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# The Biology of p21$^{Waf1/Cip1}$ - Review Paper

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**Abstract:** p21$^{WAF1/Cip1}$ belongs to the Cip/Kip family of cyclin kinase inhibitors (CKI) (p21$^{Waf1/Cip1}$, p27$^{Kip1}$, p57$^{Kip1}$). p21$^{Waf1/Cip1}$ was first described as a potent and universal inhibitor of cyclin-dependent kinases (Cdks). Two forms of functionally active p21 has been localized and described: a nuclear and a cytoplasmic forms. In this paper the structures of p21 has been described and as well as it functional biology in terms of differentiation, proliferation and apoptotic activities. The upregulation of p21 by p53-dependent and p53-independent mechanisms is also described.

**Key words:** p21$^{Waf1/Cip1}$, p53, cytoplasmic, nuclear, apoptosis, differentiation, proliferation

## INTRODUCTION

p21$^{WAF1/Cip1}$ belongs to the Cip/Kip family of cyclin kinase inhibitors (CKI) (p21$^{Waf1/Cip1}$, p27$^{Kip1}$, p57$^{Kip1}$). p21$^{Waf1/Cip1}$ was first described as a potent and universal inhibitor of cyclin-dependent kinases (Cdks)[1]. p21 functions as a checkpoint in the cell cycle by inhibiting cdks at the G1/S and G2/M interfaces[2]. p21 has been shown to bind to cyclin-cdk complexes, preventing phosphorylation of the retinoblastoma protein[3]. When this happens the E2F pathway is blocked and the cell cycle is arrested at the G1/S interface[4].

Variation in the p21 levels has been shown in epithelial cells in several lung diseases[5], upon cell-matrix disruption[6] and to be influenced by several integrins[7].

p21 expression is usually controlled at the transcriptional level by either the p53-dependent or -independent pathway[8], but p21 expression can also be regulated at the post-transcriptional level. Short wavelength UVC was found to induce p21 in a p53-dependent fashion by post-transcriptional modifications that led to enhanced stability of p21 mRNA[9]. The transcription factors C/EBPα and C/EBPβ were found to interact with p21 protein and protect it from proteolytic degradation[10]. In a number of other cell lines, post-transcriptional events strongly influence p21 expression following genotoxic stress[11]. Stress-activated kinases p38α and JNK 1 were shown to stabilise p21 by phosphorylation at serine 130[12].

p21 was shown to promote apoptosis[13], protect cells from undergoing apoptosis[14], inhibit differentiation[15] and promote differentiation.

**Structure and protein interactions of p21$^{Waf1/Cip1}$:** Cip/Kip family (p21$^{Waf1/Cip1}$, p27$^{Kip1}$, p57$^{Kip2}$ share significant sequence homology in their amino-terminal. The amino-terminal domain of p21, like the corresponding domains of p27 or p57, is both necessary and sufficient to inhibit cyclin/CDK activity. The unique carboxy-terminal domain of p21$^{Waf1/Cip1}$ associated with the proliferating nuclear antigen (PCNA), a subunit of DNA polymerase δ and can inhibit DNA replication directly, without affecting DNA repair[16].

p21$^{Waf1/Cip1}$ was identified as a mediator of p53-induced growth arrest[17] and a direct regulator of CDK activity[18]. p21$^{Waf1/Cip1}$ plays a critical role in the negative control of cell growth and is generally up-regulated in cell arrest either by cell contact, serum deprivation, differentiation or senescence[4].

The human p21 gene consists of 3 exons (68, 450 and 1600 bp) but the first exon is not translated. The human p21 protein is 164 amino acids with a molecular mass of 21 kDa and has been conserved during evolution[19]. p21$^{Waf1/Cip1}$ interacts directly with cyclins through a conserved region close to the N-terminus (Cyc 1), however it has a second weak cyclin binding near its C-terminus region (Cyc 2), which overlaps with its PCNA binding region domain. Moreover, p21 has a separate cyclin-dependant kinase domain (Cdk) binding site in its N-terminus region[20]. For optimal cyclin-Cdk inhibition, the binding to this site as well as one of the cyclins is required. p21 was shown to compete with p107 and p130 for binding to cyclin/Cdks and to disrupt already formed complexes among these molecules[21].

p21 was found to cause repression of the E2F-dependent transcription possibly by direct association with E2F factor[22]. This means that E2F could function as an anchor of p21, bringing it in juxtaposition with an E2F-dependent transcription initiation complex, thereby inhibiting its function[4].

p21 can bind to the N-terminus of c-Myc, to interfere with the c-Myc-Max association, but at the same time, the interaction between c-Myc and p21 can directly counteract p21-dependent inhibition of DNA synthesis, as c-Myc binds to the C-terminus of p21 in competition with PCNA[23]

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p21 can enhance the function of transcription co-activators such as p300[24]. The ability of p300 to interact with NF□B-dependent transcription is negatively controlled by association of p300 with active cyclin/CDK complexes. Thus the inhibition of cyclin/CDK activity could be a way of explaining the capability of p21 to activate p300-dependent transcription. It was shown that stimulation of p300 activity could occur by p21 independently of the intrinsic histone acetyltransferase activity of this co-activator and its cyclin/CDK binding region. This occurs because p21 can relieve the effects of a previously uncharacterised transcription repression domain present in p300[24].

The E7 protein of human papilloma virus 16 (HPV-16) binds to p21 and competes with PCNA for binding to the carboxy-terminus domain of p21[25,26]. The association of E7 to p21, blocks the ability of p21 to inhibit cyclin/CDK activity as well as PCNA-dependent DNA synthesis. E7 does not disrupt the association of p21 with cyclin/CDK complexes, but it is thought to relieve p21-dependent suppression of CDK activity[27].

Two DNA metabolising enzymes Fen 1 (Fap endonuclease I) and DNA-(cytosine-5) methytransferase (DNA MTase) bind to p21 in competition with PCNA. PCNA interacts physically with Fen I and stimulates its enzymatic activity. The central residues of the PCNA binding domain of p21 are the most highly conserved with Fen 1 and likely to mediate their mutually exclusive binding to PCNA[28]. DNA MTase was shown to bind directly to PCNA and the PCNA-DNA MTase association was disrupted by p21. Newly replicated DNA must be methylated before histone H1 is incorporated into the nucleosome. This suggests that p21 can control DNA methylation levels during replication and possibly during DNA repair[29].

In addition to DNA metabolising enzymes, GADD45 is also involved in p21-PCNA interactions. GADD45, like p21 is a nuclear protein and has been implicated in induction of growth arrest, apoptosis, excision repair and DNA stability[30]. Although GADD45 interacts with PCNA through a region distinct from that recognised by p21, both p21 and GADD45 compete with each other to bind PCNA[31]. GADD45 and its related homologue MyD118, can directly associate with p21[32]. GADD45 does not appear to inhibit cyclin-Cdk complexes either by itself or in combination of p21[33].

p21 binds directly to procaspase 3 at its N-terminus and is involved in death induction and suppression. p21 also bind to SAPK at its N-terminus and was found to block its phosphorylation and also the activation by the upstream MKK4 kinase[34].

p21 was shown to interact with other regulatory proteins, such as protein kinase, CK2[35], calmodulin (CaM)[36], a novel cell regulatory protein with oncogenic potential, SET[37] and the transcription factor C/EBP-α[38].

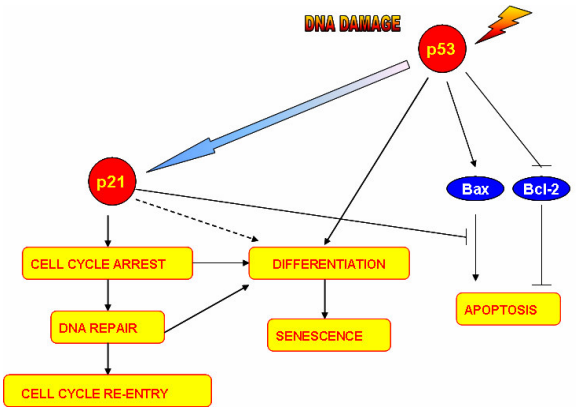


Fig. 1: p21 regulation via the p53 dependent pathway. p53 induction may lead cells down the apoptotic pathway, by upregulating Bax and downregulating a survival factor, Bcl-2. Transcriptionally induced p21 by p53 may prevent this and instead lead to cell cycle arrest. Adapted from[50]

The cell-cycle inhibitory activity of $p21^{Cip1/Waf1}$ is associated with its nuclear localisation. However a 10-amino acid truncation from the C-terminal form of p21 was reported in UV-irradiated normal diploid fibroblasts and many tumour cells mainly localised in the cytoplasm[39].

A physical association between cytoplasmic p21 and ASK1 has been described and was found to suppress the activity of ASK 1 and MAPK (SAPK/JNK) cascade activation thus preventing the cell from undergoing apoptosis[40]. It has been suggested that cytoplasmic p21 is formed by cleavage or truncation during apoptosis[41]. Other forms of cytoplasmic p21 have been described but their roles are still unclear[42].

**p53-dependent induction of p21 transcription:** The p53 tumour suppressor protein is an inducible transcription factor required for the transactivation of a number of genes involved in cell cycle control[43]. p21 expression is normal in embryos and most tissues from p53 knockout (p53 -/-) mice[44].

Although p53 is not required for p21 transcription, the regulation of p21 is p53-dependent following DNA damage by γ-radiation[44]. Cultured p21-deficient mouse embryonic fibroblasts still had the ability to undergo G1 arrest in response to DNA damage, thus p53 may induce an additional gene that participates in cell arrest[45]. p21 transcription and cell cycle arrest was observed in irradiated human cell lines[46].

The expression of p21 was found to increase via the p53-dependent pathway in murine embryonic fibroblasts following treatment with the spindle disrupter nocodazole[47]. Ribonucleotide biosynthesis inhibitors such as pyrazofurin and cyclopentenylcytosine were shown to induce p53-

dependent p21 expression in the absence of DNA damage and resulted in hypophosphorylation of retinoblastoma in normal human fibroblasts and cell growth arrest[48].

Transcriptional induction of p21 by p53 may prevent cells undergoing apoptosis and instead lead to cell-cycle arrest[49]. A summary of the induction of p21 via the p53-dependent pathway and control of apoptosis via p21 is shown in Fig. 1.

**p53-independent regulation of p21 expression:** A number of agents activate p21 transcription independent of the p53 pathway. These agents induce binding of different transcription factors to specific cis-acting elements located within the p21 promoter . The region between -119 bp and start of transcription of the human p21 gene contains six Sp1 binding sites (Sp1-1 to Sp1-6). Sp1 is a member of a multigene family that binds to DNA through C-terminal zinc-finger motifs. Sp2, Sp3 and Sp4 share extensive structural and sequence homology with Sp1[51].

Phorbol ester (PMA) and okadaic acid induce p21 through Sp1[51]. The tumour suppressor protein BRCA1 activates p21 via the region from -143 to -93 bp which contains the Sp1-1 and Sp1-2 sites and inhibits DNA synthesis[52]. Transforming growth factor-β (TGF-β)[53], calcium[54], butyrate[55], lovastatin[56], histone deacetylase inhibitor Trichostatin A (TSA)[55] and nerve growth factor (NGF)[57] has been shown to induce p21 via the Sp1-3 site in the p21 promoter. TGF-β and butyrate inhibited proliferation and induced G1 cell cycle arrest in various cells[53], calcium induced differentiation of cultured mouse keratinocytes[54] whereas lovastatin induced cell cycle arrest in p53-null human prostate carcinoma cells[56].

The addition of nerve growth factor (NGF) to PC12 cells induced p21 expression and differentiation through Sp1, Sp3 sites and p300 transcriptional co-activator[58]. Progesterone was found to increase the level of p21 and promote growth arrest. The progesterone receptor (PR) was found complexed with p300 and Sp1[59]. A variety of other transcription factors such as AP2[60], E2F[61], STATs[62] and C/EBPα[38] can induce p21 transcription in response to different signals.

**p21 and differentiation:** Exit from the cell cycle is a prerequisite for terminal differentiation and p21 expression is induced during terminal differentiation both *in vitro* and *in vivo*[63,64]. p21 expression promotes differentiation in megakaryoblast leukemia cell line (CMK)[65], in megakaryocytes cells ($CD34^+$)[66], in myelomonocytic cell line (U937)[67], in chondrosarcoma cells (SW1353)[68], in dendritic cells and macrophages from human peripheral blood monocytes[69], in skeletal muscles[70], in mice lungs[70], in peripheral nervous system neurons in response to nerve growth factor mediated by p300 protein[57], in myotube differentiation from myoblasts under the influence of the transcription factor MyoD[63] and in laryngeal tumours[71]. Interestingly, p21 was also shown to inhibit differentiation in terminally differentiated of mouse keratinocytes[72] but not those of humans[15] and decrease differentiation in human colon cancer cell line HT29[73]. The p21 was found not to be involved in the regulation of differentiation of the mouse skin tumours[74] and in mouse keratinocytes[75].

In the p21 null mice, normal differentiation has been observed thus implying that p21 is not a mutually exclusive agent that promotes differentiation[76]. Various other genes are thought to be involved and maybe cooperate with p21 to regulate differentiation including p15[76], p16[77], p18[76], p19[76], p27[77], p53[78], p57[79] and Rb[78].

**p21 and proliferation:** p21 is usually assumed to inhibit proliferation both *in vitro* and *in vivo*[80] and by the introduction of p21 expression constructs into normal[11] and tumour cell lines[19] resulted in cell cycle arrest at the G1 phase of the cell cycle[81]. Paradoxically, p21 has been shown to promote proliferation under certain circumstances[82].

Various mechanisms have been described by which p21 can regulate proliferation[83]. p21 may induce growth arrest by inhibiting the activity of cyclin-dependent kinases (Cdks) or of proliferating cell nuclear antigen (PCNA)[84]. The unique carboxy-terminal domain of p21 can associate with PCNA and DNA polymerase δ and ε and can inhibit DNA replication directly, without affecting DNA repair[20]. p21 may act as an assembly factor for Cdk/cyclin complexes. p21 was found to promotes the assembly of Cdk4/6 and cyclin D *in vitro* and was found associated with cyclinD/Cdk4 complexes during cell-cycle progression[85]. p21 was also shown to complex with cdk2 and thus lead to growth arrest[86]. The role of p21 as an assembly activator or inhibitor depends on its expression level. At low and intermediate concentrations it is an assembly factor, while at high concentrations it is an inhibitor[87].

p21 has been shown to be part of a quaternary complex that contains cyclin, Cdk and PCNA[88]. The transition between active and inactive states occurs through changes in p21 stoichiometry, particularly when multiple p21 molecules versus a single p21 molecule bind to these complexes[81].

The role of p21 to either promote or inhibit proliferation could depend on the specific cellular context[89]. Mammary tumour-susceptible MMTV-ras mice displayed higher S-phase fractions and an increase in tumours in p21 knockout mice, whereas MMTV-c-Myc exhibited lower S-phase and a decrease in tumours[89]. Summary of the regulation of proliferation by p21 is shown in Fig. 2.

**p21 and apoptosis:** p21 can both promote and inhibit apoptosis although it generally counteracts the

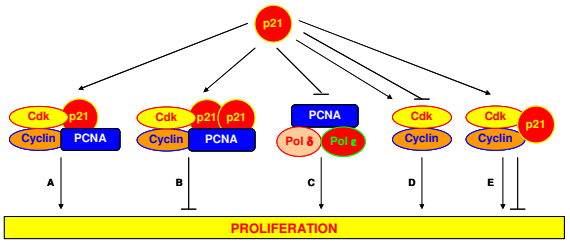


Fig. 2: Regulation of proliferation by p21. (A) p21 can form a complex with Cdk, Cyclin and PCNA. (B) At low or intermediate conc. of p21 it promotes proliferation while at high p21 conc. the complex is inactive thus inhibiting proliferation. (C) p21 can inhibit the activity of PCNA complex with DNA polymerase δ and ε and (D) Cdk/Cyclin complex or act as an assembly factor for Cdk/Cyclin complexes. (E) p21 can also associate with the Cdk/Cyclin complexes and can inhibit or promote proliferation

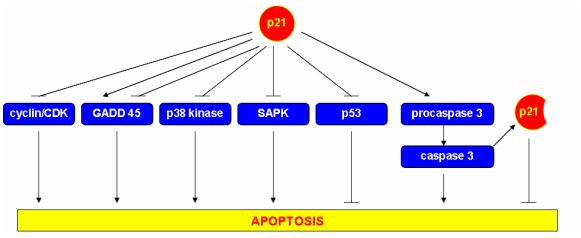


Fig. 3: Regulation of apoptosis by p21. p21 can inhibit apoptosis by complexing with cyclin/CDK, Gadd45, p38 kinase, SAPK and can inhibit the p53-dependent apoptosis pathway. p21 can also regulate Gadd45 and procaspase 3 leading to apoptosis. Caspase 3 can truncate nuclear p21 at the C-terminal thus losing the nuclear localisation signal and is located in the cytoplasm, whereby it cannot suppress growth arrest as well as apoptosis

apoptotic process[4]. p53-dependent apoptosis occurs normally in cells from p21 knockout mice[90]. Expression of p21 appears to protect colorectal carcinoma and melanoma cells from p53-induced apoptosis[91], while suppression of p21 expression by anti-sense technology and homologous recombinations was shown to shift cells from the cell-cycle arrest pathway to apoptosis[49]. While the majority of evidence supports a role of p21 in protection against apoptosis, over-expression of p21 has been associated with the induction of apoptosis in human retinoblastoma cells lines[92] and T-cells[93].

Cells from p21 knockout mice were shown to contain very high levels of apoptosis after γ-irradiation[94]. These findings might suggest that p21 protein normally protects cells from undergoing p53-mediated apoptosis by holding them in cell-cycle arrest[95] (Fig. 2).

Activation of the MAPKs of the SAPK (JNK) and p38 kinase families are also key events in the apoptotic response of many cells and p21 has been found to associate and control both types of molecules[12]. p21 was also found to form complexes with caspase 3[41] and MEKKs (ASK1)[40]. The association with these molecules could be favoured by the fact that p21 itself is a caspase substrate and becomes localised to the cytoplasm as a consequence of caspase-dependent cleavage of its nuclear localisation C-terminus domain and is unable to suppress growth as well as apoptosis[13]. This truncation would also compromise the ability of p21 to promote cyclin/CDK nuclear localisation, with the same biological end point effect[14].

p21 can also protect cells from undergoing apoptosis due to the cyclin/CDK inhibition[16]. Direct interaction between p21 and GADD45 is thought to promote apoptosis rather than cell cycle arrest[30].

Two novel proteins, p21B and p21C, which are variants of p21 were found to be induced by DNA damage, p53 and p73. p21B have two unique exons and encodes a protein that is not homologous to p21 or any other known protein. p21B was found to induce cell cycle arrest and apoptosis. p21C uses an extended version of the p21B exon I, but is spliced to the second and third exons of p21 and encodes the p21 protein[96]. Summary of the regulation of apoptosis by p21 is shown in Fig. 3.

## CONCLUSION

p21 can be localised in both nuclear and cytoplasic regions and yet have different functional roles. It is not easy to describe the exact function of p21 since of its vast array of regulatory pathways and interactions. Although traditionally p21 was believe to be localised only in the nucleus, cytoplasmic p21 seems to be an interesting missing link and need to be studied further in order to fully understand the full biological significance of p21.

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