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# p21<sup>CDKN1A</sup> and DNA Repair Systems: Recent Findings and Future Perspectives

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## 1. Introduction

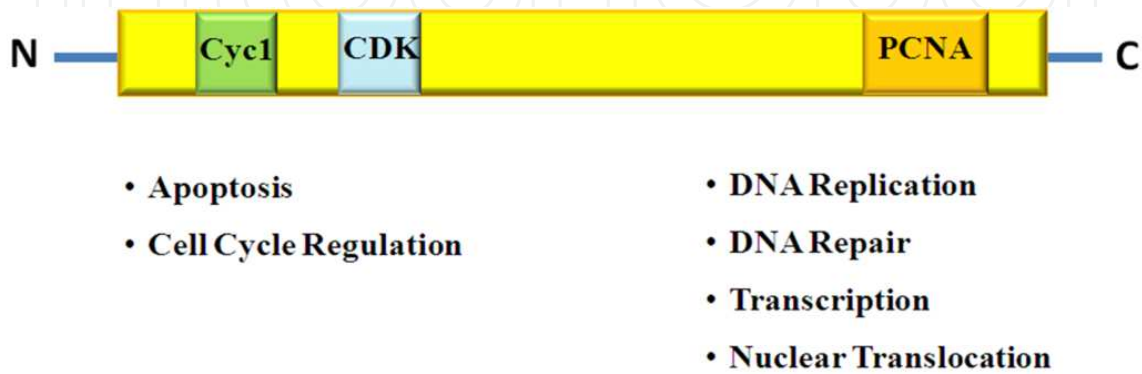
After exposure to genotoxic agents, cells activate DNA damage response pathways consisting of a signaling cascade (cell cycle checkpoints), and of DNA repair processes able to recognize and remove a great number of DNA lesions [1].

DNA repair is characterized by an impressive high number of different proteins necessary to perform specialized biochemical reactions, which are different according to the type of lesion to be repaired [2]. Thus, the nucleotide excision repair (NER) mechanism will repair bulky lesions, such as the cyclobutane pyrimidine dimers (CPDs) produced by UV-C irradiation, or other types of adducts produced by the interaction of chemicals with DNA. Base excision repair (BER) is instead involved in the removal of bases damaged by alkylating, or oxidative agents, while the repair of single and double strand breaks is performed through the pathway of homologous recombination, or via the non homologous end-joining (NHEJ) repair. In addition, cells repair errors introduced during DNA replication with the mechanism of mismatch repair (MMR).

Among the many factors involved in these defense processes against DNA damage, p21<sup>CDKN1A</sup> protein – known also as p21<sup>(WAF1/CIP1/SDI1)</sup> – plays a key role in several fundamental biological processes, such as cell cycle control, DNA replication/repair, gene transcription, apoptosis, and cell motility [3-6]. This protein is a cyclin-dependent kinase (CDK) inhibitor belonging to the Cip/Kip family; it was first described as a potent inhibitor of cell proliferation and DNA replication, both in physiological conditions and after DNA damage [7,8]. Homologs are found in several organisms, including *Xenopus* (Xic1), *Drosophila* (Dacapo), as well as *C. Elegans* (CKI-1). In mammals, p21 was previously known as CDK-interacting pro-

tein 1 (CIP1), wild type p53-activated fragment (WAF1), senescent cell-derived inhibitor 1 (SDI1), and melanoma differentiation-associated protein 6 (MDA-6); all these names have been substituted by a new terminology including all CDK inhibitors, and p21 is now named CDKN1A.

Due to the lack of a defined tertiary structure, p21 protein may adopt an extended conformation [9], which may explain its ability to interact with a number of proteins involved in several important biological processes [3-6] (Figure 1).



**Figure 1.** Schematic structure of p21 protein showing the regions responsible for binding to Cyclins, CDK and PCNA. Below the N- and C-terminal regions are indicated the processes in which they are involved, respectively.

## 2. p21 biology and functions

The main role of p21 is cell-cycle regulation, performed by inhibiting the activity of cyclin-CDK complexes thanks to direct interaction through specific sequences (termed CDK and Cy motifs) in the N-terminal domain of the protein [10-13]. Cell cycle progression may be also regulated, independently of cyclins and CDKs, thanks to the strong affinity binding to proliferating cell nuclear antigen (PCNA) [14-17], a protein playing a central role in DNA replication and repair, as well as in other processes of DNA metabolism [18,19]. This association may interfere with PCNA-dependent enzyme activities involved in DNA synthesis [18,19]. In contrast with the negative cell-cycle regulation, p21 may also serve as an assembly factor for cyclin D-CDK4/6 complexes, thus promoting cyclin D-dependent events, and downstream activation of cyclin E-CDK2 [7,8].

*CDKN1A* gene inactivation studies performed with experimental models, and in particular with knock-out mice, have confirmed the tumor suppressor functions of this protein [20,21]. The p21-null mice showed a normal development and did not show any spontaneous tumor formation until 7-month of age [20]. However, embryonic fibroblasts derived from these animals were deficient in G1 checkpoint arrest following DNA damage [20]. Subsequent studies in this model were extended to a longer time frame and the observations reported that p21-deficient mice developed spontaneous tumors at a median age of 16 months. The most

common malignancies occurring in these animals were hemopoietic (B-cell lymphoma), endothelial, and epithelial tumors [21]. In addition, accelerated tumor formation and an increased capacity of tumor metastasis, respectively induced by urethane or by gamma radiation, were found in p21<sup>-/-</sup> mice [22,23]. Accelerated tumorigenesis, and promotion of lung metastasis was also found in correlation with cytoplasmic p21 in the mammary epithelium of mice expressing the MMTV/*neu* oncogene [24]. Tumor suppression functions of p21 were also confirmed by studies in the skin and in the colon of p21-deficient mice [25,26]. Furthermore, spontaneous tumor formation in p21-null mice was also found to occur in combination with other knock-out genetic backgrounds, such as Muc2<sup>-/-</sup> (mice lacking mucin 2), and Apc<sup>1638+/-</sup> (mutant allele of the adenomatosis polyposis gene) mice [27,28].

In addition to enhanced tumor formation, further investigations showed that loss of p21 caused exhaustion of blood stem cells [29], and induced development of Systemic Lupus Erythematosus in female animals [30]. Thus, the results obtained from transgenic mice, clearly indicated the tumor suppressor role of p21, although other studies have provided contrasting results [6,31]. As an example, p21-null mice crossed with knock-in PML-RAR mice, showed an oncogenic role of p21 in maintaining self-renewal of leukemic stem cells [32]. The dual behaviour of p21 most probably occurs because of its participation in several cellular processes, and it is dependent on different factors [6,31].

An important aspect for determining the target of p21 activity is the intracellular localization. Early studies indicated that lack of p21 expression, or cytoplasmic localization of the protein, promoted anchorage-independent growth, and drug resistance [5,6,31]. Human p21 protein is located predominantly in the nucleus; however, it is also present in the nucleolus and in the cytoplasm. In the nucleus, in addition to inhibit CDK2 and binding to PCNA, p21 may also associate with transcriptional regulators [4]. In the nucleolus, p21 was found to co-localize with cyclin E [33], and to accumulate after DNA damage, as a consequence of inhibition of nuclear export [34]. Interestingly, growing body of evidence indicates that the cytoplasmic localization of p21 is linked to drug resistance [6,31], thus suggesting that in this compartment the protein may have a tumor-promoting function [35]. Cellular localization of p21 is regulated mainly by post-translation modifications. In fact, nuclear translocation appears to be counteracted by different kinases phosphorylating Thr145 and Ser146 residues located near the NLS region of p21 [36-38]. These modifications are responsible for cytoplasmic localization of p21, as well as for the loss of interaction with PCNA [39]. An important role in p21 phosphorylation is played by AKT1/PKB, which also mediates stability of the protein [36,37]. Another relevant modification of p21 (i.e ubiquitination) regulating its degradation, has been shown to occur predominantly in the nucleus, because p21 mutant in the NLS region exhibited enhanced stability [40].

A summary of the most important functions performed by p21 protein is reported in the following paragraphs.

### Cell-cycle regulation

As the principal mediator of cell cycle arrest in response to DNA damage, p21 not only acts by inactivating G<sub>1</sub>-phase cyclins/CDKs complexes, but also by inhibiting cell cycle progres-

sion through other mechanisms. These possibly include direct interaction with PCNA to inhibit DNA replication, and indirect effects mediated by interaction with other cell cycle regulators. In addition, p21 has been shown to play a role in the maintenance of G<sub>2</sub>-phase arrest, through multiple mechanisms [3,5,6].

The demonstration that p21 is involved in cell response to DNA damage, mediated through transcriptional activation by p53, was first obtained in mammalian cells [41,42]. The main role of p21 in the G<sub>1</sub> checkpoint resides in its ability to inhibit the activity of cyclin E, and cyclin A/CDK2 complexes required for the G<sub>1</sub>/S phase transition, thereby contributing to G<sub>1</sub>-phase arrest [43]. Accordingly, mouse embryonic fibroblasts (MEFs) obtained from p21-null mice fail to arrest in G<sub>1</sub> phase, in response to DNA damage [20,44]. Recently, it has been demonstrated that CDK2<sup>-/-</sup> MEFs, as well as regenerating liver cells in CDK2<sup>-/-</sup> mice, are able to arrest at the G<sub>1</sub>/S checkpoint in response to  $\gamma$ -irradiation. This response has been found to depend on the ability of CDK1 to substitute for CDK2, and on p21, which may associate with, and inhibit nuclear CDK1 at the G<sub>1</sub>/S transition [45].

p21 potentially participates in the G<sub>1</sub>/S checkpoint also by blocking directly DNA synthesis, thanks to its ability to bind the central region (interdomain connecting loop) of PCNA [46,47]. *In vitro* studies showed that the C-terminal domain of p21 is sufficient to displace DNA replication enzymes from PCNA, thereby blocking processive DNA synthesis [47,48]. *In vivo* expression of C- vs N-terminal truncated forms of p21, as well as of CDK- or PCNA-binding deficient p21 mutants, indicated that p21 interaction with PCNA could indeed arrest cell cycle [49–51]. In particular, interaction with PCNA localized at DNA replication sites could prevent loading of DNA polymerase  $\delta$ , but occurrence of this mechanism was observed in a limited number of cells [52], and never proved with endogenous p21, whose levels are significantly reduced in S phase [53,54]. Other mechanisms of p21-mediated G<sub>1</sub>/S checkpoint activation after DNA damage have been reported. A direct interaction between p21 and the p50 non-catalytic subunit of human DNA polymerase  $\delta$  was found both *in vitro* and *in vivo* [55]. It was concluded that p21 might be recruited to the DNA replication complex via direct interaction with p50, thereby facilitating the binding to PCNA. However, this interpretation does not take into account p21 degradation in S phase [53,54]. Another suggested explanation for p50–p21 interaction was the inhibition of cyclinA/CDK2 complex associated with DNA polymerase  $\delta$  [55]. An additional mechanism of p21-mediated arrest at the G<sub>1</sub>/S transition was described in HCT116 cells treated with adriamycin. ICBP90 (Inverted CCAAT box binding protein) is a 90 kDa nuclear protein that binds to the promoter of topoisomerase II $\alpha$  gene, and that was suggested to be important in the G<sub>1</sub>/S transition, due to partial colocalization with PCNA [56]. Expression of p21 directly down-regulated the levels of ICBP90 protein, both through the reduction of E2F-mediated transcription and the promotion of ubiquitin-dependent proteolytic degradation [56]. Thus, downregulation of ICBP90 by p21 might constitute another level of checkpoint control of S-phase entry.

It has been shown that p21 is also essential to sustain the G<sub>2</sub> phase checkpoint after DNA damage in human cells, as well as in preventing G<sub>2</sub>-arrested cells from undergoing additional S-phase [57–59].



Cyclin B-CDK1 complex has a relatively low affinity for p21 when compared with the other cyclin-CDK complexes [60], and a low amount of cyclin B/CDK1 was found to be associated with p21 after activation of the G<sub>2</sub> checkpoint [61]. However, p21 has been demonstrated to contribute to CDK1 inactivation by inhibiting the CDK-activating kinase (CAK) and, consequently, the CDK1-activating Thr161 phosphorylation. Thus, p21/CAK pathway appears to be essential in sustaining the G<sub>2</sub> arrest in response to DNA damage [61]. Other likely targets of p21 in G<sub>2</sub> phase are cyclin A-CDK1/2 complexes [62,63]. As an additional mechanism of G<sub>2</sub> arrest, p21 was also suggested to mediate nuclear retention of cyclin B1-CDK1 complex in response to genotoxic stress, thus preventing its activation by Cdc25 and CAK [64]. Recently, it has been also proposed that p21 contributes to G<sub>2</sub> arrest by mediating cyclin B degradation in response to DNA damage [65]. Furthermore, a new p21-dependent mechanism to maintain G<sub>2</sub> arrest after DNA damage has been shown to involve Emi1 protein, an inhibitor of the Anaphase Promoting Complex (APC) whose destruction controls progression through mitosis to G<sub>1</sub> phase [66]. It has been reported that p21 down-regulates Emi1 in cells arrested in G<sub>2</sub> by DNA damage, thereby contributing to APC activation and degradation of key substrates, including cyclins A2 and B1. Thus, p21 controls positively this checkpoint preventing G<sub>2</sub>-arrested cells from entering mitosis [66].

Another important function of p21 is related to the control of basal proliferation in specific cell types. In particular, the stem cell self-renewal of keratinocytes [67], of the haematopoietic system [29], and of the mouse forebrain and hippocampus [68,69], have been shown to depend on p21 protein. In fact, studies in CDKN1A knock-out mice showed that p21 restricts the self-renewal potential of stem cell population, and promotes their irreversible commitment to differentiation [67]. In the absence of p21, an increase in stem cell proliferation with a consequent exhaustion of the population was observed in different cell types [67-70]. Interestingly, p21 is also able to maintain the self-renewal potential of leukemic stem cells, and to protect them from DNA damage accumulation, thereby demonstrating an oncogenic activity of the protein [32].

Cell quiescence and senescence are other processes in which p21 plays a fundamental role by keeping cells arrested in G<sub>0</sub>, or G<sub>0</sub>-like state, in order to prevent untimely DNA replication [71,72]. Accordingly, loss of p21 has been shown to facilitate cell cycle entry from a quiescence state, at the expense of replication stress [73]. Interestingly, lack of p21 expression has been found to link cell cycle control with appendage regeneration in mice, since p21<sup>-/-</sup> animals showed a phenotype similar to that of regenerating mouse strains [74].

p21 also plays a complex role in cell differentiation. In fact, its expression is induced in differentiating cells of the skin and of the intestinal epithelium, as well as in cultured epidermal cells, while down-regulation has been observed at late stages of differentiation [75,76]. However, p21 appears to play a positive role in promoting differentiation of human promyelocytic leukaemia cells [77], mouse skeletal muscle and cartilage cells [78,79], and oligodendrocytes [80]. The whole body of evidence indicates that p21 plays either positive or negative roles in differentiation, independently of cell cycle control, but depending on cell type and specific stage of differentiation. This regulatory function may involve specific interactions of p21 with critical regulators of differentiation [3,6].

In contrast with the CDK inhibitory function, a cell growth promoting effect has also been demonstrated [81]. In fact, p21 may serve as an assembly factor for cyclin D/CDK4 complex, thereby promoting its nuclear translocation, kinase activation, and cell proliferation [81]. This function has been suggested to potentially confer an oncogenic activity to p21 [6,31,35].

### **Transcriptional regulation**

In addition to the role of CDK inhibitor, p21 functions as a transcriptional cofactor that may regulate transcription, either positively or negatively [3-5,82]. This activity of p21 may occur through three different mechanisms: i) by inhibition of cyclin/CDK complexes; ii) by direct binding to several transcription factors, such as NF- $\kappa$ B, Myc, E2F, STAT3, and estrogen receptors [2-5]; iii) by regulating the activity of transcriptional co-activators, such as p300/CBP [5,82]. According to the first mechanism, CDK inhibition will prevent the phosphorylation of Rb-family proteins, thereby inactivating E2F-dependent transcription [4,5]. In the second mechanism, p21 acts as a co-factor that physically interacts with, and represses the activity of transcription factors. As an example, interaction of p21 with STAT3 proteins inhibits their transcriptional activity; overexpression of p21 was shown to reduce the transcriptional activity of STAT3 proteins, without modifying their DNA binding activity [83]. In addition, it was shown that p21 may specifically repress E2F-dependent transcription [84], not only through inhibition of cyclin/CDK activity and substrate association, but also through a direct interaction with E2F factor [85], which could function as an anchor for p21 [3]. Another important example is the binding of p21 to the N-terminus of c-Myc, resulting in the interference of c-Myc-Max association, and in the suppression c-Myc-dependent transcription. At the same time, the interaction between c-Myc and p21 may directly counteract p21-dependent inhibition of DNA synthesis, as c-Myc binds p21 in competition with PCNA [86]. A general correlation has been observed between p21 inhibitory effects and specific DNA sequences in the promoter of some genes showing a cell cycle-dependent transcriptional regulation by p21 [87]. For example, it has been shown that p21 functions as transcriptional repressor of the *myc* and *cdc25A* genes upon DNA damage, being recruited to the promoter of these genes. This was associated with inhibition of p300 recruitment, and down-regulation of histone H4 acetylation [88]. p21 may also bind to other transcription factors and modulate positively their function. An example is given by the estrogen receptor (ER $\alpha$ )-dependent transcription which may be enhanced by p21 through CDK-dependent and independent mechanisms [89,90]. The third mechanism occurs by modulation of a repression domain in p300, which occurs independently of the CDK inhibitor effect on the phosphorylation of p300 [91,92]. This protein is an essential co-activator that stimulate gene expression through its acetyl transferase activity, or through its ability to interact with components of the transcriptional machinery [93]. It has been shown that p21 prevents the recruitment of p300, causing histone hypoacetylation and transcriptional repression [94].

After UV-induced DNA damage, p21 has been shown to directly interact and to regulate the histone acetyl transferase activity (HAT) activity of p300 [95], which provides accessibility of NER machinery to DNA damage sites through histone acetylation [96]. For this activity, full-length p21 protein is required and its binding to p300 is not dependent on interaction with PCNA [95]. It is known that both p21 and PCNA may bind p300 at basal levels, and that

PCNA inhibits the transcriptional activity of p300 [97]. After DNA damage, p21 may restore p300-HAT activity by disrupting the inhibitory interaction with PCNA, thereby allowing p300 to participate in NER [5].

Finally, p21 also up-regulates multiple genes that have been associated with senescence or implicated in age-related diseases, in which a DNA damage response seems to occur [98].

### Apoptosis

p21 is a major inhibitor of p53-dependent as well as p53-independent apoptosis [2-6,31]. In fact, reduction in p21 expression was shown to lead to apoptosis in DNA-damaged human cancer cells [99-101]. The cleavage and inactivation of p21 is mediated by caspase-3 in human normal cells, and in cancer cell lines [99,100]. However, the inhibitory function is not absolute since, under some circumstances (e.g. enforced overexpression), p21 may promote the signaling apoptotic pathway that ultimately determines cell death [99,100]. Initial work provided the evidence that in the absence of p21, DNA-damaged cells underwent cell cycle arrest followed by typical apoptotic cell death [59,102]. These findings suggested that p21 could exert an anti-apoptotic function in response to DNA damage. The mechanism by which p21 negatively regulates DNA damage-induced death machinery relies on its ability to bind key regulatory proteins involved in the apoptotic process (e.g. protease precursors and specific kinases) [100]. Indeed, p21 physically interacts, through its first N-terminal 33 aminoacids, with pro-caspase 3, i.e. the inactive precursor of the apoptotic executioner caspase 3 [103,104]; when bound to p21, the inactive pro-caspase cannot be converted into the active protease and apoptosis is inhibited [104]. Caspase 2, which acts upstream caspase 3, is also kept in a repressed status by p21 [105]. The strict relationship between p21 and caspases is also supported by the observation that p21 itself is cleaved by caspases early during DNA damage induced apoptosis; proteolysis involves the p21 NLS region, and impairs p21 translocation into the nucleus [106-108].

The p53-independent expression of p21 in several human cell lines, induce not only cell cycle inhibition, but also suppression of apoptosis [99,100]. Two mechanisms of action are responsible for this phenomenon: *i*) the interaction with pro-apoptotic regulatory proteins, such as pro-caspase-3, caspase-8 or apoptosis signal-regulating kinase-1 (ASK-1), with their consequent inhibition [103,104,109]. *ii*) the inhibition of apoptotic events, such as chromatin condensation, cell shrinkage and loss of adhesion, by targeting caspase-dependent activation of CDKs [110].

In the first case, p21 forms a complex with ASK-1 within the cytoplasm [111]. In the second one, p21 seems to have an anti-apoptotic activity through the inhibition of CDK activity required for activation of the caspase cascade downstream of mitochondria [112,113].

An important consequence of the inhibitory activity of apoptosis in a variety of systems is that p21 could dramatically impair the effectiveness of chemotherapeutic agents acting by damaging DNA. In this respect, an innovative strategy to kill cancer cells is based on the direct or indirect attenuation of p21 (obtained by different approaches) before chemotherapy [114-116].



In contrast with the anti-apoptotic role, p21 appears to possess pro-apoptotic functions under certain conditions, and in specific systems [5,6,31]. In fact, p21 overexpression in thymocytes induced hypersensitivity to p53-dependent cell death in response to X-rays and UV radiation [117]. Overexpression of p21 was shown to enhance the apoptotic response induced by a variety of stimuli and in different cell systems [5,6,31]. Other studies reported the pro-apoptotic role of p21 after targeted overexpression of the protein [118,119] or by showing a decrease in apoptosis after p21 gene disruption [99,100]. A pro-apoptotic effect of p21 was also observed in breast cancer cells treated with sodium butyrate, which is an inducer of p21 expression; interestingly, in these cells the pro-apoptotic effect required the interaction of p21 with PCNA [120]. However, the mechanism(s) by which p21 may promote apoptosis are still to be clarified.

Finally, p21 may also play an important role in regulating another type of cell death, i.e. autophagy, a process in which cell organelles are enclosed and destroyed in vesicles [121]. This mechanism appears to be regulated by p21 by maintaining autophagic proteins in an inactive state [122].

### **Cell motility**

One of the most recently described functions of p21 is the regulation of actin-based cell motility. Cytoplasmic p21 has been shown to influence cell motility and neuronal neurite outgrowth by interfering with substrate adhesion through the inhibition of Rho kinase [123]. Degradation of cytoplasmic p21 favors a nonmotile cell behavior. In tumor cells, high levels of p21 localized in the cytoplasm will favor Rho inhibition with consequent enhanced cell movement [124]. This effect has been shown to contribute to tumor metastasis and invasion, thus suggesting another mechanism by which p21 may play an oncogenic role [5,31].

### **DNA repair**

The role of p21 in DNA repair, has been debated for a long period, since both negative or absent effects, in contrast with studies supporting a positive role of p21, have been reported. Recent lines of evidence obtained using different experimental models (with and without overexpression systems), and particularly those performed with untransformed cells, support a positive role for p21 in DNA repair. As already stated, the idea that p21 could play a role in DNA repair was first suggested by the evidence showing that p21 interacts with PCNA [10-17]. Since this binding results in competition and displacement of PCNA-interacting proteins thereby inhibiting DNA synthesis [14-16,125], it was proposed that p21 could inhibit DNA repair, in a similar way as it affects DNA replication *in vitro*. However, a number of direct interactions between p21 and specific factors participating in different processes of DNA repair have indicated that p21 may mediate the DNA damage response also at this level.

As described in the introductory section, there are different mechanisms of DNA repair which are essentially able to remove specific lesions, thereby restoring the correct genetic information. Given their peculiarity, the lines of evidence suggesting the participation of p21 in each process will be described individually.

### 3. p21 and Nucleotide Excision Repair (NER)

The first biochemical studies showed that high p21 levels could inhibit the NER process in a reconstituted *in vitro* system [126,127]. A similar effect was observed when purified p21 protein was introduced into cells by electroporation [128]. Other studies performed on p21-null murine fibroblasts, or on p21<sup>-/-</sup> HCT116 tumor cell line, reported that the NER process was not significantly affected in the absence of the protein, thus implying that p21 was not involved in NER [129-132].

In contrast with these findings, a careful *in vitro* analysis showed that a reconstituted NER reaction was insensitive to p21, given the non-processive DNA synthesis of NER [133,134]. In addition, early studies using ectopic expression of the protein showed that p21 did not inhibit NER [135,136]. 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 [135]. A positive role for p21 in NER, was also suggested by the co-localization and interaction of p21 with PCNA in actively repairing normal fibroblasts [137,138], and by increased DNA repair in cells treated with DNA-damaging drugs, after p21 overexpression [139]. Accordingly, deletion of p21 gene in primary human fibroblasts resulted in increased sensitivity to UV radiation, together with reduced DNA repair efficiency, namely in the global genome excision repair sub-pathway [140]. Overall, the discrepancy of these results may be attributed to the different experimental conditions in biochemical assays (e.g. low vs high concentrations of p21 in *in vitro* reactions), and to the different cell model systems utilized (e.g. tumor vs normal cells, murine vs human cells), that could have introduced biasing factors, such as reduced NER efficiency in tumor cells, and the reduced global genome repair pathway in rodent cells [141].

Results obtained more recently with *in vivo* systems, i.e. by investigating the behavior of a p21 protein tagged with Green Fluorescent Protein (GFP) in living cells challenged with DNA damaging radiation, have shed more light on the role of p21 in DNA repair. In fact, spatio-temporal analysis of p21-GFP autofluorescence by time-lapse microscopy showed that p21 protein was rapidly recruited to nuclear regions where a local DNA damage was induced with the micropore irradiation technique, or with a laser beam [142]. Interestingly, in experimental settings in which p21-GFP was co-expressed with PCNA tagged with Red Fluorescent Protein (RFP-PCNA), the dynamics of the process of p21-GFP recruitment was temporally similar to that of RFP-PCNA. In fact, the kinetics of p21-GFP accumulation at DNA damage sites was very rapid, and closely followed (though with a little delay) that of PCNA, suggesting that p21 was required at a later step after PCNA recruitment. Interestingly, the protein accumulation at DNA damage sites was found to be dependent on the previous recruitment of PCNA since a p21 mutant protein unable to interact with PCNA (p21<sup>PCNA<sup>-/-</sup></sup>) did not accumulate at sites of DNA damage [142]. In addition, the involvement of p21 was clearly related to the DNA repair process, since p21 recruitment did not occur in NER-deficient XPA fibroblasts [142]. Another important feature of p21 is that both endogenous p21 in normal fibroblasts, as well as ectopic p21 protein expressed in HeLa cells, were found to co-localize with NER factors interacting with PCNA (e.g. XPG, DNA polymerase  $\delta$ ,

and CAF-1), and to be present in complexes containing these NER factors. Finally, conditions inducing an increase in endogenous p21 protein, or its ectopic expression, did not result in inhibition of NER [142].

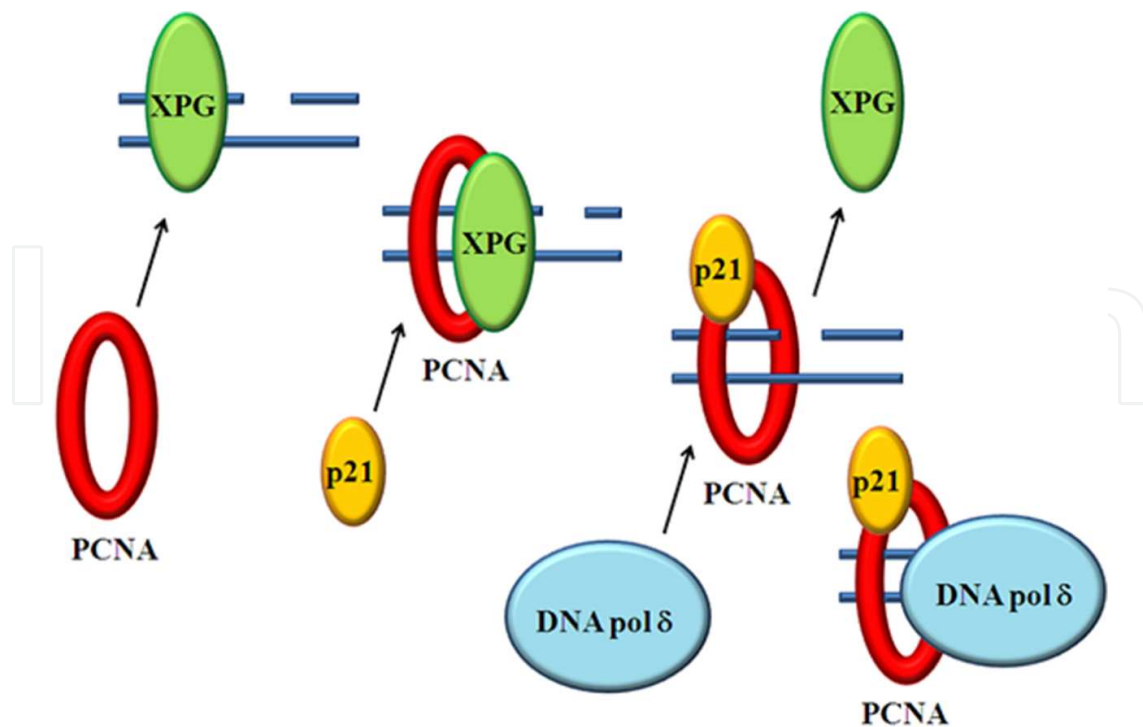
An independent confirmation that p21 does not affect NER, and that the protein co-localizes with NER factors, like XPB, has been recently obtained with a similar approach of micropore irradiation in U2OS cells expressing myc-tagged p21 protein [143]. Another study showed that the p21 recruitment after UV damage in human melanoma SK-MEL-1 and SK-MEL-2 cell lines occurred via translocation to the nucleus and interaction with PCNA, which was found to save p21 from degradation, and to enhance DNA repair [144].

A further step in clarifying what could be the role of p21 in DNA repair has been recently obtained by investigating common interactors of p21 and PCNA. One such protein was found to be p300, a transcriptional co-activator endowed with HAT activity [95]. This protein was suggested to have a role in DNA repair synthesis [145], probably acting as a p53-dependent regulator of chromatin accessibility to NER machinery [96]. p21 has been found to regulate HAT activity required during DNA repair, by dissociating the p300-PCNA interaction [95]. Since it was previously shown that PCNA inhibits both the HAT and transcriptional activity of p300 [97], it has been suggested that a function played by p21 in NER could be the removal of the inhibitory effect of PCNA on HAT activity [95]. Since p300 has been shown to acetylate a number of proteins involved in BER [5,95], our group has recently investigated whether also NER proteins are acetylated. The results have shown that XPG, the PCNA-interacting endonuclease involved in the incision step of NER, is indeed acetylated by p300, and that p21 regulates the interaction between XPG and p300 in a PCNA-dependent manner [146]. Interestingly, *in vitro* experiments have also shown that PCNA is able to inhibit the acetylation of XPG. Therefore, these results suggest that p21 may help in removing the inhibitory effect of PCNA on the acetylation of XPG. This function may serve to facilitate NER completion, since lack of XPG acetylation induced by knocking-down p300 expression and activity in human fibroblasts, has been found to result in the accumulation of the endonuclease at DNA damage sites [146]. Concomitantly, knock-down of p300/CBP expression, has been shown to significantly impair NER efficiency, suggesting that in addition to acetylate histone for chromatin accessibility, p300/CBP may also acetylate NER factors to facilitate DNA repair.

Taken together, these lines of evidence indicate that p21 accumulates at sites of DNA damage similarly to DNA repair factors [147], and suggest a regulatory role in NER based on p21 ability to control, perhaps both spatially and temporally, the interaction of repair factors with PCNA (Figure 2).

#### 4. p21 and Base Excision Repair (BER)

Further pieces of evidence suggesting that p21 is involved in other DNA repair pathways by regulating PCNA interacting proteins, were obtained by investigating the effect of p21 in the BER process. *In vitro* experiments showed that p21 inhibited PCNA-directed stimulation of



**Figure 2.** Schematic representation of interplay between PCNA, p21 and PCNA-interacting proteins, during NER. In this example, XPG endonuclease is shown. From left to right, are depicted the steps of the binding of PCNA to XPG, followed by the arrival of p21, which then displaces XPG from PCNA, to leave space for binding of the next partner, i.e. DNA polymerase  $\delta$ .

DNA polymerase  $\delta$  long-patch BER, but not in the presence of AP endonuclease 1, indicating a regulatory role of p21 in BER [148]. The requirement of p21 in BER is further supported by several findings: first, a direct physical association between p21 and poly(ADP-ribose) polymerase 1 (PARP-1), another important player in BER, was described. In particular, p21 was shown to compete with PARP-1 for binding to PCNA *in vitro*, and an association between p21 and PARP-1 was also found in normal fibroblasts treated with alkylating agents [149]. In addition, both PCNA and p21 were found to inhibit the ADP-ribosylating activity of PARP-1 [149]. We recently observed that p21-null human fibroblasts were more sensitive to DNA damage, and deficient in DNA repair induced by alkylating agents [150]. These results prompted us to investigate whether p21 might regulate the interaction of BER factors with PARP-1. The recruitment of PARP-1 and PCNA to damaged DNA was found to occur to a greater extent in p21<sup>-/-</sup> fibroblasts than in p21<sup>+/+</sup> parental cells. The PARP-1 accumulation in p21<sup>-/-</sup> cells was also accompanied by a higher activity of PARP-1, concomitantly with a persistent interaction of PARP-1 with BER factors, such as XRCC1 and DNA polymerase  $\beta$  [150]. Since an excess of PARP-1 antagonizes the activity of DNA polymerase  $\beta$ , these results suggest that prolonged association of PARP-1 with BER factors reduced the DNA repair efficiency observed in p21<sup>-/-</sup> fibroblasts [150]. These results indicate that p21 regulates the interaction between PARP-1 and BER factors, to promote efficient DNA repair.



## 5. p21 and Double-Strand Breaks Repair (DSBR)

Most of the evidence that p21 is rapidly accumulated at sites of DNA damage, have been obtained with UV-C irradiation, a typical means that primarily activates the NER pathway. However, p21 has been shown to behave in a similar way also in cells which have sustained other types of DNA lesions that are removed through different DNA repair pathways. Interestingly, the irradiation of normal human fibroblasts with heavy-ions inducing single (SSB) and double DNA strand breaks (DSB), stimulated the recruitment of p21 to sites of energy deposition [151]. Co-localization of p21 with proteins involved in double-strand break repair (i.e. Mre11, Rad50 and PCNA) was observed in these cells [151], thus lending further support to the accumulation of p21 at sites of DNA damage. This process has been shown to occur independently of p53 and core NHEJ factors (such as Ku70, Ku80, and DNA PKcs) [152]. In addition, after exposure to X-rays, recruitment of p21 was found to occur at foci spatially distinct from those containing histone  $\gamma$ -H2AX and 53BP1, suggesting no relation with DSB repair [153]. This result was explained by the production of differently types of DNA lesions, according to the energy source employed. However, p21 recruitment occurred depending on its ability to bind PCNA [153]. Since results have shown that PCNA is required for initiation of recombination-associated DNA synthesis [154], it is thus likely that the role of p21 is related to this step of DSB repair.

## 6. p21 and Translesion DNA Synthesis (TLS)

The translesion DNA synthesis (TLS) is a process taking place at arrested replication forks in a PCNA-dependent manner, and that allows the bypass of the lesion by a mechanism of DNA polymerase switch. In this process, which actually it is not a repair reaction, the high fidelity replicative DNA polymerase is replaced by a low-fidelity enzyme able to synthesize DNA past a lesion [155,156]. Independent researches investigating the mechanisms controlling this reaction obtained results indicating the participation of p21 also in this process. In particular, it was suggested that p21 was required to limit the level of mutations arising from the error-prone lesion bypass; interestingly, the interaction with PCNA was shown to be important for the regulatory role of p21 in TLS [157]. This function of p21 has been suggested to control the loading of DNA polymerase  $\eta$  on PCNA, thereby contributing to limit TLS activity and the associated mutagenesis effect [143,158]. In addition, p21 was shown to modulate the level of PCNA ubiquitination occurring during TLS. Impaired PCNA ubiquitination was observed when p21 was knocked-down by RNA interference [157], but also when a nondegradable form of p21 was expressed [159]. These apparently opposite results may be explained by the different experimental approach and model system, yet they indicate that p21 protein must be finely regulated in order to fulfill its functions in the DNA damage response.



## 7. Proteasomal degradation of p21 protein

The most important post-translational modification of p21, i.e. ubiquitination, induces its proteasomal degradation [160]. However, both ubiquitin-dependent and -independent mechanisms have been reported [53,161,162]. The ubiquitin-dependent mechanisms have been described to occur via different E3 ubiquitin ligases, namely SCF<sup>Skp2</sup>, APC/C<sup>Cdc20</sup> and CRL<sup>Cdt2</sup>, both in basal conditions (e.g. in S phase) [53,163,164], and after DNA damage induced by UV or ionizing radiation [165-167]. An ubiquitin-independent degradation of p21 has been shown to be mediated by direct association with the C8 $\alpha$ -subunit of the proteasome complex [168], or with MDM2, yet independently of its E3 ligase activity [169,170]. Degradation via the C8 $\alpha$ -subunit was protected by the interaction with PCNA [168,171]. In contrast, CRL4<sup>Cdt2</sup>-mediated (ubiquitin-dependent) degradation of p21 required the interaction with PCNA [165,166]. The relative role of these different mechanisms is not fully understood, especially in S phase [172]. To complicate these findings, p21 degradation may be dependent on the different cell model systems investigated (p21 degradation was more pronounced in transformed cell lines) [167], as well as on the overexpression system that may result in reduced degradation [167,171,173].

It was suggested that p21 destruction was required for efficient DNA repair, implying an adverse effect, in particular on the NER process [174]. However, as previously discussed, other studies have shown that p21 does not inhibit NER [142,143,173], and that p21 is required for efficient NER in normal untransformed cells [95,140]. More recently, it has been shown that degradation of p21 after DNA damage is triggered by the extent of DNA damage rather than the type of lesion, and is not required for DNA repair, in normal human fibroblasts [173]. In fact, it has been shown that by inhibiting p21 degradation with caffeine (obtained through inhibition of ATM activity [174]), the NER efficiency was not significantly reduced [174]. In agreement with these findings, a recent report showed that inhibition of p21 degradation by deletion of CUL4A (a component of the CRL4 ubiquitin ligase complex with DDB1 and DDB2), resulted in NER stimulation [175]. These lines of evidence, while indicating that p21 degradation occurs after DNA damage, still do not clarify the actual role of the process in the context of DNA repair. In fact, p21 degradation appears to be a phenomenon independent of DNA repair, since it occurs also in NER-deficient fibroblasts [176].

## 8. p21 degradation, DDB2 and DNA repair

Although there is no doubt that p21 is degraded after DNA damage, several aspects of this process suggest that it is not a pre-requisite for DNA repair, but it may be related to a more general response to DNA damage. A particular consideration to be made is that another important protein involved in NER, i.e. the UV-induced DNA damage binding protein 2 (DDB2) has been indicated as an important mediator of the cell fate following DNA damage [177]. DDB2 protein is mutated in Xeroderma pigmentosum group E patients, and cells derived from these individuals show a partial deficiency in NER [178]. DDB2 protein exhibits a

high affinity for damaged DNA and mediates binding of the CUL4A-DDB1 complex to target histone H2A ubiquitination in chromatin [179]. In addition, DDB2-DDB1-CUL4A complex ubiquitinates p21 for proteasomal degradation [165,166]. Deletion of DDB2 in mice (*DDB2*<sup>-/-</sup> cells), similarly to that of CUL4A, results in accumulation of p21 protein; however, it was also suggested that NER was restored when deleting concomitantly CDKN1A gene (*DDB2*<sup>-/-</sup> *p21*<sup>-/-</sup>) [180]. This result was again taken as the indication that p21 must be degraded for optimal DNA repair. However, it must be noted that absence of p21 resulted in an increased cell entry into S-phase [175], thus confounding the type of DNA synthesis (i.e. replicative vs repair) observed [180]. It is also worth noting that in most studies investigating p21 degradation, cells were exposed to irradiation conditions inducing extensive DNA damage [165,166,170,174]. In contrast, cell exposure to sub-lethal DNA damaging conditions, does not lead to evident p21 degradation [142,173,181]. Since p21 is also involved in the regulation of the apoptotic process, it appears evident that p21 accumulation may inhibit apoptosis. Thus, p21 degradation after extensive DNA damage may be more considered a pro-apoptotic response rather than a pre-requisite for DNA repair [5]. In fact, DDB2-deficient cells have been shown to be apoptosis-resistant [177], and to be significantly impaired in undergoing premature senescence [182]. Accordingly, p21 degradation, as stimulated after DNA damage by E3 ligases associated with MKRN1 or DDB2, has been shown to facilitate the apoptotic cell death pathway, as opposed to the cell cycle arrest and senescence [176,183,184]. Overall, these lines of evidence seem to suggest that p21 degradation is indeed induced to avoid inhibition of the apoptotic process when cells have accumulated an irreparable extent of DNA damage. In contrast, when the amount of DNA lesions are low enough to be worth attempting to repair them, p21 is not degraded and may help in DNA repair [5].

## 9. Future directions

The involvement of p21 in DNA repair processes is linked to its ability to bind PCNA which is a central hub for the majority of the factors participating in these processes. Due to its peculiar ability to displace PCNA-interacting proteins, it is likely that p21 may play a regulatory role in orchestrating the PCNA interactions. A clear example of this function is the p21 regulation of the interaction between p300 and PCNA, which has been shown to inhibit the acetyl transferase activity. The influence of p21 is useful for histone acetylation, and for chromatin remodeling function of p300 in DNA repair [95,185]. However, since also DNA repair factors are acetylated by p300/CBP [5,186], the role of p21 in this context could be to remove the inhibition exerted by PCNA. This function is important for DNA repair regulation, and the inability to perform this job is likely to impair DNA repair. In fact, in p21-null human fibroblasts the NER factor XPG (the endonuclease involved in lesion incision) accumulates at the sites of DNA damage, in a manner similar to that observed after knock-down of p300/CBP activity [146]. These results support a regulatory role by which p21 may influence XPG acetylation and consequently its retention on chromatin. Studies are under way to establish the link between XPG acetylation and NER efficiency; however, it is clear that in the absence of p21, as well as after silencing of p300/CBP, DNA repair is inefficient [140,146].

If p21 plays a regulatory role in DNA repair, how this function may be related/coupled to p21 degradation? One possibility is that p21 could be degraded after execution of its function, in order to avoid the persistence of the PCNA/p21 complex onto DNA. Prolonging the DNA residence time of this complex may be detrimental to the genome, since additional unwanted reactions might occur under these circumstances. This hypothesis is supported by findings showing that p21 has been found to co-localize with, and participate in protein complexes containing factors such as XPG, DNA polymerase  $\delta$  and CAF-1 [142], all of which are known to interact with PCNA. Therefore, coupling DNA repair with protein degradation could fulfil this function. This speculation needs a formal proof, since some DNA repair factors are ubiquitinated, while others are not. Thus, this hypothesis requires appropriated future experimentation on the effects of p21 ubiquitination on DNA repair synthesis.

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