# Circadian Control of Cell Cycle Progression

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(ABSTRACT)

Tumorigenesis is the result of uncontrolled cell growth due to the deregulation of cell cycle checkpoints [1]. Period 2 (Per2) is a tumor suppressor that oscillate in expression in a 24-hour cycle [2, 3]. Here, we show that Per2 interacts with the tumor suppressor protein p53. Both G1 and G2 checkpoint pathways involve a p53 dependent pathway which can trigger the cell to go through cell arrest or programmed cell death[4]. Understanding all the mitigating factors involved in regulating cell cycle progression under DNA damage can offer a better idea in how cells become immortal.

Initially discovered through screening of a human liver cDNA library, the novel interaction between p53-Per2 was further documented using co-precipitation. Interestingly, under genotoxic stress conditions, p53 and Per2 were not found to bind which leads us to suspect that Per2 does not affect active p53 which may possibly be due to post translational modifications of its active state. Furthermore we investigated p53's ability to act as a transcription factor in the presence of Per2, showing that the Per2-p53 complex prevents p53 from binding to DNA. This implies that the tetramerization of p53 may also be another factor in Per2's ability to bind to p53. A truncated p53 lacking the last 30 amino acids that theoretically increase p53's ability to form a tetramer showed a drastic reduction in binding to Per2 [5, 6]. On the other hand, p53 lacking the tetramerization domain showed binding similar to wildtype. Consequently we speculate that the ability of Per2 to modulate p53 and act as a tumor suppressor protein may be dependent on either the post translational modifications of p53 or its oligomeric state.

## Dedication

I would like to dedicate this to my grandmother, her kindness and presence during my early childhood continue to permeate through me. I love you.


## Acknowledgements

First and foremost I would like to express gratitude towards our post-doc Jianhua, for her infinite patience and insightfulness surrounding my project. In addition, I would like to also acknowledge her work and determination in helping me to finalize my thesis. Furthermore, I thank everyone in lab for their friendship and support, my family away from home. Last but not least I would like to acknowledge by mentor Dr. Carla V. Finkielstein, who in many ways has pushed me to reach my potential as a researcher. I felt honored to be around her passion for science and determination to achieve nothing but greatness, I could not have asked for a better thesis advisor.

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**Abbreviations**

ATM Ataxia–telangiectasia mutated
ATR Ataxia–telangiectasia and rad3-related
BMAL1 Brain and Muscle ARNT-Like 1
BRCA1 Breast cancer gene 1
CaM Calcium-calmodulin
Cdc2 Cell division cycle 2
Cdc25A Cell division cycle 25A
Cdc25C Cell division cycle 25C
Cdk2 Cyclin dependentkinase 2
Chk1 Checkpoint kinase-1
Chk2 Checkpoint kianse-2
Ck1ε Casein kinase epsilon
CreB cAMP response element binding protein
Cry Cryptochrome
EMSA Electrophoretic Mobility
FACS Flourescence activated cell sorter
Fancd2 Fanconi anemia, complementation groupu D2
Gadd45 Growth arrest DNA damage inducible
GST Glutathion-S transferase
hp53 human p53
hPer2 human Period 2
HPV Human Papillomavirus
Htt Huntington protein
MCF-7 Breast Cancer Cell Line
Mdm2 Murine double minute 2 protein
Mre11 Meiotic recombination 11
Myt1 Myelin transcription factor 1
Nbs1 Nijmegen breakage syndrome 1
Per1 Period 1
Per2 Period 2
Rev-erbα
Rb Retinoblastoma protein
RHT Retinohypothalamic tract
SCN Superchiasmatic nucleus
Smc1 Structural maintenance of chromosomes 1
ZT Zeitgeber

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## Chapter 1: Background and Significance

### A. Sensor Mechanisms in the Cell Cycle

DNA damage can occur in most, if not all cells at some point in their lifetime; what defines a cell as tumorigenic or not is how the cell cycle checkpoints resolve the DNA damage. Proper progression of the cell subsequent to genotoxic stress will depend on whether the cell is able to repair the DNA during arrest or enter programmed cell death to prevent the DNA damage from passing onto its progeny.

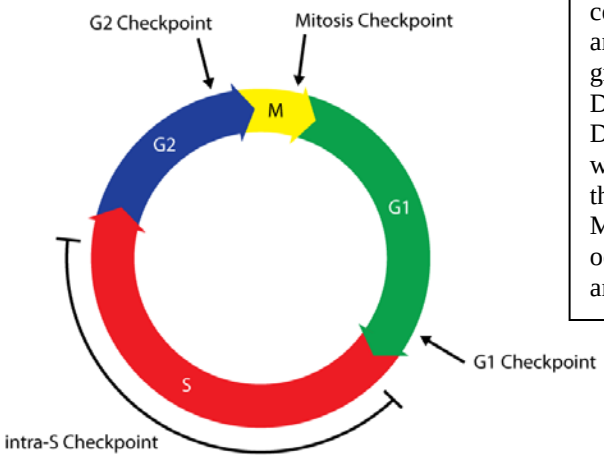


**Figure 1.1** representation of cell cycle phases (G1, S, G2 and M). G1 refers to cell growth and preparation for DNA replication. In S phase DNA replication occurs while G2 is the phase where the cell prepares for mitosis. M phase is where cytokinesis occurs. Cellular checkpoints are indicated.

### A.1 G1 phase checkpoints

In response to DNA damage, the sensor kinases ataxia–telangiectasia mutated (Atm) and ataxia–telangiectasia and rad3-related (Atr) are autophosphorylated and act on downstream tumor suppressor proteins [7]. While Atm activates in response to DNA double stranded breaks, Atr specifically senses single stranded DNA damage near replication forks by binding to lesions of DNA that have high amounts of replication protein A, a single strand DNA binding protein [8]. In an unstressed environment, cells that lack Atm still progress through the cell cycle, showing that it is a key elements only if DNA damage occurs [9]. In contrast, in the absence of Atr leads mice to early embryonic death [10].

In the event of double-strand break during G1, Atm predominantly phosphorylates the checkpoint kinase 2 (Chk2), while Atr acts on Chk1 [9]. Like Atm, Chk2 null mutation does not impact the cell cycle in the absence of genotoxic stress. Both Atm and Chk2 work together to

phosphorylate downstream substrates to activate cell cycle repair mechanisms. Among the various tumor suppressor proteins linked to the Atm/Chk2 axis is p53, a transcription factor, that either activates or inhibits genes involved in cell cycle arrest and programmed cell death. Chk2 phosphorylates p53 at Ser15 and Ser20 leading to its increased stabilization [11]. Through phosphorylation, Atm inactivates murine double minute 2 (Mdm2), a ubiquitin ligase, responsible for negatively regulating p53 and shuttling it to the cytosol for proteosomal degradation after ubiquitination [12]. In G1, cycle arrest occurs through the direct regulation of p21 by p53. p21 binds and inhibits the activity of the cyclin E-Cdk2 complex [13]. Inactive cyclin E-Cdk2 prevent the phosphorylation of the retinoblastoma protein, a factor that when unphosphorylated binds to E2F and prevents it from activating genes responsible for S-phase entry (Figure 1.2).

Cell cycle arrest in G1 is also controlled by the inhibitory phosphorylation of Cdk2, rendering it inactive to promote S phase entry. Dephosphorylation of Cdk2 is controlled by the phosphatase Cdc25A [14]. In the event of DNA damage, Chk1phosphorylates Cdc25A for degradation, the lack of Cdc25A leads to prolonged inhibition of cyclin E-Cdk2 complexes and therefore temporary arrest in G1 (Figure 1.2). Thus, DNA damage repair is facilitated through the inhibition of cyclin E-Cdk2 from activating genes for progression into S phase.

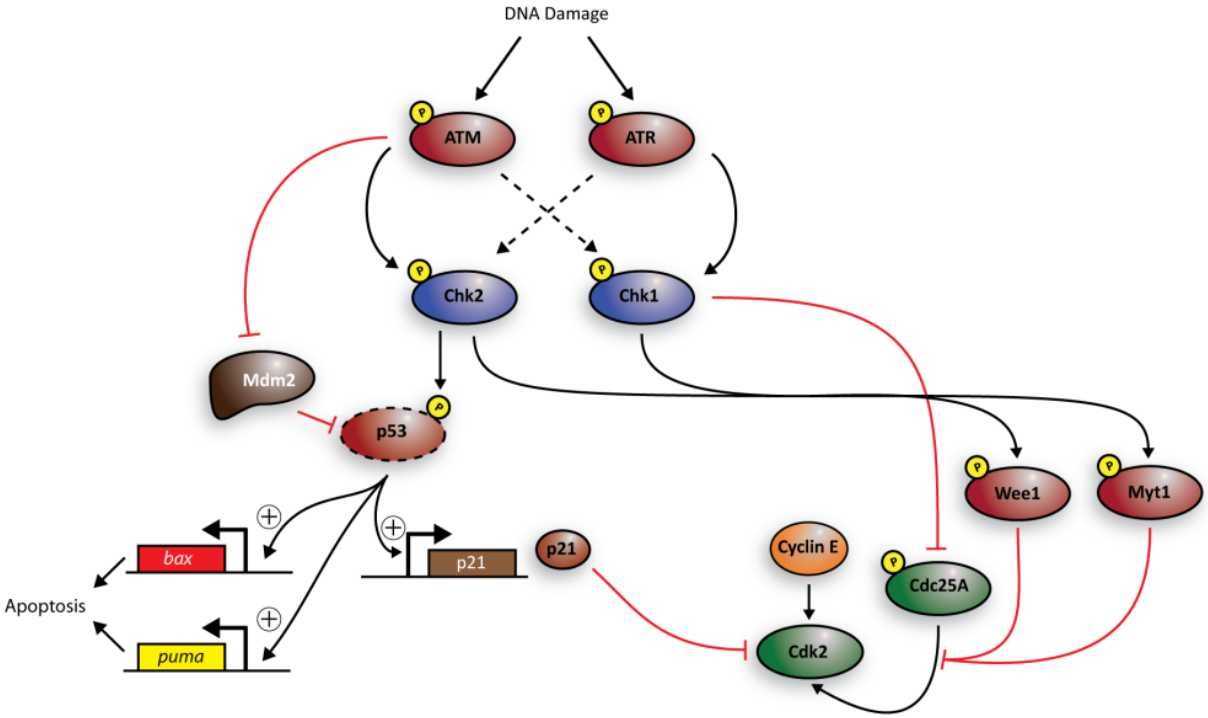


**Figure 1.2:** In response to genotoxic stress Atm and Atr phosphorylate Chk2 and Chk1, respectively. In a p53 dependent pathway, either Chk2 phosphorylates p53 increasing its stability while Mdm2 is inhibited from targeting p53 for proteasomal degradation. The tumor suppressor p53 acts on downstream genes involved in apoptosis and cell arrest. Both Chk1 and Chk2 activate kinases involved in phosphorylating Cdk2 such as Wee1 or Myt. Cdc25A is deactivated by Chk1 phosphorylation and translocated to the cytoplasm.

### A.2 S phase checkpoints

In the event of DNA damage during S phase, the cell delays DNA synthesis by preventing the firing of new origins of replication. However, any existing replication forks before the induction of damage will continue uninhibited. Therefore, the delay during S phase is more transient, also known as the intra-S phase checkpoint. The breast cancer associated 1 protein (Brca1) is a downstream effector of Atm [15]. Brca1 binds to Rad51, known for homologous recombination repair, and localizes towards the nuclei foci [16]. DNA damage during S phase is more effectively repaired through homologous recombination, due to the duplicate copy of DNA from the sister chromatids [7].

In the event of DNA double stranded breaks, along with Atm activation is the formation of a protein complex which consists of the nijmegen breakage syndrome 1 protein (Nbs1)-Rad50 and meiotic-recombination protein-11 Mre11, (MRN) [7]. The MRN complex identifies DNA lesions independent from Atm and attracts more activated Atm to the site [17]. The recruitment of Atm increases the phosphorylation of the structural maintenance of chromosomes 1 (SMC1), a protein found to be part of a complex called cohesin that prevents to premature separation of sister chromatids [18]. At the site of double stranded breakage, Atm phosphorylates histone H2AX triggering a modification of the chromatin that leads to binding of the mediator of DNA-damage checkpoint-1 (Mdc1) to the foci [7]. Mdc1 then recruits more MRN complexes, thus recruiting more Chk2 to phosphorylate Smc1 further activating the intra-S checkpoint delay (Figure 1.3) . [7]

During S phase, Cdk2-cyclin A is responsible for the firing of new origins. The intra-S phase checkpoint also mediates transient arrest through the Atr/Chk1 pathway by phosphorylating Cdc25A for proteasomal degradation which otherwise would increase Cdk2 activity. The inhibition of Cdk2 prevents the firing of new replication origins which temporarily delays DNA the completion of DNA synthesis [7].

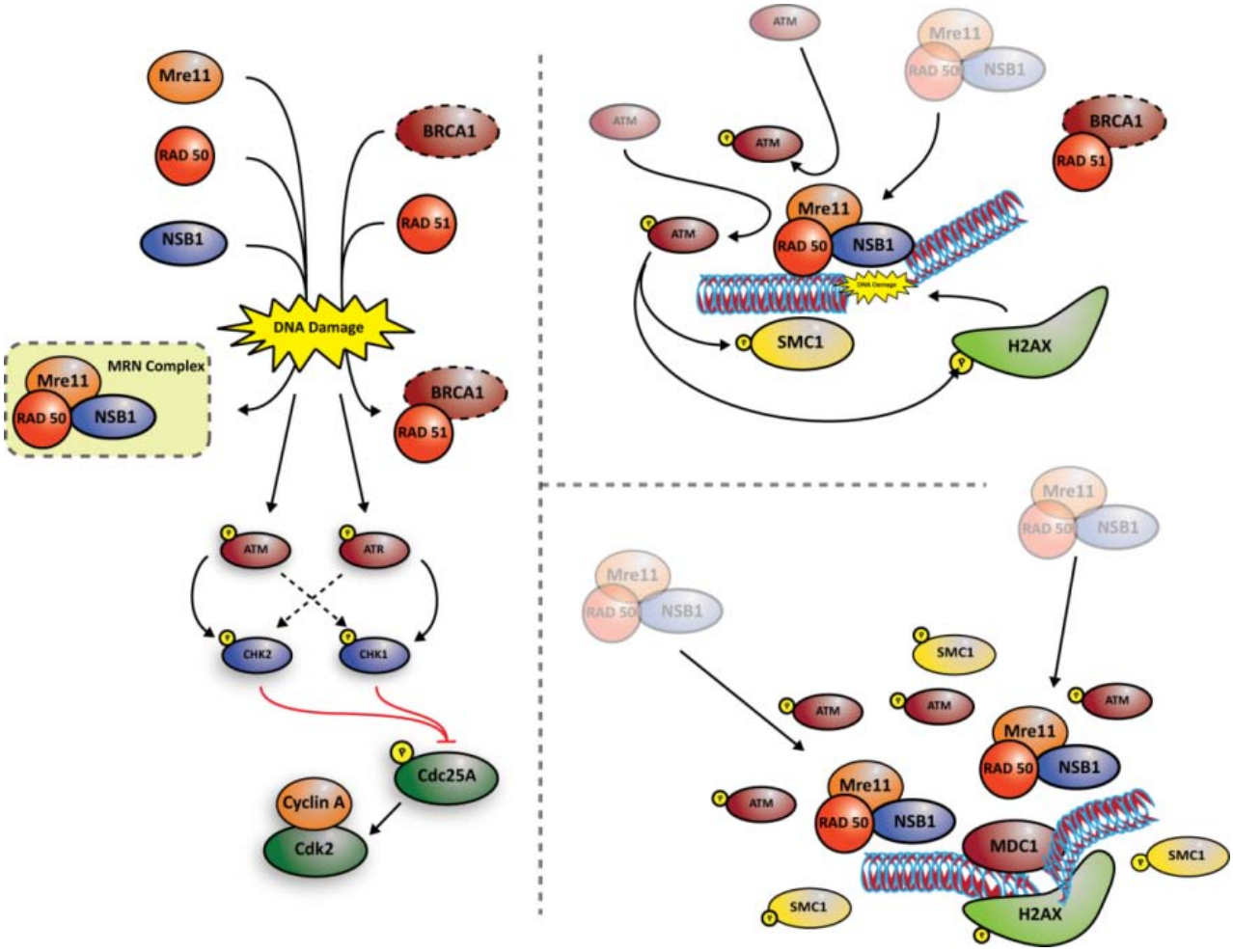


**Figure 1.3:** In response to DNA damage during S phase, Mre11, Rad50 and Nsb1 form the MRN complex and Brca1-RAD51 form a complex. The MRN complex localize to the nuclear foci and recruits more Atm for autophosphorylation which then phosphorylates Smc1 and H2AX. The chromatin modification by phosphorylated H2AX causes Mdc1 to bind to the nuclear foci recruiting more MRN complexes to amplify the checkpoint signaling. The Atm/Atr-Chk2/Chk1 pathway is involved in preventing Cdc25A's ability to release the inhibitory phosphorylation found on Cdk2.

### A.3 G2 phase checkpoints

Cells in G2 have completed DNA replication and are in preparation for mitosis, which is largely controlled by the activity of cyclin B/Cdk1 kinase [19]. Similar to Cdk2, Cdk1 contains sites for phosphorylation at Ser14 and Tyr15 that causes inhibition of the cyclin B-Cdk1 complex by kinases Wee1 and Myt1 respectively [20]. In response to DNA damage either Chk1 or Chk2 can activate the Wee1/Myt1-Cdk1 pathway by phosphorylating Wee1 and Myt1 and increasing their kinase activity similar to how Wee1/Myt1 inhibits Cdk2 in G1 phase [21, 22]. On the other hand,

phosphatase Cdc25C dephosphorylates Cdk1 kinase at the same sites Wee1 and Myt1 phosphorylate [23]. Therefore, progression to mitosis is partly governed by the ability of Cdc25C to dephosphorylate Cdk1 at Ser14 and Tyr15, but in reponse to DNA damage Chk2 can phosphorylate Cdc25C at Ser216 and promote its transport to the cytoplasm where it is ineffective [13]. After phosphorylation of Cdc25C by Chk2, 14-3-3σ, a transporter protein translocates the phosphatase out of the nucleus. In addition, p53 can repress the transcription of cyclin B, the necessary factor for Cdk1 kinase before it can activate genes for progression into mitosis (Figure 1.4).[24]

G2 phase checkpoint can also be regulated in a p53-dependent manner. Discussed earlier in G1 phase checkpoints, p53 can activate the transcription of p21 in G1 or G2 to negatively regulate the cyclin B/Cdk2 complex. Another downstream effector gene upregulated by p53 is Gadd45α, which functions by disassociating the cyclin B/Cdk2 complex [25]. Furthermore, p53 can repress the transcription of cyclin B, a factor for Cdk1 kinase to activate genes for progression into mitosis [24] (Figure 1.4).

G2 checkpoints serve as the last DNA damage sensory mechanism before mitosis occurs. Prolong arrest is mediated by manipulating the Cdk1 kinase activity. Cells that pass this stage with DNA damage unrepaired may more likely to pass mutated DNA to progeny and to increase genomic instability.

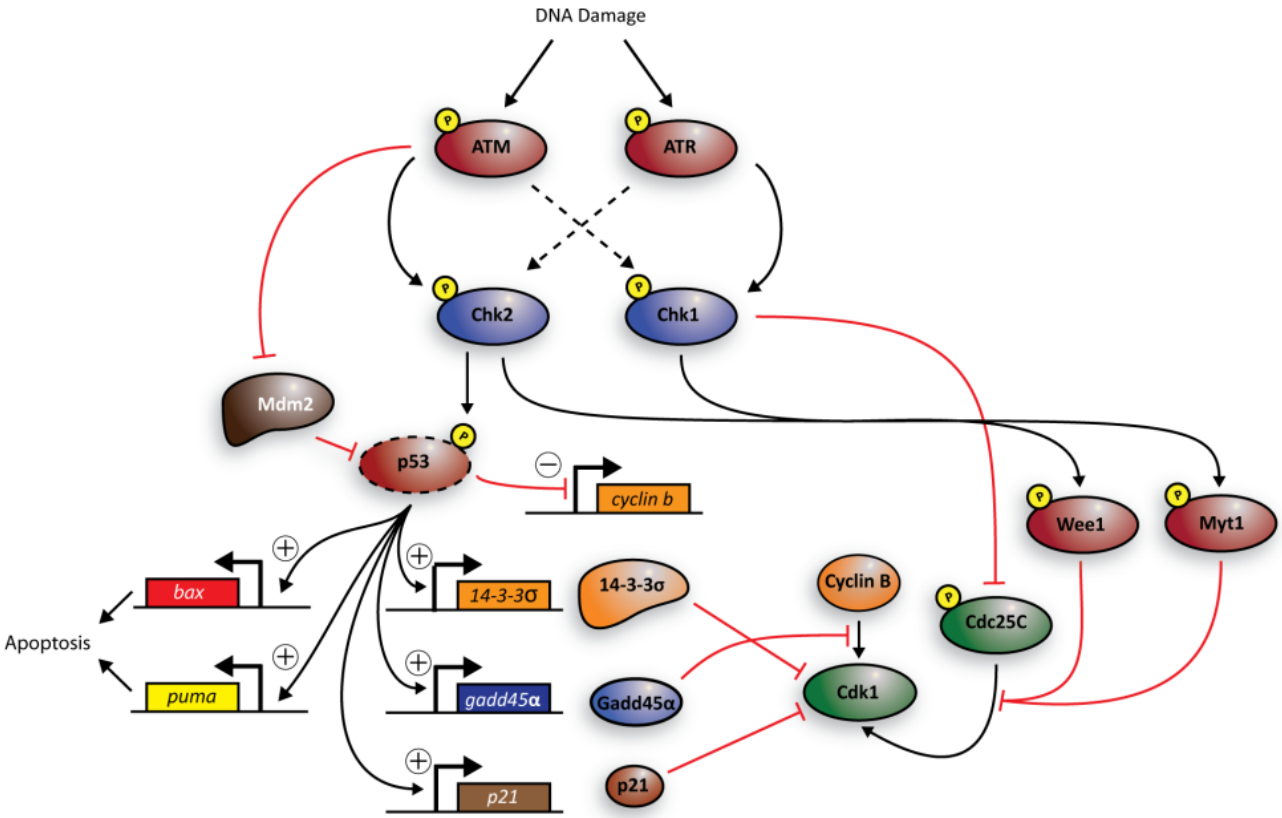


**Figure 1.4:** DNA damage during G2 phase increases the stability of p53 and therefore increases the regulation of downstream genes involved in cell arrest and apoptosis. WEE1 and MYT1 are activated in the p53-independent pathway by phosphorylating Cdk1 and inhibiting its activity for G2/M progression. CHK1phosphorylates Cdc25C, the phosphotase required to release the inhibitory phosphorylation caused by WEE1/MYT1, and translocated out of the nucleus by 14-3-3σ.

## B. The Tumor Suppressor p53

### B.1 Molecular architecture of p53

p53 is a 393 amino acid regulatory protein consisting of five domains (Figure 3.2). The first, the transactivation domain, comprise N-terminual 42 residues and serves as a site where basic transcriptional proteins can bind to and facilitate gene activation of p53's transcriptional targets [4]. A proline rich domain (residues 60-90) consists of five PXXP motifs (where P and X are proline and any residue, respectively) which are crucial to p53's ability to activitate apoptosis [26]. The domain that directly interacts with DNA resides in residues 102-292. A tetramerization domain (residues 324-355) is involved in oligomerization around p53 response elements [27]. The

fourth domain resides in the last 30 amino acids (364-393), where post translational modifications appear to change the proteins affinity for p53 response elements [28].

**B.2 Regulation of p53 protein function**

The ability of p53 to act on downstream effectors is fully dependent on its overall protein level within the nucleus, which is tightly controlled at low levels by Mdm2. In response to DNA damage, Mdm2 becomes less effective in shuttling p53 for proteasomal degradation due to its phosphorylation by Atm at Ser395 [29]. In addition, Chk2 phosphorylates p53 at Ser20 to interfere with Mdm2 binding, resulting in decreased ubiquitination of p53 [11].

Post translational modifications of p53 serve to regulate its control of cell cycle arrest or pro-apoptotic genes. The mutation of Lys120 to Arg renders the site useless for appropriate acetylation, resulting in loss of p53's ability to regulate apoptosis but not cell cycle progression [30]. Phosphorylation of p53 at Ser 46 occurs when the apoptotic pathway is induced [31].

**B.3 Diseases related to p53 malfunctioning**

About fifty percent of all human cancers contain a mutation in the p53 gene, *tp53* [32].The predominant mutations are missense mutations within the DNA binding domain, highlighting the importance of p53 as a transcription factor [33, 34]. Those diagnosed with colorectal and ovarian cancer have the highest percentage of mutations in *tp53* [34]. In addition, mutations in p53 have been implicated in lymphoid node negative breast cancers, acting as a possible independent marker for false-negative prognosis of breast cancer related diseases [35].

Li-Fraumeni's syndrome is a rare genetic disease where patients have a higher susceptibility to breast cancer, leukemia, osteosarcoma, brain tumors and colon cancer early in life. Eighty percent of those who are diagnosed with Li-Fraumeni's syndrome have mutations in *tp53* [36]. The inheritance of one copy of the mutated gene is sufficient for a high risk of early-onset of cancer. Mutations that change the consensus site for pre-mRNA splicing of p53 leading to a nonfunctional p53 also show the same symptoms of early-onset to cancer as mutations in the DNA binding domain [36].

Mutations in proteins involved in regulating p53 stability can also have a drastic effect on the cell's fate. The gene amplification of Mdm2 causes a significant decrease in levels of p53 and are associated with high incidence of sarcomas and brain tumors [37, 38]. In addition, the deletion of the gene $p14^{ARF}$, whose product binds Mdm2 and prevents ubiquitination of p53, causes brain, lung and breast cancer [37].

p53 has also been implicated in Huntington's disease, a neurodegenerative disorder, leading to motor and cognitive thinking deficits. The high glutamine repeats found in the amino terminus of the hungtington protein (Htt) is responsible for the deterioration of neurons and is also found to bind p53. It is theorized that p53's proline rich domain interacts with Htt causing p53 to gear towards apoptosis [39]. Neurodegenerative diseases such as Alzheimer's show upregulation of p53, linking possibly to the activation of premature cell programmed cell death [40, 41].

The human papillomaviruses 16 and 18 produce a protein called E6 that binds to p53 and promotes its proteasomal degradation through the ubiquitin pathway. Cells infected with either type 16 or 18 abrogate the G1 checkpoint and lose the ability to go through apoptosis in the event of DNA damage [42]. Accordingly, in the general population over 50% of women who have cervical intraepithelial neoplasia of grade 3 are infected with HPV 16 [43]. This finding underscore the importance of p53 as a tumor suppressor protein.

The tumor suppressor p53's various links to a diverse set of diseases exemplify its function for cell viability. It is essential that the sequence specific DNA binding domain remain intact, and other factors that regulate p53 remain normal otherwise p53 deregulated can either cause a cell to become immortal or enter early programmed cell death.

## C. Circadian Rhythyms

### C.1. Physiology

Circadian oscillations of proteins have been found throughout various tissues in mammals [2, 44]. While most tissues have circadian oscillating proteins, cells found in the superchiasmatic nucleus (SCN) have the capacity to synchronize all other slave oscillators within the body [45, 46].

Sleep/wake cycles, heart, liver and smooth muscles can be all entrained by the SCN through either humoral or neuronal signaling [47]. Pineal glands of chicks show circadian oscillation of melatonin secretion of an important hormone governing sleep cycles [48].

Liver extracts from mice with surgically ablated SCN showed an apparent dampening of expression peaks of clock gene transcripts versus non ablated mice (Akhtar et al., 2002). This signifies an apparent connection with how peripheral organs can be entrained indirectly by light through the functions of the mammalian pacemaker, SCN. Fibroblast culture cells such as NIH-3T3 can also be induced to oscillate clock gene expression when serum shocked, implying that some constituent of serum may act as an endocrine signal between master clocks and peripheral

clock oscillators(Balsalobre et al., 1998). Pineal glands of chicks show circadian oscillation of melatonin secretion, an important hormone for governing sleep cycles.

One of the outcomes of synchronization leads to circadian controlled expression of disaccharidases within the digestion to anticipate scheduled food uptake and more immediate breakdown of food for energy [49]. This is an example of how cells in a tissue can interpret time by changing clock protein expressions to form a scheduled output based on a 24 hour cycle.

**C.2. Core molecular signaling**

Photic entrainment is thought to begin at the retina ganglion cells located along the retinohypothalamic tract (RHT) that entrain the SCN to wavelengths between 480-511 nm [50]. Although the SCN is entrained by light, peripheral organs such as the liver can be both entrained by the SCN but more dominantly by food uptake [51].

As result of photic entrainment, glutamate is released from ganglion cells and transported towards the SCN via RHT[52]. Glutumate binds to glutamate receptors found within the neuron cells of the SCN and stimulate the release of calcium. Calcium activates Ca2+/calmodulin-dependent kinase (CaM) kinase II and IV, a kinase that phosphorylates cAMP response element binding protein (Creb) before Creb can activate downstream circadian genes [53, 54]. In response to the induction of pCreb, Period 1(Per1) and Per2, negative clock gene regulators, are acutely activated by light due to their promoters containg cAMP response elements. Patients who suffer familial advance sleep syndrome, where sleep/wake cycles are phase shifted 4-5 hours, share a mutation in Period 2 (Per2) that disrupts the stability of the protein [55, 56].

The circadian clock is composed of positive and negative feedback loops that interact with each other to form a cyclical 24 hour period of oscillating protein expression. Bmal1 binds Clock through a basic helix-loop-helix Per-Arnt-Sim (PAS) domain shared by both Clock and Bmal1. Furthermore, the Clock/Bmal1 heterodimer binds to E-boxes with nucleotide sequences of CACGTG and modulates their transcription [57]. Downstream clock genes such as *per2,* cryptochrome (*cry)*, and *rev-erbα,* all contain E-boxes that are positively regulated by Clock/Bmal1 (Figure 1.5). [58]

In contrast Per2/Cry and Rev-erbα are negative feedback regulators of their own transcription. As result of Per2 and Cry transcription, they accumulate in the cytoplasm until they form heterodimers and they re-enter the nucleus to bind to the heterodimer Clock/Bmal1 and disrupt their interaction with E-boxes found in downstream clock genes (Figure 1.5). [59] The

stability of Per2 is also governed by Casein kinase-1ε, a kinase that phosphorylates Per2 for proteasomal degradation before significant level of Cry is expressed and binds to Per2 to prevent this [60]. In addition, Rev-erbα acts by repressing Bmal1 on the transcriptional level, acting as another negative feedback pathway. As the negative feedback loop becomes dominant due to the translocation of Cry, Per and Rev-erbα to the nucleus, it will then prevent its own transcription from occurring. The reduced levels of mRNA transcription of Per, Cry, and Rev-erbα results in an eventual loss of the negative feedback loop [2]. Bmal1's rise in expression is then mediated by a competing protein of Rev-erbα called RORα. The RORα protein competes for the same ROR element that Rev-erbα binds to, but increases rather than represses Bmal1's expression [61] (Figure 1.5).

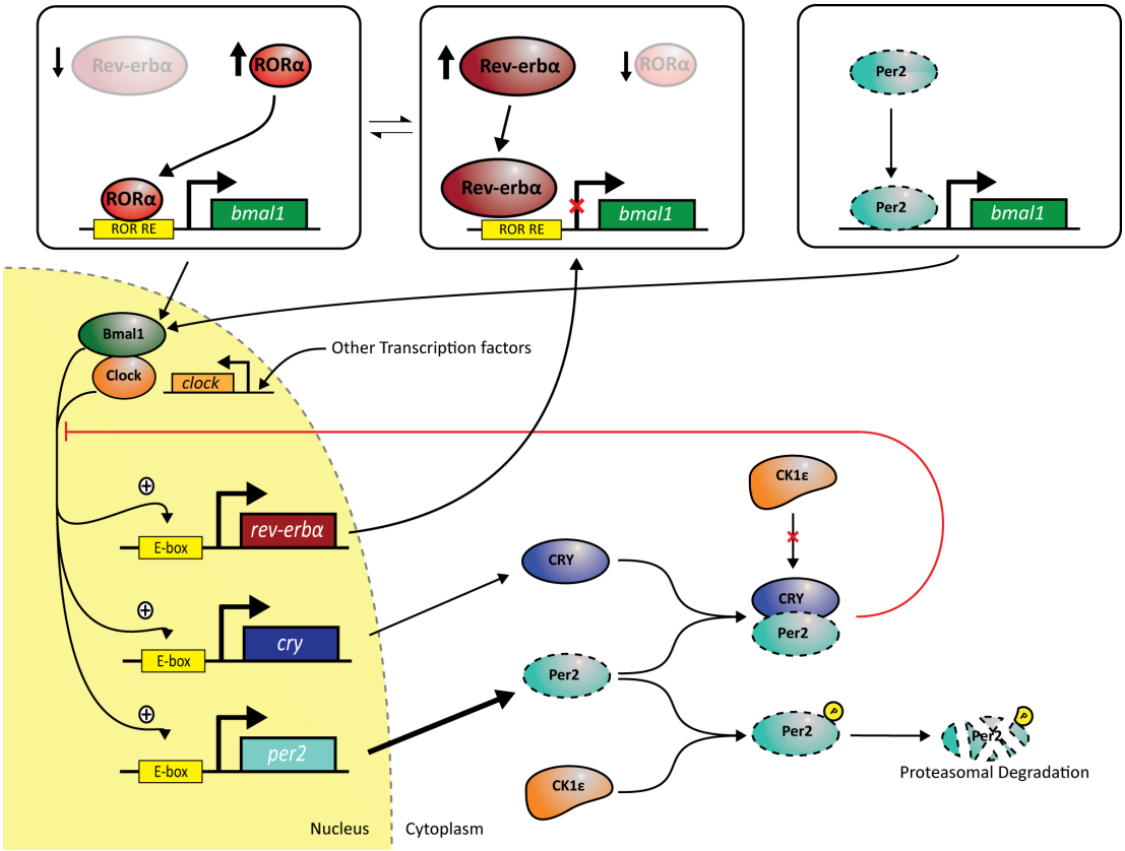


**Figure 1.5:** The mammalian circadian clock. Clock/Bmal1 form a heterodimer and bind to E-box elements found within *rev-erbα*, *per*, and *cry* genes and positively regulate transcription of these genes. Before Per2 is bound to Cry, Per2 is phosphorylated by CK1ε for proteasomal degradation. When Cry and Per2 bind they translocate to the nucleus to inhibit the activity of Clock/Bmal1's transcriptional regulation. RORα positively regulates the transcription of *bmal1* while Rev-erbα negatively regulates it by competing for the same ROR response element. Per2 alone can acutely activate the transcription of *bmal1*. Clock is constitutively expressed and controlled by other transcription factors not affiliated with circadian oscillation.

Mutations or deletions in circadian genes that lead to arrhythmicity can hamper the efficiency of cell division or even cause complications in cell viability. Mice deficient in *cry,* have increased transcription of *wee1,* a known kinase that phosphorylates Cdk1 at Tyr15 and Thr14 and arrests the cell cycle at the G2 /M boundary [62]. The promoter of *wee1* contains E-boxes that interact with Clock/Bmal1 and when bound together increase transcription [62]. The increase in phosphorylation results in an increase in the time taken to complete regeneration of the liver in *cry*-deficient mice versus wildtype mice [62]. Interestingly, the peak of mitosis is delayed 4-8 hours in livers where partial hepatectomy was done at ZT0 as opposed to ZT8 (ZT, Zeitgeber time in a 12 hour light-12 hour dark). The extra 8 hours of light that the ZT0 partial hepatectomy was exposed to correlated to the delay in its mitotic peak, implying how cell division occurs in concert with light/dark cycles. This research highlights how genes outside of circadian protein network can be controlled in a circadian manner.

Fu and colleagues showed that lack of a functional Per2 in mice led to a higher incidence of tumor formation [3]. To understand the differing sensitivity to genotoxic stress, they performed a FACS analysis showing that $Per2^{m/m}$ mice result in a loss of apoptosis leading to the perpetuation of cells becoming tumorigeneic. Secondly, the quick increase in p53 levels after IR were nonexistent in $Per2^{m/m}$ mice, inferring that the lack of apoptosis in $Per2^{m/m}$ may be the result of p53 being unable to act as a transcription factor to induce proapoptotic genes. NPAS2, paralog of Clock, when bound to Bmal1, suppresses the activity of C-Myc, an oncoprotein involved in apoptosis and cell proliferation [3]. In summary, Per2 may play a role as a tumor suppressor protein, by either indirectly acting on C-Myc through Clock/Bmal1 or possibly as a mitigating factor in p53's response to DNA damage.

Per1 overexpression causes both increased rate of apoptosis and upregulation of C-Myc. Interestingly, overexpression of Per1 causes phosphorylation of Chk2 in the absence of DNA damage. To justify Per1's indirect relationship with Chk2, it was discovered through coimmuniprecipiation, that Per1 can actually bind to either Chk2 or Atm [63]. Therefore, Per1 possibly mediates effective phosphorylation of checkpoint proteins and sensitizes cells to irradiation [63]. Real-time RT-PCR shows that Per1 mRNA is downregulated in cancer cells [63]. In conclusion, Per1 acts as a possible mediator of Chk2 phosphorylation by Atm, therefore implicating clock genes as part of the cell cycle regulation protein network.

Human Timeless protein (hTim), an orthologue of the circadian clock protein TIM in *Drosophilia,* is implicated in cell cycle regulation in mice. Interestingly, it was found that hTim

bound to endogenous Chk1 and that this interaction was significantly increased in samples exposed to DNA damage. To establish if a function exists with the interaction of hTim-Chk1, Unsal Kacmaz and coworkers repressed the translation of Tim which showed a marked decrease in Chk1 kinase activity. Furthermore, the depletion of Tim under DNA damage during S-phase resulted in no delay in DNA synthesis. This implies that yet another clock protein, Tim, is implicated in cell cycle regulation.[64].

Bioluminescence monitoring helped to discover that cell division alters with the periodicity of circadian gene expression in actively dividing cells [44]. In addition, cells active in cell division have a wider period of circadian oscillation versus nonproliferating cells [44]. Due to an inherent decrease in concentration of Cry and Per in daughter cells shortly after cytokinesis, requires more time before Per/Cry can fully repress transcription activity of Bma1/Clock [44]. This is strong evidence that shows how circadian proteins can indirectly control cell division by clock protein level dispersion and clock controlled genes such as Wee1.

**D. Significance of proposed research**

Elucidating the complex interrelationship between cell cycle regulation and circadian output pathways can prove to enhance our view of cancer related diseases. The focus on clock genes as potential cell cycle checkpoint factors will expand our scope in finding precursors to cancer beyond oncogenes and tumor suppressor genes.

The tendency of clock proteins to peak at a specific time of the day may influence the sensitivity cells are to ionizing radiation. It has been established that the circadian oscillation of proteins persists in tissues with active cell division. Therefore, we hypothesize that clock genes act upstream of cell cycle checkpoint proteins, forming another link between timing and cell cycle. Thus, tumor suppressing therapies such as chemotherapy may moderated and given at specific times of the day when cells are most susceptible to their effects.

## Chapter 2: Specific Aims

The goal of our study was to investigate the relationship between the tumor suppressor protein p53 and Per2 and how the dysfunction of this interaction is related to tumorigenesis. In relation to cell cycle, we explored how the mode of binding of these proteins took precedence over the cell to either arrest or continue to proliferate.

We hypothesize that binding of Per2 to p53 controls p53's ability to form a tetramer and become an active transcription factor, thus modulating the response to genotoxic stress. We asked i) What regions on these proteins are necessary for their interaction and what relevance do these sites have with the function of each protein? ii,) Does the response to DNA damage change how p53 interacts with Per2? iii) What effects does the Per2-p53 complex have on the overall cell cycle of mammalian cells in response to genotoxic stress? Thus we proposed to investigate the following aims:

***Aim 1. To establish the mechanism by which Per2 interacts with p53.*** Using co-precipitation assays we assessed the binding domains within Per2 and p53. We performed immunoprecipitaiton of cells subjected to IR and examined binding between Per2-p53. The main function of p53 is to form a tetramer and act as a transcription factor; therefore we studied p53's DNA binding through electrophoretic mobility shift assay in the presence of Per2.

***Aim 2. Elucidate the function of PER2-p53 complex in cell cycle progression in response to genotoxic stress.*** We used chinese hamster ovary (CHO) cells and determined how the overexpression and downregulation of hPer2 under ionizing irradiation and control settings affected cell cycle. We performd pulldown assays using Suc-1 beads and examined the protein levels of cyclin B1 and Cdk1 and whether cell arrest had occurred through the phosphorylation of Cdk1 onTyr14. We also examined cell cycle progression using flow cytometry. To further investigate the cause of any change in the cell cycle profile, we quantified changes in gene expression of those directly controlled by p53.

## Chapter 3: Results and Discussion

**A. Novel interactors of hPer2.** To identify novel interactors of Per2, we performed a two-hybrid analysis using a human liver cDNA library. The bait was full-length human Per2 (hPer2). After multiple screenings using minus histidine selective plates, we sequenced colonies that showed strong interaction. Only colonies that grew in dual selective plates containing streptomycin were considered strong interactors. Among positive interactors we found human p53 (hp53). To validate this interaction we co-transformed full length hp53 target plasmid and the hPer2 bait vector into BaterioMatch II reporter strains (Figure 3.1A). A positive control using target vector containing a known interactor (human Cry) was also tested (Figure 3.1A).

We then investigated whether this same interaction can occur in mammalian cells which contain relevant proteins that interact with hPer2 and hp53. The tumor suppressor p53 is capable of binding to various proteins, such as BRCA1, but as yet no work has been published that implicates p53 interacting with clock proteins directly. In addition, the clock protein Per1 is able to coimmunoprecipitate checkpoint proteins such as CHK2, and ATM in mammalian cells, therefore we examined if this holds true with hPer2-hp53 [63].

To establish whether an interaction between p53 and Per2 occurs in mammalian cells, we transfected Chinese hamster ovary cells with myc-hp53 and performed an immunoprecipiatation using myc-beads (Figure 3.1B). Western blot analysis using a Per2 antibody reveals hp53's ability to immunoprecipitate endogenous Per2 (Figure 3.1B). In addition, we cotransfected CHO cells with both hp53 and hPer2 and used myc beads to immunoprecipitate exogenously expressed FLAG-hp53 (Figure 3.1C). Again through immuno blot analysis binding between hp53 and hPer2 was demonstrated. CHO cells transfected with empty vector showed no binding of endogenous Per2 or the FLAG-hp53, thus showing binding is specific (Figure 3.1C). Other clock proteins have been recently associated with cell cycle regulatory proteins; for example, the human Timeless (TIM), a major component in the drosophilia circadian clock, has also shown to mediate the ATR/CHK1 pathway [64].

Collectively, these results indicate that hPer2 may be another factor that mediates DNA damage in a p53 dependent manner.

**B. Regions of hp53 binding onto hPer2.** Both bacterial and CHO cell lysates contain numerous proteins that may influence the interaction between hPer2 and p53. We used radiolabeled hPer2 and GST-p53 expressed in *E. coli* to assess whether this interaction occurs directly or indirectly. We performed a competition assay using GST-p53 bound to glutathione beads in pulldown

samples with varying amounts of untagged hp53 and constant levels of radiolabeled hPer2. Samples with higher amounts of untagged hp53 showed less binding of hPer2 onto p53-bound beads due to untagged p53's ability to also seize hPer2 (Figure 3.2C). The ability of GST-hp53 to sequester hPer2 confirms binding .The lack of lysate or other possible cofactors prove that hp53 and hPer2 without the presence of any other factor.

To identify the binding regions of hPer2-hp53 we cloned eight constructs of hp53, residues 1-363, 1-325, 1-296, 1-200, 100-310, 200-393 and 300-393 (Figure 3.2A). We performed GST-pulldown using radiolabeled hPer2 and the various fusion proteins of hp53. Interestingly, p53(1-363) showed a drastic reduction in binding capabilities compared to wildtype (Figure 3.2B). Deleting of the last 30 amino acids of hp53 favors increased binding to DNA as a tetramer which suggests that the oligomerization of p53 may act as a inhibitor of binding to hPer2 [5, 6] .On the other hand, a tetramerization domain deficient form of hp53(1-325), showed similar binding as wildtype (Figure 3.2B). This suggests that p53 cannot bind to hPer2 in the form of a tetramer. However, binding still occurs with hp53(200-393) which still contains the tetramerization domain. This may be due the lack of the first 200 amino acids which exposes DNA binding domain, where we believe hPer2 interacts with hp53 at residues 200-325 (Figure 3.2B).

The region in which we believe hPer2binds to hp53 is partially the DNA binding domain. Therefore hPer2 may impede p53's ability act as a transcription factor.

**C. Regions of hPer2 binding onto p53.** To examine the regions in which hp53 binds to hPer2 we used GST-pulldown assay with truncated contructs of hPer2 (Figure 3.3A). Pulldown experiments exposed two regions in hPer2 where p53 binds *in vitro'* residues 356-574 and 683-872, containing a nuclear localization signal (NLS) and a nuclear export signal (NES), respectively (Figure 3.3B). We speculate that hp53 binds to hPer2 and uses it as a vehicle to be translocated towards the nucleus. In addition p53 not only physically interacts with BRCA1 but it translocates p53 into the nucleus to further the cells sensitivity towards DNA damage in p53 dependent pathway [65]. Furthermore, we performed a GST-pulldown assay using constructs encoding residues 356-574 and 683-872 to further localize the binding region of hPer2 (Figure 3.3C). In conclusion, we hypothesize that hPer2 and hp53 may act on each other to transport between cytoplasm and nucleus effectively controlling their ability to act as a transcriptional factor.

**D. hPer2-p53 binding in response to genotoxic stress conditions.** In response to DNA damage, p53 is post translationally modified to prevent MDM2 mediated proteasomal degradation and act as a transcription factor for downstream cell cycle genes. We wanted to investigate whether ionizing irradiation interferes with the binding of hPer2 and p53 by cotransfection of both proteins and immunoprecipitation. Interestingly, p53 from ionized samples showed a decreased ability to pull down hPer2 (Figure 3.4). This could be either that a post-translational modification in p53 prevents physical interaction of hPer2 or the now active p53 oligomerization state prevents PER2 from binding to p53(200-325). Recent work has revealed that tetramerization of p53 functions to mask the NES signal found on the C-terminus of p53, effectively increasing the subcellular organization of p53 towards the nucleus [66]. We speculate that the binding of hPer2-p53 essentially masks both proteins' NES signals and therefore possibly prevents their shuttle back to the cytoplasm.

**E. hp53 binding to DNA compromised in the presence of hPer2.** An important aspect of p53 is its ability to regulate transcription of major cell cycle proteins such as p21, cyclin B and GADD45α [25, 42] In addition, p53 acts as a transcription factor by forming a tetramer around a p53 response element. We hypothesize that if hPer2 interacts with p53 depending on its oligomerization then this binding may play a role in how p53 acts on p53 response elements. We performed an electrophoretic mobility shift assay (EMSA), to explore p53's DNA binding affinity in the presence of hPer2. Thus, we purified fusion GST-hPer2(683-872) and observed hp53's ability to act as a DNA binding factor in the presence of increasing protein levels of GST-hPer2(683-872) (Figure 3.5).

To test whether hp53-hPer2 complex is capable of binding to DNA, we performed a two-stage binding reaction. First, hp53 and hPer2 were incubated before the addition of the radiolabeled probe. Visualized by autoradiography, GST-hPer2(683-872) was able to inhibit p53's ability to bind to DNA. Increasing hPer2(683-872) protein levels in subsequent lanes further justified our findings with gradual decrease in the intensity of the band (Figure 3.5A). Studies expose how the E6 protein which can bind directly to p53 leads to varying degrees of inhibition of p53's capacity to bind to DNA depending on the strength of the E6-p53 binding complex[67]. Therefore HPV types 16 and 18 have varying degrees of cancer risk, so we speculate that hPer2 may play a role in changing its ability to act on genes by binding to it.

In contrast, we developed an EMSA experiment to test whether the binding of hPer2 can disrupt preformed p53 tetramers already bound to a DNA response element [68]. We incubated the

DNA probe with hp53 before the addition of hPer2(683-872). The data suggests that once hp53 is bound to the DNA probe GST-hPer2(683-872) cannot disassociate the complex(Figure 3.5B). In addition no supershift of the p53-DNA complex was observed, therefore hPer2(683-872) cannot bind as a multimeric complex with p53 and DNA.

In conclusion, due to hPer2's lack of ability to release p53 from DNA, we hypothesize that hPer2 has no effect on hp53 if the tetramer has already formed around the DNA. It is also possible that the binding may change preferred DNA response elements, thus changing downstream effector responses to DNA damage which will require further experiments to test this hypothesis.

**F. Cell cycle arrest via phosporylation of Cdk1 at Tyr15 in cells overexpressing hPer2.** Arrest in G2 is governed by the phosphorylation status of Cdk1 at Tyr15 Ser14 by WEE1 and Myt1 respectively. To investigate cell cycle arrest in cells overexpresing hPer2 we performed Western blots using antibodies recognizing Cdk1 P-Tyr15 and cyclin B. To test the function of the G2 checkpoint in cells overexpressing we introduced ionizing irradiation (IR). We hypothesize that IR samples with overexpressed hPer2 will show a more prolonged cell cycle arrest in correspondence to hPer2's ability to act as a tumor suppressor protein [3]. Control samples showed a transient phosphorylation of Cdk1that decreased 36 hours after IR, while IR samples transfected with hPer2 showed a constant phosphorylation throughout the time course and a sustained high level of cyclin B (Figure 3.6). The tumor suppressor p53 acts as a negative regulator of cyclin B in the event of DNA damage. This supports our idea that hPer2 may play a role in increasing p53's localization in the nucleus but not preventing p53's ability to act as a transcription factor once modified from DNA damage checkpoint proteins within the nuclear foci. Both empty vector and transfected hPer2 non-irradiated control samples were indistinguishable (Figure 3.6).

**G. Cell cycle analysis in cells overexpressing hPer2.** Cell cycle analysis by flow cytometry can indicate the phase in which cells are arrested. Samples from cells overexpressing hPer2 showed no difference compared to controls (Figure 3.7). Irradiated samples showed the same arrest in G2 (Figure 3.7). Interestingly, overexpression of hPer2 in normal conditions did not cause CHO cells to go into arrest, whereas tumor suppressor proteins such as p53 lead to premature arrest or cell death when overexpressed [12]. We hypothesize that hPer2 acts by interacting with inactive hp53 and therefore does not induce possible downstream genes that can lead to premature arrest in normal conditions.

**H. Gene expression in the absence of Per2.** To fully understand the importance of hPer2 as a tumor suppressor in CHO cells, we knockedown mRNA expression of Per2 using siRNA to see what gene expressions depend on the presence of Per2. We hypothesized that if hPer2 interacts with hp53, that likely candidates of changes in gene expression would be genes directly controlled by hp53.

We performed RNA extraction then Reverse transcriptase-PCR and among the genes tested, expression of both *bax* an apoptotic factor transcriptionally regulated by p53, and *myc* another gene involved in either apoptosis were severely reduced [26] (Figure 3.9). Establishing a model for this regulation will require more experiments. Myc can indirectly control the protein levels of p53 through $p14^{ARF}$ which then can control *bax* expression otherwise Myc can directly modulate Bax independent from p53 (Figure 3.9).

## Chapter 4: Summary and Conclusion

We have shown that p53, a major gatekeeper for the cell cycle, can physically interact with hPer2. In addition, this binding interaction can interfere with p53's ability to bind to DNA. However, the mechanisms that control the interaction between hp53 and hPer2 remain to be determined. As a transcription factor, p53 bound to Per2 may lead to different genes transactivationally regulated, which can be explored using RT-PCR or luciferase promoter activity. The ability of p53 to form a tetramer is a key component necessary for it to act as a transcription factor. Experiments should expand on dynamics of oligomerization state of p53 in the presence of hPer2.

We have demonstrated that hPer2 binds to p53 between amino acids 200 and 325 (Figure 3.2B). A major portion of this region resides in the DNA binding domain, which we speculate acts as an antagonist to p53's ability to form a tetramer around p53 response elements (Figure 3.2A). On the other hand, p53 binds to regions of hPer2 where a nuclear localization or nuclear exportation signal exists. Future experiments can perhaps expand on subcellular organization of hp53 in the absence or overexpression of hPer2, to help define whether hp53-hPer2 complex can lead to changes in their localization.

We have shown that under genotoxic stress conditions hp53 can no longer interact with hPer2 (Figure 3.4). Upon DNA damage, hp53 is post translationally modified to resist proteasomal degradation or stabilized to bind as tetramers [11, 12]. To decipher the mechanism in which hp53 can no longer interact with hPer2, future experiments should focus on mutant forms of hp53 that either manipulate post translational modifications or its oligomerization to determine whether this is the cause.

We observed hPer2's ability to prevent hp53 from binding to a DNA response element (Figure 3.5). Interestingly, this leads us to believe that the presence of hPer2 prevents p53 from acting on its downstream genes and therefore causing abrogation of the G2 checkpoint. However, Fu and colleagues show that the absence of a functional Per2 leads to higher risks of tumors in mice, impying Per2 to be a tumor suppressor rather than an oncoprotein.[3]. We speculate that the fact that our EMSA experiment uses hp53 expressed in *E.coli* lacks the necessary proteins to modify hp53 to be truly transcriptionally active, therefore it is possible that the checkpoint system modifies hp53 to disassociate the hp53-hPer2 complex under genotoxic stress conditions. Further EMSA assays should be done by using hp53 purified from a

mammalian expression system under genotoxic stress. We consider that hPer2 may act as a tumor suppressor by mediating hp53's stability during normal conditions so that more free hp53 will be available when DNA damage does occur. In accordance to this speculation, Fu and colleagues work shows that $Per2^{m/m}$ mice showed a delayed increase in p53 levels in response to ionizing irradiation versus wildtype [3].

In summary, our data supports our hypothesis that Per2 acts on p53's by binding to it directly and inhibiting its normal cell cycle response. Per2 may act as a tumor suppressor by modulating the turn over rate of p53, subcellular organization, or transcriptional regulation of p53. We also speculate that the oligomeric state may somehow play role in the way Per2-p53 interact. Thus it is possible that p53 dependent checkpoint pathways are more sensitive to DNA damage at specific times of the day due to the nature of Per2's circadian oscillating expression and interaction with p53.

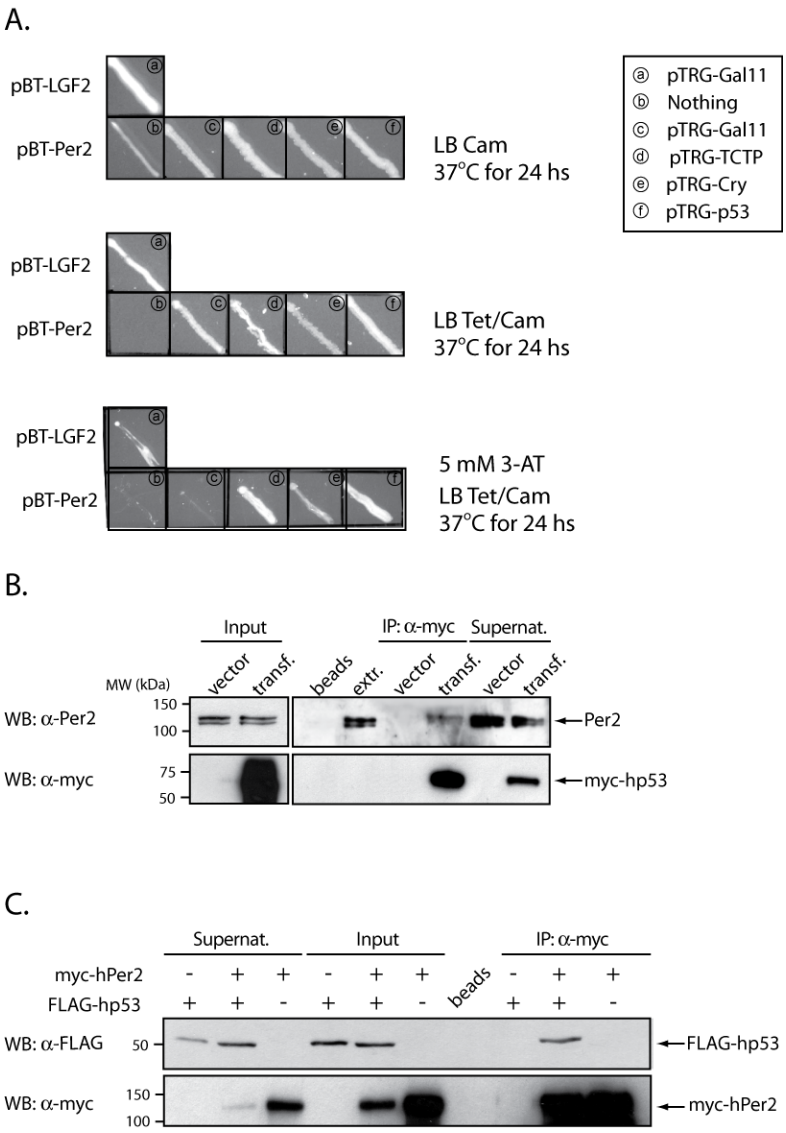


**Figure 3.1:** hPer2 interacts with hp53 in vitro. **(A)** Two-hybrid analysis using non-selective and selective plates show interactors of hPer2. **(B)** Transfected CHO extracts were immunoprecipitated with myc-hp53 using myc-beads to purify. Western blot analysis using Per2 antibody was done to examine endogenous Per2 binding to myc-hp53. **(C)** Co-transfection of myc-hPer2 and FLAG-hp53. Samples were immunoprecipitated using myc-beads and binding complexes of exogenous fusion tagged proteins were examined by western blot analysis.

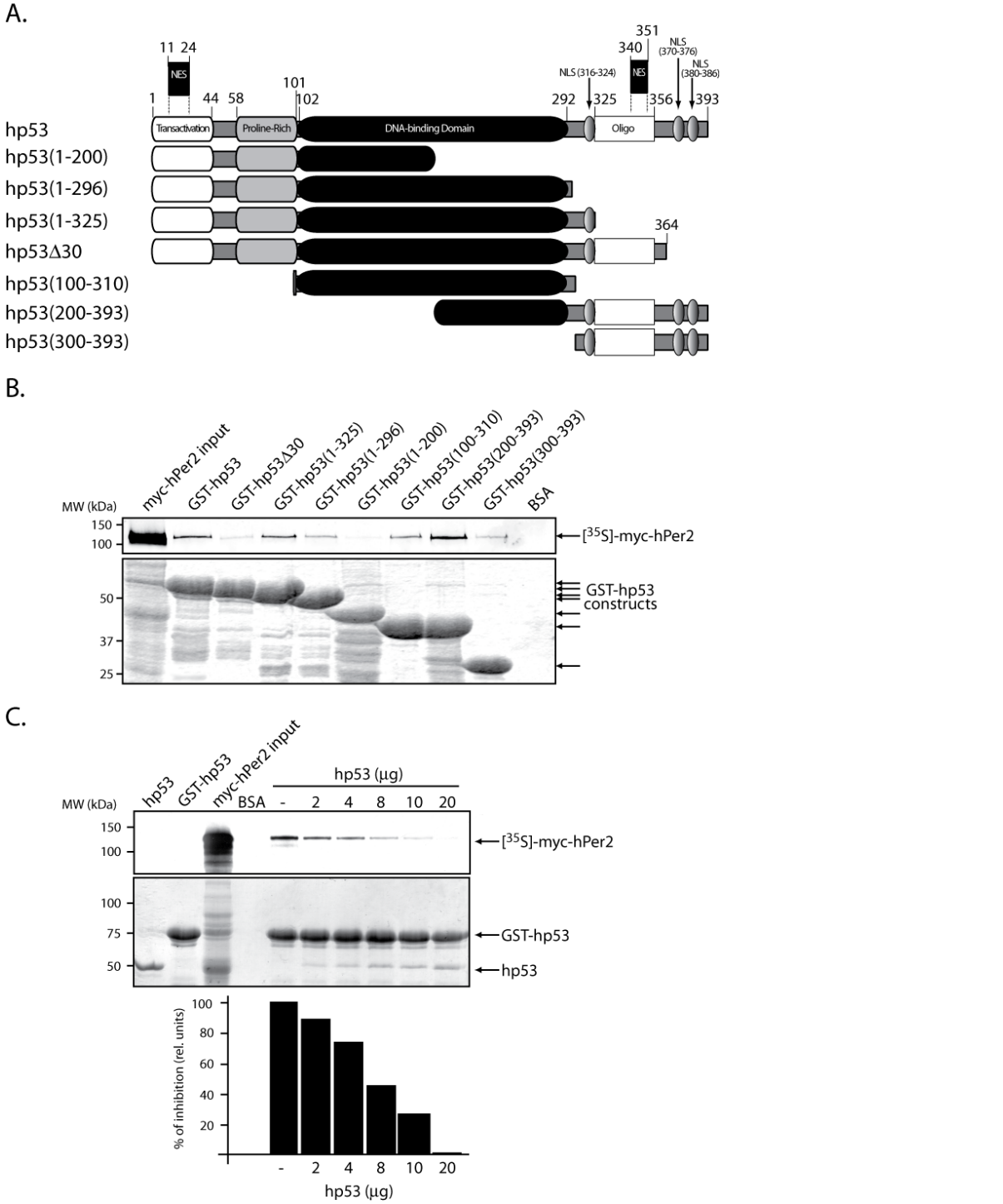


**Figure 3.2:** Binding of regions of hp53. **(A)** Domains in wildtype human p53 and constructs of hp53 used in the experiment. **(B)** GST-pulldown assay using different constructs of p53 and full length radiolabeled hPer2. **(C)** GST-competition pulldown assay using GST-hp53, untagged wt p53 and radiolabeled hPer2. Increasing amounts of untagged p53 in subsequent lanes.

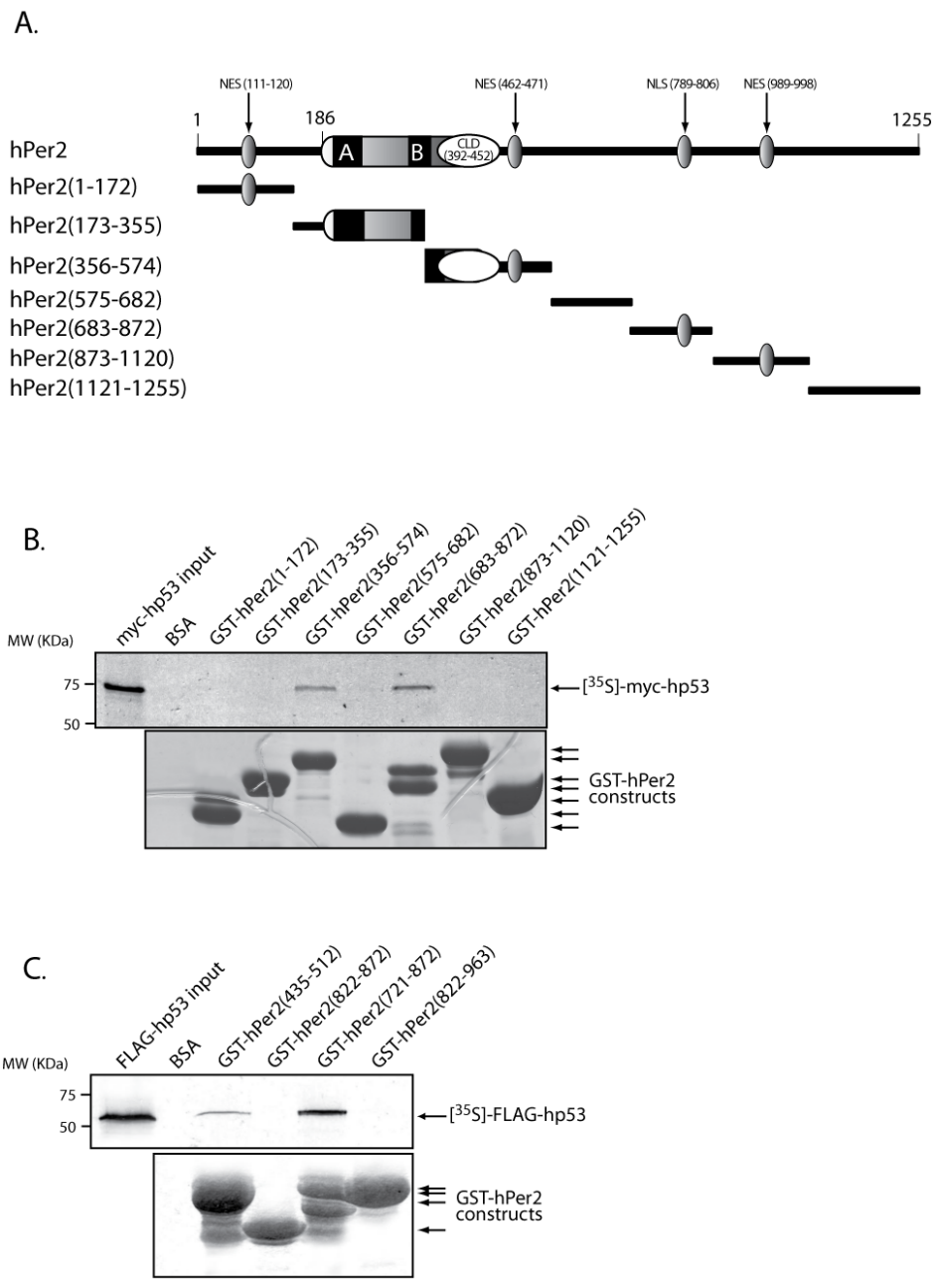


**Figure 3.3:** hp53 binding onto hPer2. **(A)** Regions of hPer2 containing the cytoplasmic localization domain, nuclear export signal (NES), and nuclear localization signal (NLS). The various constructs of hPer2 used in the experiment. **(B)** GST-pulldown using constructs of hPer2 and radiolabeled full-length hp53. **(C)** GST-pulldown using amino acid regions within fragments of hPer2(356-574) and hPer2(683-872) to further examine binding regions of hPer2/hp53.

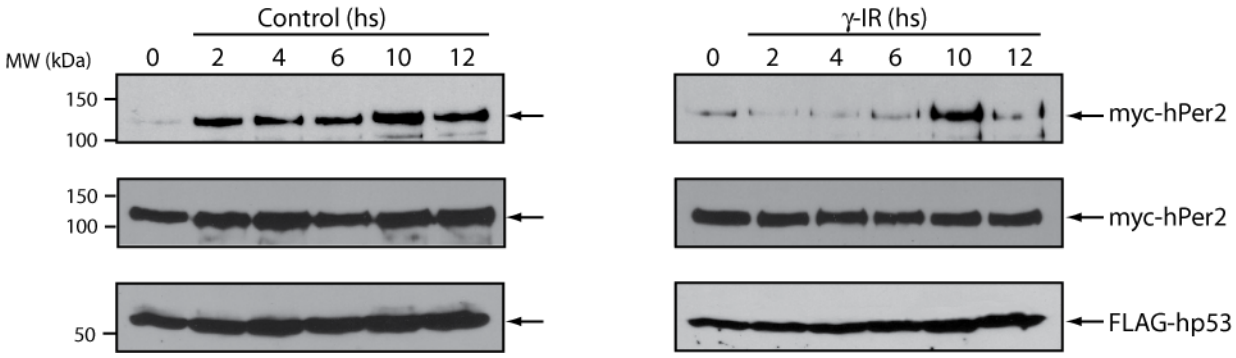


**Figure 3.4:** hPer2-p53 binding in response to genotoxic stress conditions. CHO cells were transfected with both myc-hPer2 and FLAG-hp53 and exposed to 10 Gy γ-IR. Coimmunoprecipitation was performed using myc-beads and western blot analysis was performed using myc and FLAG antibodies for binding complexes.

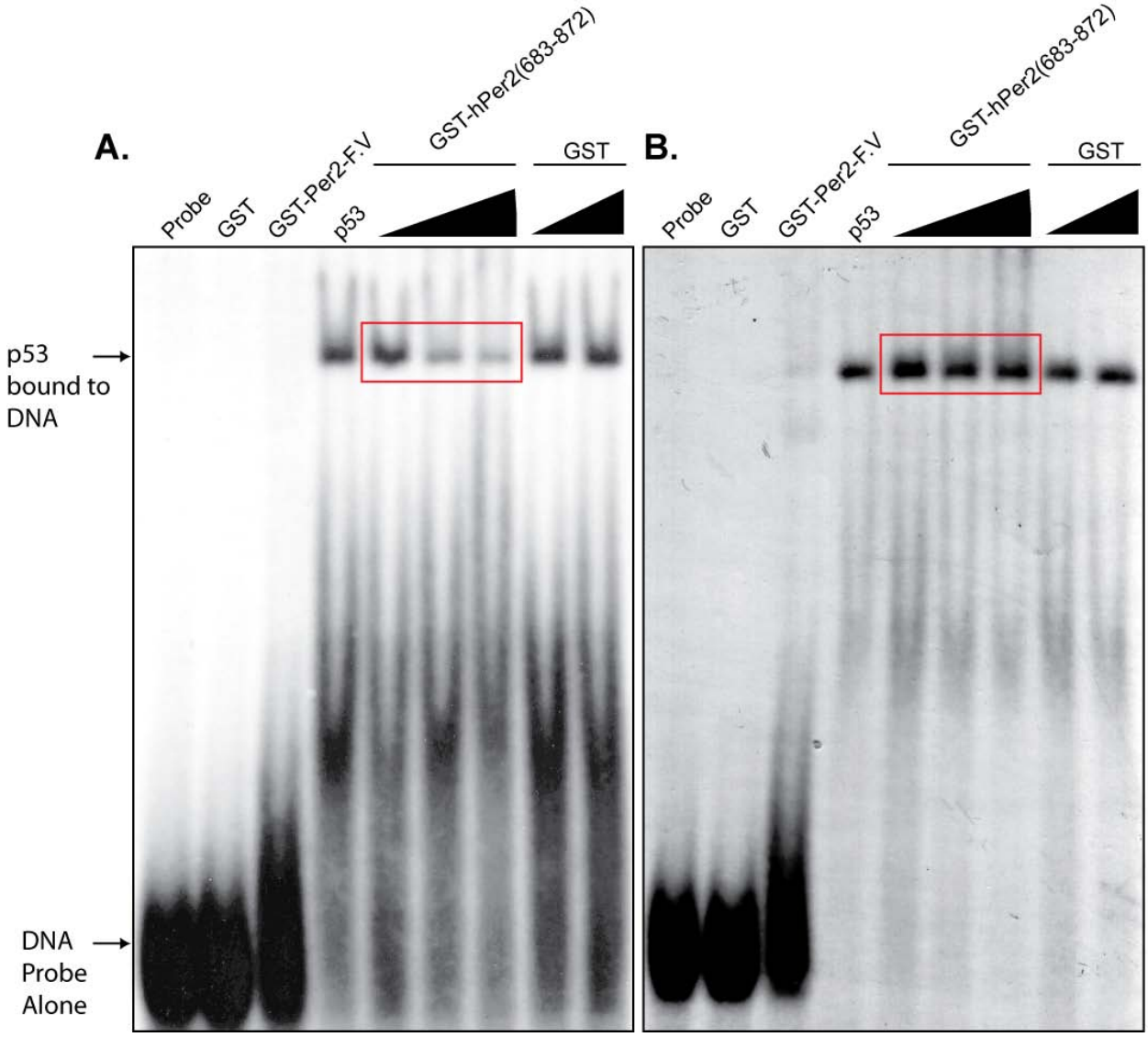


**Figure 3.5:** DNA binding capacity of hp53 in the presence of hPer2. **(A)** Electrophoretic mobility shift assay was done using untagged wt hp53 and radiolabeled DNA 5' - GTTTGC TTTCATAGACATGCCAGTTATTTATCTTG - 3'. hp53 was first incubated with hPer2(683-875) for 20 min before adding DNA probe for another 20 min. **(B)** Same as **A** except hp53 was incubated with DNA probe first before the addition of hPer2(683-875).

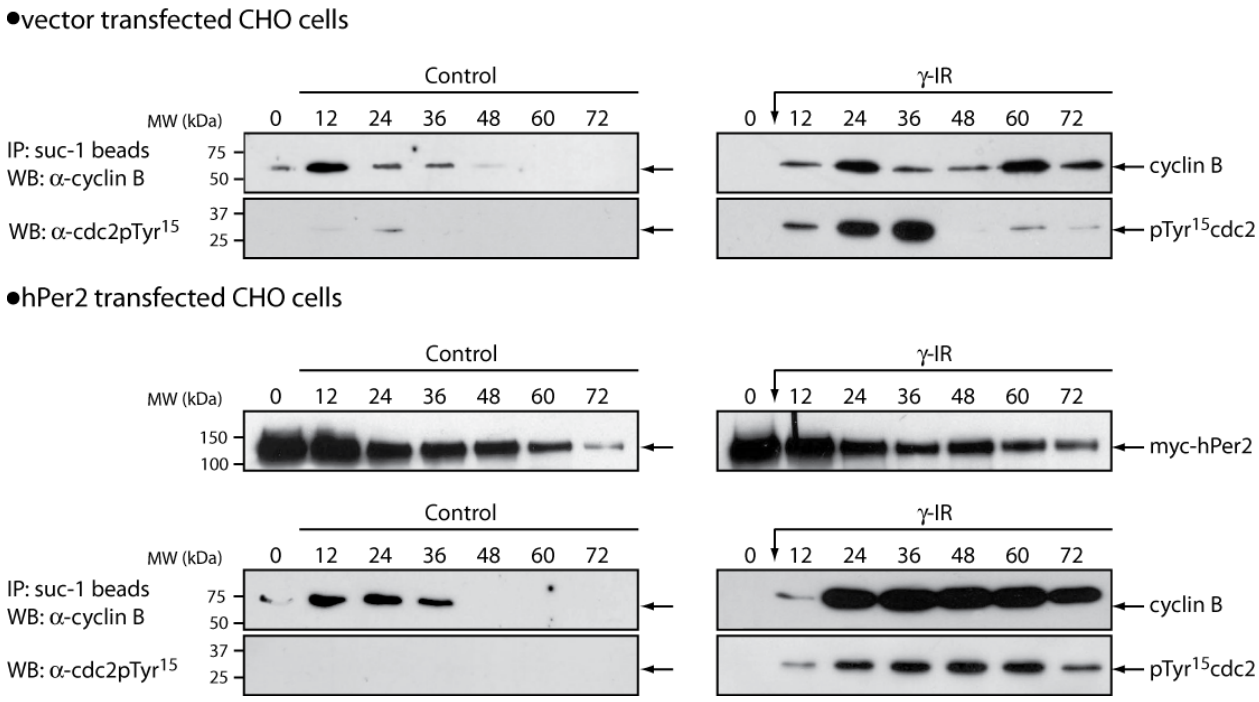


**Figure 3.6:** Cyclin B levels and Cdc2 phosphorylation at Tyr15 in cells overexpressed in hPer2. CHO cells were transfected with hPer2 and exposed to 10 Gy γ-IR. Cyclin B and Cdc2 Tyr15 levels were examined through western blot analysis.

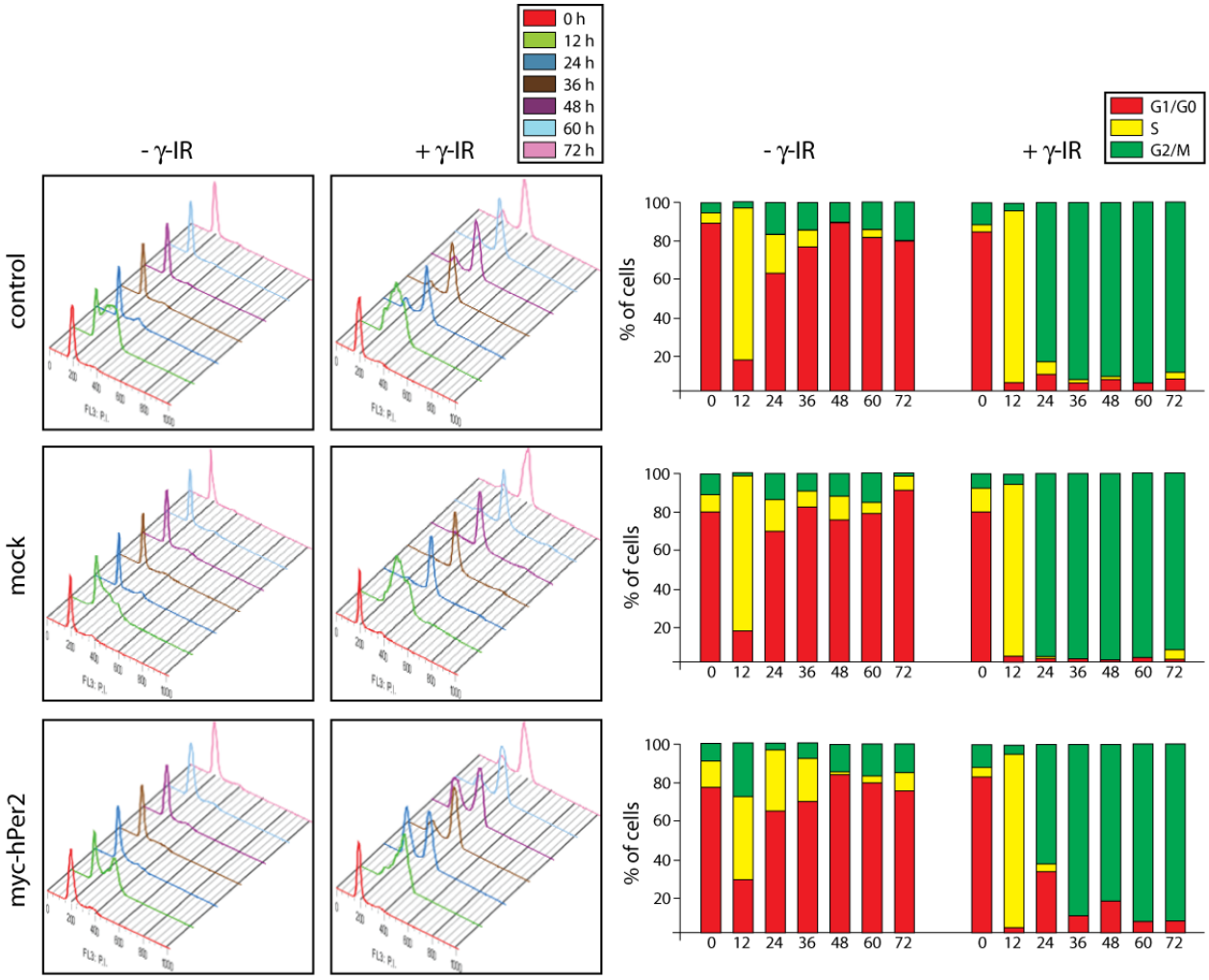


**Figure 3.7:** Cell cycle profile using FACS. CHO cells were transfected with hPer2 and exposed to 10 Gy γ-IR. Cells were cell cycle synchronized using serum deprivation. Samples were fixed with 70% ethanol before being stained with propidium idiodide.

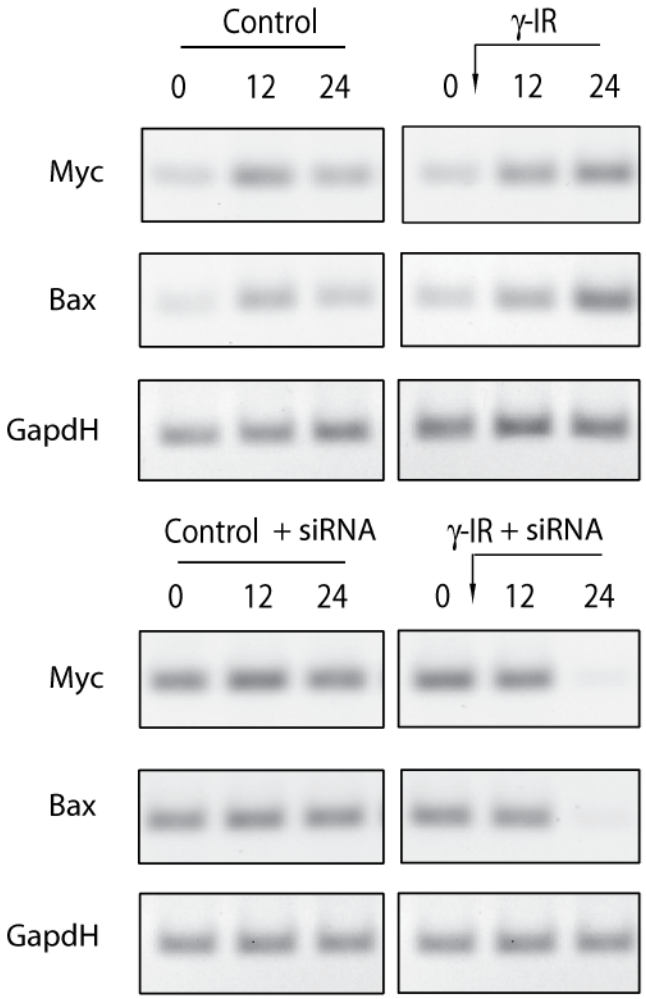


**Figure 3.8** mRNA expression levels of *myc* and *bax* in the absence of Per2 and ionizing irradiation. CHO cells were treated with siPer2 and 10 Gy γ-IR. Samples subjected to RNA extraction and then cDNA synthesis before running on DNA gel.

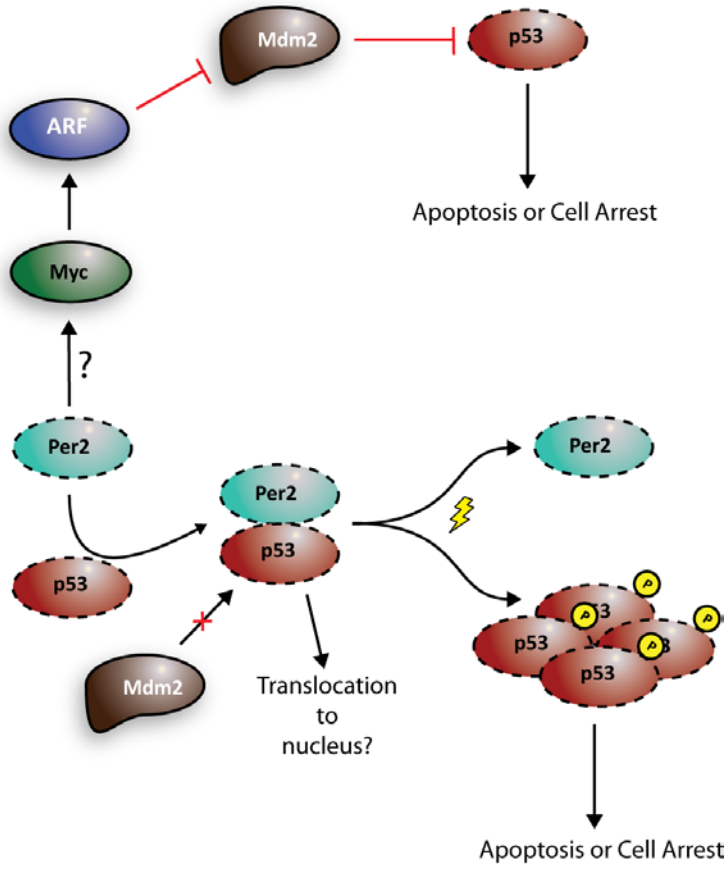


**Figure 3.9:** Model hPer2 assistance in hp53 regulated checkpoint pathways. hPer2's ability to bind directly to hp53 may prevent MDM2-degradation pathway or translocation. On the other hand Per2 may indirectly regulate p53 by modulating myc expression.

## Chapter 5: Materials and Methods

**DNA cloning, plasmids and protein expression and purification.** Human p53 and human PER2 cDNA constructs were amplified by PCR and cloned into pGEX-4T3 (GE Healthcare). GST-fusion proteins were expressed using *E.coli* Rosetta cells (Novagen) and purified using glutathione-Sepharose beads followed by manufacturer's protocol(GE Healthcare).

**Cell culture and transfection studies.** Chinese hamster ovarian (CHO) cells obtained from the American Type Culture Collections and maintained in F-12 Kaign's Modified media containing 10% fetal bovin serum. PER2 and p53 expression vector was constructed by cloning PER2 cDNA and p53 cDNA into pCS+ MT and pCS+ FT vectors respectively. Transfections were done using Lipofectamine 2000 (Invitrogen) and according to manufacturer's protocol. CHO cells were cell cycle synchronized by serum deprivation for 6 hours, F-12K media without serum. When indicated, cells will be irradiated with 10 Gy γ-IR. Time points were taken by digesting cells with trypsin-EDTA followed by trypsin inactivation, spun down at 2000 x g washed with Phosphate-buffered saline (PBS), and stored at -80 C.

**Immunoprecipitation.** Whole cell lysates were prepared using the following lysis buffer: 50 mM Tris-HCl, pH 7.4; 80 mM ß-glycerophosphate; 20 mM EDTA and protease inhibitor cocktail. Samples were sonicated then cleared for 15 min at 14,000 x g 4 ºC. Cleared lysates were then incubated with FLAG beads (Sigma) at 4ºC overnight. Two washes of low salt IP buffer (20 mM Tris-HCl, pH7.4; 5mM EDTA; 0.1% Triton-X100; 100 mM NaCl) then two washes of high salt IP buffer (20 mM Tris-HCl, pH7.4; 5mM EDTA; 0.1% Triton-X100; 1 M NaCl), finally one last wash of low salt IP buffer. Detection of myc-PER2 and FLAG-p53, the immunoprecipitates were eluted by incubation with Laemmli sample buffer at 95 C for 2 min. Run on SDS-PAGE gel for separation and transferred to a polyvinylidine difluoride membranes using a semidry apparatus.

**In vitro translation.** *In vitro* transcription-translation mixtures (TNT kit, Promega) containing [$^{35}$S]methionine were used to translate pCS+MT hPER2 and pCS+FT-hp53.

**Pull-down assays.** p53 and PER2 constructs were expressed and purified onto glutathione beads. Pulldown reaction consisted of 20 μg of GST-fusion protein bound to glutathione-Sepharose beads and radiolabeled [$^{35}$S]methionine protein and incubated at room temperature for 1 hour. Subsequent washes with IP buffer, x low salt IP buffer, 2x high salt IP

buffer, and 1x low salt IP buffer. Pulldown samples were eluted by incubation with Laemmli sample buffer at 95°C for 2 min. Samples then were run on SDS-PAGE gel and visualized by autoradiography.

**Electrophoretic mobility shift assay EMSA.** Complimentary DNA strands of a p53 consensus site were obtained and annealed by heating to 95 C for 2 min and cooled to room temperature. T4 polynucleotide kinase (Ambion) was used to end label probes with [$\gamma$-$^{32}$P]ATP and then purified using NucAway spin columns (Ambion).

Binding reactions were subjected to nondenaturing polyacrylamide electrophoresis through 4% gels in 1× Tris borate-EDTA buffer system. Gels were vacuum dried and visualized by autoradiography.

**Antibodies and Western blot analysis.** The following antibodies were used: myc (Santa Cruz); FLAG M2 (Sigma); Cdk1-Tyr15 and Cyclin B1 (Cell Signal). The membrane was blocked in Phosphate-buffered saline-Tween 20 (PBST) plus 5% milk at room temperature for one hour. Primary antibodies were incubated overnight at 4 ºC with blocking solution and membrane, followed by multiple washes in PBST. Secondary antibodies anti rabbit-horse radish peroxidase (HRP) and anit mouse-HRP were obtained from GE Healthcare. Bound anti-HRP was detected using ECL reagent kit (Pierce).

**FACS analysis** CHO cells were trypsinized washed with PBS and fixed in 70% ethanol at -20 C. Then stained with Krishan's solution (50 µg/ml propidium iodide (PI), 0.1% sodium citrate, 20 µg/ml RNase A) overnight at 4ºC.

**Reverse transcription-PCR (RT-PCR) analyses.** Total cellular RNA was extracted with Trizol reagent (Invitrogen). First-strand cDNA was synthesized using reverse transcriptase iScript cDNA kit (BioRad). The PCR conditions was as follows: hot start at 94°C for 5 min; 25 amplification cycles, each consisting of 94°C for 60 s, 55°C for 60 s, and 72°C for 30 s; and a final extension step at 72°C for 5 min. PCR products were separated on 2% agarose gels and visualized by staining with ethidium bromide.

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