THE ROLE OF p21 IN G2 CELL CYCLE ARREST IN RESPONSE TO DNA DAMAGE

by

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Abstract

Cell cycle arrest is a critical defense tactic in response to DNA damage as it allows time for DNA repair or induction of apoptosis. When a cell cannot efficiently induce cell cycle arrest, the result is the inappropriate progression through the cell cycle and the accumulation of mutated or damaged DNA. The p53-activated gene $p21^{Cip1/WAF1}$ is the principal mediator of cell cycle arrest in response to DNA damage. Cancer cells unable to mediate cell cycle arrest in response to DNA damage (cells lacking functional p53 or p21) are more sensitive to cell death and thus have a heightened sensitivity to DNA-damaging cancer therapeutics (i.e.: chemotherapies and radiation).

p21, a cyclin-dependent kinase inhibitor, has long been demonstrated to play a critical role in G1 cell cycle arrest in response to DNA damage through its interaction with G1-associated cyclin/Cdk complexes. Work described in this thesis suggests that p21 may also play a significant role in G2 cell cycle arrest. Experiments demonstrate that in response to DNA damage G2 cell cycle arrest is aided by the p21-mediated degradation of the G2-associated cyclin, cyclin B1. Cells lacking or impaired in the ability to upregulate p21 in response to DNA damage are unable to mediate the degradation of cyclin B1, cannot sustain G2 cell cycle arrest and eventually succumb to cell death. Cell death primarily occurs through mitotic catastrophe resulting from aberrant entry into M phase in the presence of DNA damage. Furthermore, preliminary experiments suggest p21 may play a direct role in mediating cyclin B1 degradation. In conclusion, these results suggest p21-mediated cyclin B1 degradation is necessary to sustain G2 cell cycle arrest and protect cells from death in response to DNA damage.

List of Abbreviations and Symbols Used

µg	Micrograms
µM	Micromolar
ATM	Ataxia Telangiectasia Mutated Gene
ATP	Adenosine Triphosphate
ATR	ATM and RAD3-related
BSA	Bovine Serum Albumin
Cdc	Cell Division Cycle
Cdk	Cyclin-dependent Kinase Inhibitor
CdkI	Cyclin-dependent Kinase Inhibitor
Cip/Kip	Cell Cycle Inhibitor
CO_2	Carbon Dioxide
C-terminus	Carboxy-terminus
Cy1	Cyclin Binding Region 1
Cy2	Cyclin Binding Region
DDR	DNA Damage Response
DMEM	Dulbecco's Modified Eagle's Medium
DNA	Deoxyribonucleic Acid
DNA-PK	DNA-dependent Protein Kinase
E2F	Group of Transcription Factor Genes
ECL Plus®	Enhanced Chemiluminescence
EDTA	Ethylenediaminetetraacetic Acid
Etp	Etoposide
FACS	Florescence Associated Cell Sorting
GADD45	Growth Arrest and DNA Damage 45 Gene
GAPDH	Glyceraldehyde-3-phosphate Dehyodrogenase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIFBS	Heat Inactivated Fetal Bovine Serum
HU	Hydroxyurea
IP	Immunoprecipitation

IR	Ionizing Radiation
K site	Cdk Binding Region
MC	Mitotic Catastrophe
MDM2	Murine Double Minute 2
MPF	Maturation/M-phase-Promoting Factor
N-terminus	Amino-terminus
NaCl	Sodium Chloride
NaF	Sodium Fluoride
Na_3VO_4	Sodium Orthovanadate
NLS	Nuclear Localization Signal
PBS	Phosphate Buffered Saline
PBST	Phosphate Buffered Saline 0.5% Tween 20
PCNA	Proliferating Cell Nuclear Antigen
PI	Propidium Iodide
PIC	Protease Inhibitor Cocktail
PUMA	p53 Upregulated Modulator of Apoptosis
PVDF	Polyvinyldifluoride
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
Rb	Retinoblastoma
Ros	Roscovitine
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis
siRNA	Small Interfering Ribonucleic Acid
UV	Ultraviolet Radiation

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Chapter 1: Introduction

1.1. Mammalian DNA Damage Response (DDR) and Cancer

Maintenance of the human genome is paramount to the controlled and productive life of a cell. The genome is under constant stress applied by both endogenous and exogenous sources. To protect against these stresses cells have evolved a number of complex mechanisms to ensure the integrity of their genomic material. One such mechanism is the mammalian DNA damage response (DDR). The DDR is a series of protein signaling pathways that act to detect and eliminate damaged or mutated DNA (Figure 1.1). The DDR either directs the repair of the damaged DNA or eliminates the damaged DNA through the induction of cell death.

The DDR is critical to cellular defence against carcinogenesis. In fact, in most established cancers the DDR pathway is severely compromised by either the downregulation or loss of essential signaling proteins.[1-4] The DDR is an important factor to consider not only when examining cancer prevention but also cancer treatment. In normal cells the DDR can be indirectly triggered through the inappropriate activation of oncogenes that occurs during carcinogenesis. In most cases oncogene activation causes an increase in the rate of cell proliferation. At this increased rate of cell division the cell is more prone to the development of "mistakes" or mutations as the genome replicates. These mutations are recognized as DNA damage and activate the DDR.

As mentioned above, most cancer cells have some impairment in the DDR pathway.[1-4] This impairment is exploited by conventional cancer therapies (i.e.: chemotherapy and irradiation), which act by directly or indirectly causing breaks or mutations within the genomic DNA (Figure 1.2). These treatments are regarded as cancer cell selective. For example, normal cells with an intact DDR are able to repair the DNA damage caused by the cancer treatments and continue to grow normally. Conversely, cancer cells with an impaired DDR become overwhelmed with DNA damage and are unable to sustain cell survival,

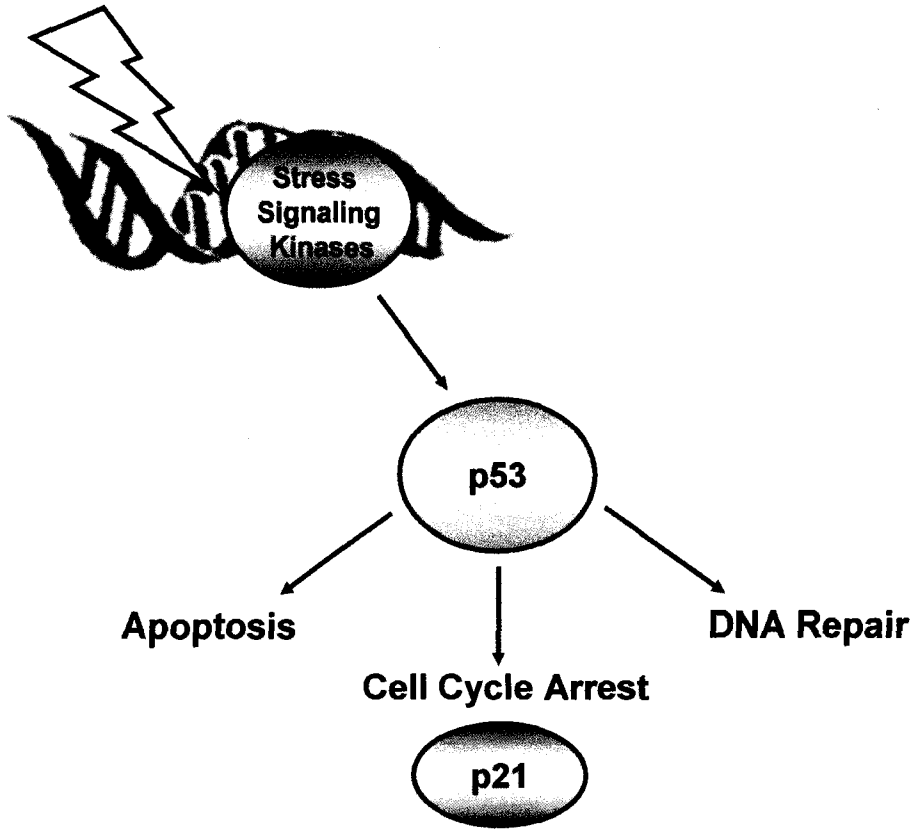


Figure 1.1. Mammalian DNA Damage Response. DNA damage is sensed by DNA damage/stress sensing proteins/kinases. These kinases/proteins activate a signaling cascade that results in the phosphorylation/activation of the tumour suppressor protein p53. p53 can then act to transcriptionally upregulate a variety of effector genes involved in cell cycle arrest, DNA repair or apoptosis. These genes act to repair the damaged DNA or eliminate the cell containing the damaged DNA through induction of cell death. The key mediator of p53-mediated cell cycle arrest in response to DNA damage is the cyclin-dependent kinase inhibitor p21.

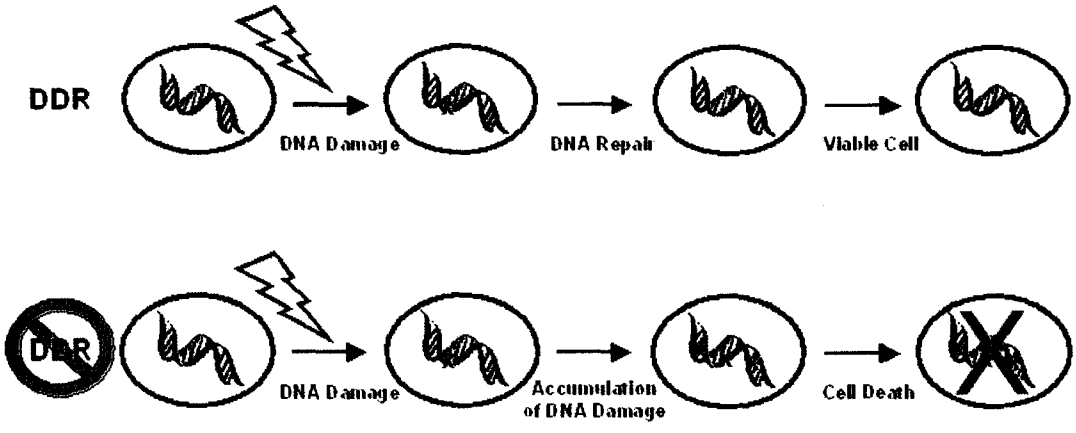


Figure 1.2. DNA-Damaging Cancer Therapeutics. Conventional cancer treatments (i.e.: chemotherapeutics and irradiation) exploit the fact that most cancer cells have an impaired DDR. Normal cells within the body have a functional DDR and are able to repair DNA damage caused by genotoxic therapeutic agents. In contrast, cancer cells with an impaired DDR are unable to repair the DNA damage caused by therapeutic agents, accumulate mutations, are eventually overwhelmed with DNA damage and succumb to cell death.

resulting in elimination of the cancerous cells. Thus, it is evident that a detailed understanding of the DDR and the proteins involved is of great consequence to the study of cancer.

1.2. The Tumour Suppressor p53

Central in the DDR pathway is the tumour suppressor protein, p53 (Figure 1.1). First identified in 1979, p53 is referred to as both the "guardian of the genome" and the "policeman of the oncogenes".[5-9] Activated in response to DNA damage and/or oncogene activation, the importance of p53 is highlighted by the high incidence of p53 mutations that occur in human cancers. In fact, p53 is the most frequently mutated gene in human cancers with greater than 50% of cancerous cells having lost p53 or expressing an inactive, mutant, p53 and thus also possessing a deficient DDR.[10-13]

p53 is a sequence-specific DNA-binding protein that activates the transcription of numerous effector genes in response to stressful stimuli.[14] These effector proteins are primarily involved in mediating cell cycle arrest, DNA repair or apoptosis to prevent the continued growth of DNA-damaged cells.[8] Cells which posses p53 mutations or disruption in the p53 signaling pathway indirectly promote tumour development by allowing the growth of damaged or mutated cells.[15,16]

1.2.1. p53 Activation

DNA damage is detected by DNA damage sensing proteins (i.e.: ATM, ATR and DNA-PK). These DNA damage sensing proteins activate signaling cascades that ultimately result in the phosphorylation and transcriptional activation of p53. Phosphorylation of serine-15 on p53 is thought to be particularly important for initiation of p53 transcription and is viewed as the principal indicator of p53 activation. Serine-15 phosphorylation results in increased levels of p53 within the cell by disrupting p53's interaction with its negative regulator MDM2.[17-20] Under normal cell cycle conditions MDM2, an E3

ubiquitin ligase, ubiquitinates p53 resulting in its degradation through the proteasomal pathway.

In addition to DNA damage, p53 activation also occurs in response to oncogene activation leading to increased apoptosis or premature senescence. The adenovirus *EIA* oncogene has been shown to induce p53 accumulation and promote apoptosis when introduced into primary cells.[21-24] Furthermore, *EIA* activation has also been shown to enhance cell sensitivity to DNA-damaging agents.[25] Endogenous oncogene activation also results in p53 activation, with *c-myc* activation leading to increased apoptosis and *ras* activation resulting in p53-induced senescence (a permanent form of cell cycle arrest).[26,27] To date it is unknown by what mechanism oncogene activation activates p53 but it is thought to be indirectly through creation of DNA damage (discussed above).

1.2.2. p53 Target Genes and Cell Fate

Some of the more well known p53 effector genes are *PUMA, Noxa* and *BAX* which are involved in apoptosis and *GADD45* which is important in DNA repair. By far the most extensively studied of all p53 target genes is the cyclin-dependent kinase inhibitor (cdkI) p21 which mediates cell cycle arrest (discussed in detail below).

The factors that influence the decision of a cell to undergo cell cycle arrest, repair its DNA or eliminate itself through apoptosis are not completely understood. Although, it does appear these outcomes are not mutually exclusive. It has been suggested that the extent of DNA damage may be an important factor in deciding the fate of the cell. For example, if the DNA damage is minor the cell may induce cell cycle arrest to allow time for DNA repair and continuation through the cell cycle. In contrast, if the DNA damage is extensive, the cell may arrest for a short period of time while it upregulates genes involved in apoptosis to trigger cell death. Regardless of the eventual outcome, it appears as though p53-mediated cell cycle arrest is the initial response to cellular stress and is then followed by p53-mediated upregulation of genes involved in DNA repair or apoptosis.[3,10,28] This suggestion is supported by observations that indicate p53 is

"sitting and primed" on the promoter of the cell cycle arrest gene p21. It has been shown that in response to stress stimuli this "primed" p53 is rapidly triggered to initiate transcription of p21, with p21 subsequently mediating cell cycle arrest.[29]

1.2.3. Effect of p53 Mutations on Cancer Therapies

Undisputedly an important factor in the DDR, the exact role of p53 as a mediator of cell cycle arrest has recently become somewhat confusing and controversial.[5,30,31] Originally p53 was thought to be critical for the induction of apoptosis and efficient cell death, and thus cells containing mutant p53 were regarded as apoptosis resistant.[30,32] However, closer examinations have revealed p53 to have some protective properties, particularly through its role in mediating p21-dependent cell cycle arrest (Figure 1.3).[30] In fact, there are now many cases where, somewhat contradictorily, p53-defective cells are demonstrated to be more sensitive to DNA-damaging therapeutics than are p53 wild-type cells.[30] For example, when normal (non-cancerous) cells with p53 mutations encounter DNA damage they are unable to upregulate p21 to mediate cell cycle arrest. This inability to arrest the cell cycle can contribute to the development of cancerous cells as the cell cannot mediate efficient DNA repair or apoptosis and mutant cells continue to propagate (discussed above). However, in cancerous cells with intact p53, p21-mediated cell cycle arrest can act detrimentally as a protective mechanism against DNA-damaging cancer therapeutics by allowing time for DNA repair to take place. Therefore, it is apparent that p53's role as a tumour suppressor gene is context-dependent and p53 status must be taken into account when considering therapeutic options.

1.3. Cell Cycle and Cyclin/Cdk Complexes

The term "cell cycle" refers to a strictly controlled series of events within a eukaryotic cell, and is comprised of four distinct phases: 1) G1 phase, in which the cell grows and prepares to replicate its DNA, 2) S phase or Synthesis, where the cell replicates its DNA, 3) G2 phase, where the cell continues its growth and

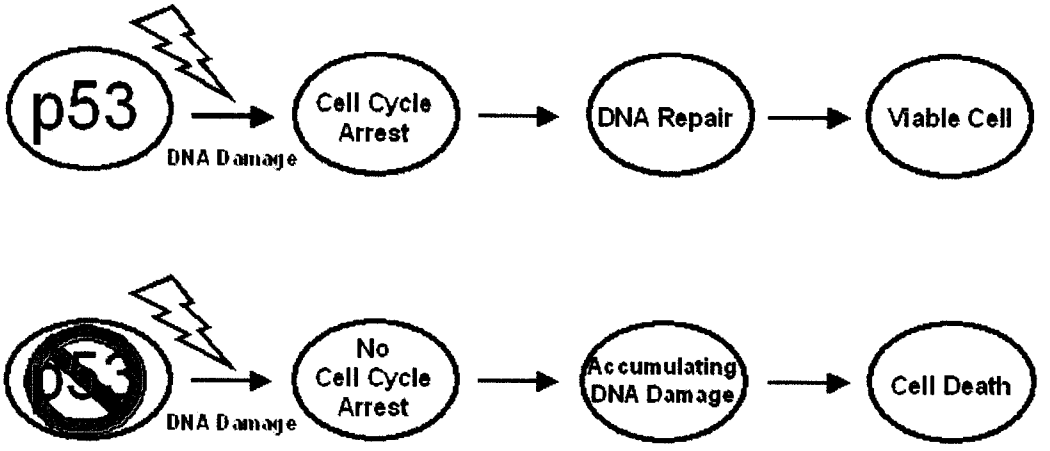


Figure 1.3. Effect of p53 Mutations on Cancer Cell Treatment. p53 is known as a tumour suppressor protein but when treating cancer cells with wild-type p53 it can detrimentally act to protect the cancer cells from cell death. In response to DNA-damaging cancer therapeutics p53 can mediate cell cycle arrest, allowing time for the cancer cells to repair its damaged DNA and continue productively proliferating. In contrast, cancer cells deficient for p53 or possessing mutant p53 cannot mediate cell cycle arrest in response to DNA-damaging therapeutics and accumulate an extensive amount of DNA damage which ultimately results in cell death.

metabolism in preparation for mitosis, and 4) M phase or Mitosis, in which the cell segregates its chromosomes and cell division occurs.

Progression through these individual phases is regulated by the sequential activation and deactivation of distinct protein complexes (Figure 1.4). These protein complexes are composed to two groups of proteins: cyclins and cyclin-dependent kinases (Cdk). Cyclins are the regulatory subunit of the cyclin/Cdk complex.[33-36] Cyclin protein expression and protein stability fluctuate in accordance with specific cell cycle phases (Figure 1.5). Cyclin D1 expression begins in early G1 and peaks in early S. Cyclin E is important in the progression from G1 to S with its expression peaking at the G1/S transition, while cyclin A expression begins in S phase followed by cyclin B1 expression which peak in late G2 and early M, respectively.[37-39] Stringent control of the expression and degradation of cyclins within their proper phases is critical to productive progression through the cell cycle.

Contrasting with the highly organized expression of the cyclin regulatory subunits is that of the catalytic subunits, the Cdks, which are constitutively expressed throughout the cell cycle.[40] Cdk2, Cdk4, and cdc2 (Cdk1) are the most important Cdks in the cell cycle. These Cdks specifically interact with and are activated by cyclin proteins as they become available, driving the cell cycle forward (Fig. 2).[40] Cdk2 is the most indiscriminate Cdk that interacts with cyclin D1, cyclin E and cyclin A, whereas Cdk4 and Cdk1 are more selective, predominately interacting with cyclin D1 and cyclin B1, respectively.[37,40-42]

1.3.1. Cyclin B1

Progression from G2 to M phase of the cell cycle is driven by the activation of cyclin B1/Cdk1 complexes.[43] Alternatively termed "M-phase/maturation-promoting factor" (MPF), cyclin B1/Cdk1 complex activation mediates the reorganization of subcellular structures by phosphorylation of cell structure proteins such as lamins, histones, condensins and mictotubule-associated proteins.[43,43-49] These events are essential for nuclear envelope breakdown and

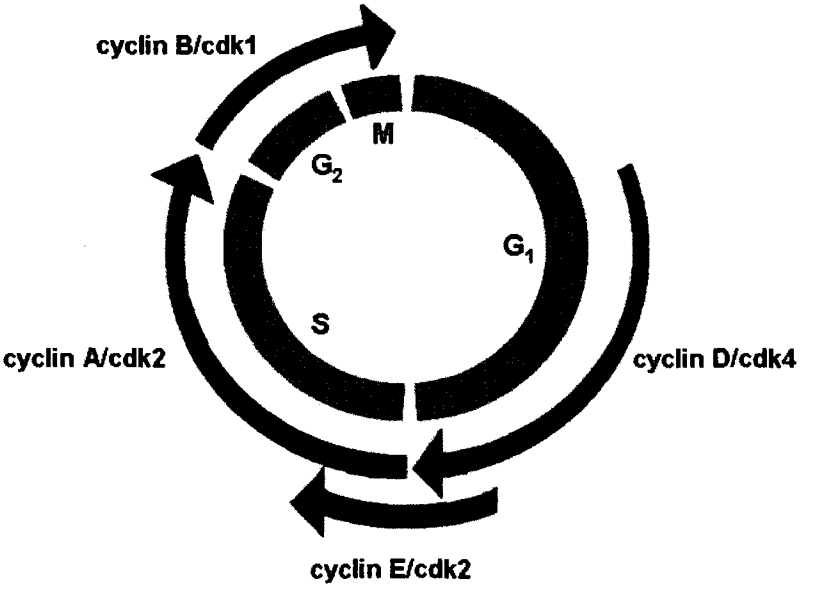


Figure 1.4. Differential Cyclin/Cdk Activation Regulates Cell Cycle Transitions. The cell cycle is a strictly controlled series of events that ensure productive replication of a cell's genomic material and cell division. G1 is where the cell grows in size and prepares to replicates its DNA; during this phase cyclin D/Cdk4 are activated. Transition from G1 to S phase, where the DNA is replicated, is triggered by the activation of cyclin E/Cdk2 complexes. Throughout S phase cyclin A/Cdk2 complexes are activated. When DNA replication is complete the cell enters G2, where it prepares to undergo mitosis or cell division. Cyclin B1/Cdk1 complexes become activated in early/mid G2 and stay activated until exit from mitosis where the rapid deactivation of cyclin B1/Cdk is required.

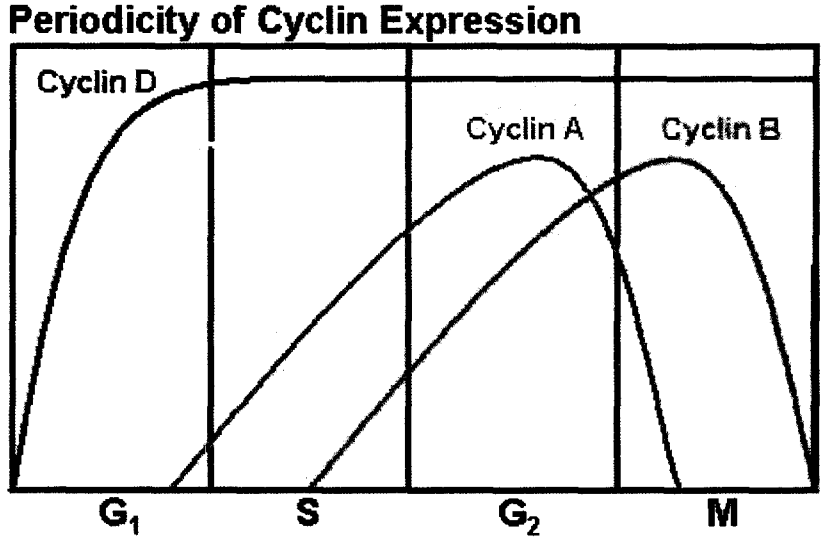


Figure 1.5. Regulation of Cyclin Expression Through the Normal Cell Cycle. Cyclin expression is strictly regulated throughout the cell cycle allowing for controlled association/activation of cyclin/Cdk complexes depending on cell cycle phase. Cyclin D expression begins in early G1 and is stable throughout the cell cycle. Cyclin E protein expression, which is important for entry into S phase, begins in late G1 and peaks at the G1-to-S transition. Cyclin A protein expression increases throughout late G1/S peaking in later G2. Cyclin B1 expression begins in S phase, increases throughout G2, peaks in M phase and is rapidly decreased prior to exit from mitosis.

entry into mitosis. Microinjection studies have shown cyclin B1 expression alone can drive G0/G1 cells into mitosis.[50] Furthermore, cyclin B1 has been shown to be necessary for survival, as cyclin B1 nullizygous mice die *in utero*.[51]

Under normal cell cycle conditions cyclin B1 protein levels are highest at late G2/early M phase and cyclinB1/Cdk1 activity is sustained from prophase through to metaphase in the cytoplasm.[34,46,50] However, subsequent entry into anaphase and exit from mitosis requires rapid deactivation of cyclinB1/Cdk1 activity and translocation into the nucleus. This rapid deactivation is reportedly achieved through proteasome-mediated destruction of the cyclin B1 protein.[39,52]

Under normal cell cycle conditions cyclin B1 protein turnover is mediated through interaction with the anaphase-promoting complex/cyclosome (APC).[39,52-57] The APC is a highly conserved multisubunit E3 ubiquitin ligase that targets cell cycle regulators, such as cyclins, for degradation. The APC controls the passage through the cell cycle by specifically targeting proteins for proteolysis. Two activating receptor subunits can be associated with the APC, cdc20 and cdh1, and are able to control APC substrate specificity.[39,53,55] Substrates for the APC contain one or more short sequences in their N-terminus called a destruction box (D-box). Both APC^{cdc20} and APC^{cdh1} have been shown to target cyclin B1 for ubiquitination (and thus degradation) during normal cell cycle progression.[53,56,58]

1.3.2. Cyclin B1 and Cancer

Increasing evidence indicates that the deregulation of cyclin B1 correlates with both tumour formation and aggressiveness.[59-67] Overexpression of cyclin B1 has been shown to force the bypass of the G2/M checkpoint.[50] This promotes aberrant entry into mitosis which results in the formation of cells with damaged or mutated tetraploid DNA content. Interestingly, although cells overexpressing cyclin B1 are more aggressive in tumourigenesis they have been demonstrated to be more sensitive to DNA damage-induced apoptosis in response to chemotherapeutic and radioactive agents than are cells with normal cyclin B1 expression. Presumably this sensitivity is a result of the cell's inability to protectively arrest the cell cycle in G2. Indeed, a new type of cell death has been

identified, termed mitotic catastrophe (MC), that describes this specific mode of cell death. [68-70] MC is characterized by the inappropriate activation of cyclin B1/Cdk1 and the formation of multinucleate, giant cells with uncondensed chromosomes and greater than 4N DNA content.[39,69,71] Thus, much like apoptosis, MC represents a hurdle to tumourigenesis.

1.4. The Cyclin-Dependent Kinase Inhibitor p21

The DDR activates a number of "checkpoint control proteins". In response to stressful stimuli these proteins act to alter the normal progression of the cell cycle. Through regulation of cyclin/cyclin-dependent kinase (Cdk) complex activity, these checkpoint proteins allow cell cycle arrest and DNA repair. Failure of such checkpoint control proteins allows for inappropriate progression through the cell cycle which ultimately results in the generation of mutant cells or cell death.

One such checkpoint control protein is the cyclin-dependent kinase inhibitor p21 (CDKN1A). First identified in 1995, p21 plays a pivotal role in the DDR as a downstream mediator of p53-induced cell cycle arrest. In this respect p21 itself is often described as a "tumour suppressor" protein.[72,73] p21 mediates both transient and permanent forms of cell cycle arrest by binding to and inhibiting the activation of cyclin/Cdk complexes which are responsible for cell cycle progression.[74,75] Primarily studied with regard to its interaction with Cdk2-containing cyclin/Cdk complexes (specifically cyclin A/Cdk2 and cyclin E/Cdk2), p21 is thought to be the principal mediator of DNA damage-induced G1 cell cycle arrest. [76-81] However, more recent reports have also suggested p21 may play a role in DNA damage-induced G2 cell cycle arrest.[79,82-85]

Although p21 is generally regarded to be a tumour suppressor protein initial experiments in mice did not support this hypothesis, with studies finding p21 null mice to remain tumour free.[86] However, closer examination revealed that p21 null mice did develop spontaneous tumours around 16 months of age as compared to control animals that remained tumour free for over 2 years.[87] With

regard to human cancers, p21 mutations are exceedingly rare although many tumours are deficient in p21 since they posses mutant and/or deficient p53.[88]

1.4.1. Cip/Kip Family Members

There are three members of the Cip/Kip family of cyclin-dependent kinase inhibitors: $p21^{(WAF1,Cip1)}$, $p27^{(Kip1)}$ and $p57^{(Kip2)}$. [89-91] They constitute a family because they all share a homologous Cdk inhibitory domain which is both necessary and sufficient for binding and inhibition of Cdk2- and Cdk4-containing complexes.[76,92-96] p21 was the first cdkI to be identified and has subsequently been shown to be a critical negative regulator of the cell cycle in response to DNA damage. p27 has been demonstrated to play an important role in the normal cell cycle by regulating cyclin/Cdk activity, particularly in the G0-to-G1 transition. Like p21, p27 is regarded as a tumour suppressor protein and, indeed, reduced p27 levels in primary cancers have been highly correlated with decreased patient survival.[97] Less is known about p57. Discovered simultaneously by two groups looking for homologues of p21 and p27, p57 has been linked to Wilms tumours and Beckwith-Wiedemann syndrome.[98-101]

1.4.2. p21 Structure

The p21 protein itself consists of two principal domains (Figure 1.6). The amino-terminal domain shares sequence homology with the other Cip/Kip family members. This domain consists of a cyclin-binding region, Cy1, close to the N-terminus and a Cdk-binding region, termed the K site.[76,96] Unlike the other members on the Cip/Kip family, p21 possesses a second, weaker cyclin-binding region, Cy2, in its carboxy-terminal domain.[76] Although Cy2 binds cyclins more weakly than Cy1 its presence in conjunction with Cy1 and the K site has been shown to strengthen p21's interaction with some cyclin/Cdk complexes.[76] In addition to Cy2 the carboxy-terminal domain of p21 also contains a Nuclear Localization Signal (NLS), necessary for trafficking into the nucleus, and a Proliferating Cell Nuclear Antigen (PCNA) binding region which allows p21 to bind to PCNA and inhibit DNA replication without affecting DNA repair.[102-106]

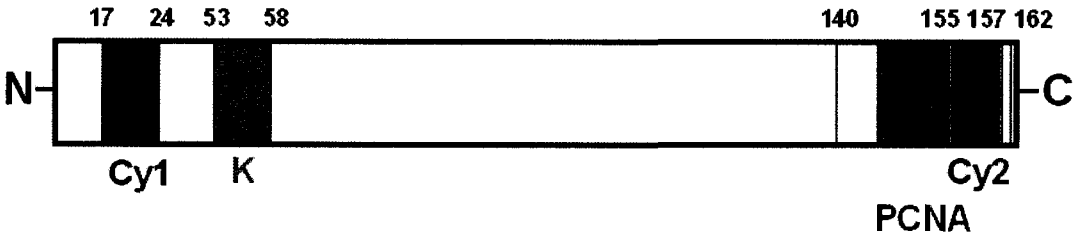


Figure 1.6. Structure of p21. The N-terminus of p21 contains a cyclin-binding region (Cy1) and a cyclin-dependent kinase-binding region, termed the K site. The C-terminus of p21 contains a Nuclear Localization Signal (NLS) that overlaps a Proliferating Cell Nuclear Antigen (PCNA)-interacting region and a second cyclin-binding region (Cy2).

1.4.3. p21 as a Mediator of Cell Cycle Arrest

In solution p21 is an unstructured protein theoretically capable of adopting multiple conformations depending on the targeted protein.[102] In this respect p21 has been described as a universal inhibitor of Cdks. However, p21 has predominately been shown to associate with cyclin A/Cdk2, cyclin E/Cdk2, cyclin D1/Cdk4 and, in some instances, cyclin A/Cdk1 complexes.[94-96,102-108] p21 does not inactivate cyclin/Cdk complexes by dissociation; instead its acts as a stoichiometric inhibitor.[91,97] The α-helix of Cip/Kip proteins initiates first contact with the cyclin, then the second helix inserts deep inside the catalytic cleft of the Cdk subunit blocking ATP loading. [81,89,109] In the case of Cdk2-containing complexes, Cdk2 conformational changes further lock the catalytic cleft in inactive form.[89] Further consequences of Cdk inhibition also include dephosphorylation of Rb and downregulation of a large group of E2F-dependent genes involved in DNA replication and cell cycle progression which aids in cell cycle arrest.[110]

Having been demonstrated by some groups to have low affinity to Cdk1 (as compared to Cdk2), p21 is thought to play a marginal role in the inactivation of the G2-associated cyclin B1/Cdk1 complex.[74,107,111-113] Nevertheless, p21 overexpression studies have shown that p21 can inhibit cyclin B1-associated kinase activity by blocking the activating phosphorylation on Cdk1; however, these studies have shown only a minor fraction of cyclin B1/cdk1 complexes to be associated with p21.[114] Also, recent studies have described a role for p21 in the nuclear retention of cyclin B1/Cdk1 complexes in response to DNA damage. [115] This nuclear retention is thought to prevent the activation of cyclin B1/Cdk1 complexes by sequestering them away from activating kinases present in the cytoplasm.

Regardless of the disputed role of p21 in G2 cell cycle arrest, it is clear that p21 plays an important role in the mammalian DDR.

1.5. Summary

In cases where DNA damage is present and it is not beneficial to replicate DNA, the cell can 1) induce cell cycle arrest, allowing for time to repair the damaged DNA, or 2) induce apoptosis. The decision between these two outcomes is mediated by the DDR through the tumour suppressor protein p53 and its down-stream transcriptional targets. p21 is the most well-known downstream transcriptional target of p53. In response to DNA damage, p53 is stabilized and activated. This enables p53 to up-regulate genes such as p21 which control cell cycle arrest and apoptosis. p21 has been shown to be the key mediator of p53-dependent G1 cell cycle arrest after DNA damage. p21 exerts G1 cell cycle arrest through direct interaction with G1 cyclin/Cdk complexes (specifically cyclinA/Cdk2 and cyclinE/Cdk2) with its amino-terminal domain and also by binding to the proliferating cell nuclear antigen (PCNA) with its carboxy-terminal domain. This cell cycle arrest is essential to prevent the propagation of damaged or mutated DNA which can promote tumourigenesis.

Recent studies, included those contained in this thesis, have suggested that p21 may also play a more important role in G2 cell cycle arrest than previously thought. The work in this thesis is centered around understanding the role of p21 in the DDR and G2 cell cycle arrest. More specifically, this work assess the correlation between p21 expression and cyclin B1 regulation in response to DNA damage. Of note, the majority of studies contained in this thesis have been published in the form of a journal article in *Cell Cycle*.[116]

Chapter 2: Materials and Methods

2.1. Cell Culture

Isogenic human colon carcinoma cell lines, HCT116 (WT, p53-/- or p21-/-) were provided by Bert Vogelstein (John Hopkins, Baltimore, USA). They were grown in DMEM (Gibco), 10% HIFBS, 1X streptomycin/penicillin (Invitrogen) at 37°C and 5% CO_2. Cells were seeded to be 60% confluent at time of DNA damage treatment.

HCT116 cell were chosen because they are frequently used in the study of the mammalian DNA damage pathway. Their frequent use is due to the high number of established cell lines with specific gene deletions, particularly genes involved in the DNA damage pathway (i.e.: p53 and p21).[72,83]

2.2. DNA-Damaging Agents

Cell lines were treated with DNA-damaging agent indicated: 5 μg/mL etoposide (Etp) (Sigma), 150 μg/mL hydroxyurea (HU) (Sigma), 20 J/m^2 UV (C), or 12 Gy Gamma-irradiation (IR), for the times indicted (0-72 h). Concentrations were selected based on previous DNA damage studies using HCT116 cells.[79,117,118]

2.3. Cell Cycle Analysis

2.3.1. Detection of Cell Death

Medium was collected and cells were trypsinzed at indicated times (0 h-48 h) and pelleted by centrifugation at 500 g for 5 min at 4°C. The cell pellet was washed with PBS and pelleted again at 500 g for 5 min at 4°C. The cell pellet was resuspended in 70% ethanol to fix the cells and stored at 4°C overnight. Cells were pelleted as before, washed with PBS contain 0.2 mg/mL Rnase A

(Invitrogen) and pelleted again. Finally cells were suspended in 50 µg/mL propidium iodine (PI) (Sigma) in 0.2 mg/mL Rnase A containing PBS. Cells were subjected to flow cytometry using fluorescence-activated cell sorter (FACS) (BD Bioscience) and cell cycle analysis was performed using Modfit LT™ Flow Cytometry Modeling Software. Cell death was calculated as the percentage of cells in the sub-G1 population.

2.3.2. Cell Cycle Analysis

Medium was discarded and cells were washed with PBS two times prior to trypsinization. Cell cycle analysis preformed as described above.

2.4. Preparation of Whole Cell Lysate

Cells were washed two times with PBS at the indicated times post treatment, trypinsized and pelleted by centrifugation at 500 g for 5 minutes at 4°C. Pellet was resuspended in lysis buffer (5 times the volume of the pellet) [20 mM HEPES, 150 mM NaCl, 1% Triton-X 100, 5 mM EDTA, 5 mM NaF, 25 mM β- glycerophophate, 1 mM Na_3VO_4, 10 mM okadaic acid and 1X protease inhibitor cocktail (PIC) (Sigma)] and incubated on ice for 10 minutes. Lysates were then syringed with 26-gauge needle 10-20 times and centrifuged at 23 000 g for 15 min at 4°C. The supernatant was collected and glycerol was added to a final concentration of 10%. Protein concentration was determined using Coomasie reagent and bovine serum albumin (BSA) standards following manufacturer's instructions (Pierce).

2.5. Induction of Artificial Cell Cycle Arrest

Cells were treated with Etp alone, Rocovitine (Ros) alone (10 µM), or Etp and Ros simultaneously. Cells were harvested and FACS was performed as described above. Cell cycle analysis was performed using Modfit LT™ Flow

Cytometry Modeling Software and cell death was calculated as the percentage of cells in the sub-G1 population.

2.6. Senescence Detection

Senescence was detected using the Senescent Cells Staining Kit (Sigma). Briefly, cells were seeded in a 6-well plate and treated for the indicated times with Etp then washed twice in PBS. 1.5 mL of Fixation Buffer® was added and incubated 7 min at room temperature and rinsed 3 times in PBS. 1mL of Staining Mixture® was added per well and Parafilm-sealed plates were incubated at 37°C without CO_2 (from 2 h - overnight). Plates were then examined under the microscope for the presence of blue (senescent) cells.

This senescence detection assay was developed by Dimri *et al.* in 1995 while looking for biomarkers of senescent cells.[119] Briefly, senescent cells were found to increase expression of the beta-galactosidase gene. Addition of a Staining Mixture and incubation at pH 6 will turn cells expressing beta-galactosidase blue; indicative of senescent cells.

2.7. Hoechst Nuclear Staining and Microscopy

Cells were grown on coverslips and treated as indicated. Cells were washed with PBS for 5 min, fixed in 4% formaldehyde at room temperature for 15 min and washed 3 times in PBS for 5 min each time. Cells were then stained with 1 mg/mL Hoechst 33258 for 10 min, washed 3 times with PBS for 5 min and sealed onto slides. Slides were examined using confocal microscopy.

2.8. SDS-PAGE and Western Blot Analysis

Laemmli buffer with 5% β-mercaptoethanol was added 1:1 with cell lysates (25 µg) and samples boiled for 5 min. Samples were resolved alongside pre-stained protein markers (BioRad) using 12% Tris-HCl acrylamide gels for

SDS-PAGE. Gels were transferred to 0.2 μm polyvinyldifluoride (PVDF) membrane (BioRad) for 70 min at 110V. Membrane were blocked overnight at 4°C in 5% BSA PBST while shaking. Membranes were incubated with the indicated primary antibodies diluted in 5% BSA PBST for 1 h at room temperature, washed in PBST 5 times for 5 min, incubated with appropriate secondary antibody for 1 h at room temperature, washed in PBST 3 times for 5 min and then treated with chemiluminescent reagent ECL Plus® (Amersham Biosciences). Membranes were developed using a Typhoon scanner (Amersham Biosciences)

2.9. Antibodies

Primary antibodies recognized human p53(DO-1) (Santa Cruz), p21(F-5)(C-19) (Santa Cruz), cyclin B1(D-11)(GNS1)(H-433) (Santa Cruz), β-actin (Sigma). Secondary antibodies were goat anti-mouse and goat anti-rabbit conjugated horseradish peroxidase (HRP) (Jackson Immunochemicals). Antibodies were used for Western Blot analysis and co-immunoprecipitation experiments.

2.10. p21 Retroviral Add Back

p21-expressing retroviral vector was created by cloning p21 cDNA from pCEP4-p21 (Bert Vogelstein) into pBMN IRES Puromycin vector (Craig McCormick).[120] Retrovirus was then made by transfecting Phoenix amphotroptic packaging cell line using calcium phosphate. Virus-containing medium was collected after 72 h, Sequa-brene (Sigma) was added to a final concentration of 4 μg/mL and filtered through a 0.45 μm filter syringe. 50% confluent HCT116 p21-/- cells were then infected for 24 h with retroviral-containing medium. Virus was removed, cells given 24 h to recover from infection, selected for 48 h in 1 μg/mL puromycin (Sigma) and then harvested for immunoblot analysis.

2.11. siRNA Knockdown

Knockdown of p21 in HCT116 p21+/+ cells was achieved by transfection of pooled sequences of siRNA targeting p21 or a non-specific sequence (Dharmacon) using Interferin transfection reagent (Polyplus Transfection Inc). 24 h after transfection of siRNA, cells were treated with 5 μg/mL Etp for the indicated times.

2.12. Quantitative PCR (Q-PCR) and Statistical Analysis

RNA extraction was carried out using an RNeasy Extraction Kit® (Qiagen). Production of cDNA from cytoplasmic RNA (1 μg) was done using the Superscript II Reverse Transcription Kit® (Invitrovrogen) with random hexamers (250 μg) for priming amplification. Q-PCR analysis was carried out on the MxPro 3000P QPCR machine using Brilliant SYBR Green Q-PCR Master Mix® (Stratagene) according to manufacturers instructions. *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)* was used as an internal control for all samples. QPCR was performed for the amplification of total p53, p21 and cyclin B1 mRNA levels.

Experiments were completed in replicates of 3 and statistical analysis involved t-tests and determination of p-values was done using GraphPad Prism Software (GraphPad Software Inc). Statistical significance was assigned to t-tests resulting in a p-value ≤ 0.05.

2.13. Inhibition of Protein Degradation

Cells treated for the indicated times with 5 μg/mL Etp alone or with Etp and 1 μM proteasome inhibitor MG132 (Sigma) added for the last 10 h pre-harvest. Lysates were collected as described above and subject to SDS-PAGE and Western blot analysis with the indicated antibodies.

2.14. Co-Immunoperception (co-IP) Experiments

Cell lysates (500 μg) were pre-cleared using 4 μg mouse or rabbit normal IgG (Calbiochem) conjugated to ProteinG Dynal Beads (Invitrogen) for 1 h mixing end over end for 1 h at 4°C. Cleared lysates were then subject to immunoprecipitation with 2 μg of indicated antibody conjugated to ProteinG Dynal Beads for 1h mixing end over end at 4°C. Beads were then washed 5 times with lysis buffer, resuspended in 50 μl Laemmli protein sample buffer with 5% β-mercaptoethanol and boiled for 5 min. Samples were then subject to SDS-PAGE and Western blot analysis with specified antibodies.

2.15. Immunofluorescence (IF)

Cells were grown on coated glass coverslips and treated as indicated. Cells were washed once in PBS for 5 min, fixed in 4% formaldehyde for 15 min at room temperature and washed 3 times in PBS for 5 min. Cells were then blocked in 10% normal goat serum diluted in 0.03% Triton X-100 in PBS for 1 h at room temperature. Coverslips were then incubated in the primary antibodies indicated diluted in 0.01% BSA in 0.03% Triton-X100 in PBS overnight at 4°C. Coverslips were then washed 3 times in PBS for 5 min, incubated for 1 h in appropriate secondary at room temperature and washed again in PBS 3 times for 5 min. Coverslips were then sealed on slides and examined using the conflocal microscope.

Chapter 3: Results

3.1. Cells with an Impaired DNA Damage Response (DDR) are More Sensitive to Etoposide-Induced Cell Dath than Cells with an Intact DDR.

As mentioned above, many cancer cells possesses mutated p53.[11] From a "cancer development" prospective this is not beneficial, as it impairs the DDR which acts to protect against cancer development and tumourigenesis. Somewhat paradoxically, recent studies have suggested that from a "cancer treatment" prospective an impaired DDR maybe beneficial, as the cell has no means to combat DNA-damaging cancer therapeutics (Figure 1.2 and Figure 1.3).[5,30,121] To test this hypothesis, isogenic HCT116 cells specifically deleted for p53 or p21 were compared in their responses to the DNA-damaging agent Etp. Etp is a topoisomerase II inhibitor that impairs the religation of newly synthesized DNA, causing double-strand DNA breaks.

HCT116 wild-type (hereafter referred to as p21+/+), p53-/- and p21-/- cells were treated with Etp for 0 h, 24 h or 48 h. Cells were then collected and subjected to FACS Analysis to detect cell death (% of cells in Sub-G1). Cells with an impaired DDR pathway (i.e. cells lacking p53 or p21) were noticeably more sensitive to Etp-induced cell death at 48 h as compared to cells with an intact DDR (HCT116 p21+/+ cells) (Figure 3.1). Given that p21 is downstream of p53 in the DDR pathway and that HCT116 p53-/- and HCT116 p21-/- were comparably more sensitive to DNA damage than HCT116 p21+/+, p21 was identified as the main mediator of DNA damage sensitivity.

Interestingly, HCT116 p21+/+ cells with p21 knockdown were particularly sensitive to cell death, even more so than the HCT116 p21-/- cells. This finding suggests that continuous culture of the HCT116 p21-/- cell line has allowed the selection of cells that are somewhat more resistant to DNA damage-induced cell death than newly p21-deficient cells.

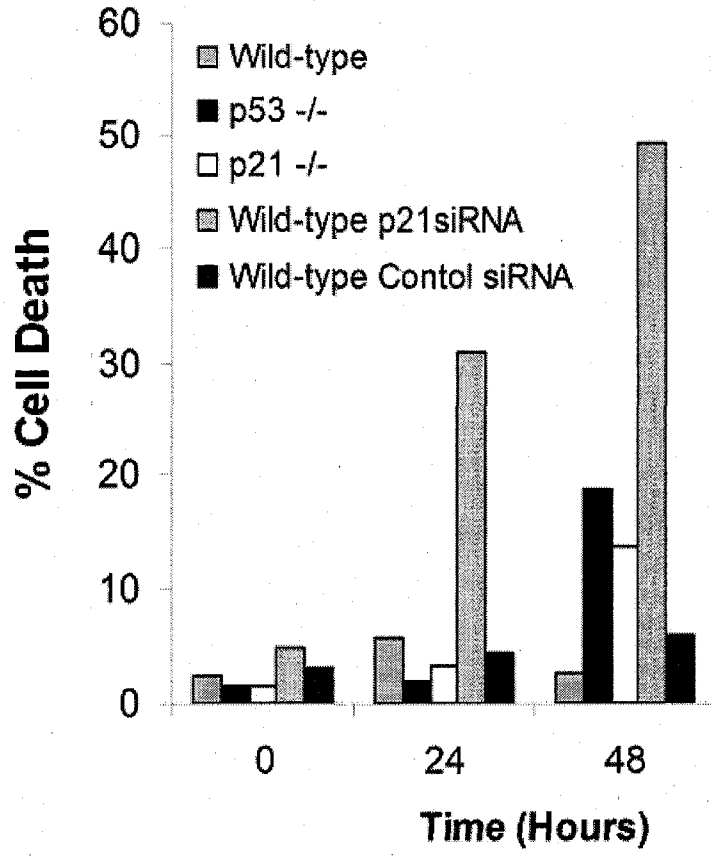


Figure. 3.1. Cells Impaired in the Ability to Upregulate p21 in Response to DNA Damage are Sensitive to Etoposide-Induced Cell Death. HCT116 wild-type (p21+/+), p53-/- and p21-/- cells were treated with 5 μg/ml etoposide (Etp) for 0 h, 24 h or 48 h. siRNA was used to knockdown (kd) p21 or a non-specific sequence in HCT116 p21+/+ and cells were treated for 0 h, 24 h or 48 h. After Etp treatment cells were collected and subject to FACS analysis to detect cell death as indicated by percent of cell in sub-G1. When treated with Etp for 48 h both HCT116 p53-/- and p21-/- cells show increased sensitivity to DNA dmage-induced cell death as compared to HCT116 wild-type cells. HCT116 p21+/+ p21kd cells were particularly sensitive to Etp-induced DNA damage with 50% of the cell population succumbing to cell death. The discrepancy between HCT116 p21+/+ p21kd and HCT116 p21-/- cell sensitivity to DNA damage-induced cell death is likely due to the long-term propagation of HCT116 p21-/- in culture which has allowed selection of more stress-resistant cells. Figure shown is one representative experiment of three repeated experiments.

Together these results suggest that cells with an impaired DDR pathway are more sensitive to DNA damage-induced cell death than are cells with an intact DDR.

3.2. Cells Lacking p21 are Unable to Sustain Arrest in G2 and Accumulate Greater than 4N DNA Content in Response to Etp-induced DNA Damage.

Having determined that sensitivity to DNA damage-induced cell death was correlated with p21 status, and given that p21 is a known mediator of cell cycle arrest, cells treated with Etp were subjected to cell cycle analysis.

HCT116 p21+/+ and p21-/- cells were subjected to Etp treatment for 0 h, 24 h or 48 h. Cell cycle analysis was performed using florescence-activated cell sorting (FACS) and analysized using ModFit LT software (Figure 3.2.). HCT116 p21+/+ cells were predominately arrested in G2 after 24 h and 48 h of Etp treatment. Interestingly, although the majority of HCT116 p21-/- cells were in G2 at 24 h post-Etp treatment, by 48 h a significant proportion of cells showed greater than 4N DNA content. Thus, it appears cells lacking p21 are unable to sustain G2 cell cycle arrest in response to DNA damage and inappropriately continue through the cell cycle.

3.3. Cell Cycle Arrest Protects Cells from Etp-Induced Cell Death.

To determine whether the inability to sustain cell cycle arrest is responsible for the increased cell death seen in cells lacking p21, roscovitine (Ros), an inducer of cell cycle arrest, was employed. Ros is a purine analog that is a potent inhibitor of cyclin-dependent kinases (Cdk).[122] In particular, it is a competitive inhibitor of cyclin B/Cdk1, cyclin A/Cdk2 and cyclin E/Cdk2.[122] Thus, it acts to "artificially" arrest the cell cycle.

Wild-type HCT116 cells, p53-/- cells and p21-/- cells were treated either with Etp and Ros alone or both agents simultaneously for up to 48 h and then subject to FACS analysis to determine the amount of cell death (Figure 3.3). As

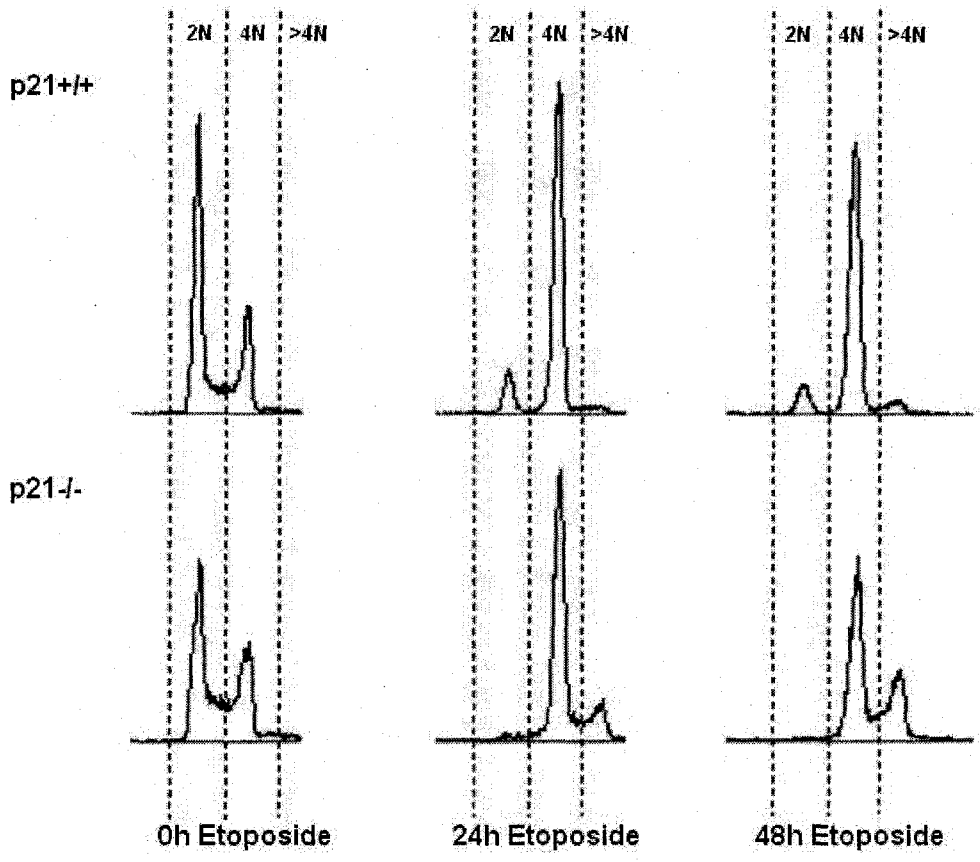


Figure. 3.2. Cells Lacking p21 are Unable to Sustain G2 Cell Cycle Arrest in Response to Etoposide-Induced DNA Damage. HCT116 p21+/+ and HCT116 p21-/- were treated with Etp for 0 h, 24 h or 48 h, collected and subject to FACS analysis to determine cell cycle profile. HCT116 p21+/+ cells were arrested primarily in G2 24 h and 48 h post Etp treatment. HCT116 p21-/- cells were arrested in G2 at 24 h after Etp treatment but by 48 h a significant number of cells had accumulated greater than 4N DNA content suggesting they were unable to sustain G2 cell cycle arrest. Figure shown is one representative experiment of three repeated experiments.

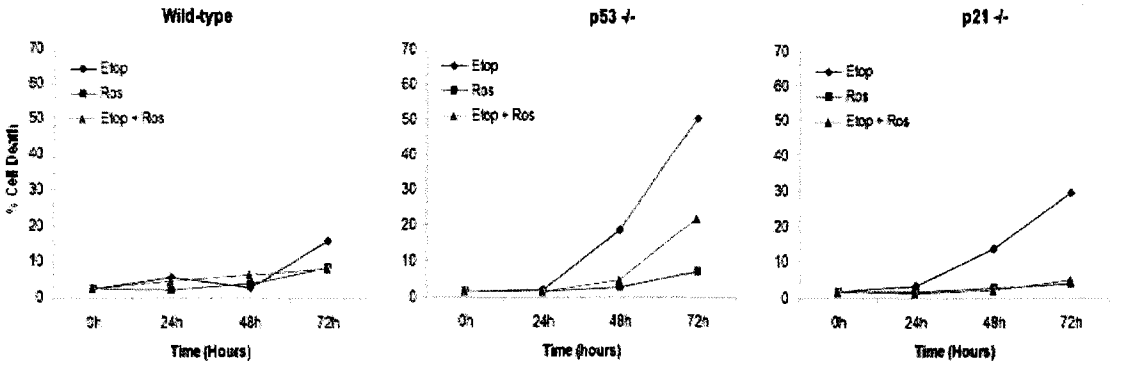


Figure 3.3. Cell cycle arrest protects cells from etoposide-induced cell death. HCT116 p21+/+, p53-/- or p21-/- cells were treated with Etp alone, roscovitine (Ros) alone or both agents simultaneously. Ros is a cyclin/cdk inhibitor that acts to artificially induce cell cycle arrest. Cells were collected at 0h, 24 h, 48 h or 72 h and subject to FACS analysis to determine percent of cell death as indicated by sub-G1 population. When treated with Etp alone HCT116 p53-/- and p21-/- displayed increased cell death 48 h and 72 h post treatment as compared to HCT116 p21+/+ cells. Addition of Ros at the time of Etp treatment reduced the amount of cell death seen in HCT116 p53-/- and p21-/- cells. Ros alone had a negligible effect on cell death. Figure shown is one representative experiment of three repeated experiments.

noted previously HCT116 p53-/- and p21-/- cells were sensitive to Etp-induced cell death. Interestingly, simultaneous treatment of Ros with Etp resulted in decreased cell death compared to Etp alone. This finding suggests that cell cycle arrest can act as a barrier to cell death in response to DNA-damaging agents.

3.4. Prolonged Exposure to DNA Damage Results in the Induction of a Senescence-Like Phenotype.

Some literature suggests cell cycle arrest is only a temporary hurdle to cell death. Studies have shown that in some instances after prolonged periods of cell cycle arrest cells (to their own detriment) re-nter the cell cycle in the presence of DNA damage, eventually succumbing to cell death.[123] Conversely, more recent studies have suggested that in response to prolonged exposure to DNA damage cells can enter a senescence-like phenotype protecting the cell from death.[124,125]

With this in mind, the reponse of HCT116 p21+/+ cells to prolonged exposure to DNA damage was examined. HCT116 p21+/+ and HCT116 p21-/- cells were treated with Etp for up to 72 h and subjected to staining for senescence (a permanent form of cell cycle arrest) detection (Figure 3.4). HCT116 p21-/- cells displayed no evidence of senescence induction (blue staining) up to 72 h Etp treatment. In contrast, by 72 h Etp treatment the majority of HCT116 p21+/+ cells were stained blue, indicating the induction of senescence.

Thus, the ability to induce cell cycle arrest is important to the induction of senescence and protection against cell death in response to prolonged exposure to DNA-damaging agents. This finding has significant implications when discussing DNA-damaging cancer therapies for patients harbouring tumours with intact DDR pathways.

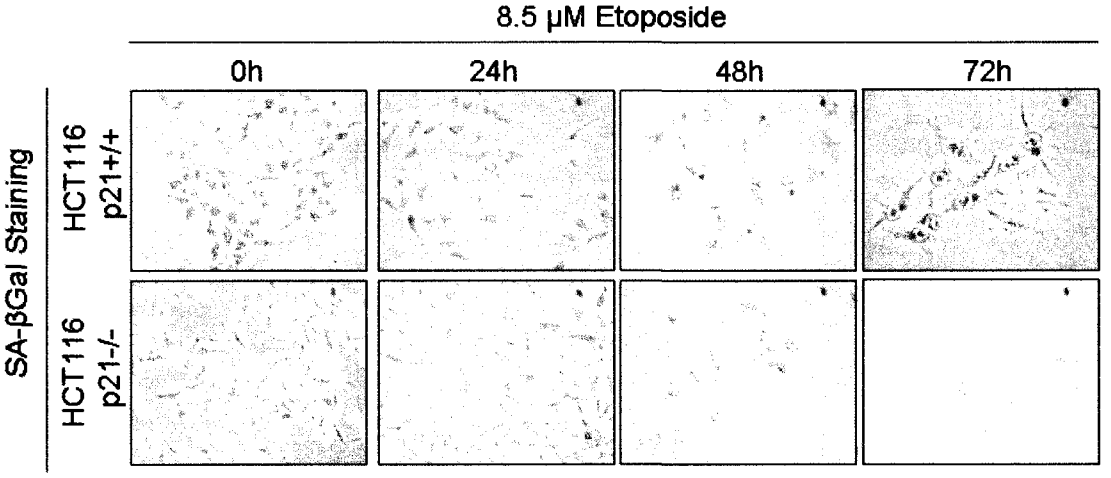


Figure 3.4. Prolonged exposure to DNA damage induces a senescence-like phenotype in HCT116 p21+/+ cells. HCT116 p21+/+ and p21-/- cells were treated with Etp for 0 h, 24 h, 48 h, or 72 h. Cells were then stained using a senescence detection kit and examined under light microscope. Senescent positive-staining cells are blue in colour whereas senescence-negative cells show no colour change. 48-72 h post Etp treatment HCT116 p21+/+ cells stained positive for senescence. In contrast, even up to 72 h post Etp treatment HCT116 p21-/- cells did not stain positive for senescence. These results suggest p21 is necessary for the induction of a senescence-like phenotype in response to DNA damage. Figure shown is one representative experiment of three repeated experiments.

3.5. Cells Unable to Mediate Cell Cycle Arrest Die Through Mitotic Catastrophe (MC) in Response to Etp-Induced DNA Damage.

Having demonstrated that cell cycle arrest protects cells with an intact DDR from cell death the next step was to determine the mechanism by which HCT116 p21-/- cells were undergoing cell death. Literature suggests cells that are unable to sustain G2 arrest inappropriately enter mitosis and die through a mechanism known as mitotic catastrophe.[69] Mitotic catastrophe is characterized by formation of small fragmented micronuclei and uncontrolled activation of cyclinB1/Cdk1 complexes.[69]

The nuclei of HCT116 p21+/+ and HCT116 p21-/- cells were observed by staining with Hoechst 33258 dye and examined using confocal microscopy (Figure 3.5). While HCT116 p21+/+ cells displayed large condensed nuclei following 48 h of Etp treatment (consistent with G2 cell cycle arrest), HCT116 p21-/- cells displayed small fragmented nuclei consistent with descriptions of mitotic catastrophe. Thus, it appears cells lacking p21 are unable to mediate cell cycle arrest in response to DNA damage, inappropriately continue through the cell cycle and die through mitotic catastrophe.

3.6. Cells Impaired in the Ability to Upregulate p21 in Response to Etp-Induced DNA Damage are Unable to Properly Mediate Cyclin B1 Expression.

Since death by mitotic catastrophe results from uncontrolled activation of the mitosis-promoting factor (MPF), cyclin B1/Cdk1, cyclin B1 protein levels were examined in response to Etp treatment.[69]

HCT116 p21+/+ and HCT116 p21-/- cells were treated for 0 h, 24 h or 48 h with Etp, harvested and subjected to western blot analysis for p53, p21 and cyclin B1 protein expression (Figure 3.6A).

HCT116 cells with intact p21 showed upregulated p21 expression in response to Etp and displayed decreased levels of cyclin B1 at 48 h post DNA damage. In contrast, HCT116 p21-/- cells showed no change in cyclin B1 levels,

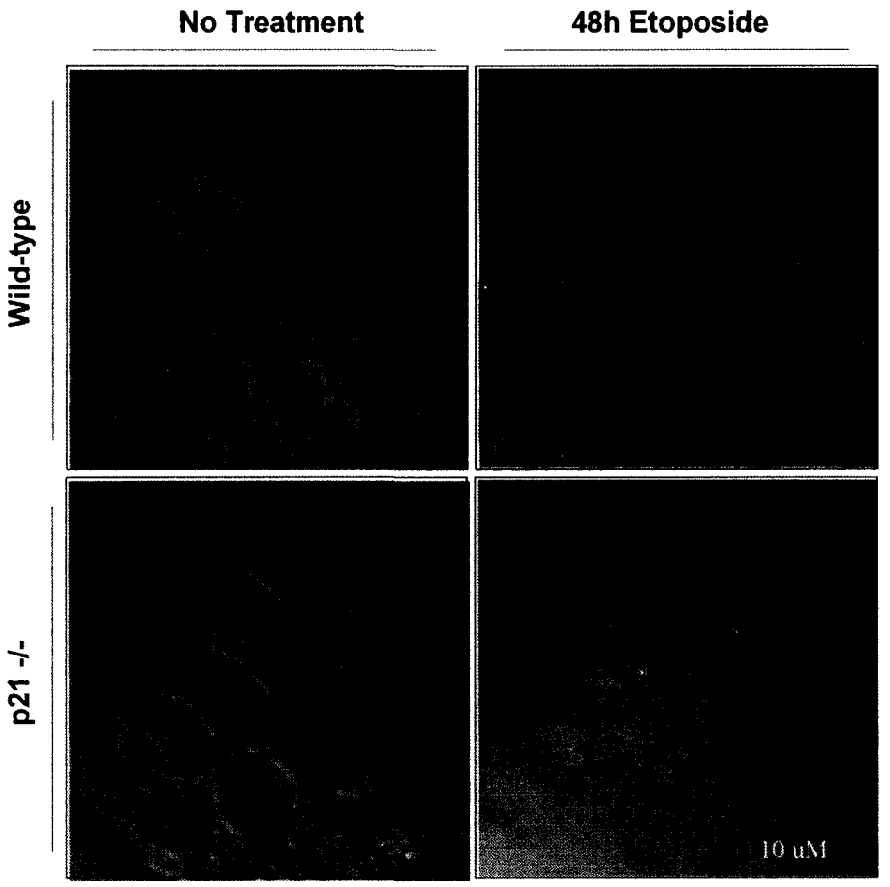
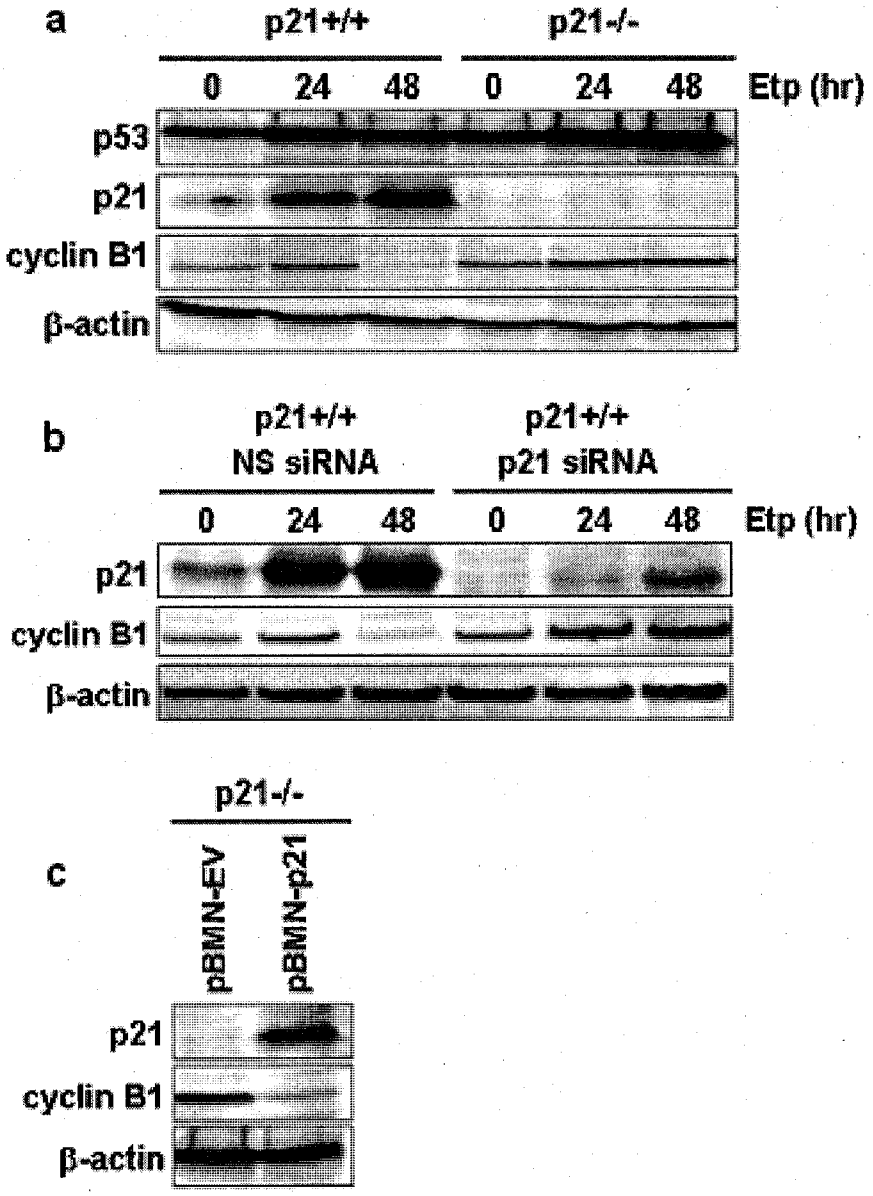


Figure 3.5. Cells lacking p21 are dying though mitotic catastrophe in response to etoposide-induced DNA damage. HCT116 p21+/+ and HCT116 p21-/- cells were grown on coverslips and treated with Etp for 0 h or 48 h. After Etp treatment cells were washed, fixed and stained with Hoechst 33258 to visualize nuclei. Coverslips were then sealed to slides and examined by confocal microscopy. 48 h post Etp treatment nuclei of HCT116 p21+/+ cells displayed an enlarged condensed morphology consistent with G2 cell cycle arrest. Conversely, 48 h post Etp treatment the nuclei of HCT116 p21-/- cells displayed small fragmented micronuclei consistent with descriptions of mitotic catastrophe. Pictures shown are representative of three repeated experiments.

Figure 3.6. Cells Lacking p21 or Impaired in the Ability to Upregulate p21 in Response to Etoposide-Induced DNA Damage Display Deregulated Cyclin B1 Protein Levels. (A) HCT116 p21+/+ and HCT116 p21-/- were treated with Etp for 0 h, 24 h or 48 h. Lysates were collected and subject to western blot analysis to detect p53, p21 and cyclin B1 protein. At 48 h post Etp treatment HCT116 p21+/+ cells showed decreased levels of cyclin B1 as compared to 0 h and 24 h after Etp treatment. HCT116 p21-/- cells show no change in cyclin B1 levels from 0 h-48 h of Etp treatment. These results suggest p21 is necessary for cyclin B1 regulation in response to DNA damage. **(B)** Pooled siRNA sequences against p21 or a non-specific control sequence were transfected into HCT116 p21+/+ cells. Cells were simultaneously treated with Etp for 0 h, 24 h or 48 h. Lysates were harvested and subjected to western blot analysis to detect p21 and cyclin B1 protein. While the control siRNA had no effect on p21 levels siRNA against p21 resulted in significantly decreased p21 upregulation in response to Etp-induced DNA damage. Inhibition of p21 upregulation in HCT116 p21+/+ cells impaired the ability of these cells to regulate cyclin B1 protein levels. **(C)** HCT116 p21-/- cells were transduced with p21-containing (pBMN-p21) or empty (pBMN-EV) reterovirus. 48 h after cell recovery from infection lysates were harvested and subjected to western blot analysis to detect p21 and cyclin B1 protein. While transduction with pBMN-EV had no effect on cyclin B1 levels in HCT116 p21-/- cells, transduction with pBMN-p21, even in absence of DNA damage, resulted in decreased cyclin B1 protein levels. Together these results suggest p21 is necessary for proper cyclin B1 regulation in response to DNA damage. β-actin was used as a loading control in all experiments.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cyclin B1
β-actin
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pBMN-p21
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cyclin B1
β-actin

suggesting p21 is likely involved in cyclin B1 regulation in response to DNA damage.

To further strengthen the connection between p21 upregulation in response to DNA damage and the decrease in levels of cyclin B1, siRNA was used to knock down p21 expression in HCT116 wild-type cells (Figure 3.6B). siRNA knockdown of p21 in HCT116 p21+/+ cells significantly impaired their ability to decrease cyclin B1 in response to Etp-induced DNA damage, whereas the control knockdown had no effect. Additionally, reintroduction and overexpression of p21 into HCT116 p21-/- using a retroviral vector resulted in a decrease in cyclin B1 expression as compared to the control vector (Figure 3.6C).

Together these results suggest that in response to DNA damage p21 expression is necessary for cyclin B1 downregulation, and explain why cells lacking p21 are unable to sustain G2 cell cycle arrest.

3.7. Cells Lacking p53 are Impaired in the Ability to Mediate Cyclin B1 because they Cannot Upregulate p21 in Response to DNA Damage.

It has been suggested that decreased cyclin B1 levels seen in wild-type cells in response to DNA damage may be the result of p53-mediated transcriptional repression at the cyclin B1 promoter.[126-128] However, HCT116 p21-/- cells (which possess a functional p53) show no decrease in cyclin B1 levels in response to DNA damage. Additionally, although HCT116 p53-/- failed to downregulate cyclin B1 after 48 h of Etp treatment, reintroduction of p21 into these cells restored the ability of these cells to downregulate cyclin B1 in the absence of p53 (Figure 3.7). Collectively, these data suggest that the deregulated cyclin B1 expression observed in DNA-damaged HCT116 p53-/- cells is due to the inability of these cells to upregulate p21 and not due to p53 transcriptional repression at the cyclin B1 promoter.

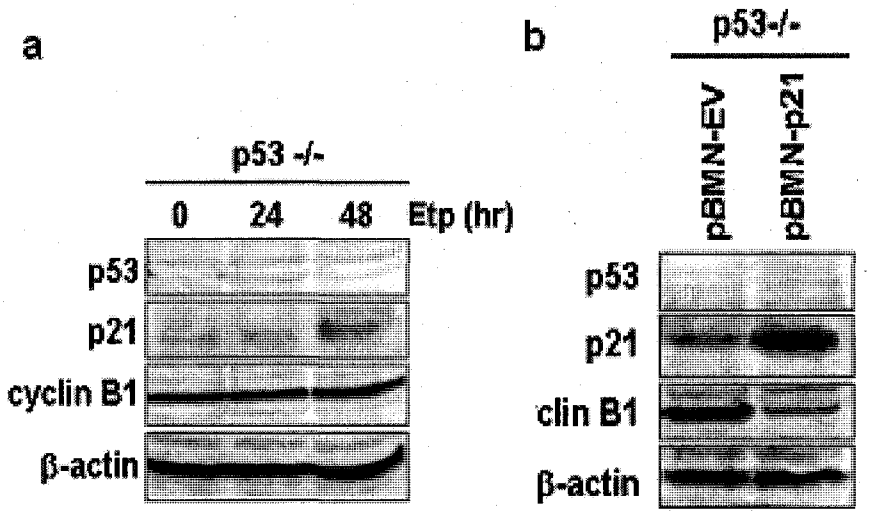


Figure 3.7. Cells Lacking p53 are Impaired in the Ability to Upregulate p21 in Response to DNA Damage and Cannot Properly Regulate Cyclin B1 Protein Levels. (A) HCT116 p53-/- cells were treated with Etp for 0 h, 24 h or 48 h. Lysates were collected and subject to western blot analysis to detect p53, p21 and cyclin B1 protein. HCT116 p53-/- cells were impaired in the ability to upregulate p21 at all time points compared to HCT116 p21+/+ cells. **(B)** HCT116 p53-/- cells were transduced with p21-containing (pBMN-p21) or empty (pBMN-EV) retrovirus. 48 h after cell recovery from infection lysates were collected and subjected to western blot analysis to detect p53, p21 and cyclin B1 protein. While transduction with EV-pBMN had no effect on cyclin B1 levels in HCT116 p53-/- cells, transduction with pBMN-p21, even in the absence of DNA damage, resulted in decreased cyclin B1 protein levels. These results suggest p21, but not p53, is necessary for proper cyclin B1 regulation in response to Etp-induced DNA damage. β-actin was used as a loading control in all experiments.

3.8. p21 Mediates the Degradation of Cyclin B1 in Response to Etp-Induced DNA Damage.

Two possibilities could explain the cyclin B1 protein downregulation observed in response to Etp-induced DNA damage: 1) transcriptional repression at the cyclin B1 promoter, or 2) increased cyclin B1 protein degradation. Quantitative Real Time PCR (Q-PCR) analysis of cyclin B1 mRNA levels in HCT116 p21+/+ revealed no significant change in the transcription of cyclin B1 after Etp treatment as compared to non-damaged cells suggesting transcriptional repression is not the cause of the observed decrease in cyclin B1 protein levels (Figure 3.8).

Under normal cell cycle conditions the destruction of cyclin B1 occurs through the proteasomal pathway and is mediated by an E3 ubiquitin ligase complex, anaphase-promoting complex/cyclosome (APC/C).[39,52-57] Consequently, we tested the effect of the proteasomal inhibitor MG132 on cyclin B1 levels in response to DNA damage. HCT116 p21+/+ cells were treated with Etp for 0 h, 24 h or 48 h with MG132 added 10 h before harvest (Figure 3.9.). The addition of MG132 prior to the 48 h time point abolished the decrease in cyclin B1 protein levels seen with Etp alone, confirming the decrease in cyclin B1 protein levels is due to proteasomal destruction.

These results suggest that in response to Etp treatment p21 promotes the degradation of cyclin B1 protein.

3.9. p21-Mediated Cyclin B1 Degradation is a General Response to DNA Damage that Results in p21 Upregulation.

To determine whether p21-mediated cyclin B1 degradation is a general response to DNA damage, HCT116 p21+/+ cells were treated with a variety of DNA-damaging agents for 48 h (Figure 3.10). Etp, gamma-irradiation (IR), hydroxyurea (HU) and UV were selected because they differ in the ability to induce p21 expression. Like Etp, IR is able to induce p21 expression and resulted in cyclin B1 degradation 48 h post treatment. In contrast, treatment with

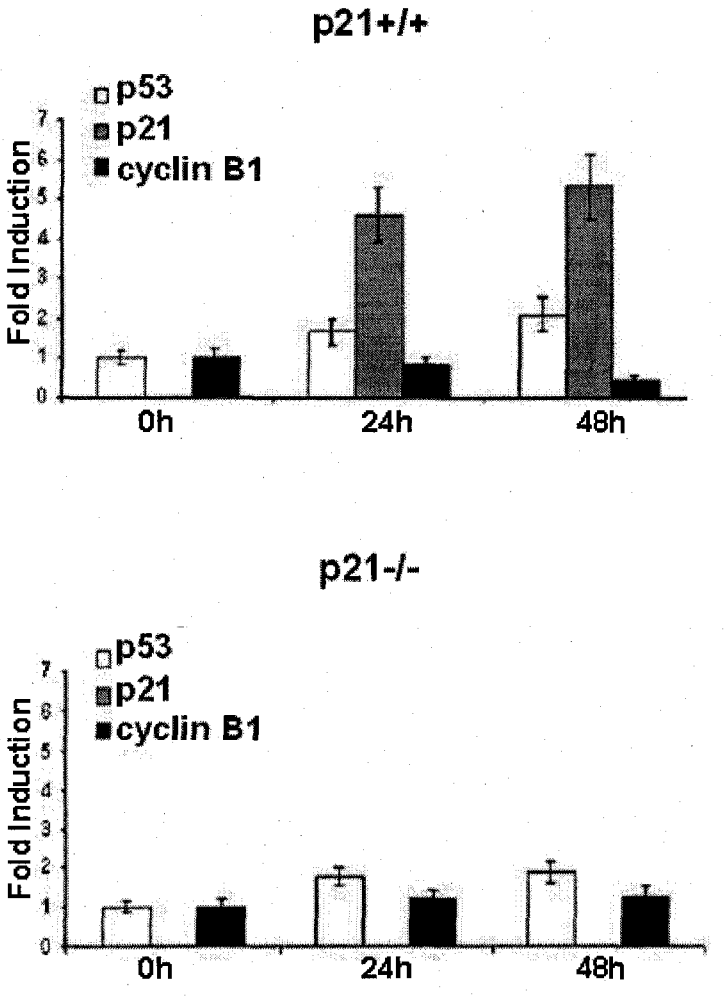


Figure 3.8. Cyclin B1 Decrease in Response to DNA Damage is Not Due to Transcriptional Repression. HCT116 p21+/+ and p21-/- cells were treated with the DNA-damaging agent Etp for the times indicated. Isolated RNA was subjected to Q-PCR to measure transcript levels for p53, p21 and cyclin B1 with GAPDH used as a normalizing control. In response to Etp, HCT116 p21+/+ trancriptionally upregulated p53 and p21 as expected. However, no significant change in cyclin B1 transcription was observed. HCT116 p21-/- displayed increased levels of p53 in response to DNA damage with no change in cyclin B1. These results suggest that transcriptional repression is not responsible for the decrease in cyclin B1 seen in HCT116 p21+/+ cells in response to DNA damage. Experiments were completed in replicates of 3 and statistical analysis involved t-tests and determination of p-values was done using GraphPad Prism Software. Statistical significance was assigned using t-tests resulting in a p-value≤ 0.05.

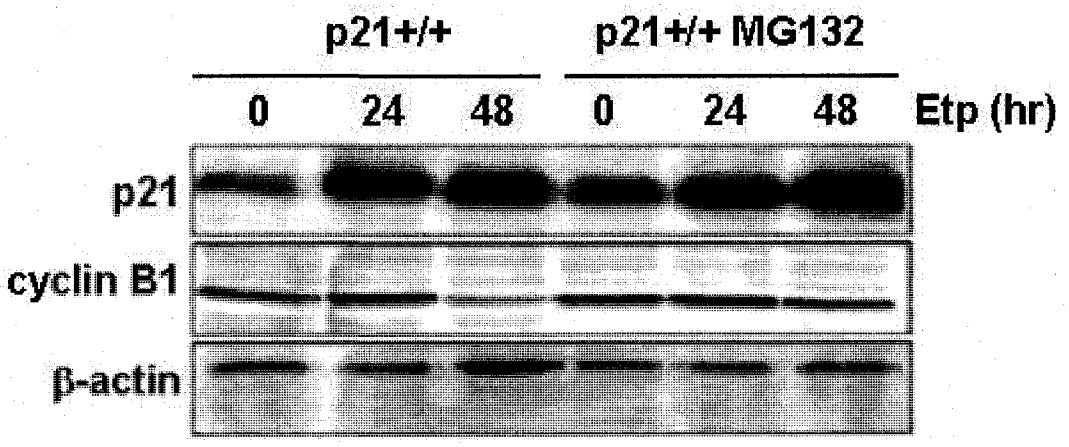


Figure 3.9. p21 Mediates Cyclin B1 Protein Degradation in Response to Etp-Induced DNA Damage. HCT116 p21+/+ cells were treated with Etp for 0 h, 24 h or 48 h. The proteasome inhibitor MG132 was added 10 h prior to harvest. Lysates were collected and subject to Western blot analysis to detect p21 and cyclin B1 protein levels. β-actin was used as a loading control. HCT116 p21+/+ treated with Etp alone showed decreased levels cyclin B1 48 h after treatment. In contrast, HCT116 p21+/+ cells treated with Etp and MG132 did not show a decrease in cyclin B1 48 h after Etp treatment. These results suggest that cyclin B1 levels are decreased through increased protein degradation.

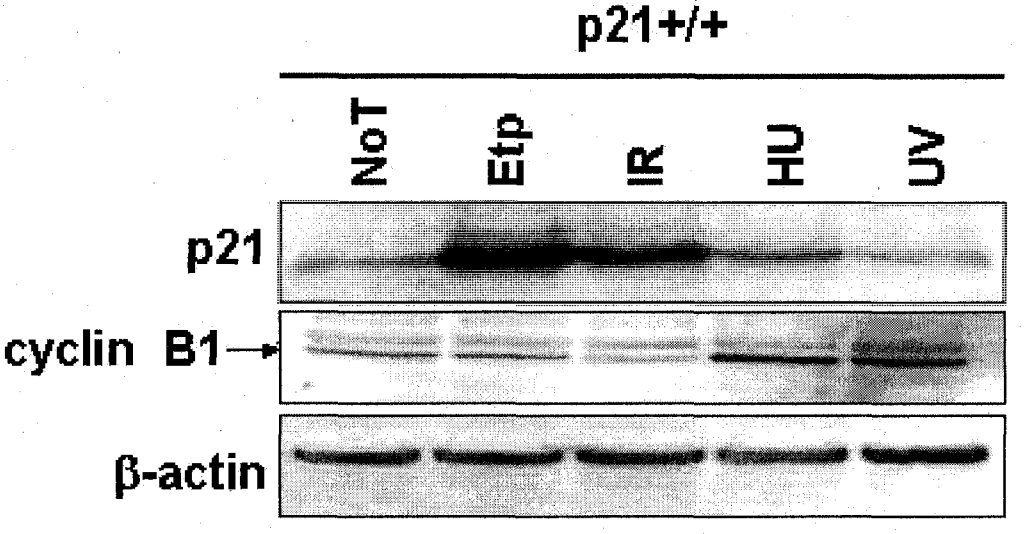


Figure 3.10. p21 Upregulation is Necessary to Mediate Cyclin B1 Degradation in Response to DNA Damage. HCT116 p21+/+ were left untreated (NoT) or treated for 48 h with DNA-damaging agents (Etp, gamma-irradiation, HU or UV) differing in their ability to upregulate p21. Lysates were harvested and subject to western blot analysis to detect p21 and cyclin B1 protein. β-actin was used as a loading control. HCT116 p21+/+ treated with Etp and gamma-irradiation upregulated p21 in response to DNA damage and were subsequently able to mediate the degradation of cyclin B1. Conversely, treatment of HCT116 p21+/+ cells with HU or UV (C) did not result in p21 upregulation and no deceases in cyclin B1 protein levels were observed. These results suggest that p21-mediated cyclin B1 degradation is a general response to DNA damage that results in p21 upregulation.

HU or UV, which did not induce p21 expression, did not result in degradation of cyclin B1. These results confirm the role of p21 in mediating cyclin B1 degradation in response to DNA damage and highlight this as a general response to DNA damage that upregulates p21.

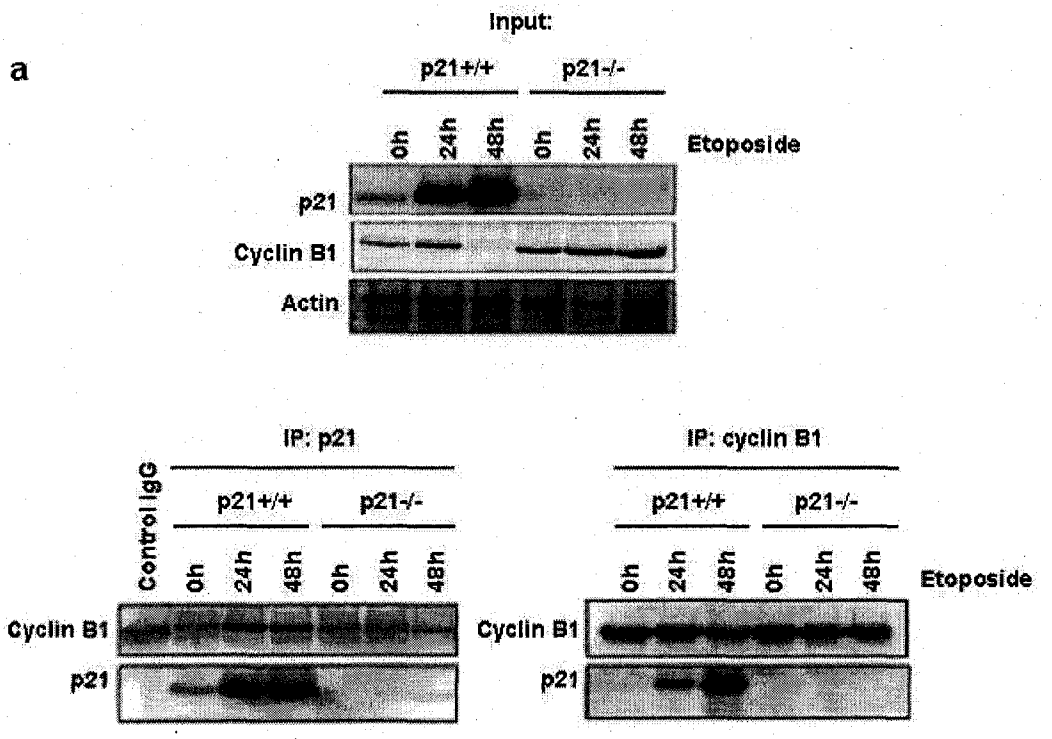
3.10. p21 Interacts with Cyclin B1 in Response to Etp-Induced DNA Damage.

Given that p21 upregulation in response to DNA damage was confirmed as necessary for cyclin B1 degradation, the next objective was to determine the mechanism of p21-mediated cyclin B1 degradation. Past studies have demonstrated p21 to have a relatively low affinity to cyclin B1/Cdk1 complexes compared to Cdk2-containing cyclin/Cdk complexes.[107] Surprisingly, when HCT116 p21+/+ cells were treated with Etp and subjected to immunoprecipitation experiments, a significant amount of p21 interacted with cyclin B1 (Figure 3.11A). Interestingly, even at 48 h of Etp treatment, when cyclin B1 levels were very low, p21 co-immunoprecipitated with cyclin B1.

To confirm that cyclin B1 and p21 were indeed interacting in response to DNA damage, immunofluorescence was performed (Figure 3.11B). HCT116 p21+/+ cells were grown on slides, treated with Etp for 0 h, 24 h or 48 h and p21 and cyclin B1 were detected using specific fluorescent secondary antibodies. Slides were then examined by confocal microscopy for protein localization. Consistent with immunoprecipitation experiments, immunofluorescence showed cyclin B1 and p21 colocalizing after 24 h of Etp-induced DNA damage.

Together these results indicate that in response to DNA damage p21 interacts with cyclin B1. Further experimentation is needed to determine whether this interaction is necessary for cyclin B1 degradation in response to DNA damage.

Figure 3.11. p21 Interacts with Cyclin B1 in Response to DNA Damage. (A) HCT116 p21+/+ cells were treated with Etp for 0 h, 24 h or 48 h. Lysates were harvested and subject to immunoprecipitation experiments followed by Western blot analysis to detect cyclin B1 and p21 protein levels. Immunoprecipitations were carried out using either cyclin B1 or p21. 24 h after Etp treatment p21 and cyclin B1 co-immunoprecipitated. Interestingly, even at 48 h when levels of cyclin B1 are relatively low, p21 still co-immunoprecipitated. **(B)** HCT116 p21+/+ cells were grown on coverslips, treated for 0 h or 24 h with Etp then fixed to coverslips. Cells were then stained for p21 and cyclin B1 localization. Consistent with immunoprecipitation experiments, immunoflorescence showed cyclin B1 and p21 localizing together outside the nucleus (indicated by arrows) at 24 h after Etp treatment. Together these results suggest that in response to DNA damage p21 and cyclin B1 interact and this interaction may be important for cyclin B1 degradation.


a
Input:
p21+/+
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Control IgG
p21+/+
p21-/-
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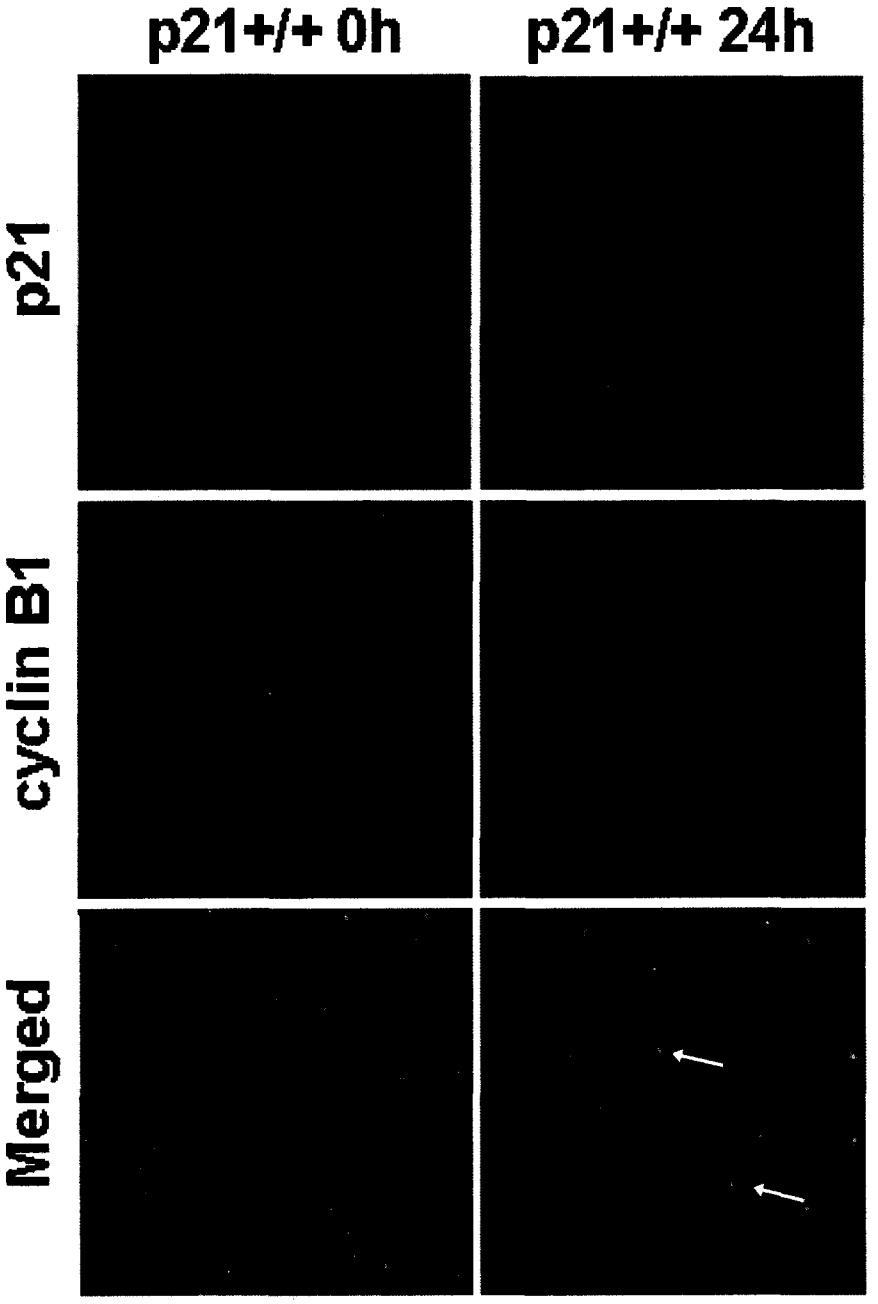
p21+/+ 0h
p21+/+ 24h
p21
cyclin B1
Merged

3.11. Summary.

Cell cycle arrest is a barrier to cell death induced by DNA damage. When a cell cannot efficiently induce cell cycle arrest, the result is inappropriate progression through the cell cycle. A key mediator of DNA damage-induced cell cycle arrest is the cyclin-dependent kinase inhibitor p21. The results contained within this thesis highlight a previously uncharacterized role for p21 in mediating the degradation of the G2-associated cyclin, cyclin B1.

In response to Etp-induced DNA damage p21 is upregulated in a p53-dependent manner. In cells with an intact p53-p21 pathway the primary response is G2 cell cycle arrest characterized by a decrease in cyclin B1 protein levels. With prolonged exposure to DNA damage this cell cycle arrest is shown to progress to a senescence-like phenotype, protecting cells from death. In contrast, cells with an impaired p53-p21 DNA damage-sensing pathway (i.e. HCT116 p21-/- and HCT116 p53-/- cells) are unable to mediate a decrease in cyclin B1 levels and cannot to sustain G2 cell cycle arrest. The inability to sustain G2 cell cycle arrest resulted in the formation of small fragmented micronuclei with the cells eventually undergoing cell death through a process known as mitotic catastrophe.

The inability of HCT116 p21-/- cells to sustain G2 cell cycle arrest was due to inappropriate levels of cyclin B1. Experimentation revealed that p21 is necessary for the degradation of cyclin B1 in response to DNA damage. Thus, cells impaired in the ability to upregulate p21 in response to DNA damage (i.e. HCT116 p21+/+ p21kd, HCT116 p21-/- and HCT116 p53-/-) cannot efficiently mediate G2 cell cycle arrest and are consequently more sensitive to DNA damage-induced cell death. Furthermore, although further experimentation is needed, preliminary immunoprecipitation and immunofluorescence experiments suggest p21 may play a direct role in mediating cyclin B1 degradation.

In conclusion, these results suggest p21-mediated cyclin B1 degradation is necessary to sustained G2 cell cycle arrest and protect cells from death in response to DNA damage.

Chapter 4: Discussion

4.1. Cells with an Impaired DDR are Impaired in the Ability to Mediate Cell Cycle Arrest in Response to DNA Damage and are More Sensitive to DNA Damage-Induced Cell Death.

A study done in 1997 consisting of a 60-cell-line panel of cells differing in their p53 status found that cells lacking p53 continued to metabolize a growth indicating reagent while cells with intact p53 did not metabolize the indicator reagent in response to DNA damage.[32] The original interpretation of this study was that cells lacking p53 continued to metabolize the indicator reagent because they were resistant to cell death while cells with intact p53 did not metabolize the indicator because they were dying through apoptosis. Based on this study most research was then conducted subscribing to the idea that p53 was necessary for induction of apoptosis. This theory prevailed for a long time in the study of the mammalian DNA damage response (DDR) in cancer cells. Since the majority of cancer cells are deficient in p53 (either through deletion or mutation of the gene) they should, in accordance with the original hypothesis, be resistant to apoptosis. However, in recent years the hypothesis has changed and the original study has been subject to re-interpretation. Work described in this thesis, in conjunction with other studies, suggests that p53 may aid in protecting cancer cells from cell death by inducing cell cycle arrest.[31] With regard to the above-mentioned study, it appears as though cells lacking p53 continued to metabolize the indicator reagent because they were unable to arrest the cell cycle and continued grow in the presence of DNA damage which eventually resulted in cell death. In contrast, cells with intact p53 failed to metabolize the indicator reagent because they underwent cell cycle arrest in response to DNA damage.

Thus, consistent with this new hypothesis, the work described in this thesis suggests that p53 may play a protective role by inducing p21-mediated cell cycle arrest in response to DNA damage.

4.2. Cells Lacking p53 or p21 are More Sensitive to DNA Damage-Induced Cell Death because they Cannot Mediate Cell Cycle Arrest.

Consistent with the above-mentioned hypothesis, cells lacking p53 were found to be more sensitive to DNA-damage induced cell death than cells with intact p53 (Figure 3.1). By 48 h a significant number of p53 null cells had undergone cell death compared to p53 wild-type cells. Since it is proposed that p53 wild-type cells are able to resist cell death by inducing cell cycle arrest, cells lacking the cyclin-dependent kinase inhibitor p21 were also tested. p21 has been shown to be the principal mediator of p53-dependent cell cycle arrest in response to DNA damage.[72] Cells lacking p21 were also found to be more sensitive to DNA damage-induced cell death compared to wild-type cells at 48 h post-damage.

Since HCT116 p21-/- cells have been propagated in culture for many years, and given that p21 is a critical protein in regulation of cell propagation, it is plausible that the culture as a whole has undergone some adaptations that allow it to be more resistant than cells with a newly acquired p21 mutation might be. With this in mind, siRNA was used to knockdown p21 in wild-type cells and cells were then subjected to DNA damage. Interestingly, wild-type cells with p21 knockdown were extremely sensitive to DNA damage-induced cell death compared to control knockdown cells. This finding suggests that the inability of p53 to upregulate p21 in response to DNA damage sensitizes cells to cell death.

Evidence also exists that suggests p21 my play a role in regulating p53 activity through a negative feedback mechanism. This hypothesis is supported by findings that demonstrate cells lacking p21 have a higher basal level of p53. [129] Furthermore, even under normal conditions this increased level of p53 harbours activating phosphorylations without having encountered additional DNA damage stimuli.[130] Thus, wild-type cells, with intact p53, may be more resistant to cell death because of both p21-induced cell cycle arrest and the p21-mediated negative regulation of p53 activation and its apoptosis-inducing functions.

To confirm that it is indeed cell cycle arrest that is protecting cells from DNA damage-induced cell death roscovitine (Ros), a chemical cyclin-dependent kinase inhibitor, was used. Ros mimics the action of p21 by arresting the cell cycle. Addition of Ros and the DNA-damaging agent Etp simultaneously in both cells lacking p53 or cells lacking p21 resulted in significantly decreased levels of cell death as compared to cells treated with Etp alone (Figure 3.3). This finding confirms cell cycle arrest is an efficient method of protection against cell death.

4.3. Cells Lacking p21 are Unable to Maintain G2 Cell Cycle Arrest and Die Through Mitotic Catastrophe in Response to DNA Damage.

Cell cycle analysis of wild-type and p21 null cells exposed to DNA damage revealed that, consistent with previous studies, p21 null cells are unable to mediate G1 cell cycle arrest.[72] More interestingly, cells lacking p21 displayed a deficiency in maintaining G2 cell cycle arrest (Figure 3.2). Since its discovery p21 has been known to play a major role in G1 cell cycle arrest through its interaction and inhibition of Cdk2-containing cyclin/Cdk complexes.[74] However, due to its relatively low affinity to Cdk1-containing cyclin/Cdk complexes p21 has been thought to play little or no role in G2 cell cycle arrest. More recent studies along with the work in this thesis suggests that p21 plays a more significant role in G2 cell cycle arrest than previously described.[82,83,113,114,116,128]

A study published by the Vogelstein group at the Johns Hopkins Oncology Center showed that cells lacking p53 are unable to sustain G2 cell cycle arrest in response to DNA damage, with cells bypassing the G2 checkpoint, inappropriately entering mitosis and accumulating greater than 4N DNA content.[83] They suggest that the inability of these cells to upregulate p21 in response to DNA damage results in an impaired G2 checkpoint. The FACS analysis displayed in Figure 3.2 is consistent with this suggestion, with p21 null cells unable to maintain G2 cell cycle arrest and accumulating a greater than 4N DNA content. Vogelstein and co-workers suggest that p21 may indeed play a role in regulating the mitosis-promoting cyclin B1/Cdk1 complex and demonstrate

p21 null cells to have inappropriately activated cyclin B1/Cdk1 kinase activity in response to DNA damage. Cyclin B1 data contained in this thesis also support this hypothesis and are discussed below.

Since cells lacking p21 cells were shown to be more sensitive to DNA damage-indcued cell death than wild-type cells the mechanism of cell death was examined. A particular type of cell death, termed mitotic catastrophe (MC), is characterized by the inappropriate progression from G2 to M phase in the presence of DNA damage.[69] MC is also characterized by the aberrant activation of cyclin B1/Cdk1 complexes and the formation of small fragmented micronuclei.[69] Nuclear staining of DNA-damaged p21 null cells revealed the presence of multiple fragmented micronuclei (Figure 3.5). Conversely, when treated with DNA damaging agents wild-type cells displayed large condensed nuclei consistent with G2 cell cycle arrest.

Together, these observations suggest that cells lacking p21 are unable to sustain G2 cell cycle arrest in response to DNA damage, continue through the cell cycle, accumulating greater than 4N DNA content and eventually die through MC. These findings highlight p21 as a potential target molecule to increase cell death in response to DNA-damaging cancer therapeutics. The ability to upregulate p21 to mediate cell cycle arrest in response to DNA damage allows for the protection of the cell from cell death. With this said, inhibition of cell cycle arrest, through direct targeting of p21, coupled with DNA damage therapy may be a more effective means of triggering cancer cell death.

4.4. Prolonged Exposure to DNA Damage Leads to Senescence in Wild-Type Cells.

Having determined that wild-type cells are not dying but undergoing cell cycle arrest in response to DNA damage, the fate of these cells was examined. Emerging studies have suggested that in response to prolonged exposure to DNA damage cells can undergo "DNA damage-induced senescence".[131]

Senescence was originally described with respect to cellular aging.[132,133] As a cell divides its telomeres shorten. After a certain number of cell divisions the shortened telomeres trigger a signaling pathway that induces a permanent form of cell cycle arrest also known as senescence. In fact, most cells in the human body have already undergone senescence.[131] More recently senescence has been associated with oncogene activation and prolonged exposure to DNA damage.[134-136] p21 is believed to be an important protein in mediating the senescence response.[137] In regards to oncogene-induced senescence (OIS) it is believed the low levels of DNA damage caused by oncogene activation activates the DDR, protecting the cell from continuing transformation.[137] This sustained activation of the DDR in response to oncogene activation is akin to prolonged exposure to DNA-damaging agents.

Exposure of wild-type cells with an intact DDR to the DNA-damaging agent Etp for 72 h resulted in the induction of senescence as indicated by positive staining of cells in response to a senescence-detecting agent (Figure 3.4). In contrast, when treated with Etp for 72 h and then subjected to senescence detection p21 null cells stained negative, indicating the absence of a senescence phenotype. These findings are consistent with oncogene activation studies, involving ras activation, which demonstrate p21 to be necessary for senescence induction.[110]

Senescence induction as a means of combating cancer is somewhat controversial.[138] The true definition of senescence, as described with respect to aging, is a form of irreversible cell cycle arrest, essentially rendering the cell inactive. OIS and DNA damage-induced senescence display features consistent with true senescence but the idea of having cancerous cells - which are characterized by the ability to surpass cellular defense mechanisms - present within the body is unappealing to some. Regardless, most cancer cells possess a defective DDR and thus favor cell death over senescence in response to DNA damage, and consequently this is not a major concern when treating cells with DNA-damaging therapeutics.

4.5. Cells with an Impaired DDR Cannot Properly Regulate Cyclin B1 Expression in Response to DNA Damage.

Under normal cell cycle conditions cyclin B1 expression is tightly regulated. Peaking in late G2, cyclin B1/Cdk1 complexes are responsible for entry into mitosis.[39] However, to exit mitosis cyclin B1 levels must quickly be reduced.[39] This reduction occurs through proteolytic degradation of cyclin B1 (discussed further below). In response to DNA damage the regulation of cyclin B1 is more complicated.

Having determined that p21 is necessary for sustained G2 cell cycle arrest in response to Etp-induced DNA damage the effects of DNA damage on the G2-associated cyclin, cyclin B1, were then examined. As seen in Figure 3.6A, p53 is upregulated in response to DNA damage and this upregulation/activation results in the increase of p21 as indicated by western blot analysis. Additionally, increased cyclin B1 levels are seen at 24 h post DNA damage compared to untreated cells. This increase can be accounted for by the increased number of cells present in the G2 phase of the cell cycle (Figure 3.2). More interestingly, although the majority of cells were arrested in G2 at 48 h post Etp treatment these cells had reduced levels of cyclin B1 compared to untreated cells and cells treated for only 24 h. Since this decrease cannot be accounted for by decreased numbers of cells arrested in G2, cyclin B1 levels must be downregulated in response to DNA damage. In contrast to wild-type cells with an intact DDR, p21 null cells with an impaired DDR response are unable to downregulate cyclin B1 levels in response to DNA damage. This inability to downregulate cyclin B1 in p21 null cells is consistent with the inability of these cells to mediate G2 cell cycle arrest and their subsequent death by mitotic catastrophe, as inappropriate activation of the cyclin B1/Cdk1 mitosis-promoting complex has been shown to force the bypass of the G2/M checkpoint and allow cells to accumulate greater than 4N DNA content. These results suggest that p21 is necessary for proper cyclin B1 regulation in response to DNA damage.

To further define the relationship between cyclin B1 and p21, complementary experiments involving p21siRNA knockdown and p21 add back were conducted. When treated with Etp to induce DNA damage, wild-type cells with p21 knockdown were impaired in the ability to downregulate cyclin B1 while control knockdown cells were unaffected in the ability to downregulate cyclin B1 (Figure 3.6B). Additionally, p21 add back into p21 null cells restored their ability to downregulate cyclin B1 (Figure 3.6C). Of note, DNA damage was not necessary for p21 null cells with p21 add back for cyclin B1 regulation. This suggests DNA damage-induced post-translational modifications of p21 are not necessary to mediate cyclin B1 downregulation. Together these experiments confirm p21 is necessary for proper cyclin B1 regulation in response to DNA damage.

Cells lacking p53 or harbouring p53 mutations have also been demonstrated to be impaired in the ability to respond to DNA damage. Specifically, it is reported that p53 null cells are unable to properly mediate cyclin B1 regulation in response to DNA damage.[126,127] In these reports it is suggested that decreased cyclin B1 levels seen in wild-type cells in response to DNA damage are the result of p53-mediated transcriptional repression at the cyclin B1 promoter.[126,127] However, our demonstration that p21 null cells - which possess a functional p53 - show no decrease in cyclin B1 levels in response to DNA damage suggests that this mechanism is not likely. Additionally, while we noted the lack of cyclin B1 downregulation in cells deficient for p53 as reported by others, etopic p21 expression in p53 null cells resulted in downregulation of cyclin B1 protein levels (Figure 3.7).[126,127] Collectively, our data suggest that the deregulated cyclin B1 expression observed in DNA-damaged p53 null cells is due to the inability of these cells to upregulate p21 and subsequently mediate cyclin B1 degradation, and not due to p53 transcriptional repression at the cyclin B1 promoter.

4.6. p21 Mediates the Degradation of Cyclin B1 in Response to DNA Damage.

Although other groups have similarly observed cyclin B1 downregulation in response to DNA damage, this thesis is the first to link this response to p21 upregulation. Two hypotheses could explain the p21-mediated cyclin B1 protein downregulation observed in response to DNA damage, one of which is transcriptional repression at the cyclin B1 promoter and the other involving increased cyclin B1 protein degradation.

As mentioned above, previous studies have suggested p53-mediated transcriptional repression may be responsible for reduced cyclin B1 levels observed in response to DNA damage.[126,127] This hypothesis was rejected since in was found that p21 null cells, which possess functional p53, are impaired in the ability to regulate cyclin B1 and p21 add back restores the ability to regulate cyclin B1. However, it is possible that another protein mediates cyclin B1 transcriptional repression in response to DNA damage. To this end, Quantitative Real Time PCR (Q-PCR) analysis was performed. Q-PCR showed no significant change in the transcription of cyclin B1 after DNA damage as compared to non-damaged cells (Figure 3.8). This suggests transcriptional repression is not the cause of the observed decrease in cyclin B1 protein.

Under normal cell cycle conditions the destruction of cyclin B1 occurs through the proteasomal pathway and is mediated by an E3 ubiquitin ligase complex, anaphase-promoting complex/cyclosome (APC/C). Addition of the proteasomal inhibitor MG132 blocked the decrease of cyclin B1 in DNA damaged cells. Thus, results suggest that increased proteasomal degradation is responsible for the decease in cyclin B1 levels in response to DNA-damage (Figure 2.9). These results suggest p21 mediates the degradation of cyclin B1 in response to DNA damage.

Of note, although HCT116 p21+/+ showed no significant change in cyclin B1 transcription at 24 h and 48 h post Etp-induced DNA damage (as assessed by Q-PCR) it appears as though there is a slight decreasing trend in

transcription. This finding was not completely unexpected, as it has been demonstrated that HCT116 p21+/+ cells begin to enter a senescence-like state at 72 h (discussed above) post Etp treatment. Senescence requires that downregulation of the majority of cell cycle genes and this has primarily been shown to occur through transcriptional repression.[139] Most probably by 72 h post Etp-induced DNA damage QPCR analysis of cyclin B1 would display a significant decrease in cyclin B1 transcription levels as compared to untreated cells. Thus it appears that at earlier time points post DNA damage (i.e.: 24 h and 48 h) protein degradation is the main factor in decreasing cyclin B1 levels but at later time points (i.e.: 72 h+) transcriptional repression becomes more important in cyclin B1 decrease,helping to aid in the induction of senescence.

Different forms of DNA damage differ in the ability to induce p21 upregulation. If p21 is necessary in mediating cyclin B1 degradation, then cells treated with DNA-damaging agents that do not upregulate p21 should display increased cyclin B1 protein levels as compared to cells treated with DNA-damaging agents that do upregulate p21. Consistent with this hypothesis, cells treated with hydroxyurea or UV, which do not induce p21 expression, displayed no changes in levels of cyclin B1 (Figure 3.10.). In contrast, cells treated with Etp or gamma-irradiation (IR), which do induce p21 expression, displayed decreased levels of cyclin B1. These results confirm the necessity of p21 in mediating cyclin B1 downregulation and suggest p21-mediated cyclin B1 degradation is a general response to DNA damage that results in p21 upregulation.

The cellular benefit of complete cyclin B1 degradation in response to DNA damage as opposed to cyclin B1/Cdk1 inhibition is twofold. Firstly, prolonged inhibition of cyclin B1/Cdk1 in response to DNA damage is relatively unreliable and can to lead to slippage through the G2/M checkpoint resulting in the formation of mutant cell with greater >4N. Secondly, depletion of cyclin B1 is required for permanent cell cycle arrest known as senescence (discussed above). In this situation the majority of cell cycle proteins are downregulated, ensuring a permanent G2 phase cell cycle arrest.[139]

Of particular note, recent evidence has implicated p21 in the promotion of the degradation of another important cell cycle protein, Rb.[140] Work by Broude *et al.* has shown that in response to DNA damage, Rb is degraded in a p21-dependent manner, facilitating G1 phase cell cycle arrest.[140] Although the mechanism by which p21 promotes this degradation is yet to be determined, preliminary evidence suggests p21 enhances the proteasomal turnover of Rb, since proteasomal inhibitors blocked Rb degradation. These results show that p21 does in fact have the ability to promote the degradation of cell cycle proteins, further strengthening the hypothesis proposed in this thesis.

4.7. p21 Interacts with Cyclin B1 Following DNA Damage.

Having determined that p21 is necessary for cyclin B1 degradation it is still unclear whether p21 plays a direct or indirect role in the mechanism of cyclin B1 degradation. In this regard, preliminary studies demonstrate p21 co-immunoprecipitates with cyclin B1 at 24 h and 48 h post Etp-induced DNA damage (Figure 3.11A). Furthermore, immunofluorescence studies confirm p21 and cyclin B1 to increase their co-localization following DNA damage. These results suggest p21 may play a direct role in cyclin B1 degradation (Figure 3.11B).

Studies conducted by Charrier-Savournin *et al.* have also demonstrated a p21/cyclin B1 interaction in response to a number of different DNA-damaging agents.[115] They suggest that in response to DNA damage p21 interacts with cyclin B1 and that this interaction localizes cyclin B1/Cdk1 complexes to the nucleus, preventing cyclin B1/Cdk1 activation by Cdc25. However, in response to Etp-induced DNA damage cyclin B1 and p21 seem to co-localize at the outside the nucleus, perhaps at the centrosome. The centrosome is where cyclin B1/Cdk1 complexes become activated to mediate their mitosis promoting activity.[141-143] Given that the majority of cells treated with Etp are in the G2 phase of the cell cycle, it is conceivable that cyclin B1/Cdk1 complexes would be in close proximity to where they function in the G2 phase of the normal cell cycle.

Although many questions are still to be addressed, it may be that in addition to preventing cyclinB1/Cdk1 activation, p21 interaction with cyclin B1 enhances cyclin B1 interaction with the APC/C by putting the two in close proximity and consequently promoting cyclin B1 degradation (discussed further below).

4.8. Summary

Cell cycle arrest is a critical defense tactic in response to DNA damage. When a cell cannot efficiently induce cell cycle arrest, the result is the inappropriate progression through the cell cycle and the accumulation of mutated or damaged DNA – the defining features of cancer. The work described herein suggests that while cells with an impaired DDR are more prone to the development of cancer this impairment can be exploited when treating cancer cells with DNA-damaging cancer therapeutics.

This thesis confirms that cells unable to mediate cell cycle arrest (those with an impaired DDR) in response to DNA damage are more sensitive to DNA-damage-induced cell death. Cell death primarily occurs through mitotic catastrophe, characterized by inappropriate progression through the cell cycle and the formation of small fragmented micronuclei within the cells that cannot protectively arrest the cell cycle in response to DNA damage.

Importantly, the experiments contained within this thesis highlight a previously uncharacterized role for p21 in the degradation of cyclin B1, necessary to sustain G2 cell cycle arrest in response to DNA damage. The degradation of cyclin B1 is proteasome-mediated as its decrease is blocked by the addition of the proteasomal inhibitor MG132. Although the necessity for p21 in cyclin B1 degradation in response to DNA damage is without question, it is still unclear as to whether p21 plays a direct role or indirect role in the mechanism of cyclin B1 degradation. In this regard, preliminary studies described in this thesis demonstrate p21 to immunoprecipitate with cyclin B1 following DNA damage. Consistently, p21 and cyclin B1 were also demonstrated to co-localize following DNA damage. Together these results suggest p21 may play a direct role in cyclin

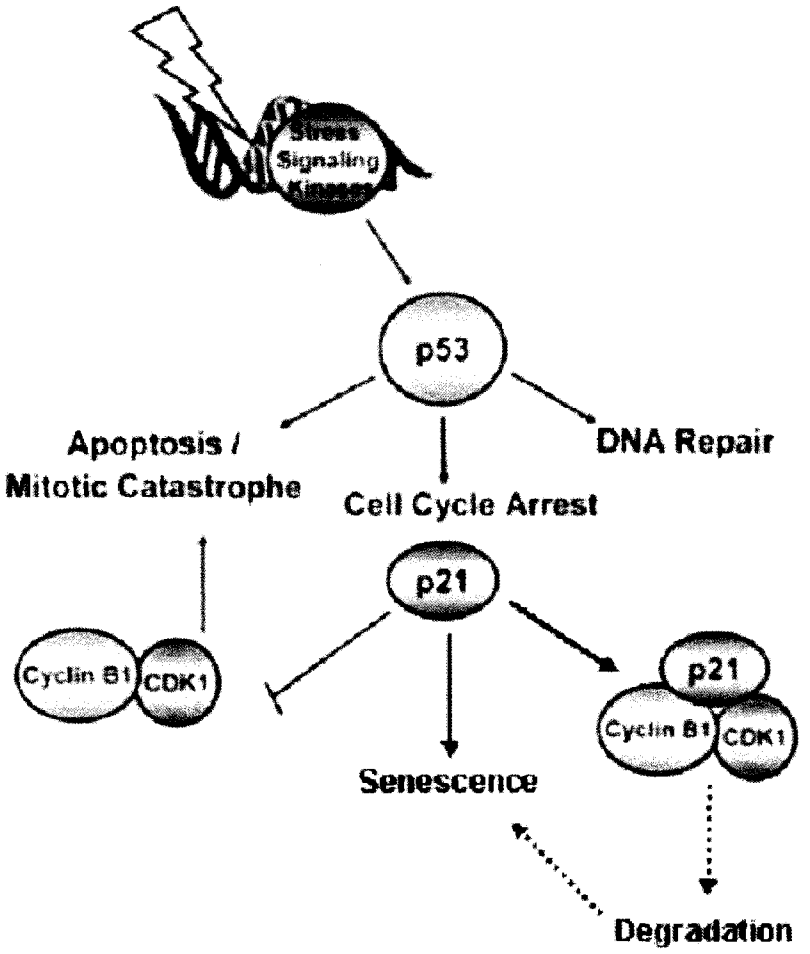

Figure 4.1. p21 Role in the Mammalian DNA Damage Response (DDR). In response to DNA damage, stress-signaling kinases/proteins are activated and signal to the tumour suppressor protein p53. p53 is activated to mediate the transcription of various p53 effector genes that are involved in cell cycle arrest, apoptosis and DNA repair. The cyclin-dependent kinase inhibitor p21 is the principal mediator of cell cycle arrest. p21 upregulation is necessary for G1 cell cycle arrest and maintenance of G2 cell cycle arrest in response to DNA damage. p21 aids in G2 cell cycle arrest by promoting the degradation of the G2-associated cyclin, cyclin B1, thus preventing inappropriate continuation through the cell cycle resulting in mitotic catastrophe. Cyclin B1 degradation may require it's interaction of p21 but the exact mechanism of cyclin B1 degradation in response to DNA damage is yet to be determined. Prolonged exposure to DNA damage results in the induction of p21-dependent senescence.

B1 degradation necessary for sustained G2 cell cycle arrest in response to DNA damage (Figure 4.1).

4.9. Future Directions: Determining the Mechanism of Cyclin B1 Degradation

4.9.1. Separating Cell Cycle Arrest and Cyclin B1 Degradation Functions of p21.

Having demonstrated that p21 and cyclin B1 interact following DNA damage it is still unclear if this interaction is necessary for cyclin B1 degradation. It may be that that p21-mediated cell cycle arrest alone is enough to trigger cyclin B1 degradation. p21 interaction with cyclin B1 observed following DNA damage may be a result of p21's ability to bind a number of different cyclins and of little consequence to the degradation of cyclin B1.

To address this question it is essential to separate the cell cycle arrest function of p21 from its ability to interact with cyclin B1. To this end, p21 protein mutants can be created. As mentioned in the Introduction (Chapter 1), p21 possesses two cyclin-binding regions and a Cdk-binding region (the K site) (Figure 1.6). These regions can be manipulated to destroy the cell cycle arresting properties of p21. These p21 mutants can then be transfected into p21 null cells to determine if p21 can interact with cyclin B1 and if cyclin B1 degradation is restored in response to DNA damage. Of note, addition of DNA-damaging agents may not be necessary, as add back of p21 into p21 null cells did not require DNA damage to mediate cyclin B1 downregulation (Figure 3.6B). If a mutant can be generated that does not mediate cell cycle arrest but still binds cyclin B1 and cyclin B1 levels are decreased in response to DNA damage, then cell cycle arrest is not necessary for cyclin B1 degradation.

In theory these experiments can be easily conducted, however; closer inspection of p21 function reveals some problems that may arise. p21 mediates cell cycle arrest by binding cyclin/Cdk complexes with its Cdk-binding and cyclin-binding regions. Hence it is conceivable that p21 uses these same regions to

bind cyclin B1. Thus, abolishing p21's cell cycle arresting properties may also abolish its ability to bind cyclin B1 and these two functions may not be separable. More extensive experiments may have to be designed.

4.9.2. Determine if Cyclin B1 is Degraded by the APC in Response to DNA Damage.

As mentioned above, under normal cell cycle conditions cyclin B1 protein turnover is mediated through interaction with an E3 ubiquitin ligase, APC.[39,52-57] The APC controls the passage through the cell cycle by specifically targeting cell cycle proteins for proteolysis. Two activating receptor subunits can be associated with the APC, cdc20 and cdh1, and are able to control APC substrate specificity.[39,53,55] Both APC^{cdc20} and APC^{cdh1} have been shown to target cyclin B1 for ubiquitination (and thus degradation) during normal cell cycle progression, suggesting they may also play a role in DNA damage-induced degradation of cyclin B1.[53,56,58]

Following DNA damage, IP experiments can be conducted to determine whether cyclin B1 is indeed interacting with the APC or its components. Additionally, ubiquitination studies can be conducted to demonstrate cyclin B1 ubiquitination in response to DNA damage.

4.9.3. Determine if p21 Promotes the Interaction of Cyclin B1 with the APC in Response to DNA Damage.

Interestingly, a new report by Amador *et al.* has found that p21 is able to interact with APC^{cdc20}.[144] They show that in the normal cell cycle APC^{cdc20} binds p21 in the G2 phase. This interaction results in the alleviation of p21-mediated transient arrest in the G2 phase by triggering p21 ubiquitination and degradation, allowing for the continued passage from G2 to M phase. Given that p21 has the ability to interact with APC^{cdc20} and that APC^{cdc20} is known to mediate cyclin B1 degradation, it may be that in cases of DNA damage p21 promotes the interaction of cyclin B1 with APC^{cdc20} and thus promotes cyclin B1 degradation.

This would explain why no cyclin B1 degradation occurs in response to DNA damage in cells lacking p21.

To explore this hypothesis further IP studies can be conducted with p21 and APC components including the adaptor protein cdc20. Having determined that a population of p21 interactes with cyclin B1 following DNA damage (Figure 3.11), further co-IP studies involving p21, cyclin B1 and APC components in various combinations would provide evidence as to the protein complex formed within the cell in response to DNA damage.

4.10. Concluding Remarks

In conclusion, although p21 mutations are relatively rare in cancers p53 mutations are very common, and cells with mutant p53 are impaired in the ability to upregulate p21 in response to DNA damage. This thesis highlights the implications of an intact p53-p21 DNA damage-sensing pathway in the treatment of cancers with DNA-damaging agents and provides insight into the dual role of p21 as both a tumour suppressor and an oncogene.[145]

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