

# Spatiotemporal dynamics of p21<sup>CDKN1A</sup> protein recruitment to DNA-damage sites and interaction with proliferating cell nuclear antigen

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## Summary

The cyclin-dependent kinase inhibitor p21<sup>CDKN1A</sup> plays a fundamental role in the DNA-damage response by inducing cell-cycle arrest, and by inhibiting DNA replication through association with the proliferating cell nuclear antigen (PCNA). However, the role of such an interaction in DNA repair is poorly understood and controversial. Here, we provide evidence that a pool of p21 protein is rapidly recruited to UV-induced DNA-damage sites, where it colocalises with PCNA and PCNA-interacting proteins involved in nucleotide excision repair (NER), such as DNA polymerase  $\delta$ , XPG and CAF-1. In vivo imaging and confocal fluorescence microscopy analysis of cells coexpressing p21 and PCNA fused to green or red fluorescent protein (p21-GFP, RFP-PCNA), showed a rapid relocation of both proteins at microirradiated nuclear spots, although dynamic measurements suggested that p21-

GFP was recruited with slower kinetics. An exogenously expressed p21 mutant protein unable to bind PCNA neither colocalised, nor coimmunoprecipitated with PCNA after UV irradiation. In NER-deficient XP-A fibroblasts, p21 relocation was greatly delayed, concomitantly with that of PCNA. These results indicate that early recruitment of p21 protein to DNA-damage sites is a NER-related process dependent on interaction with PCNA, thus suggesting a direct involvement of p21 in DNA repair.

Supplementary material available online at

<http://jcs.biologists.org/cgi/content/full/119/8/1517/DC1>

Key words: p21<sup>waf1/cip1</sup>, PCNA, DNA repair, Nucleotide excision repair, UV irradiation

## Introduction

The cyclin-dependent kinase (CDK) inhibitor p21<sup>CDKN1A</sup> (also known as p21<sup>waf1/cip1</sup>) plays an important role in several cellular pathways in response to intracellular and extracellular stimuli. In particular, p21 is involved in growth arrest induced by cell-cycle checkpoints, senescence, or terminal differentiation (Dotto, 2000). In addition, p21 has been shown to interact directly, or indirectly with proteins regulating gene expression, thus suggesting a role for p21 in regulation of transcription (Coqueret, 2003).

Although its activity is usually associated with CDK inhibition, p21 is also able to interact directly with proliferating cell nuclear antigen (PCNA), thereby inhibiting DNA replication (Gulbis et al., 1994). PCNA is a cofactor of DNA polymerases  $\delta$  and  $\epsilon$ , that is necessary both for DNA replication and repair (Tsurimoto, 1999; Warbrick, 2000). However, PCNA plays a major role in coordinating DNA metabolism with cell-cycle control (Prospero, 1997) by interacting with other DNA replication and repair factors, as well as with cell-cycle proteins (Paunesku et al., 2001; Vivona and Kelman, 2003). The binding of p21 to PCNA results in competition and displacement of PCNA-interacting proteins,

thereby inhibiting DNA synthesis (Oku et al., 1998). Given that PCNA is also involved in DNA repair, the effects of p21 on this process are more controversial. In fact, biochemical studies suggest that high p21 levels inhibit DNA repair (Pan et al., 1995; Podust et al., 1995), and similar results are obtained on electroporated cells (Cooper et al., 1999). However, other studies showed that nucleotide excision repair (NER) was insensitive to p21 in vitro (Shivji et al., 1994; Shivji et al., 1998), and that p21 did not inhibit NER in vivo (McDonald et al., 1996; Sheikh et al., 1997). In particular, cells expressing a p21 mutant form unable to bind PCNA were deficient in NER, but when the wild-type protein was expressed, cells became proficient for repair (McDonald et al., 1996). A positive role for p21 in NER, was also suggested by the colocalisation and interaction of p21 and PCNA in actively repairing normal fibroblasts (Li et al., 1996; Savio et al., 1996), and by cell resistance to cytotoxic drugs after p21 expression (Ruan et al., 1998). Other studies performed on p21-null murine fibroblasts, or on tumour cell lines lacking p21 protein, report that the NER process is not significantly affected (Smith et al., 2000; Adimoolan et al., 2001; Wani et al., 2002). However, deletion of p21 gene in human normal fibroblasts results in reduced

DNA repair capacity (Stivala et al., 2001). Thus, although p21 is required for the successful cellular response to DNA damage, its participation in NER is still debated. It is known that p21 must be degraded for S-phase entry (Bornstein et al., 2003; Gottifredi et al., 2004), to prevent PCNA binding and consequent inhibition of DNA replication. Similarly, it has been recently shown that ubiquitin-dependent proteolysis of p21 is triggered after UV-induced DNA damage, and that this degradation is required for PCNA recruitment to DNA-repair sites (Bendjennat et al., 2003). However, it is well known that PCNA is also recruited to DNA-damage sites with fast kinetics in quiescent cells (Toschi and Bravo, 1988; Proserpi et al., 1993; Aboussekhra and Wood, 1995; Savio et al., 1998), which show delayed p21 proteasomal degradation (Bendjennat et al., 2003). Thus, p21 removal may not be directly required for this step of the repair process.

In this study we have investigated whether the induction of p21 may inhibit DNA repair by preventing PCNA recruitment, and analyzed the spatiotemporal dynamics of p21 recruitment to DNA-damage sites directly on living cells. We show that a pool of p21 was rapidly recruited to, and colocalised with PCNA and other DNA-repair proteins at DNA-damage sites. By coexpressing p21 fused to green fluorescent protein (p21-GFP) and PCNA fused to red fluorescent protein (RFP-PCNA), we further show by dynamic fluorescence measurements that p21-GFP was recruited to damaged sites with slower kinetics than that of RFP-PCNA. Relocation of p21 was found to depend on prior recruitment of PCNA to DNA-damage sites.

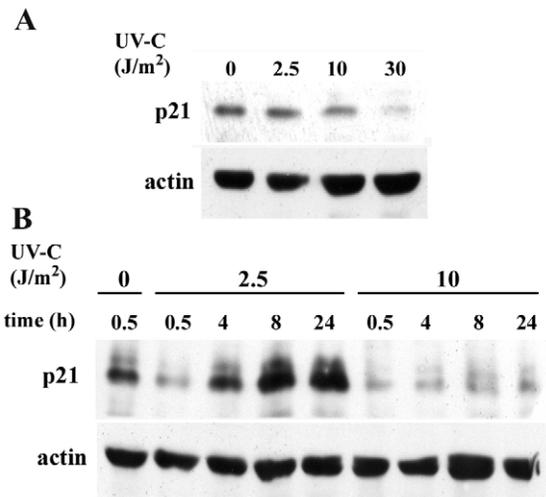
## Results

### p21 protein is not completely degraded after UV irradiation

To investigate to what extent removal of p21 protein was required for DNA repair, human fibroblasts were exposed to different UV-C doses, and collected at various periods of time after irradiation. Fig. 1A shows that 6 hours after irradiation, p21 levels were unchanged in samples exposed to a relatively low dose (2.5 J/m<sup>2</sup>). By contrast, the protein was significantly degraded (by about 55% as quantified by band densitometry versus actin loading), at a high dose (10 J/m<sup>2</sup>). These doses corresponded to clonogenic survivals of about 80% and 10%, respectively. A more significant decrease in p21 protein (by about 85%) was observed after a dose of 30 J/m<sup>2</sup>. The time-course study (Fig. 1B) showed that after an initial reduction (by about 60%) observed 30 minutes after exposure to 2.5 J/m<sup>2</sup> UV-C, p21 levels increased, reaching at 24 hours, about twice the amount of the untreated control samples. A consistent decrease was observed at each time point after irradiation with 10 J/m<sup>2</sup>, though p21 was not completely degraded, because about 20% of the protein was still detected 24 hours after irradiation.

### p21 is recruited together with PCNA to DNA repair sites

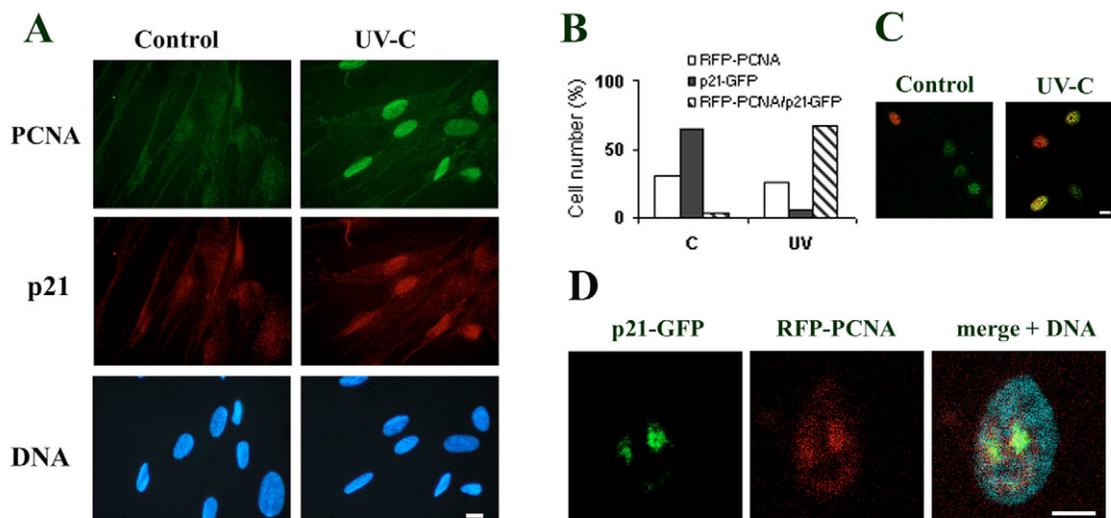
Previous studies on the involvement of p21 in NER focus only on a time scale of hours after DNA damage (Li et al., 1996; Savio et al., 1996). Thus, we first asked whether p21 protein surviving degradation could relocate to DNA-damage sites within a short interval after UV irradiation, similarly to PCNA. Fibroblasts were synchronised in G1 phase, to avoid the presence of S-phase cells containing high levels of chromatin-bound PCNA. Samples collected 30 minutes after irradiation



**Fig. 1.** p21 is not completely degraded after UV-induced DNA damage. (A) Dose response analysis of p21 degradation after UV-induced DNA damage in LF1 human fibroblasts. Cells were lysed directly in loading buffer 6 hours after UV-C irradiation at the indicated doses. Samples were analysed by western blot for p21 protein levels versus actin as a loading control. (B) Time-course analysis of p21 degradation after UV irradiation at 2.5 or 10 J/m<sup>2</sup>. Samples were analysed for p21 and actin, as above.

were processed for indirect immunostaining of chromatin-bound PCNA, and three-step amplification with streptavidin-Texas-Red for detection of p21. The results clearly indicate that after DNA damage, early recruitment of p21 occurs similarly to PCNA (Fig. 2A).

To further test that this was an active process induced by exposure to UV-C radiation, p21 and PCNA were coexpressed in HeLa cells as GFP and RFP fusion proteins, p21-GFP and RFP-PCNA respectively. Coexpression levels of the two fusion proteins were similar in a high proportion (60-80%) of transfected cells. Previous analysis showed that p21-GFP arrested HeLa cells mainly in G1, and partly in G2 phase (Cazzalini et al., 2003). Thus, in nonirradiated control samples chromatin-binding of fluorescent proteins was dependent on the cell-cycle phase. About 65% of transfected cells showed chromatin-bound p21-GFP, whereas RFP-PCNA was chromatin-bound (in about 30% of cells), only in S phase (Leonhardt et al., 2000), as verified by BrdU incorporation (not shown). The concomitant presence of the two proteins bound to chromatin, was found in a very low number of cells (about 4%), that were probably at the G1-S transition, as previously observed for p21-GFP and endogenous PCNA (Cazzalini et al., 2004). By contrast, after UV irradiation, both proteins were chromatin-bound in about 70% of transfected cells (Fig. 2B). In these cells, the two proteins were already colocalised 30 minutes after irradiation, as indicated by the yellow colour of the merged confocal images (Fig. 2C). To further test the recruitment of both p21-GFP and RFP-PCNA at DNA repair sites, cotransfected HeLa cells were also exposed to local UV irradiation (10 J/m<sup>2</sup>) through 3- $\mu$ m-pore filters. Fig. 2D shows confocal sections of green and red fluorescence signals that are localised to the exposed areas. The merged image shows the distribution of the two proteins in the nucleus, as visualised by DNA staining.



**Fig. 2.** Early recruitment of p21 to DNA repair sites. (A) LF1 human fibroblasts were irradiated with UV-C ( $10 \text{ J/m}^2$ ) and 30 minutes later samples were extracted in situ and fixed for indirect immunofluorescence determination, or biotin-streptavidin amplification of chromatin-bound PCNA (green fluorescence) and p21 (red fluorescence), respectively. DNA (blue fluorescence) was stained with Hoechst 33258. (B) HeLa cells were cotransfected with p21-GFP and RFP-PCNA expression vectors, and 24 hours later exposed to UV-C radiation ( $10 \text{ J/m}^2$ ). After 30 minutes, control (C) and irradiated (UV) cells were extracted in situ and fixed for detection and counting of cells showing only chromatin-bound RFP-PCNA (empty bars), p21-GFP (solid bars), or both (dashed bars). The percentages of cells in a representative experiment are shown. (C) Confocal sections of merged green and red fluorescence signals, of untreated control and UV-C-irradiated cells. (D) HeLa cells expressing p21-GFP and RFP-PCNA were exposed to local UV-C radiation ( $10 \text{ J/m}^2$ ) through  $3 \mu\text{m}$  pores. Confocal sections of green (p21-GFP) and red (RFP-PCNA) fluorescence signals are displayed, together with the merged image showing also the blue fluorescence (Hoechst) of DNA counterstaining. Bars,  $10 \mu\text{m}$  (A,C);  $5 \mu\text{m}$  (D).

### Spatiotemporal dynamics of p21-GFP recruitment to DNA-damage sites

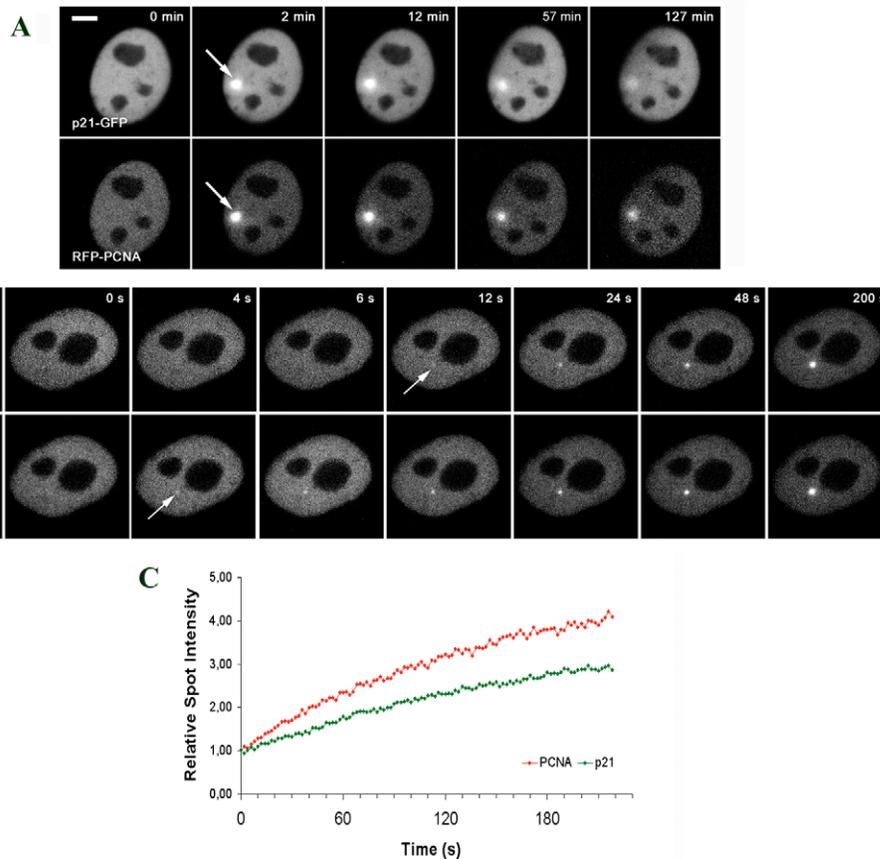
To directly compare p21 relocation with that of PCNA, and to demonstrate that this process occurred independently of the cell type, we analysed the recruitment kinetics of p21-GFP and RFP-PCNA in living C2C12 myoblasts, after inducing cyclobutane pyrimidine dimers (CPDs) DNA damage with a  $405 \text{ nm}$  laser (supplementary material Fig. S1). The dynamics of p21-GFP and RFP-PCNA fluorescent signals observed at DNA-damage sites in single C2C12 living cells, is shown in Fig. 3A. The irradiated spot shows maximal fluorescence intensities of both proteins within 2–15 minutes of irradiation, and then a decrease reaching basal levels 1–2 hours later. A detailed analysis showed that both proteins started to accumulate at the irradiated spot within a few seconds, but p21-GFP fluorescence appeared with a short delay after that of RFP-PCNA (Fig. 3B). Also, a direct comparison of fluorescence intensities at irradiated spots revealed a slightly but consistent faster recruitment of RFP-PCNA than p21-GFP (Fig. 3C). A similar behaviour was also observed in HeLa cells (supplementary material Fig. S2).

### p21 is recruited to UV-damaged sites together with DNA-repair proteins

In order to clarify whether the levels of p21 may be a crucial determinant negatively influencing the recruitment of PCNA to DNA-damage sites, human fibroblasts were treated with trichostatin (TSA), a histone deacetylase inhibitor that is known to induce transcription of the p21 gene thereby increasing p21 protein levels (Richon et al., 2000). TSA-treated and mock-treated cells were exposed to UV-C irradiation, and

30 minutes later were collected for determination of total and chromatin-bound levels of PCNA and p21, as well as of other proteins participating in DNA repair. Western blot analysis shows that the total amount of PCNA, DNA ligase I (Lig I), or DNA polymerase  $\delta$  (pol  $\delta$ ), were not appreciably modified by TSA or UV-C exposure, either alone or in combination (Fig. 4A). As expected, TSA induced an increase in p21 protein levels (by about 35%), whereas UV-C reduced the levels to about 15% of the untreated control sample. Interestingly, cells treated with TSA and then irradiated with UV-C also showed reduced levels (about 20% of the TSA-treated sample), indicating that p21 was degraded to a similar extent, notwithstanding the higher starting levels. The levels of the above proteins in the chromatin-bound fraction were undetectable in the control and in the TSA-treated cells, whereas UV-C induced a significant relocation of all proteins, including p21 itself. Pre-treatment with TSA did not induce any significant decrease in the amount of chromatin-bound PCNA, or of the other proteins, even if the levels of chromatin-bound p21 were apparently increased (Fig. 4B).

To further test whether p21 relocation occurred concomitantly with PCNA, and did not interfere with the recruitment of other repair factors, HeLa cells transfected with p21-GFP expression vector, were exposed to UV-C irradiation through filters with  $3 \mu\text{m}$  pores. Thirty minutes later, cells were processed for in situ hypotonic lysis, fixed and immunostained with antibodies to pol  $\delta$ , XPG, or CAF-1. Fig. 5A shows confocal sections of p21-GFP fluorescence (green) and immunofluorescence (red) signals relative to pol  $\delta$ , and the merged image of both signals, together with that of DNA. Similarly, Fig. 5B,C shows the presence of XPG or CAF-1, respectively, together with that of p21-GFP, at

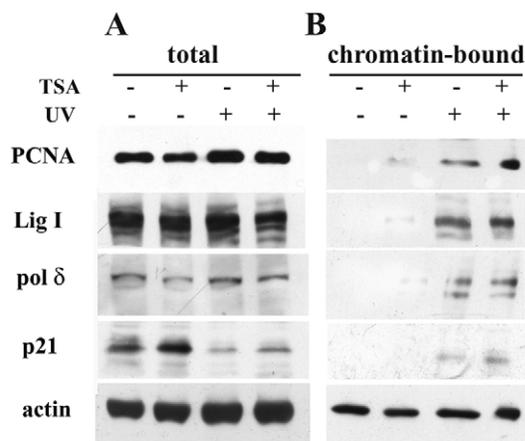


**Fig. 3.** Dynamics of p21 recruitment to DNA-repair sites in living cells. (A) C2C12 myoblasts expressing both p21-GFP and RFP-PCNA were exposed to 405 nm laser microirradiation and fluorescence signals were acquired after the indicated times. Maximum projections of confocal mid sections show the accumulation of p21-GFP and RFP-PCNA fluorescence signals at sites of DNA damage (arrows). (B) Short-term kinetic analysis of p21-GFP and RFP-PCNA fluorescence after 405 nm laser microirradiation. Signals were acquired every 2 seconds and confocal sections are shown of images taken at the indicated times. The arrows indicate the site of irradiation. (C) Plot of the relative fluorescence intensity of p21-GFP (green) and RFP-PCNA (red) at the irradiated spot. Fluorescence intensities acquired every 2 seconds at the irradiated region, were corrected for background, and for total nuclear loss of fluorescence over the time course, and normalised to the pre-irradiation value. Bars, 5  $\mu$ m (A,B).

locally irradiated sites. Each protein analysed was detected (though with variable intensity) at virtually every spot containing p21-GFP. These results indicate that in addition to PCNA, p21-GFP also colocalises with these proteins involved in different steps of DNA repair.

#### Interaction of p21 with PCNA and DNA pol $\delta$ after UV-C irradiation

Previous studies showed that p21 does not influence the RFC-mediated PCNA loading to DNA replication sites, yet it prevents or destabilises further binding of PCNA-interacting proteins, such as pol  $\delta$  (Waga and Stillman, 1998; Cazzalini et al., 2003). To understand whether this was also the case during DNA repair, native p21 or p21-GFP were immunoprecipitated from normal fibroblasts, or from HeLa cells, respectively using antibodies to p21 or to GFP. After immunoprecipitation, bound peptides were analysed by western blot for the presence of PCNA and pol  $\delta$  (p125 subunit). Fig. 6A shows the results of



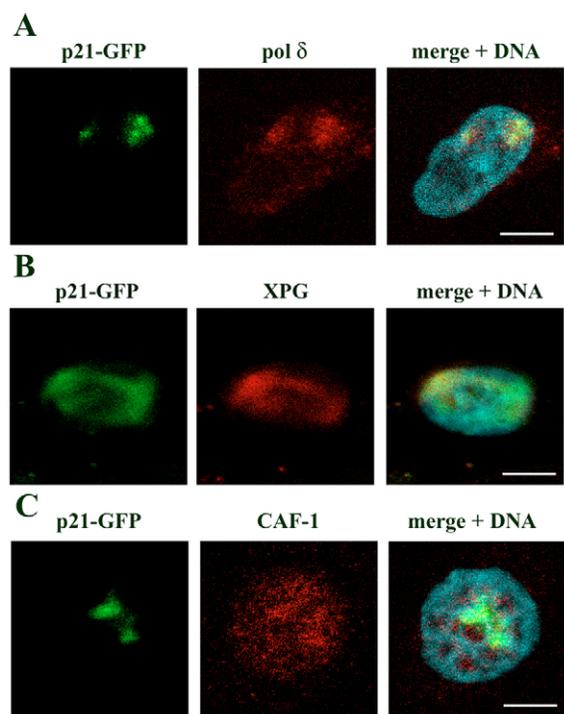
**Fig. 4.** Induction of p21 expression does not inhibit recruitment of PCNA and DNA repair proteins. LF1 fibroblasts were treated for 16 hours with TSA to induce expression of p21, as described in the Materials and Methods. 30 minutes after exposure to UV-C radiation (10 J/m<sup>2</sup>), cells were directly lysed in loading buffer, for determination by western blot of total cellular content (total) of p21, PCNA, pol  $\delta$  (p125 subunit) and Lig I. (A). Parallel samples were fractionated for western blot analysis of proteins in the chromatin-bound fraction (B). Actin was also determined as a loading control.

p21 immunoprecipitation from fractionated cell extracts (detergent-soluble or chromatin-bound fractions) of LF1 fibroblasts. It can be seen that in the detergent-soluble fraction, PCNA was coimmunoprecipitated with p21 from both control and UV-treated samples. Interestingly, the pol- $\delta$ -p125 subunit was also present, being immunoprecipitated to a higher extent in UV-treated cells than in the control samples, though protein levels in the input soluble extracts were not significantly different. For a positive control, an aliquot of the soluble fraction was immunoprecipitated with a polyclonal antibody anti-p125 subunit. As expected, PCNA was coimmunoprecipitated with p125 (Riva et al., 2004).

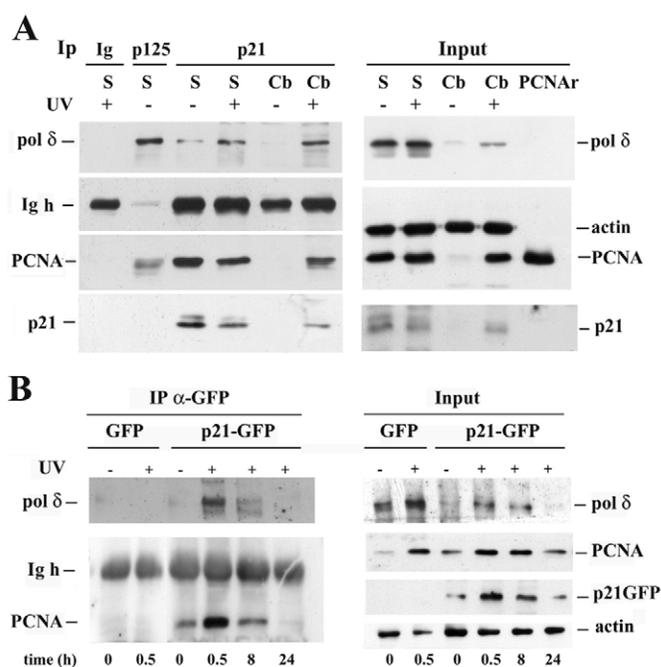
No detectable signal of PCNA or p125 could be observed in the immunoprecipitation from the chromatin-bound fraction of untreated control cells, in which p21 levels were not detectable. By contrast, PCNA and pol  $\delta$  were clearly immunoprecipitated by p21 antibody from the chromatin-bound fraction of UV-treated samples, in which p21 levels before immunoprecipitation were readily detected.

To investigate the kinetics of the p21-PCNA interaction, HeLa cells expressing p21-GFP or pEGFP, were UV irradiated

and collected at various periods of time. After fractionation, immunoprecipitation was performed with anti-GFP antibody on chromatin-bound extracts. Fig. 6B shows that both PCNA and pol  $\delta$  could be immunoprecipitated from the chromatin-bound fraction of cells transfected with the p21-GFP expression vector, but not from that of cells transfected with empty vector (pEGFP). In particular, PCNA coimmunoprecipitated with p21-GFP from untreated control and from UV-irradiated samples, whereas pol  $\delta$  was found only in the immunoprecipitates from UV-irradiated cells. The levels of PCNA and pol  $\delta$  immunoprecipitating together with p21-GFP decreased with time. This result may be attributed to a reduction in the levels of pol  $\delta$  in the chromatin-bound extract. The levels of PCNA were not concomitantly reduced, probably because they represent the sum of chromatin-



**Fig. 5.** Colocalisation of p21-GFP with DNA repair proteins recruited to DNA-damage sites. HeLa cells expressing p21-GFP were locally irradiated with UV-C ( $10 \text{ J/m}^2$ ) through  $3 \mu\text{m}$  pores. After 30 minutes, cells were extracted in situ and fixed for determination of chromatin-bound p21-GFP and immunofluorescence staining of DNA repair proteins. (A) Confocal sections of p21-GFP (green) and pol  $\delta$  (red) fluorescence signals are displayed together with the merged image also showing DNA counterstaining with Hoechst 33258 (blue). (B) confocal sections showing the single and merged images of p21-GFP (green), and XPG (red) at the irradiated sites. DNA was counterstained with Hoechst 33258 (blue). (C) Confocal sections showing the recruitment of p21-GFP (green) and CAF-1 (red), and DNA counterstaining (blue). Bars,  $5 \mu\text{m}$ .



**Fig. 6.** p21 does not displace pol  $\delta$  from binding to PCNA after UV-C DNA damage. (A) Immunoprecipitation (Ip) was performed on detergent-soluble (S), or chromatin-bound fraction (Cb) obtained from LF1 fibroblasts irradiated or not with UV-C ( $10 \text{ J/m}^2$ ), and harvested after 30 minutes. Samples were immunoprecipitated with anti-p21, or with anti-p125 (pol  $\delta$ ) polyclonal antibodies, or with purified rabbit immunoglobulins (Ig) for specificity control. The immunoprecipitated material was analysed by western blot for the presence of PCNA, pol  $\delta$  (p125 subunit), and p21. The position of each protein is shown together with Ig heavy chains (Ig h). Fractionated extracts (Input) were loaded (1/30 and 1/15 for S and Cb fractions, respectively) together with recombinant PCNA (PCNAr), and analysed by western blot for pol  $\delta$ , PCNA, p21, and actin as a loading control. (B) Immunoprecipitation (IP) was performed on HeLa chromatin-bound extracts with anti-GFP antibody. Cell extracts were obtained from cells expressing pEGFP (GFP), or p21-GFP, irradiated or not with UV-C ( $10 \text{ J/m}^2$ ) and harvested at times indicated below each panel. Western blot analysis of PCNA and pol  $\delta$ , was performed on immunoprecipitated material. The position of each protein is shown together with Ig heavy chains (Ig h). Chromatin-bound extracts (Input) were loaded (1/15) on a parallel gel for western blot analysis of pol  $\delta$ , PCNA, p21-GFP, and actin as a loading control.

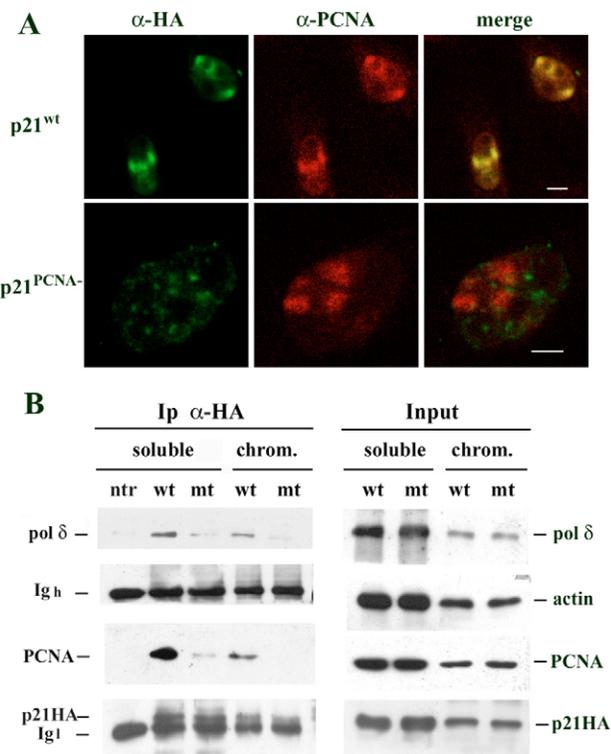
bound PCNA in transfected and non-transfected cells (see Discussion).

### Relocation of p21 to DNA-damage sites depends on the interaction with PCNA

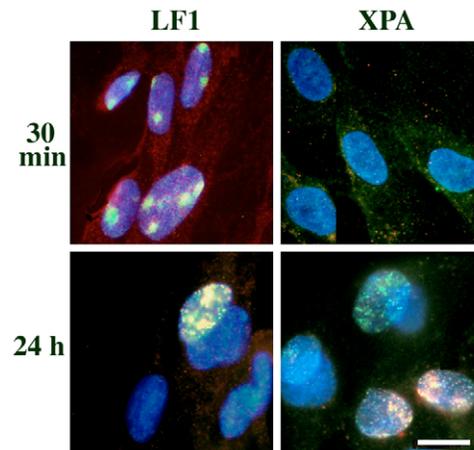
Although it had been suggested that p21 interaction with PCNA is important for DNA repair (MacDonald, 1996; Stivala et al., 2001), the mechanism underlying this aspect was not previously elucidated. Thus, we investigated whether the presence of p21 at DNA-damaged sites was dependent on the interaction with PCNA. To this purpose, HeLa cells were transfected with constructs for the expression of HA-tagged wild-type p21 (p21<sup>wt</sup>-HA), or a mutant form unable to bind PCNA (p21<sup>PCNA-</sup>-HA) (Cayrol and Ducommun, 1998). These constructs were chosen because a similar p21 mutant protein

fused to GFP was previously found to be unstable (Cazzalini et al., 2003). After localised UV irradiation, cells were immunostained with anti-PCNA and anti-HA antibodies for detection of chromatin-bound PCNA and p21-HA, respectively. Analysis by fluorescence confocal microscopy showed that p21<sup>wt</sup>-HA colocalised with PCNA at the locally irradiated sites, as shown by the merged images of p21-HA (green fluorescence) and PCNA (red fluorescence). By contrast, the p21<sup>PCNA-</sup>-HA mutant form showed a heterogeneous, punctuate distribution, but was not recruited with PCNA to the irradiated areas (Fig. 7A). Whole-cell exposure to UV-C was also performed for immunoprecipitation analysis with anti-HA antibody, after cell fractionation. Fig. 7B shows that PCNA and pol  $\delta$  were immunoprecipitated from both the soluble and chromatin-bound fractions, obtained from UV-exposed cells expressing p21<sup>wt</sup>-HA. In the immunoprecipitate obtained from the soluble fraction of cells expressing p21<sup>PCNA-</sup>-HA, a faint band was detected at the position relative to pol  $\delta$  or PCNA, probably because of an interaction with CDK2 (Cazzalini et al., 2003). Remarkably, no band corresponding to PCNA or pol  $\delta$  could be detected in the immunoprecipitate performed on the chromatin-bound fraction, despite the presence of both proteins together with p21<sup>PCNA-</sup>-HA, in the extract. Immunoprecipitation with anti-HA antibody from non-transfected cells showed the absence of any of these proteins in the immunoprecipitate, indicating the specificity of the antibody reaction.

As a further step to understand whether p21 recruitment was dependent on the DNA-repair process, and not a consequence of checkpoint activation, we used NER-deficient XP-A fibroblasts (DeLaat et al., 1999). These cells do not recruit PCNA with the fast kinetics shown by normal cells (Aboussekhra and Wood, 1995; Miura, 1999). Fig. 8 shows that



**Fig. 7.** p21 recruitment to DNA repair sites requires interaction with PCNA. HeLa cells were transfected with HA-tagged constructs for expression of wild-type p21 (p21<sup>wt</sup>-HA) or a mutant form (p21<sup>PCNA-</sup>-HA) unable to bind PCNA (p21<sup>PCNA-</sup>-HA). (A) 24 hours after transfection, cells were exposed to local UV irradiation (15 J/m<sup>2</sup>) through filters with 3  $\mu$ m pores, extracted in situ 30 minutes later and fixed for immunofluorescence staining with anti-HA (green fluorescence) or anti-PCNA (red fluorescence) antibody. Confocal sections of each signal, together with the merged images, are shown. Bars, 5  $\mu$ m. (B) Immunoprecipitation was performed with anti-HA antibody on detergent-soluble (soluble), and chromatin-bound (chrom.) fractions obtained from non-transfected (ntr) cells, and from cells expressing p21<sup>wt</sup> (wt), or p21<sup>PCNA-</sup> (mt) proteins. Immunoprecipitated material was analysed by western blot with anti-pol  $\delta$ , anti-PCNA, and anti-HA antibodies. The position of each protein is shown together with Ig heavy (Ig h), and light (Ig l) chains. For detergent-soluble and chromatin-bound extracts, 1/30 and 1/15 respectively, were loaded (Input) on a parallel gel for western blot analysis of pol  $\delta$ , PCNA, p21HA-tagged proteins, and actin as a loading control.



**Fig. 8.** p21 recruitment to DNA-damage sites depends on DNA repair activity. LF1 and XP20PV (XPA) fibroblasts were exposed to local UV-C irradiation (15 J/m<sup>2</sup>) through filters with 3  $\mu$ m pores, extracted in situ, and fixed at the indicated times for determination of chromatin-bound PCNA and p21. Samples were immunostained with anti-PCNA polyclonal, and anti-p21 monoclonal antibody, detected respectively with secondary antibody conjugated with Alexa Fluor 488 (green fluorescence) or Alexa Fluor 594 (red fluorescence); DNA was counterstained with Hoechst 33258 (blue fluorescence). Bar, 10  $\mu$ m.

30 minutes after local exposure to UV-C irradiation, immunofluorescence signals related to PCNA (green) and p21 (red) were present at damaged sites in normal (LF1), but not in XPA fibroblasts, as indicated by the yellow spots (merged green and red signals). This result was not dependent on the lack of lesions at the irradiated areas, since CPDs were detected with a specific antibody (supplementary material Fig. S3). Chromatin-bound PCNA and p21 associated to DNA-repair sites could be detected in XPA cells, 24 hours after irradiation.

### p21 does not inhibit PCNA-dependent DNA-repair synthesis

In order to test whether the presence of p21 did not inhibit DNA repair, unscheduled DNA synthesis (UDS) was assessed in normal LF1 fibroblasts, as well as in p21-GFP-expressing HeLa cells. In normal fibroblasts, UDS can be visualised by BrdU incorporation and discriminated from DNA replication, in which much higher levels of BrdU are normally incorporated (Nakagawa et al., 1998). Fig. 9A shows the spots of BrdU incorporation detected in LF1 fibroblasts after exposure to UV-C (10 J/m<sup>2</sup>) and further incubated for 3 hours in medium containing 100 μM BrdU. For comparison, samples containing high levels of p21 induced by TSA treatment (see Fig. 4A)

were also included (TSA and TSA+UV). An overexposed S-phase cell is visible in the control sample. UDS in irradiated samples is denoted by the presence of fluorescent nuclear foci. Quantification by flow cytometry revealed that G1-phase cells treated with TSA before irradiation (TSA+UV) showed BrdU incorporation levels 1.5 times higher than untreated but UV-irradiated cells (Fig. 9B). In HeLa cells, UDS was assessed by autoradiography of [<sup>3</sup>H]thymidine incorporation in cells transfected with p21-GFP, or pEGFP, and detected by immunoperoxidase staining (brown colour) with anti-GFP antibody (Fig. 9C). The results showed that the number of autoradiographic granules relative to UDS, detected after UV irradiation in p21-GFP transfected cells, was not significantly different from that in pEGFP, or in non-transfected cells (Fig. 9D).

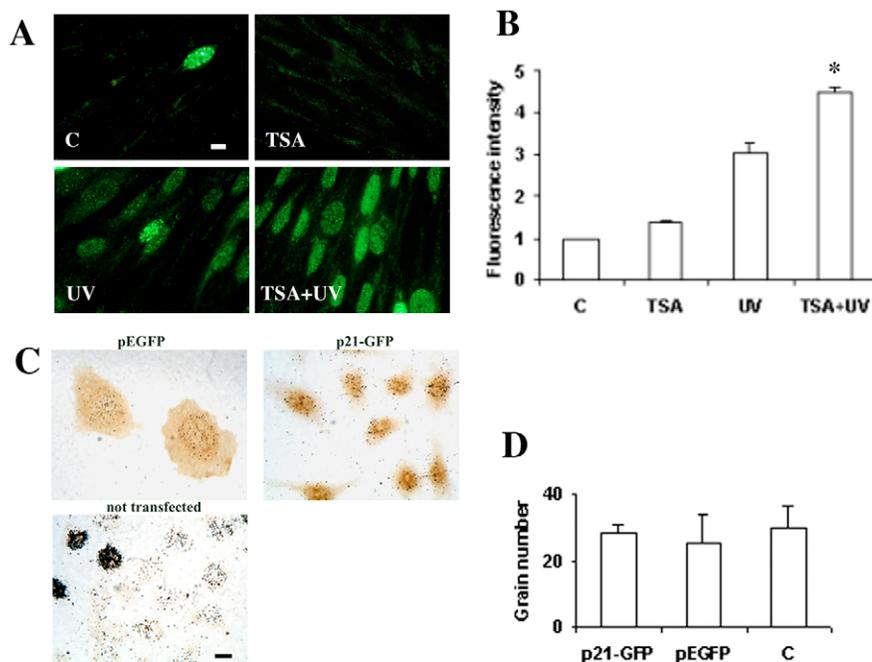
### Discussion

#### Fast recruitment of p21 and PCNA to DNA repair sites independently of p21 degradation

In this study we provide evidence showing that UV-induced DNA damage elicits two different immediate responses regarding p21 protein. In normal fibroblasts, UV irradiation induced a drastic reduction (~60-80%) in p21 protein levels.

However, a detectable pool (~5-10%) of the remaining protein was rapidly recruited together with PCNA at the irradiated sites. It was recently reported that p21 was degraded by the proteasome within hours after UV irradiation, in order to promote PCNA-dependent DNA repair (Bendjennat et al., 2003). In our system, p21 protein was not completely degraded either at low (2.5 J/m<sup>2</sup>) or high (10 J/m<sup>2</sup>) UV doses. In fact, other mechanisms regulating gene expression after UV damage (Barley et al., 1998; McKay et al., 1998), or after other types of DNA lesions (Frouin et al., 2003), may influence p21 turnover. An important factor limiting p21 proteasomal degradation is the interaction with PCNA, because the C8 proteasome subunit binds p21 at its C-terminal region, where the PCNA binding site is located (Touitou et al., 2001). In fact, a p21 mutant protein unable to bind PCNA showed proteasomal degradation faster than wild-type protein (Cayrol and Ducommun, 1998).

Dose-response and time-course studies previously showed that low levels of p21 become chromatin-bound within 2 hours of UV damage (Pagano et al., 1994; Savio et al., 1996). Here we have clearly detected chromatin association of p21 as an immediate response to DNA damage. The time course of p21-GFP and RFP-PCNA accumulation in living murine and human cells, showed that both proteins were recruited within seconds of UV irradiation and persisted for more than 2 hours, consistent with in vivo dynamics of



**Fig. 9.** p21 does not inhibit UDS repair activity. (A) Untreated or TSA-treated LF1 fibroblasts were irradiated with UV-C (10 J/m<sup>2</sup>), and incubated with 100 μM BrdU for 3 hours. Cells were then fixed and immunostained with anti-BrdU antibody, a secondary biotinylated antibody followed by streptavidin-FITC. Fluorescence images of untreated (C), or TSA-treated cells (TSA) are shown together with samples exposed to UV radiation (UV), or exposed after TSA treatment (TSA+UV). (B) Normalised fluorescence intensity of BrdU immunofluorescence in G1-phase cells, measured by flow cytometry. Mean values ± s.d. (n=3) are reported. \*P<0.05 compared with levels in the UV sample (Student's *t*-test). (C) HeLa cells expressing pEGFP, or p21-GFP, were UV-C irradiated (20 J/m<sup>2</sup>), incubated for 2 hours in [<sup>3</sup>H]thymidine and then fixed. Cells were immunostained with anti-GFP primary antibody and HRP-conjugated secondary antibody, and detected by immunoperoxidase staining (brown precipitate). UDS is denoted by the presence of nuclear autoradiographic granules. (D) Quantification of UDS grains in non-S-phase nuclei. Mean values of grain number (± s.d.) in duplicate samples, are reported. Bars, 10 μm (A,C).

other repair factors, such as ERCC1-XPF or TFIIH (Houtsmuller et al., 1999; Hoogstraten et al., 2002; Rademakers et al., 2003; Moné et al., 2004). This time period is in agreement with estimated repair time under local irradiation conditions (Houtsmuller et al., 1999), with the time course of endogenous PCNA recruitment (Toschi and Bravo, 1988; Prosperi et al., 1993), and with the evidence that PCNA recruited to sites of DNA damage shows a very low turnover (Solomon et al., 2004). Initial measurements indicated that RFP-PCNA was relocated slightly faster than p21-GFP, suggesting that p21 binding to DNA-damage sites followed that of PCNA.

Rapid recruitment of p21 was previously observed after heavy-ion-induced DNA damage in human fibroblasts, supporting a role for p21 in early processing of double-strand breaks (Jakob et al., 2002). We also observed rapid p21 relocation with irradiation conditions (337 nm laser) producing double-strand breaks (not shown). However, we used 405 nm laser irradiation to induce CPDs, a typical NER substrate (supplementary material Fig. S1). Although it cannot be excluded that under these conditions other DNA lesions were also produced, our results were confirmed by microirradiation experiments with UV-C. Thus, the p21 response seems to be independent of the type of lesion, but related to PCNA-dependent repair pathways.

#### Interaction of p21 with PCNA at DNA repair sites does not displace PCNA-interacting proteins

Our results also showed that p21 relocation after DNA damage occurred concomitantly with the recruitment of other proteins directly involved in DNA repair, such as pol  $\delta$  and Lig I (Aboussekhra et al., 1995). This relocation was not affected in TSA-treated fibroblasts, which after DNA damage exhibited chromatin-bound p21 levels higher than those in samples exposed only to UV irradiation. In addition, in HeLa cells we found that p21-GFP was not significantly degraded (not shown), and colocalised with pol  $\delta$ , and with factors known to interact with PCNA, required at different steps of the NER process, such as XPG (Gary et al., 1997), and CAF-1 (Green and Almouzni, 2003). These results indicate that p21 does not inhibit the recruitment of repair factors to DNA-damage sites.

In the present study, immunoprecipitation experiments have shown that in UV-irradiated samples, pol  $\delta$  could interact with chromatin-bound p21 and PCNA. These results are in contrast to previous findings showing that p21 disrupts the interaction of PCNA-associated proteins involved both in DNA replication and repair, such as FEN-1 (Chen et al., 1996), Lig I (Levin et al., 1997), DNA methyltransferase (Chuang et al., 1997), XPG (Gary et al., 1997), or pol  $\delta$  (Cazzalini et al., 2003; Riva et al., 2004). However, in those studies the interaction was mainly assessed with purified proteins, or by overexpressing p21 in cells not exposed to DNA damaging agents. Thus, although high p21 levels may saturate PCNA binding, this condition may not have occurred in repairing normal cells. We have also shown that in UV-damaged cells, chromatin-bound pol  $\delta$  remained associated with PCNA, even in cells expressing exogenous p21 (either as GFP- or HA-tagged proteins). Moreover, a p21 mutant form (p21<sup>PCNA-</sup>) unable to bind PCNA (Cayrol and Ducommun, 1998), was not able to coimmunoprecipitate detectable levels of chromatin-bound PCNA, or pol  $\delta$ , further supporting the evidence that

p21 binds in vivo to PCNA complexed with pol  $\delta$  at DNA-damage sites.

The role of a p21-PCNA-pol- $\delta$  complex during DNA repair is still unclear. It could be hypothesised that p21 is required for PCNA-pol- $\delta$  interaction during DNA repair. From this point of view, p21-null human fibroblasts showed substantially normal recruitment of PCNA after UV irradiation, whereas the repair efficiency was significantly reduced (Stivala et al., 2001). Alternatively, this complex could represent a transition state in which p21 binding to PCNA will enable the disassembly of pol  $\delta$ , thereby promoting the next PCNA-dependent steps (Riva et al., 2004). In fact, in p21-null human fibroblasts, or in cells with mutant p53, an accumulation of chromatin-bound PCNA was observed at late repair times (Stivala et al., 2001; Riva et al., 2001). Similar behaviour of PCNA was observed here in p53-deficient HeLa cells (Fig. 6B).

#### p21 recruitment depends on binding to PCNA involved in NER activity

The evidence that p21<sup>PCNA-</sup> mutant protein was not able to relocate to DNA-damage sites strongly suggests that interaction with PCNA is responsible for p21 recruitment. Thus, the slightly slower kinetics of p21-GFP accumulation may indicate that PCNA is recruited first, and soon after p21 follows. In NER-deficient XPA cells, PCNA recruitment was previously described only at late times after DNA damage (Miura, 1999). In agreement with these findings, we also observed a delayed relocation of p21 concomitant with that of PCNA. The lack of early recruitment of both proteins in XPA cells supports the conclusion that p21 interacts with PCNA in a process dependent on ongoing DNA repair, and not as an immediate consequence of checkpoint activation. Accordingly, early p21 recruitment after UV damage was also observed in normal quiescent fibroblasts (not shown), which have p21 levels higher than those in proliferating cells (Itahana et al., 2002).

#### p21 does not inhibit DNA-repair activity in living cells

The presence of p21 did not inhibit DNA repair, as determined by the UDS assay, because both TSA-treated human fibroblasts and HeLa cells containing detectable levels of p21, showed UDS activity equivalent to that of cells containing low physiological levels (untreated fibroblasts), or low-to-undetectable amounts (untransfected or pEGFP-transfected HeLa cells) of endogenous p21. Thus, it is possible that only relatively high p21 levels, such as those reached in cells expressing a p21 mutant protein not degraded after UV irradiation, may reduce or abolish the recruitment of PCNA (Bendjennat et al., 2003). Interestingly, an increase in UDS activity was observed after UV exposure in fibroblasts treated with TSA, an inducer of p21 (Richon et al., 2000). It is known that TSA also increases histone acetylation, thereby favouring the accessibility of DNA-repair machinery to DNA-damage sites (Rubbi and Milner, 2003), which was not hindered by higher p21 levels.

We have shown that, as an immediate response to DNA damage, cells do not completely degrade p21, and that low p21 levels interacting with PCNA accumulate at DNA-damage sites, without inhibition of DNA repair. Possible effects of p21 on the composition and/or activity of PCNA complexes in DNA repair remain to be clarified by future studies.

## Materials and Methods

### Cells, transfections and treatments

HeLa S3 cell line was grown in Dulbecco's modified Eagle's medium (DMEM, Sigma) supplemented with 10% foetal bovine serum (FBS, Gibco BRL), 4 mM L-glutamine (Gibco BRL), 100 U/ml penicillin, 100 µg/ml streptomycin in a 5% CO<sub>2</sub> atmosphere. Expression constructs coding for p21 wt or a C-terminal mutant (p21<sup>PCNA-</sup>) protein deficient for PCNA interaction (Cayrol and Ducommun, 1998) were cloned in pEGFP-N1 (Clontech), or pCDNA3 vectors, for expression of p21-GFP or p21-HA fusion proteins respectively, as previously described (Cazzalini et al., 2003). The expression construct encoding mRFP-PCNAL2 was obtained by replacing EGFP with mRFP1 (Sporbert et al., 2005). Cells seeded on coverslips or Petri dishes, were transiently transfected with Effectene transfection reagent (Qiagen) 24 hours after seeding (70% confluence), and irradiation was usually performed 24 hours after transfection.

Mouse C2C12 myoblasts were cultured in DMEM supplemented with 25 mM HEPES, 50 µg/ml gentamicin and 20% FBS (Sporbert et al., 2002). Cells grown on gridded coverslips, or on Lab-Tek® chamber slides (Nunc), were either microinjected with plasmid DNA using an automated microinjection system (Eppendorf), or cotransfected with TransFectin® transfection reagent (Bio-Rad), according to manufacturers instructions. Cells were subsequently incubated overnight before microirradiation and live-cell analyses.

Human embryonic lung fibroblasts (LF1), kindly provided by J. Sedivy (Brown University, Providence, RI), were grown in Earle's minimal essential medium (Invitrogen) supplemented with 10% FBS (Invitrogen), 100 U/ml penicillin and 100 µg/ml streptomycin in a 5% CO<sub>2</sub> atmosphere. The XP20PV (XPA) primary fibroblasts were provided by M. Stefanini (IGM-CNR, Pavia, Italy), and grown in HAM F-10 medium, supplemented with 10% FBS.

For cell synchronisation in G1 phase, fibroblasts were serum starved (0.5% FBS) for 72 hours, and then re-incubated in complete medium for 8 hours. In some experiments, cells were treated for 16 hours with trichostatin A (TSA) at the final concentration of 200 ng/ml (Rubbi and Milner, 2003). Cell exposure to UV-C was performed with a lamp (Philips TUV-9) emitting mainly at 254 nm, at doses ranging from 2.5 to 30 J/m<sup>2</sup>, as measured with a DCRX radiometer (Spectronics). Localised irradiation was performed by laying Isopore polycarbonate filters (Millipore) with 3-µm pores (Katsumi et al., 2001) on top of the cells.

### Laser microirradiation and time-lapse microscopy

Microirradiation experiments were essentially performed as described (Mortusewicz et al., 2005). In brief, C2C12 cells were seeded on coverslips and sensitised for microirradiation by incubation in medium containing BrdU (10 µg/ml) for 20 hours. For live-cell microscopy and irradiation, coverslips were mounted in FCS2 (Biotech), or in POC (VisiTron Systems) live-cell chambers and maintained at 37°C. In some experiments, microirradiation was carried out with a microdissection system (P.A.L.M.) using a pulsed N<sub>2</sub> laser (337 nm) coupled to a Zeiss LSM410 confocal laser-scanning microscope. However, to induce the formation of cyclobutane pyrimidine dimers (CPDs), microirradiation was carried out with a 405 nm Diode laser coupled to a Leica TCS SP2/AOBS confocal laser scanning microscope. The laser was set to maximum power at 100% transmission, and cells were irradiated for 1 second. For evaluation of the recruitment kinetics, fluorescence intensities of the irradiated region were corrected for background and for total nuclear loss of fluorescence over the time course and normalised to the pre-irradiation value.

For long time-lapse analysis, light optical sections were acquired with a Zeiss LSM410 confocal laser-scanning microscope using the 488 nm Ar laser line and the 543 nm HeNe laser line. Six mid z-sections at 0.5 µm intervals were taken every 3-10 minutes and cells were followed up to several hours. Focus drift over time was compensated with a macro, as described (Mortusewicz et al., 2005). After image acquisition, a projection of all six z-sections was performed from each time point using ImageJ 1.34. Short time series were taken with a Leica TCS SP2/AOBS confocal laser-scanning microscope using the 488 nm Ar laser line and the 561 nm DPSS laser line. Before and after microirradiation, confocal image series of one mid z-section were recorded every 2 seconds.

### Immunofluorescence and confocal microscopy

HeLa cells seeded on coverslips were transfected as described above. After 24 hours, cells were locally irradiated, and re-incubated in whole medium for the required period of time. Cells on coverslips were then washed twice in PBS, dipped in cold physiological saline and lysed for 10 minutes at 4°C in hypotonic buffer: 10 mM Tris-HCl (pH 7.4), 2.5 mM MgCl<sub>2</sub>, 0.1% Nonidet NP-40, 0.2 mM phenylmethylsulfonyl fluoride (PMSF) and 0.2 mM Na<sub>3</sub>VO<sub>4</sub>. Thereafter, samples were washed in PBS, fixed in 2% formaldehyde for 5 minutes at room temperature (RT), and then post-fixed in 70% ethanol. After re-hydration, samples were blocked in PBST buffer (PBS, 0.2% Tween 20) containing 1% bovine serum albumin (BSA), and then incubated for 1 hour with specific monoclonal antibodies. anti-PCNA (PC10, Dako), anti-DNA polymerase δ (pol δ) p125 subunit (clone 22, BD Biosciences), anti-CAF-1 (Ab-2, Oncogene Research), or anti-XPG (Ab-1, NeoMarkers), all diluted 1:100 in PBST buffer/BSA. After washing, each reaction was followed by incubation for 30 minutes with anti-mouse antibody conjugated

with Alexa Fluor 594 (Molecular Probes). Cells expressing p21-HA fusion proteins were incubated with anti-HA monoclonal antibody (clone H7, Sigma), and with FL261 rabbit polyclonal antibody to PCNA (Santa Cruz Biotech.), diluted 1:500 or 1:100, respectively. After three washes with PBST buffer, coverslips were incubated for 30 minutes with goat anti-rabbit and anti-mouse antibodies labeled with Alexa Fluor 488 or 594 (Molecular Probes), respectively. After immunoreactions, cells were incubated with Hoechst 33258 dye (0.5 µg/ml) for 2 minutes at RT and washed in PBS. Slides were mounted in Mowiol (Calbiochem) containing 0.25% 1,4-diazabicyclo-[2,2,2]-octane (Aldrich) as antifading agent. LF1 fibroblasts grown on coverslips were irradiated as above, dipped in cold double-distilled H<sub>2</sub>O before lysis in hypotonic buffer, and fixation as above (Savio et al., 1998). Samples were incubated for 1 hour in FL261 polyclonal antibody to PCNA, and with monoclonal antibody to p21 (clone DCS 60.2, NeoMarkers), both diluted 1:100. After washing, samples were incubated for 30 minutes in goat anti-rabbit and anti-mouse antibodies conjugated with Alexa Fluor 488 (1:200), and Alexa Fluor 594 or biotin, respectively. In the latter case, incubation with streptavidin-Texas-Red (Amersham Biosciences) diluted 1:100 was performed.

For determination of laser-induced CPDs, C2C12 cells were fixed with 3.7% formaldehyde in PBS and permeabilised with 0.2% Triton X-100 for 4 minutes. DNA was denatured by incubation in 0.5 M NaOH for 5 minutes, and then coverslips were stained with anti-CPDs monoclonal antibody (Kamiya Biomedical) diluted 1:1000 in PBS containing 2% BSA, and detected with Cy3-conjugated goat anti-mouse antibody (Amersham) diluted 1:400. Cells were counterstained with DAPI and mounted in Vectashield (Vector Laboratories).

Fluorescence signals were acquired with a Leica TCS SP2 confocal microscope, at 0.3 µm intervals. Image analysis was performed using the LCS software. Images of fixed cells were taken with a Zeiss Axiophot 2 widefield epifluorescence microscope equipped with a cooled CCD camera (Visitron Systems), or with a BX51 Olympus fluorescence microscope equipped with a C4040 digital camera.

### Immunoprecipitation and western blot analysis

For western blot analysis, cells were directly lysed in SDS sample buffer (65 mM Tris-HCl pH 7.5, 1% SDS, 30 mM DTT, 10% glycerol, 0.02% Bromophenol Blue), or fractionated in soluble and chromatin bound fraction, as previously described (Riva et al., 2004) with minor modifications. Cells were lysed in hypotonic buffer containing 10 mM Tris-HCl (pH 7.4), 2.5 mM MgCl<sub>2</sub>, 1 mM PMSF, 0.5% Nonidet NP-40, 0.2 mM Na<sub>3</sub>VO<sub>4</sub> and a mixture of protease and phosphatase inhibitor cocktails (Sigma). After 10 minutes on ice, cells were pelleted by low-speed centrifugation (200 g, 1 minute), and the detergent-soluble fraction was recovered. Lysed cells were washed once in hypotonic buffer, followed by a second wash in 10 mM Tris-HCl buffer (pH 7.4), containing 150 mM NaCl, and protease/phosphatase inhibitor cocktails. Cell pellets were then incubated with DNase I (20 U/10<sup>6</sup> cells) in 10 mM Tris-HCl (pH 7.4), 5 mM MgCl<sub>2</sub> and 10 mM NaCl for 15 minutes at 4°C. After a brief sonication on ice, samples were again centrifuged (13,000 g, 1 minute), and the supernatant containing the chromatin-bound fraction was collected.

For immunoprecipitation, about 10<sup>7</sup> cells were re-suspended in 1 ml lysis buffer and fractionated as above. Equal amounts of each extract were incubated with anti-GFP rabbit polyclonal antibody (Molecular Probes), N-19 rabbit polyclonal antibody to p21 (Santa Cruz), or with H7 anti-HA antibody pre-bound to protein A Sepharose CL-4B (Pharmacia). Half the amount of each antibody was used for chromatin-bound fractions. In some experiments, C20 polyclonal antibody to pol δ p125 subunit (Santa Cruz) was also used. Reactions were performed for 3 hours at 4°C under constant agitation. The samples were then centrifuged at 14,000 g (30 minutes, 4°C), and immunocomplexes were washed with ice-cold 50 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 0.5% Nonidet NP-40. Immunoprecipitated peptides were eluted in SDS sample buffer and resolved by 7.5% or 12% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were electrotransferred to nitrocellulose, then membranes were blocked for 30 minutes in 5% non-fat milk in PBST buffer, and probed with primary antibodies anti-PCNA, or anti-HA (H7) diluted 1:1000. Anti-pol δ p125 (clone 22), and anti-DNA ligase I (1A9, NeoMarkers) were diluted 1:500. Membranes were then washed in PBST, incubated for 30 minutes with appropriate HRP-conjugated secondary antibodies (Amersham), and revealed using enhanced chemiluminescence.

### Analysis of DNA repair by UDS determination

DNA repair was assessed by determination of unscheduled DNA synthesis (UDS). After irradiation, LF1 fibroblasts were incubated for 3 hours in medium containing 100 µM BrdU, then fixed in 70% ethanol (Nakagawa et al., 1998). After DNA denaturation in 2 N HCl for 30 minutes, and neutralisation for 15 minutes in 0.15 M Na<sub>2</sub>B<sub>4</sub>O<sub>7</sub>, samples were blocked in TBST/BSA. Incubation (1 hour) in anti-BrdU antibody (Amersham) was followed by a biotinylated anti-mouse antibody, and streptavidin-FITC (Amersham). Immunofluorescence of G1-phase cells was measured with an Epics XL flow cytometer, as described (Stivala et al., 2001).

In p21-GFP, or pEGFP-expressing HeLa cells, UDS was determined after irradiation (20 J/m<sup>2</sup>), by incubating cells for 2 hours in medium containing 1 ml [<sup>3</sup>H]-thymidine (NEN, 10 µCi/ml specific activity), then chased for 1 hour in medium containing 10 µM each cold thymidine and cytidine. Cells were then fixed

in 4% formaldehyde and post-fixed in 70% ethanol. Detection of cells expressing p21-GFP or pEGFP was performed by incubation in anti-GFP antibody, followed by immunoperoxidase staining with diaminobenzidine. Samples were processed for autoradiography using an Ilford K2 emulsion, exposed for 4 days at 4°C, and then developed and fixed before mounting on microscope slides. Autoradiographic granules were counted in 50 non-S phase cells showing GFP staining, in duplicate experiments.

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