., 2004). Moreover, the Y family polymerases contain ubiquitin-binding domains, termed UBM and UBZ, responsible for the increased affinity of these polymerases for ubiquitylated PCNA (Bienko et al., 2005; Parker et al., 2007). In this context, it seems contradictory

that the 6Mycp21 (CDK–) mutant, which inhibits pol η recruitment, fails to impair PCNA ubiquitylation. However, both domains of p21 could collaborate to modulate the polymerase switch at the replication fork. Moreover, the real contribution of PCNA ubiquitylation to TLS is a field of continuing controversy (Haracska et al., 2006; Lehmann et al., 2007; Parker et al., 2007; Prakash et al., 2005) and more work will be necessary to shed light on the role of PCNA ubiquitylation in vivo.

It has been proposed that p21 acts as a positive regulator of TLS because the transient downregulation of p21 positively modulates PCNA ubiquitylation after UV irradiation (Avkin et al., 2006; Livneh, 2006). Here, by contrast, we show that p21, via its PCNA-interacting domain, impairs PCNA-pol η interaction and pol η recruitment to stalled replication foci after UV irradiation. These data indicate that p21 might act as a negative regulator of TLS, controlling both the loading of pol η to PCNA and the PCNA ubiquitylation status (Soria et al., 2006). This model provides an alternative scenario in line with the finding that $p21^{-/-}$ cell lines show increased TLS efficiency and associated mutagenesis (Avkin et al., 2006).

Taken together, these data highlight the importance of appropriate cellular levels of p21, which might play a crucial role in the management of pol η loading. In the absence of DNA damage, p21 might impede the accidental loading of pol η and the consequential mutagenesis (see Fig. 5; Fig. 6C; Table 2, ANOVA for 4C). After UV, when TLS plays a decisive role, progressive p21 degradation might allow pol η gradual access to replication forks, thus averting the replication fork blockage that could trigger cell death.

Future perspectives

Our findings raise a wide range of questions regarding the impact of p21 on PCNA-dependent DNA synthesis and regarding the significance of PCNA and pol η foci formation throughout the cell cycle. The data in Figs 3 and 4 suggest that PCNA and pol η also organize into foci in the G1/G2 phases of the cell cycle after UV irradiation. Since no TLS events are expected to take place outside of S phase, the biological significance of these PCNA/pol η foci remains to be determined. Furthermore, recent papers suggest the involvement of pol η and other Y polymerases in additional processes, such as gene conversion, homologous recombination and cell death (Kawamoto et al., 2005; Liu and Chen, 2006; McIlwraith et al., 2005), suggesting the existence of as yet unknown functions of Y polymerases. Our findings provide new insights into the potential role of p21 as a regulator of TLS polymerases, which merits further exploration.

Materials and Methods

Cell culture, transfection and UV irradiation

U2OS cells were obtained from ATCC and grown in DMEM (Invitrogen) supplemented with 10% FBS. HCT116 and HCT116 *p21*$^{-/-}$ were obtained from B. Vogelstein (Johns Hopkins University, Baltimore, MD). GM00500 (XPA wild type), GM00544 (XPA-deficient) and GM002911 (XPA-deficient) cell lines were purchased from Coriell Repositories. Transfections were performed using Lipofectamine 2000 (6 μl/10^6 cells). p21 constructs were described previously (Soria et al., 2006). GFP-PCNA was kindly provided by Dr M. C. Cardoso (Max Delbrück Center for Molecular Medicine, Berlin, Germany) and has been described previously (Leonhardt et al., 2000). GFP-pol η was a gift of Dr A. Lehmann and is described elsewhere (Kannouche et al., 2001). UVC irradiation was delivered with a CL-1000 ultraviolet cross-linker equipped with 254 nm tubes (UVP). For full-cell irradiation, doses from 10 to 40 J/m^2 were delivered after removal of the culture medium. For local irradiation, polycarbonate filters containing multiple 5 μm pores (Millipore, TMTP01300) were positioned in direct contact with cells and subjected to 80 J/m^2 [equivalent to a lower dose as reported in Green and Almouzni (Green and Almouzni, 2003)]. Genotoxic agents used were daunorubicin, 0.22 μM (Oncogene Research Products) and actinomycin D, 5 nM (Calbiochem).

Cell-cycle analysis

Cells were fixed with ice-cold ethanol and samples resuspended in PBS containing RNase I (50 mg/ml) and propidium iodide (PI) (25 mg/ml, Sigma). Stained samples were subjected to FACS (FACScalibur, Becton Dickinson) and data were analyzed using Summit 4.3 software (DakoCytomation). The profiles shown were obtained by gating the GFP-PCNA-positive cells by dual-channel FACS analysis.

BrdU-incorporation assays

For detection of replicative DNA synthesis, cells were incubated for 30 minutes in DMEM/10% FBS containing 10 μM BrdU (Sigma). For detection of repair-associated DNA synthesis, 100 μM BrdU was added to the culture medium and incubated for 4 hours post-UV irradiation. Prior to immunofluorescence, cells were subjected to a denaturing step with 1.5 M HCl for 4 minutes in order to expose the BrdU epitope for antibody detection.

Immunostaining and microscopy

Cells were plated on 10-mm diameter coverslips, transfected and fixed. For imaging, cells were fixed in 4% paraformaldehyde/sucrose for 15 minutes at room temperature, followed by a 10-minute incubation with 0.1% Triton X-100. This fixation method does not alter the GFP-PCNA/pol η distribution when compared with that observed in vivo (data not shown) and enables colocalization analysis with p21, which is highly extractable and frequently lost after other fixation protocols. For quantifying the percentage of cells with GFP-PCNA/pol η foci, cells were incubated in ice-cold methanol for 20 minutes at –20°C followed by a 30-second pulse of ice-cold acetone (Ogi et al., 2005). This method allows detection of only well-assembled GFP-PCNA/pol η foci. Blocking was performed overnight in PBS/2% donkey serum (Sigma). Coverslips were incubated for 1 hour in primary antibodies: anti-p21 AB1 (Oncogene Research Products), anti-p21 C19 (Santa Cruz), anti-BrdU (Amersham), anti-γH2AX (Upstate) and anti-XPB (Santa Cruz). Secondary anti-mouse Cy2/Cy3-conjugated antibodies were from Jackson ImmunoResearch. GFP-PCNA and GFP-pol η were detected by GFP autofluorescence. DAPI (Sigma) staining was used to visualize nuclei. Images were obtained with a Zeiss Axioplan confocal microscope. Live cell imaging and FLIP/FRAP experiments were performed at 37°C using a Zeiss Axiovert 100M confocal microscope as described (Essers et al., 2002; Essers et al., 2005).

Protein analysis

For Triton extractability experiments, cells were incubated for 60 seconds in PBS containing 1% Triton X-100. The Triton-soluble fraction was collected and the remaining insoluble fraction was solubilized by resuspension in an equal volume of sample buffer. For immunoprecipitations, cells were grown on 10-cm plates, transfected and lysed as described (Soria et al., 2006). For immunoprecipitations of chromatin-associated PCNA, a previously described protocol was used (Bi et al., 2006). Immunoprecipitations were performed using anti-p21 C19 and anti-PCNA PC10 (Santa Cruz). For direct western blot analysis, samples were lysed in Laemmli buffer. Western blots were performed using a combination of anti-p21 antibodies (C19 and AB1); polyclonal Ab 1801 against human p53; SMP14, 2A10, 3F3 against MDM2 (generously provided by A. Levine, Rockefeller University, New York, NY); a rabbit polyclonal against PIG3 (kindly provided by D. Hill, Oncogene Research Products, Cambridge, MA); a monoclonal Ab against GFP (Santa Cruz); a polyclonal Ab against pol η (Santa Cruz); a polyclonal Ab against pol δ (Abcam); and a polyclonal Ab against actin (sigma). Incubation with secondary antibodies (Sigma) and detection (ECL, Amersham) were performed according to manufacturers' instructions.

Statistical analysis

Student's *t*-test and analysis of variance (ANOVA) were performed using the GraphPad InStat software. Other calculations and graphics were performed using Microsoft Excel 2003.

We thank A. Lehmann and M. C. Cardoso for the generous gift of GFP-pol η and GFP-PCNA plasmids; T. Zimmermann for generous advice regarding the FLIP/FRAP set-ups; and Kristine McKinney for helpful discussion. Funding was provided by Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT) PICT22052/PICT01322, CONICET (National Council of Sciences) and The Academy of Sciences for the Developing World to V.G., Fundación Barón to O.L.P. and the National Institute of Health (RO3-TW007440) to C.P. V.G. and O.L.P. are researchers from CONICET. G.S. and J.S. are supported by fellowships from CONICET and ANPCyT, respectively.

References

Avkin, S., Sevilya, Z., Toube, L., Geacintov, N., Chaney, S. G., Oren, M. and Livneh, Z. (2006). p53 and p21 regulate error-prone DNA repair to yield a lower mutation load. *Mol. Cell* **22**, 407-413.

Bendjennat, M., B. J., Jascur, T., Brickner, H., Barbier, V., Sarasin, A., Fotedar, A. and Fotedar, R. (2003). UV irradiation triggers ubiquitin-dependent degradation of p21WAF1 to promote DNA repair. *Cell* **114**, 599-610.

Bi, X., Barkley, L. R., Slater, D. M., Tateishi, S., Yamaizumi, M., Ohmori, H. and Vaziri, C. (2006). Rad18 regulates DNA polymerase kappa and is required for recovery from S-phase checkpoint-mediated arrest. *Mol. Cell. Biol.* **26**, 3527-3540.

Bienko, M., Green, C. M., Crosetto, N., Rudolf, F., Zapart, G., Coull, B., Kannouche, P., Wider, G., Peter, M., Lehmann, A. R. et al. (2005). Ubiquitin-binding domains in Y-family polymerases regulate translesion synthesis. *Science* **310**, 1821-1824.

Bruning, J. B. and Shamoo, Y. (2004). Structural and thermodynamic analysis of human PCNA with peptides derived from DNA polymerase-delta p66 subunit and flap endonuclease-1. *Structure* **12**, 2209-2219.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W. and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. *Science* **282**, 1497-1501.

Burgers, P. M. (1991). Saccharomyces cerevisiae replication factor C. II. Formation and activity of complexes with the proliferating cell nuclear antigen and with DNA polymerases delta and epsilon. *J. Biol. Chem.* **266**, 22698-22706.

Cayrol, C., Knibiehler, M. and Ducommun, B. (1998). p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. *Oncogene* **16**, 311-320.

Cazzalini, O., Perucca, P., Riva, F., Stivala, L. A., Bianchi, L., Vannini, V., Ducommun, B. and Prosperi, E. (2003). p21CDKN1A does not interfere with loading of PCNA at DNA replication sites, but inhibits subsequent binding of DNA polymerase delta at the G1/S phase transition. *Cell Cycle* **2**, 596-603.

Chen, J., Jackson, P. K., Kirschner, M. W. and Dutta, A. (1995). Separate domains of p21 involved in the inhibition of Cdk kinase and PCNA. *Nature* **374**, 386-388.

Chen, U., Chen, S., Saha, P. and Dutta, A. (1996). p21Cip1/Waf1 disrupts the recruitment of human Fen1 by proliferating-cell nuclear antigen into the DNA replication complex. *Proc. Natl. Acad. Sci. USA* **93**, 11597-11602.

Cooper, M. P., Balajee, A. S. and Bohr, V. A. (1999). The C-terminal domain of p21 inhibits nucleotide excision repair In vitro and In vivo. *Mol. Biol. Cell* **10**, 2119-2129.

Davis, A. F. and Clayton, D. A. (1996). In situ localization of mitochondrial DNA replication in intact mammalian cells. *J. Cell Biol.* **135**, 883-893.

Eissenberg, J. C., Ayyagari, R., Gomes, X. V. and Burgers, P. M. (1997). Mutations in yeast proliferating cell nuclear antigen define distinct sites for interaction with DNA polymerase delta and DNA polymerase epsilon. *Mol. Cell. Biol.* **17**, 6367-6378.

Essers, J., Houtsmuller, A. B., van Veelen, L., Paulusma, C., Nigg, A. L., Pastink, A., Vermeulen, W., Hoeijmakers, J. H. and Kanaar, R. (2002). Nuclear dynamics of RAD52 group homologous recombination proteins in response to DNA damage. *EMBO J.* **21**, 2030-2037.

Essers, J., Theil, A. F., Baldeyron, C., van Cappellen, W. A., Houtsmuller, A. B., Kanaar, R. and Vermeulen, W. (2005). Nuclear dynamics of PCNA in DNA replication and repair. *Mol. Cell. Biol.* **25**, 9350-9359.

Flores-Rozas, H., Kelman, Z., Dean, F. B., Pan, Z. Q., Harper, J. W., Elledge, S. J., O'Donnell, M. and Hurwitz, J. (1994). Cdk-interacting protein 1 directly binds with proliferating cell nuclear antigen and inhibits DNA replication catalyzed by the DNA polymerase delta holoenzyme. *Proc. Natl. Acad. Sci. USA* **91**, 8655-8659.

Garg, P. and Burgers, P. M. (2005). DNA polymerases that propagate the eukaryotic DNA replication fork. *Crit. Rev. Biochem. Mol. Biol.* **40**, 115-128.

Gary, R., Ludwig, D. L., Cornelius, H. L., MacInnes, M. A. and Park, M. S. (1997). The DNA repair endonuclease XPG binds to proliferating cell nuclear antigen (PCNA) and shares sequence elements with the PCNA-binding regions of FEN-1 and cyclin-dependent kinase inhibitor p21. *J. Biol. Chem.* **272**, 24522-24529.

Gottifredi, V., McKinney, K., Poyurovsky, M. V. and Prives, C. (2004). Decreased p21 levels are required for efficient restart of DNA synthesis after S phase block. *J. Biol. Chem.* **279**, 5802-5810.

Green, C. M. and Almouzni, G. (2003). Local action of the chromatin assembly factor CAF-1 at sites of nucleotide excision repair in vivo. *EMBO J.* **22**, 5163-5174.

Haracska, L., Johnson, R. E., Unk, I., Phillips, B., Hurwitz, J., Prakash, L. and Prakash, S. (2001). Physical and functional interactions of human DNA polymerase eta with PCNA. *Mol. Cell. Biol.* **21**, 7199-7206.

Haracska, L., Acharya, N., Unk, I., Johnson, R. E., Hurwitz, J., Prakash, L. and Prakash, S. (2005). A single domain in human DNA polymerase iota mediates interaction with PCNA: implications for translesion DNA synthesis. *Mol. Cell. Biol.* **25**, 1183-1190.

Haracska, L., Unk, I., Prakash, L. and Prakash, S. (2006). Ubiquitylation of yeast proliferating cell nuclear antigen and its implications for translesion DNA synthesis. *Proc. Natl. Acad. Sci. USA* **103**, 6477-6482.

Hoege, C., Pfander, B., Moldovan, G. L., Pyrowolakis, G. and Jentsch, S. (2002). RAD6-dependent DNA repair is linked to modification of PCNA by ubiquitin and SUMO. *Nature* **419**, 135-141.

Johansson, E., Garg, P. and Burgers, P. M. (2004). The Pol32 subunit of DNA polymerase delta contains separable domains for processive replication and proliferating cell nuclear antigen (PCNA) binding. *J. Biol. Chem.* **279**, 1907-1915.

Kannouche, P., Broughton, B. C., Volker, M., Hanaoka, F., Mullenders, L. H. and Lehmann, A. R. (2001). Domain structure, localization, and function of DNA polymerase eta, defective in xeroderma pigmentosum variant cells. *Genes Dev.* **15**, 158-172.

Kannouche, P. L., Wing, J. and Lehmann, A. R. (2004). Interaction of human DNA polymerase eta with monoubiquitinated PCNA: a possible mechanism for the polymerase switch in response to DNA damage. *Mol. Cell* **14**, 491-500.

Kaur, M., Pop, M., Shi, D., Brignone, C. and Grossman, S. R. (2007). hHR23B is required for genotoxic-specific activation of p53 and apoptosis. *Oncogene* **26**, 1231-1237.

Kawamoto, T., Araki, K., Sonoda, E., Yamashita, Y. M., Harada, K., Kikuchi, K., Masutani, C., Hanaoka, F., Nozaki, K., Hashimoto, N. et al. (2005). Dual roles for DNA polymerase eta in homologous DNA recombination and translesion DNA synthesis. *Mol. Cell* **20**, 793-799.

Lee, H., Zeng, S. X. and Lu, H. (2006). UV Induces p21 rapid turnover independently of ubiquitin and Skp2. *J. Biol. Chem.* **281**, 26876-26883.

Lee, J. Y., Yu, S. J., Park, Y. G., Kim, J. and Sohn, J. (2007). Glycogen synthase kinase 3beta phosphorylates p21WAF1/CIP1 for proteasomal degradation after UV irradiation. *Mol. Cell. Biol.* **27**, 3187-3198.

Lehmann, A. R. (2006). Translesion synthesis in mammalian cells. *Exp. Cell Res.* **312**, 2673-2676.

Lehmann, A. R., Niimi, A., Ogi, T., Brown, S., Sabbioneda, S., Wing, J. F., Kannouche, P. L. and Green, C. M. (2007). Translesion synthesis: Y-family polymerases and the polymerase switch. *DNA Repair (Amst.)* **6**, 891-899.

Leonhardt, H., Rahn, H. P., Weinzierl, P., Sporbert, A., Cremer, T., Zink, D. and Cardoso, M. C. (2000). Dynamics of DNA replication factories in living cells. *J. Cell Biol.* **149**, 271-280.

Li, R., Waga, S., Hannon, G. J., Beach, D. and Stillman, B. (1994). Differential effects by the p21 CDK inhibitor on PCNA-dependent DNA replication and repair. *Nature* **371**, 534-537.

Lin, J., Reichner, C., Wu, X. and Levine, A. J. (1996). Analysis of wild-type and mutant p21WAF-1 gene activities. *Mol. Cell. Biol.* **16**, 1786-1793.

Liu, G. and Chen, X. (2006). DNA polymerase eta, the product of the xeroderma pigmentosum variant gene and a target of p53, modulates the DNA damage checkpoint and p53 activation. *Mol. Cell. Biol.* **26**, 1398-1413.

Livneh, Z. (2006). Keeping mammalian mutation load in check: regulation of the activity of error-prone DNA polymerases by p53 and p21. *Cell Cycle* **5**, 1918-1922.

Luo, Y., Hurwitz, J. and Massague, J. (1995). Cell-cycle inhibition by independent CDK and PCNA binding domains in p21Cip1. *Nature* **375**, 159-161.

Maga, G. and Hubscher, U. (2003). Proliferating cell nuclear antigen (PCNA): a dancer with many partners. *J. Cell Sci.* **116**, 3051-3060.

Maga, G., Jonsson, Z. O., Stucki, M., Spadari, S. and Hubscher, U. (1999). Dual mode of interaction of DNA polymerase epsilon with proliferating cell nuclear antigen in primer binding and DNA synthesis. *J. Mol. Biol.* **285**, 259-267.

Marti, T. M., Hefner, E., Feeney, L., Natale, V. and Cleaver, J. E. (2006). H2AX phosphorylation within the G1 phase after UV irradiation depends on nucleotide excision repair and not DNA double-strand breaks. *Proc. Natl. Acad. Sci. USA* **103**, 9891-9896.

McDonald, E. R., 3rd, Wu, G. S., Waldman, T. and El-Deiry, W. S. (1996). Repair Defect in p21 WAF1/CIP1 –/– human cancer cells. *Cancer Res.* **56**, 2250-2255.

McIlwraith, M. J., Vaisman, A., Liu, Y., Fanning, E., Woodgate, R. and West, S. C. (2005). Human DNA polymerase eta promotes DNA synthesis from strand invasion intermediates of homologous recombination. *Mol. Cell* **20**, 783-792.

Medema, R. H., Klompmaker, R., Smits, V. A. and Rijksen, G. (1998). p21waf1 can block cells at two points in the cell cycle, but does not interfere with processive DNA-replication or stress-activated kinases. *Oncogene* **16**, 431-441.

Moldovan, G. L., Pfander, B. and Jentsch, S. (2007). PCNA, the maestro of the replication fork. *Cell* **129**, 665-679.

Nakanishi, M., Robetorye, R. S., Pereira-Smith, O. M. and Smith, J. R. (1995). The C-terminal region of p21SDI1/WAF1/CIP1 is involved in proliferating cell nuclear antigen binding but does not appear to be required for growth inhibition. *J. Biol. Chem.* **270**, 17060-17063.

Ogi, T., Kannouche, P. and Lehmann, A. R. (2005). Localisation of human Y-family DNA polymerase kappa: relationship to PCNA foci. *J. Cell Sci.* **118**, 129-136.

Ogryzko, V. V., Wong, P. and Howard, B. H. (1997). WAF1 retards S-phase progression primarily by inhibition of cyclin-dependent kinases. *Mol. Cell. Biol.* **17**, 4877-4882.

Oku, T., Ikeda, S., Sasaki, H., Fukuda, K., Morioka, H., Ohtsuka, E., Yoshikawa, H. and Tsurimoto, T. (1998). Functional sites of human PCNA which interact with p21 (Cip1/Waf1), DNA polymerase delta and replication factor C. *Genes Cells* **3**, 357-369.

Pan, Z. Q., Reardon, J. T., Li, L., Flores-Rozas, H., Legerski, R., Sancar, A. and Hurwitz, J. (1995). Inhibition of nucleotide excision repair by the cyclin-dependent kinase inhibitor p21. *J. Biol. Chem.* **270**, 22008-22016.

Parker, J. L., Bielen, A. B., Dikic, I. and Ulrich, H. D. (2007). Contributions of ubiquitin- and PCNA-binding domains to the activity of Polymerase eta in Saccharomyces cerevisiae. *Nucleic Acids Res.* **35**, 881-889.

Perucca, P., Cazzalini, O., Mortusewicz, O., Necchi, D., Savio, M., Nardo, T., Stivala, L. A., Leonhardt, H., Cardoso, M. C. and Prosperi, E. (2006). Spatiotemporal dynamics of p21CDKN1A protein recruitment to DNA-damage sites and interaction with proliferating cell nuclear antigen. *J. Cell Sci.* **119**, 1517-1527.

Plosky, B. S., Vidal, A. E., Fernandez de Henestrosa, A. R., McLenigan, M. P., McDonald, J. P., Mead, S. and Woodgate, R. (2006). Controlling the subcellular localization of DNA polymerases iota and eta via interactions with ubiquitin. *EMBO J.* **25**, 2847-2855.

Podust, V. N., Podust, L. M., Goubin, F., Ducommun, B. and Hubscher, U. (1995). Mechanism of inhibition of proliferating cell nuclear antigen-dependent DNA synthesis by the cyclin-dependent kinase inhibitor p21. *Biochemistry* **34**, 8869-8875.

Prakash, S., Johnson, R. E. and Prakash, L. (2005). Eukaryotic translesion synthesis DNA polymerases: specificity of structure and function. *Annu. Rev. Biochem.* **74**, 317-353.

Prosperi, E. (2006). The fellowship of the rings: distinct pools of proliferating cell nuclear antigen trimer at work. *FASEB J.* **20**, 833-837.

Sheikh, M. S., Chen, Y. Q., Smith, M. L. and Fornace, A. J., Jr (1997). Role of p21Waf1/Cip1/Sdi1 in cell death and DNA repair as studied using a tetracycline-inducible system in p53-deficient cells. *Oncogene* **14**, 1875-1882.

Shivji, M. K., Ferrari, E., Ball, K., Hubscher, U. and Wood, R. D. (1998). Resistance of human nucleotide excision repair synthesis in vitro to p21Cdn1. *Oncogene* **17**, 2827-2838.

Smith, M. L., Ford, J. M., Hollander, M. C., Bortnick, R. A., Amundson, S. A., Seo, Y. R., Deng, C. X., Hanawalt, P. C. and Fornace, A. J., Jr (2000). p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. *Mol. Cell. Biol.* **20**, 3705-3714.

Solomon, D. A., Cardoso, M. C. and Knudsen, E. S. (2004). Dynamic targeting of the replication machinery to sites of DNA damage. *J. Cell Biol.* **166**, 455-463.

Soria, G., Podhajcer, O., Prives, C. and Gottifredi, V. (2006). P21Cip1//WAF1 downregulation is required for efficient PCNA ubiquitination after UV irradiation. *Oncogene* **25**, 2829-2838.

Sporbert, A., Gahl, A., Ankerhold, R., Leonhardt, H. and Cardoso, M. C. (2002). DNA polymerase clamp shows little turnover at established replication sites but sequential de novo assembly at adjacent origin clusters. *Mol. Cell* **10**, 1355-1365.

Stelter, P. and Ulrich, H. D. (2003). Control of spontaneous and damage-induced mutagenesis by SUMO and ubiquitin conjugation. *Nature* **425**, 188-191.

Volker, M., Mone, M. J., Karmakar, P., van Hoffen, A., Schul, W., Vermeulen, W., Hoeijmakers, J. H., van Driel, R., van Zeeland, A. A. and Mullenders, L. H. (2001). Sequential assembly of the nucleotide excision repair factors in vivo. *Mol. Cell* **8**, 213-224.

Waga, S., Hannon, G. J., Beach, D. and Stillman, B. (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* **369**, 574-578.

Warbrick, E. (1998). PCNA binding through a conserved motif. *BioEssays* **20**, 195-199.

Warbrick, E. (2000). The puzzle of PCNA's many partners. *BioEssays* **22**, 997-1006.

Watanabe, K., Tateishi, S., Kawasuji, M., Tsurimoto, T., Inoue, H. and Yamaizumi, M. (2004). Rad18 guides poleta to replication stalling sites through physical interaction and PCNA monoubiquitination. *EMBO J.* **23**, 3886-3896.

Xu, H., Zhang, P., Liu, L. and Lee, M. Y. (2001). A novel PCNA-binding motif identified by the panning of a random peptide display library. *Biochemistry* **40**, 4512-4520.

Zhang, P., Mo, J. Y., Perez, A., Leon, A., Liu, L., Mazloum, N., Xu, H. and Lee, M. Y. (1999). Direct interaction of proliferating cell nuclear antigen with the p125 catalytic subunit of mammalian DNA polymerase delta. *J. Biol. Chem.* **274**, 26647-26653.