Chapter 11

Assessing Cell Cycle Independent Function of the CDK Inhibitor p21^{CDKN1A} in DNA Repair

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Abstract

The cyclin-dependent kinase (CDK) inhibitor p21^{CDKN1A} is a small protein that is able to regulate many important cell functions, often independently of its activity of CDK inhibitor. In addition to cell cycle, this protein regulates cell transcription, apoptosis, cell motility, and DNA repair. In particular, p21 may participate in different DNA repair processes, like the nucleotide excision repair (NER), base excision repair (BER), and double-strand breaks (DSB) repair, because of its ability to interact with DNA repair proteins, such as proliferating cell nuclear antigen (PCNA), a master regulator of many DNA transactions. Although this role has been debated for a long time, the influence of p21 in DNA repair has been now established. However, it remain to be clarified how this role is coupled to proteasomal degradation that has been shown to occur after DNA damage. This chapter describes procedures to study p21 protein recruitment to localized DNA damage sites in the cell nucleus. In particular, we describe a technique based on local irradiation with UV light through a polycarbonate filter with micropores; an in situ lysis procedure to detect chromatin-bound proteins by immunofluorescence; a cell fractionation procedure to study chromatin association of p21 by Western blot analysis, and p21 protein–protein interactions by an immunoprecipitation assay.

Key words DNA repair, Localized DNA damage, p21^{CDKN1A}, p21 degradation, p21 recruitment, PCNA

1 Introduction

The cyclin-dependent kinase (CDK) inhibitor p21$^{waf1/cip1}$ plays important roles in several cellular pathways in response to intracellular and extracellular stimuli. In particular, p21 was identified as a target of p53 tumor-suppressor gene in response to DNA damage [1, 2]. However, it was afterwards recognized that p21 exerts also its functions in physiological conditions, such as in cellular quiescence and senescence, and in the control of cell differentiation of different cell types [3–5]. In addition to cell cycle control, p21 is involved, independently of CDK inhibition, in the regulation of: (1) transcription, (2) apoptosis and possibly autophagy, (3) cell motility, and (4) DNA repair processes [5–9].

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p21 is the main intermediary of cell cycle arrest in response to DNA damage, not only by inactivating the G_1-phase CDK/cyclin complexes but also through the direct interaction with proliferating cell nuclear antigen (PCNA), a master regulator of important DNA transactions [10–12]. This activity occurs thanks to the high affinity binding to PCNA, through which p21 may displace relevant PCNA partner proteins involved in DNA replication [13, 14]. However, this mechanism has been demonstrated in vitro, and in overexpression systems, while its occurrence in normal cells has never been demonstrated, likely because p21 is degraded in S phase [9, 15].

As a transcription regulator, p21 may act not only through the inhibition of CDK [6, 7] but also with a CDK-independent mechanism, by direct binding to transcription factors, or transcriptional co-activators [7, 16]. An active role of p21 is also found in the regulation (mainly inhibition) of the apoptotic process, through the interaction with procaspase 3, with the apoptosis signal-regulating kinase-1 (ASK-1), and with stress-activated MAP kinases [5, 17].

More recent is the discovery of the involvement of p21 in cell motility regulation through the inhibition of Rho kinase, and by interfering with substrate adhesion, as reviewed in ref. [5].

Another important function of p21 is the participation in DNA repair, due to its ability to interact with PCNA. However, this matter has been debated for a long time, since both negative and absent effects have been reported in contrast with findings supporting a positive role of p21 in the process [5, 9, 18]. Furthermore, it was suggested that p21 proteasomal degradation is necessary for DNA repair [19]. In contrast, the findings for a positive role for p21 in nucleotide excision repair (NER) are based on the evidence that p21-null fibroblasts showed a reduced DNA repair efficiency [20], that endogenous p21 protein co-localized with NER factors interacting with PCNA (like XPG, DNA polymerase δ, and CAF-1), and that p21 was present in complexes with these proteins without inhibiting DNA repair [21, 22]. Finally, p21 has been shown to play a role in NER by restoring the acetyltransferase activity of p300 [23], which is required for DNA repair [24], but is inhibited by PCNA [25].

The interaction of p21 with PCNA has been also reported to be important in the Translesion DNA Synthesis (TLS), in order to limit a mutagenic load effect [22, 26]. In addition, p21 has been suggested to play a role in the base excision repair (BER) process by associating with poly(ADP-ribose) polymerase-1 (PARP-1) to regulate its interaction with BER factors [27, 28]. More recently, a PCNA-dependent accumulation of p21 into foci after ionizing irradiation inducing double-strand breaks (DSB), has been also reported [29, 30], further indicating that p21 function in DNA repair is strictly related to PCNA [9, 18]. However, a CDK-dependent function of p21 in DSB repair, has been also described [31, 32].

In the S phase of the cell cycle and after UV irradiation, an ubiquitin-dependent degradation of p21 occurs when it is bound

to chromatin-associated PCNA [33]; this process is mediated by the interaction with E3 complex Cul4-DDB1^{Cdt2} [34, 35]. Degradation of p21 in S phase and after DNA damage has been explained with the need to avoid inhibition of the PCNA-mediated DNA synthesis step of the repair process. However, p21 degradation does not appear to be strictly required for efficient DNA repair in normal cells [9]. Interestingly, in vivo kinetic analysis has shown that p21 recruitment to DNA damage sites follows that of PCNA after a short delay [21, 30]. This behavior suggests that p21 degradation might occur after regulation of the interaction between PCNA with relevant partners participating in DNA repair [36].

Analysis of protein recruitment to DNA damage sites has provided useful information on the orchestration of the DNA damage response [37]. Although expensive instrumentation is required to follow this process in vivo, more cheap techniques are also available to perform similar studies in fixed cells or in cell extracts.

In this chapter we describe some techniques which allow to investigate the behavior of p21 in the DNA repair process, by analyzing its recruitment and localization at DNA repair sites, and its interaction with relevant proteins (e.g., PCNA) participating in the process. The micropore irradiation technique [38, 39] allows DNA damage to be localized within restricted regions of the cell nucleus, thus enabling the recruitment process to be precisely analyzed. This approach may be coupled with an in situ extraction procedure necessary for analyzing chromatin-bound proteins by immunofluorescence (or autofluorescence) detection [21, 40]. Biochemical techniques of chromatin release may be also used to investigate the process of protein recruitment by western blot, and also to perform immunoprecipitation assays. Experimental conditions for using inhibitors of proteasomal activity (such as MG132), in order to study the effect of p21 protein accumulation at sites of DNA damage, are also described.

2 Materials

1. UV-C germicidal lamp T-UV9 (Philips, emission peak at ~254 nm).
2. UV-C radiometer (e.g., DRC-100X, Spectronics).
3. Isopore polycarbonate filters (Millipore) with 3 μm pores of diameter.
4. Fluorescence microscope (e.g., Olympus BX51).
5. Standard polyacrylamide gel electrophoresis (PAGE) and protein transfer equipments.
6. Culture media for untransformed human cells (e.g., fibroblasts), or transformed cells (e.g., HeLa).

7. Carbobenzoxy-l-leucyl-l-leucyl-l-leucinal (MG132), proteasome inhibitor (Sigma or Calbiochem). Prepare a 25 mM stock solution in DMSO.
8. Protease inhibitor cocktail solution in DMSO without EDTA (Sigma). Store in aliquots at −20 °C.
9. Formaldehyde (supplied as 37 % solution stabilized with methanol).
10. p21 monoclonal antibodies: DCS 60.2 (NeoMarkers); CP-74 (Sigma); p21 polyclonal antibodies C-19, or N-20 (Santa Cruz Biotech.).
11. Goat anti-mouse and anti-rabbit secondary antibodies conjugated with Alexa 488 or 594 fluorochromes (Life Technologies), or antibodies conjugated with DyLight 488 or 594 (KPL).
12. Goat anti-mouse or anti-rabbit antibodies conjugated with horseradish peroxidase (HRP).
13. Protein G-agarose magnetic beads and magnet (Dynal, Life Technologies).
14. Citrate-Phosphate buffer: 24.5 mM citric acid, 5.2 mM Na_2HPO_4, pH 5.0.
15. Bovine serum albumin (BSA).
16. Peroxidase substrates for enhanced chemiluminescence (ECL) detection (Cyanagen)
17. Phosphate buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10.6 mM Na_2HPO_4, 1.4 mM KH_2PO_4, pH 7.4.
18. Physiological saline: 154 mM NaCl.
19. Hypotonic lysis buffer: 10 mM Tris–HCl, pH 7.4, 2.5 mM $MgCl_2$, 0.5 % Igepal. Use freshly prepared solution, and immediately before use add 1 mM DTT (dithiothreitol; stock is 1 M, store in aliquots at −20 °C), 1 mM PMSF (phenylmethylsulfonyl fluoride; stock is 200 mM in isopropanol or DMSO, store at 4 °C or r.t.), 0.2 mM sodium vanadate (Na_3VO_4; stock is 100 mM; store in aliquots at −20 °C), and 100 μL/10^7 cells of protease inhibitor cocktail. Keep supplemented buffer on ice.
20. Washing buffer: 10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM PMSF, and 50 μL/10^7 cells of protease inhibitor cocktail.
21. DNA digestion buffer: 2× solution is 20 mM Tris–HCl, pH 7.4, 20 mM NaCl, 10 mM $MgCl_2$, 20 μL/10^7 cells of protease inhibitor cocktail. Prepare fresh solution each time.
22. DNase I solution: DNase I (Sigma, D-4527, or equivalent) dissolved in 20 mM NaCl and 0.1 mM PMSF just prior to use.

23. SDS-loading buffer: 65 mM Tris–HCl, pH 7.4, 100 mM DTT, 1 % SDS (sodium dodecyl sulfate), 10 % glycerol, 0.02 % bromophenol blue. Store at −20 °C.
24. PBS-Tween 20 solution: PBS containing 0.2 % Tween 20.
25. PBT solution: PBS containing 1 % BSA and 0.2 % Tween 20. Prepare fresh solution each time.
26. Ponceau S: 0.1 % in 5 % acetic acid solution. Store at room temperature.
27. Blocking solution: 5 % nonfat dry milk plus 0.2 % Tween 20 in PBS. Prepare fresh solution each time.
28. Bisbenzimide H 33258 (Hoechst 33258): stock solution is 1 mM in bidistilled H_2O. Store at 4 °C.
29. Mounting medium (e.g., Mowiol) containing 0.25 % antifading agent (1,4-diazabicycloctane).

3 Methods

The methods described here allow to study the recruitment of p21 protein to localized sites of cell nucleus where DNA damage is induced. To accomplish this step, a microirradiation technique, based on the use of filter with micropores, which is layered on top of cells so that the damaging radiation (in this case, UV light) will pass only through the filter pores. DNA repair is triggered in the nuclear regions exposed to the light and accumulation of DNA repair proteins will become detectable (*see* Fig. 1). To obtain reliable results, accurately determined conditions of exposure of the cells to UV light, are first described (*see* Subheading 3.1).

Recruitment of p21 protein to DNA damage sites may be detected as chromatin-bound protein by means of immunocytochemical techniques and fluorescence microscopy (*see* Subheading 3.2), which allow direct visualization of the protein associated with DNA repair sites.

The same process may be investigated with biochemical techniques by using cell fractionation procedures allowing to isolate p21 protein associated with chromatin in a detergent-resistant form (*see* Subheading 3.3). The extraction of the detergent-soluble protein is first obtained with a hypotonic solution in the presence of a non-ionic detergent [20, 21]. The subsequent release of chromatin-bound proteins is obtained by DNA digestion [20, 21]. Detection of p21 in each cell extract fraction is obtained by Western blot analysis. Immunoprecipitation studies using commercially available p21 antibodies can also be easily performed [21, 27].

3.1 Induction of DNA Damage with Irradiation

The pathway of DNA repair induced by DNA damage greatly depends on the type of lesion. In order to detect chromatin-bound p21 involved in DNA repair, one of the most suitable procedures

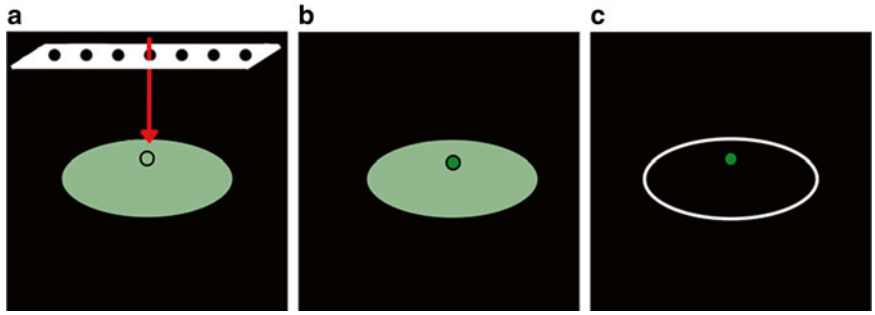

Fig. 1 Schematic representation of the protocol to obtain localized damage in specific regions of DNA: (**a**) the cells are irradiated with UV-C light (*arrow*) through a polycarbonate filter (*white*) with micropores. A localized damage is thus obtained in a nuclear region corresponding to the micropore (*small circle*). (**b**) The protein of interest, distributed across the whole nucleus, is then accumulated only in the region corresponding to the irradiated area (*darker circle*). (**c**) Accumulation of the protein at the site of DNA damage is better visualized after in situ hypotonic lysis allowing to detect only the protein bound to these sites (*small circle*)

is based on the cell exposure to UV-C light (*see* **Note 1**). Best results are obtained with quiescent cells (e.g., fibroblasts growth-arrested by serum starvation) since DNA synthesis proteins involved in DNA repair (e.g., PCNA) also participate in DNA replication, and cells in S phase may give confounding results. However, studies with proliferating cells may be also performed to obtain information otherwise not available on quiescent cells. Studies may also be undertaken with transformed cells, but their DNA repair capacity must be taken into account, depending particularly on the p53 status [1, 2].

To investigate the effect of p21 degradation on DNA repair, an inhibitor of the proteasome (e.g., MG132) may be used before UV-irradiation. This step is described below.

3.1.1 Exposure to Proteasomal Inhibitors

1. Cells grown in petri dishes (*see* below), are treated with proteasomal inhibitors (e.g., MG132, final concentration 25 μM in culture medium) for times ranging from 30 min to 5 h, depending on the type of the experiment. If cells must be grown for periods longer than 2–3 h after UV exposure, the cells should be previously incubated with MG132 for 30 min, and then re-incubated in the presence of the inhibitor also after irradiation.
2. When the effect of p21 on DNA repair is to be investigated, the cells may be treated for 5 h in the presence of MG132, in order to allow accumulation of p21 protein. Then, the cells are exposed to UV light and collected 30 min later.

3.1.2 UV Light Irradiation

UV-C irradiation (254 nm) is one of the most used methods to induce DNA damage and to induce DNA repair by the NER pathway: typical lesions produced are cyclobutane pyrimidine dimers, and 6–4 photoproducts. Since the emission spectrum of commonly

used UV lamps (often used are germicidal lamps) may be different, an accurate determination of the energy supplied to the cells, must be performed.

Particular care must be taken to avoid accidental exposure to the UV-C light of the lamp. Wear appropriate gloves and eye protection. As the optimal condition, the lamp is placed under a three-side closed wooden box, with the open side covered with a black sheet of paper or cloth, which can be lifted to introduce the petri dishes. If possible, perform all subsequent steps under a laminar flow hood to avoid contamination during exposure. The glass window of the hood will also protect from UV light reflected from the steel working level. To avoid reflection, a sheet of black paper may be used.

1. Monitor the energy of the lamp (e.g., T-UV9, Philips) in the UV-C region by using a radiometer (e.g., Spectronics, USA) equipped with a detector sensitive in the 200–280 nm range.
2. Set the distance of the UV source from the working level, so that the fluence of UV light will be 0.5–1 $J/m^2/s$. Check that the UV energy will be uniformly distributed throughout the surface to be exposed.
3. Use cells grown on petri dishes (e.g., 10 cm diameter). For immunofluorescence analysis, grow the cells attached on coverslips (24 × 24 mm) placed into 35-mm diameter dishes. Cells in suspension may be also used, but care must be taken to layer into a petri dish as a thin film in PBS, so that irradiation will be as much as possible uniform.
4. Remove the culture medium and wash the cells twice with warm sterile PBS. Aspirate almost all PBS, leaving just a thin liquid layer on top of the cells.
5. Remove the dish lid and expose the cells to UV light for the period of time required to supply the chosen dose (e.g., 10 J/m^2).
6. Add back the culture medium and re-incubate cells at 37 °C for the desired period of time (a suitable time course ranges from 30 min to 24 h).

3.1.3 Localized Micropore Irradiation

1. To obtain a damage localized to a limited portion of nuclear DNA, the cells on the slides are covered with polycarbonate filters. In this way only the regions corresponding to the pores will be exposed to the radiation, since polycarbonate will stop the majority of UV-C light [38, 39]. Filters are available with pores (randomly distributed) of 3, 5, or 8 μm in diameter. The choice may be based both on the nuclear size of the cells under investigation, or on the extent of the nuclear area that it is to be irradiated.

2. The filter is previously rinsed with PBS to ensure that all pores are open. This operation may be performed by placing the filter into an appropriate filter holder and flushing energetically the solution with a syringe.
3. Under a hood, remove the lid of the petri dish, as in previous section (**step 4**) and lay the filter on top of the cells on the coverslip, taking care to avoid exposure to the UV lamp.
4. Irradiated the cells, as described in the previous section (**step 5**) and expose the cells to a dose ranging from 10 to 30 J/m^2 (*see* **Note 2**).
5. Stop irradiation and add back the culture medium so that the filter will float. Remove the filter with a small clamp. If the cells must be kept alive from periods longer than a few hours, use all sterilized materials (including filters and clamp).
6. Re-incubated cells at 37 °C for the period required and then proceed to the extraction-fixation procedure (next section).

3.2 Detection of p21 Recruitment to DNA Repair Sites by Immunofluorescence Microscopy

To determine the recruitment of p21 protein to DNA damage sites by immunofluorescence microscopy, an in situ hypotonic extraction procedure is necessary to remove the soluble protein not associated with DNA repair sites [20, 21].

3.2.1 In Situ Extraction for Immunofluorescence Microscopy Analysis of Chromatin-Bound p21

1. Seed cells (about 5×10^4) on coverslips or microscope slides (previously cleaned with a 1:1 mixture ethanol/ether, and sterilized). Grow the cells to an 80 % density before use.
2. Rinse the coverslips with PBS, then dip into cold bi-distilled H_2O for about 4 s. For some cells dipping in cold physiological saline may be preferable (*see* **Note 3**).
3. Transfer the coverslips to a petri dish containing cold hypotonic lysis buffer (containing 0.1 mM PMSF), in which the detergent (Igepal) concentration has been reduced to 0.1 %, to avoid detachment of cells (*see* **Note 4**). Keep the petri dishes at 4 °C for about 10 min, with gentle agitation every 3–4 min.
4. Remove the lysis solution and wash the cells carefully with cold PBS or physiological saline, then aspirate and replace with fresh PBS (e.g., 2 mL for a 35-mm dish).
5. Add an equal volume of 2 % formaldehyde (*see* **Note 5**) to reach a final 1 % concentration in PBS, and fix the permeabilized cells for 5 min at r.t. under continuous agitation (*see* **Note 6**).
6. Wash the coverslips again with PBS and further post-fix in cold 70 % ethanol. The samples can be stored in this solution at −20 °C for 1–2 months before further processing for immunocytochemical staining.
7. Remove the fixative and wash the cells with PBS. All subsequent steps are done at r.t.

8. Block the unspecific staining sites by incubation for 15 min with PBT solution.
9. Incubate the coverslips for 1 h at r.t. with anti-p21 monoclonal (DCS 60.2) or polyclonal antibody (C-19 or N-20), diluted 1:100 in PBT solution. The coverslips are placed with cells facing upside-down, onto a drop (50 μL) of antibody on a flat Parafilm strip, so that a sandwich is formed (*see* **Note 7**).
10. Stop the incubation by returning the coverslips into petri dishes with the cells facing up, and wash three times (10 min each) with PBS-Tween 20.
11. The following steps are performed in the dark to avoid photobleaching of fluorochromes. Incubate the coverslips for 30 min with appropriate secondary antibody (diluted 1:200 in PBT solution) by placing again the cells upside-down onto a drop of antibody, as described above. Choose a secondary antibody conjugated with suitable fluorochromes (e.g., Alexa or DyLight 488 for green fluorescence, Alexa or DyLight 594 for red fluorescence), depending on the color emission required (*see* **Note 8**).
12. Remove the coverslips and wash them in petri dishes three times (10 min each) with PBS-Tween 20.
13. If the fluorescent signal is too weak, a third antibody against the secondary one (e.g., donkey anti-goat) labeled with same fluorochrome may be used. In this case, after the step with secondary antibody, incubate for 30 min with the third antibody (increase dilution to 1:300 dilution to avoid high background fluorescence), and then wash three times with PBS-Tween 20, as in **step 12**.
14. Counterstain DNA with Hoechst 33258 or DAPI (0.2 μM in PBS) for 2 min, then remove the dye and wash the coverslips twice (5 min each) with PBS.
15. Mount the coverslips in aqueous mounting medium (e.g., Mowiol) containing anti-fading.
16. After mounting, view slides with a fluorescence microscope (*see* Fig. 2) equipped with filter sets for UV, blue, and green excitation of fluorescence.
17. Seal the coverslips with transparent nail polish to prevent them from drying, and to allow storage of slides at –20 °C.

3.3 Biochemical Analysis of p21 Associated with DNA Repair Sites

The analysis of p21 associated with DNA repair sites by biochemical techniques includes: (1) extraction of the detergent-soluble protein; (2) release of chromatin-bound protein by DNA digestion with DNase I; when necessary, (3) immunoprecipitation of both detergent-soluble and DNase-released fractions with anti-p21 antibodies.

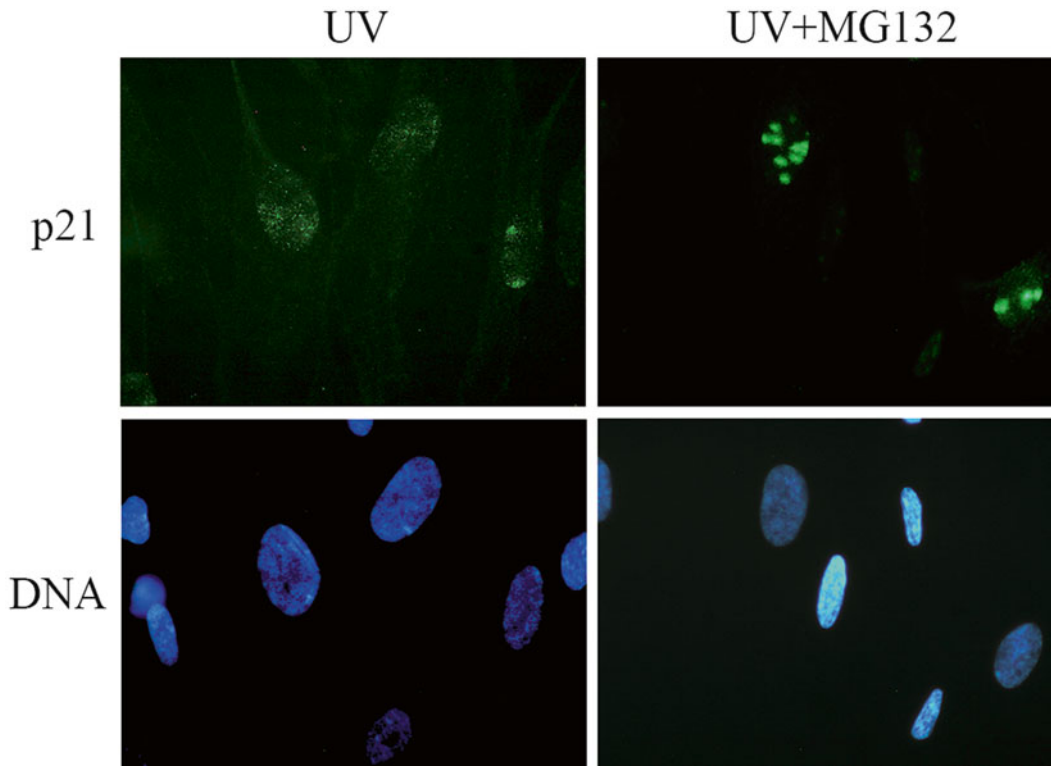

Fig. 2 Immunofluorescence analysis of p21 recruiment to localized sites of UV-induced DNA damage. Mock and MG132-treated human fibroblasts grown on coverslips were exposed to local UV-C irradiation (30 J/m^2) and then re-incubated in culture medium for 30 min. Cells were then treated with hypotonic lysis solution and fixed. Immunofluorescence staining with polyclonal p21 antibody (N-20) is shown in the *upper panels*, while the corresponding fields showing DNA staining with Hoechst dye is shown in the *lower panels*

3.3.1 Extraction of Detergent-Soluble p21 Protein

Both fresh cells and frozen pellets in 2 mL-Eppendorf tubes can be used with this extraction procedure.

1. Chill fresh cells, or thaw frozen samples to 0 °C on ice and resuspend in 1 mL of hypotonic lysis buffer. The volume of lysis buffer (1 mL) is given for extraction from a cell pellet of about 10^7 cells, but it can be scaled down for smaller samples (*see* **Note 9**). However, for an efficient extraction of soluble nuclear proteins, add about 10 volumes of lysis buffer to the cell pellet.
2. Gently resuspend the cell pellet by pipetting the sample about 3–4 times and allow lysis to occur for 10 min on ice (*see* **Note 10**).
3. Pellet the samples by low-speed centrifugation (300 × *g*, 1 min, 4 °C). Collect the supernatant containing the detergent-soluble protein fraction, and measure protein content with the

Bradford method. For normal human fibroblasts, about 2 mg/mL of soluble proteins are released from 10^7 cells. If this amount is lower, other soluble proteins may be released in the next washing step (*see* below), meaning that extraction has not been complete (*see* **Note 11**). Determine the protein concentration also in the fraction released in the washing buffer, and if necessary, add a further washing step. The detergent-soluble proteins may be analyzed by western blot, or used for subsequent immunoprecipitation studies, to be compared to the chromatin-bound protein fraction.

3.3.2 Extraction of Chromatin-Bound p21 by DNA Digestion with DNase I

After extraction of the detergent-soluble protein fraction, chromatin-bound proteins must be solubilized for Western blot analysis. The procedure described below, which is also suitable for immunoprecipitation studies [21, 27], allows the release of DNA-bound protein complexes, and it is based on DNA digestion with DNase I (*see* **Note 12**).

1. Resuspend pelleted permeabilized cells in washing buffer in order to remove as much as possible any trace of soluble proteins.
2. Centrifuge the samples (300×*g*, 1 min), and then resuspend the pellets in half-volume of DNase I solution (e.g., 250 μL for a final digestion volume of 500 μL) containing 100–200 DNase I units/10^7 cells. After thorough resuspension, add the second-half volume of 2× digestion buffer (*see* **Note 13**). The amount of DNase I (*see* **Note 14**) to be added is dependent on the cell type (e.g., cells with a DNA content higher than diploidy, require proportionally higher amounts of DNase I, as compared with normal diploid cells).
3. Carry out the digestion for 15–30 min at 4 °C, with constant agitation in order to avoid cell sedimentation and clumping (*see* **Note 15**).
4. Pellet the samples by high-speed centrifugation (13,000×*g*, 1 min) and collect the resulting supernatants, containing the DNase-released proteins, for subsequent use.
5. Load 40–60 μg of detergent-soluble proteins, and a similar, or higher volume (*see* **Note 16**) of the DNase-released fraction, on a 12 % polyacrylamide gel for standard Western blot analysis.

3.3.3 Immunoprecipitation of Detergent-Soluble and DNase-Released Proteins

If fractionated extracts will be used for immunoprecipitation studies, perform the following steps:

1. Take 100–200 μL of protein G-agarose magnetic beads with a wide-bore pipet from the 50 % suspension; choose the bead volume as a function of the amount of antibody to be used, according to the manufacturer instructions.

2. Resuspend the beads and wash (three times) in citrate-phosphate buffer (pH 5.0).
3. Incubate the p21 antibody with dried protein G-agarose beads in citrate-phosphate buffer (pH 5.0) for at least 1 h at r.t., in a total volume of 100–150 μL. Usually, 4 μg of antibody are sufficient to immunoprecipitate p21 protein from the soluble fraction. Since the amount of p21 protein in the DNase-fraction is generally lower, the amount of antibody to be added may be reduced (e.g., 2 μg).
4. Take a sample of soluble cell lysate containing 1–1.5 mg total protein, and use the whole sample of DNase-released proteins. Set apart a small volume (30–50 μL) of each fraction to check the amount of protein input.
5. Add a suitable amount of p21 antibody pre-bound to protein G-agarose magnetic beads to each cell extract. Incubate for 3 h at 4 °C on a rotating wheel.
6. Precipitate the beads by placing the tubes on a magnet for 2 min at 4 °C. Remove the supernatant by gentle aspiration with a Pasteur pipette, being careful not to touch the beads (if the beads are touched on, place the tube again on the magnet and repeat the step).
7. Wash the immune complexes bound to beads three times with washing buffer. Each time, add 0.5–1 mL of washing buffer and resuspend the beads by gentle vortexing. The final wash should be removed completely.
8. For SDS-PAGE, add 60 μL of SDS-loading buffer to each sample.
9. Denature the proteins in the sample by heating to 100 °C for 5 min. Centrifuge for 15 s at 10,000 × *g* and load half of the supernatant (30 μL) on the gel.

3.3.4 Immunoblot Analysis of Fractionated Cell Extracts, or Immunoprecipitated Proteins

1. After SDS-PAGE, transfer the proteins to a nitrocellulose (or PVDF) membrane with a conventional apparatus (semidry or immersion).
2. Check the protein transfer to the membrane by Ponceau S staining.
3. Remove excess stain with several washes in bi-distilled H_2O.
4. Block the membranes for 30 min at r.t. with blocking solution.
5. Incubate the membranes for 1 h at r.t. with monoclonal (CP-74) or polyclonal antibody to p21 (diluted 1:500 in PBS-Tween 20), then wash 4–6 times (10 min each) with PBS-Tween 20, under continuous agitation.

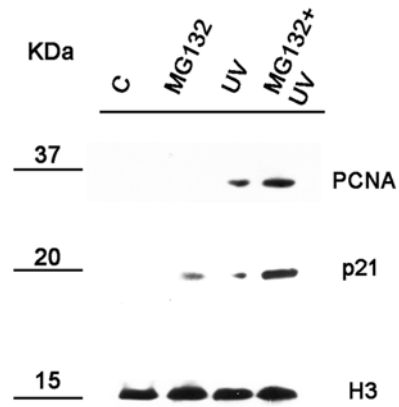

Fig. 3 Western blotting analysis of chromatin-bound proteins at the DNA damage sites. Mock and MG132-treated (5 h) human fibroblasts were exposed to UV-C irradiation (10 J/m^2), and then re-incubated in culture medium for 30 min. Cells were then detached and kept frozen at −80 °C until use. Hypotonic lysis and DNAse I digestion were performed to extract chromatin-bound proteins. Western blot analysis was performed with anti-p21, and anti-PCNA antibodies, to visualize association of these proteins to chromatin after DNA damage. Immunostaining with anti-histone H3 antibody was performed to check nuclear protein extraction and equal loading of samples

6. Incubate the membranes (30 min, r.t.) in a secondary HRP-conjugated antibody (diluted 1:2000 in PBS-Tween 20), then repeat washings as in the previous step.
7. Incubate the nitrocellulose (or PVDF) membranes with peroxidase substrates for ECL detection and expose to autoradiographic film (*see* Fig. 3).

4 Notes

1. In the case of UV light, the main DNA repair process which is initiated at these sites, is the NER process. However, if another source of focused radiation (e.g., a laser beam) is available, different DNA repair activities (e.g., DSB repair) may be investigated. In this case, the energy provided by the laser (whose emission must be properly chosen to induce DNA damage) is coupled with a photosensitizer to induce DNA lesions similar to those obtained with UV light, or to induce DSB [21, 40].
2. In the literature, local UV irradiation with doses as high as 100 J/m^2 may be found. However, we noticed that it is not necessary to use these conditions when studying recruitment of p21 and PCNA.

3. Dipping of the cover slips in cold ddH_2O is particularly useful with cells with large cytoplasm (e.g., fibroblasts) since this will favor the extraction of detergent-soluble proteins. However, when using cells with a small cytoplasm (e.g., some tumor cells, such as HEK 293 cells), the hypotonic stress is likely to result in a substantial detachment of the cells from the coverslip.
4. Avoid pipetting the hypotonic lysis solution directly onto the cells, since this will increase the risk of cell detachment from the coverslip.
5. Formaldehyde vapors are toxic. Solutions containing formaldehyde should be prepared in a chemical hood.
6. For some cell types (e.g., HeLa cells), the final formaldehyde concentration may be increased up to 4 % because it has been noticed that cells fixed with lower concentrations will lose their nuclear morphology after freezing–thawing of slides stored at −20 °C.
7. The antibody sandwich formed with the coverslip on the Parafilm strip will not dry under normal RT conditions, so that is not necessary to prepare a humid chamber. This has the advantage of reducing the incubation volume, thus saving antibody.
8. The choice of secondary antibody depends on the fluorescence emission to be detected under the microscope. In same case, it may be useful to perform a double staining with two primary antibodies (i.e., a monoclonal and a polyclonal). Choose secondary antibodies (e.g., anti-mouse and anti-rabbit) made in the same specie (goat), to avoid unwanted cross-reactions.
9. It is worth using not fewer than 5×10^6 cells to easily detect the chromatin-bound fraction of p21, and consider about 3.5×10^6 cells as the lower limit for detection.
10. Resuspension of the cell pellet by pipetting may produce foam due to the presence of detergent in the hypotonic lysis buffer. However, this will not compromise extraction of soluble p21 protein. Care must be taken if cells with small cytoplasm (e.g., lymphocytes) are used, since pipetting could result in nuclear damage and loss. In this case, pipette only two or three times.
11. When the extraction of detergent-soluble proteins is efficient, the amount of proteins released in the subsequent washing buffer should not be higher than 20 % of the initial release.
12. An alternative procedure to release chromatin-bound proteins is based on the production of DNA strand breaks by sonication. This procedure is equally effective for releasing chromatin-bound proteins in a form suitable for immunoprecipitation experiments. This would not be otherwise possible with protocols using SDS to release nuclear-bound proteins.

However, when using sonication, care must be taken to reduce protein degradation by using a "low" setting and short pulses, and by performing the entire procedure on ice.

13. Good resuspension of the cell pellet in the DNase solution is a requisite for efficient DNA digestion and consequent release of DNA-bound proteins. The addition of 2× digestion buffer after cell resuspension is performed because of the Mg^{2+} contained in the digestion buffer, which will favor cell clumping, thereby reducing the extent of DNA digestion.
14. The quality of DNase I is very important, since contaminants present in different preparations (e.g., chymotrypsin) may be responsible for proteolytic degradation. A chromatographically purified preparation (e.g., Sigma D-4527) is recommended.
15. Digestion conditions (e.g., time and temperature) should be determined depending on the experiment. For coimmunoprecipitation studies, possible degradation of proteins may be reduced by shortening both incubation time and temperature. If cell clumping occurs during DNA digestion, a very brief sonication will help to disrupt cellular aggregates and facilitate DNA breakage.
16. Given that p21 bound to chromatin constitutes only a minor fraction of the total cellular amount of the protein, loading of proportionally higher volumes of DNase extracts, as compared to the soluble fraction, may be required to improve detection by western blot. It is advisable to check the protein loading of different samples, by detecting another protein (e.g., actin or histone H3) as an internal standard.

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