Serine-15 Phosphorylated p53 in Complex with Mdm2 is Inhibited from Activating p53 Effector Pathways

By

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A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy, The City University of New York

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Abstract

Serine-15 Phosphorylated p53 in Complex with Mdm2 is Inhibited from Activating p53 Effector Pathways

By

Tamara Gopen

Adviser: Dr. Jill Bargonetti

We have studied a previously unidentified pathway for the inactivation of wild type *p53* by comparing the p53 response of two different cell lines; one that undergoes the expected tumor suppressor mediated response (ML-1) and one that does not (MANCA). Both the ML-1 and MANCA cell lines contain wild type *p53*. The signal transduction pathway causing accumulation of nuclear p53 protein is intact in both cell lines as treatment with camptothecin, etoposide, and mitomycin C cause p53 levels to increase. While the ML-1 p53 growth arrest and apoptosis pathways are functional, the same pathways in the MANCA cell line are impaired. The MANCA p53 protein is phosphorylated at Serine-15, however the p53 dependent induction of *waf1, gadd45, mdm2, fas,* and *noxa* are strikingly compromised. Surprisingly, there is extensive p53 DNA binding activity in the MANCA cells and this binding remains unchanged after DNA damage. We have found that MANCA cells have an abundance of the Mdm2 protein, and this same protein was barley detectable in the ML-1 cell line. Co-immunoprecipitation experiments with p53 and Mdm2 antibodies have shown a specific interaction with the MANCA p53 and Mdm2 proteins, even in the presence of DNA damage. Interestingly, there was no such interaction detected in the ML-1 cell line upon

DNA damage. In the presence of DNA damage there was also a change in the phosphorylation state of the Mdm2 protein in the ML-1 cell line, and this same change was not seen in the MANCA cell line. Our data suggests a model in which the p53-Mdm2 protein-protein interaction in the MANCA cell line may render the p53 protein inactive. This complex may not allow for the transcriptional activation of p53 target genes mediating growth arrest and apoptosis.

Key words: p53; Mdm2; protein-protein complex; apoptosis

This thesis is dedicated to my mother for all of her unconditional love, support, and devotion.

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Chapter 1
Introduction

The p53 Tumor Suppressor Protein

The p53 tumor suppressor protein plays a central role in the prevention of cancer development by its ability to activate a growth arrest or apoptotic program (Bargonetti and Manfredi, 2002). In the presence of functional p53 the cell is protected from several different types of stress, which may be present in the cellular environment (carcinogens, hypoxia, free radicals, and oncogenic stimulation) (Pluquet and Hainaut, 2001). When the p53 pathway is inhibited, tumorigenesis is accelerated. The p53 protein exerts its effects as a tumor suppressor by acting as a transcription factor (Raycroft et al., 1990, Farmer et al., 1992).

The p53 tumor suppressor protein consists of four functional domains each serving a different role in the function of the p53 protein **(Figure 1.1)** (Levine, 1997). It is the combined efforts of these domains that allows for the p53 protein to initiate a growth arrest or apoptotic pathway in the presence of a DNA damage signal. The amino terminus of the protein has been shown to have transcription activity (Fields, and Jang, 1990). Additionally, the amino terminus contains many phosphorylation sites, and modification at these sites may lead to a conformational change of the p53 protein, and subsequent interaction with several different transcription factors (Horikoshi et al, 1995). The carboxy terminus can bind DNA non-specifically, which may help to regulate the activity of p53 (Wang et al., 1994). The tertramerization domain also resides within the carboxy terminus (amino acids 324-355). It is believed that the tetramerization domain

may be responsible for enhancement of p53 specific DNA binding (McLure, and Lee., 1998). It is the central core region of the p53 protein that is responsible for the sequence specific DNA binding activity (Bargonetti., et al., 1993; Pavletich, et al., 1993; Wang, et al., 1994). The p53 protein binds specifically to genes which contain p53 response elements with a consensus binding site (5'-PuPuPuC(A/T)(T/A)GPyPyPy-3') (El-Deiry et al., 1992). Oftentimes there are mutations within the p53 DNA binding domain, which lead to loss of activity, and deregulation of cell growth (Bargonetti et al., 1993, Cho et al., 1994). The central region of the p53 protein is highly conserved and most mutations occur within this region, which ultimately affect p53's ability to bind DNA and therefore inhibit its tumor suppressor function (Ko and Prives, 1996). These mutations allow for the progression of cancer development.

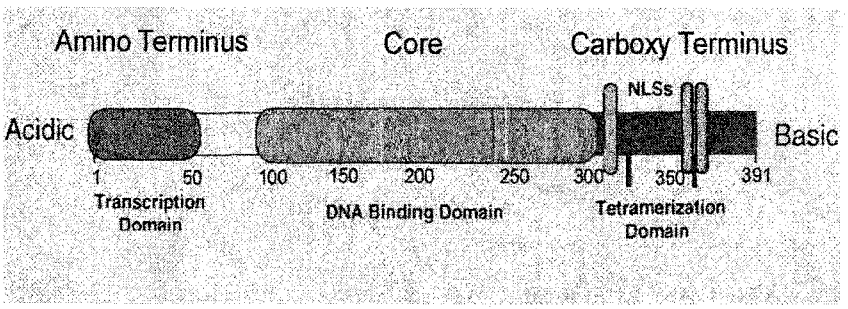


Figure 1.1 A model representing the p53 protein domains.

Several studies have shown that p53 DNA binding can be activated when cells are treated with radiation, hydrogen peroxide, actinomycin D, etoposide, camptothecin, 5-florouracil, mitomycin C, and cisplatin (Tishler et al., 1993). The presence of these substances in the cellular environment leads to DNA strand breaks which are sufficient to allow for an increase in p53 levels. Specifically, camptothecin allows for p53 to have an extended half-life, as well as increased protein levels (Tishler et al., 1993). Similar to camptothecin, etoposide has also been shown to cause double stranded DNA breaks, which are the initiators of signaling pathways that can ultimately lead to activation of

apoptosis (Kaufmann, 1998). p53 can also be induced either by nucleotide depletion, or hypoxia (Alarcon et al., 1999; Linke et al., 1996;Chernova et al., 1998). Many different types of cellular stress cause initiation of post-translational modifications of p53 which allow for stabilization of the p53 protein (Giaccia and Kastan, 1998).

Post-translational Modifications and Increased Stability of the p53 Protein

A complete discussion of p53 must include a brief understanding of how the protein becomes stabilized to act as a transcription factor. The multiple roles of p53 suggest that there must be multiple levels of control to regulate its activity. These levels of control exist because of the different modifications that occur within the different domains of the p53 protein, as discussed previously. The following discussion will take a closer look at the post-translational modifications of p53, and how these modifications effect the half-life of the p53 protein. In the absence of a stimulus the levels of p53 are very low, and without modification the protein may exist in a transcriptionally inactive form (Kastan, et al., 1991; Giaccia and Kastan, 1998).

The amino terminal transactivation domain is composed of several serine and threonine residues that become phosphorylated in the presence of DNA damage or cellular stress (Bean and Stark, 2002). Serine 15 is phosphorylated by the ATM kinase or DNA dependent protein kinase, and it is a key residue in allowing for the increased half-life of the p53 protein (Shieh et al., 1997; Siliciano et al., 1997). When p53 is phosphorylated at serine 15, the Mdm2 protein can no longer interact with p53, and p53 is then freed from possible degradation by ubiquitin ligase. Although, recent data has

shown that the phosphorylation of serine 20 and threonine 18 may be the crucial residues involved in disrupting the p53-Mdm2 protein-protein interaction (Unger et al., 1999; Shieh et al., 1999; Bean and Stark, 2002). Specifically, when Serine 20 of the p53 protein is phosphorylated in response to stress, the negative regulation imposed by Mdm2 binding is removed. The presence of cellular stress is believed to initiate a phosphorylation cascade which ultimately inhibits the p53-Mdm2 interaction. p53 is then free to activate transcription of downstream target genes (Sakaguchi et al., 2000).

The carboxy terminal domain of p53 plays a multifunctional regulatory role. The carboxy terminus contains serine and lysine residues that are modified in the presence of DNA damage and cellular stress. The acetylation of p53 at lysine 382 in the presence of DNA damage has been shown to enhance the sequence specific DNA binding activity of p53 (Sakaguchi et al., 1998). Near to this residue is serine 392 whose phosphorylation has been shown to enhance tetramerization formation (Sakaguchi et al., 1997). Although, it is the de-phosphorylation of the p53 protein at serine 376 that enhances its interaction with the ubiquitous transcription factor, p300 **(Figure 1.2)** (Giaccia and Kastan, 1998).

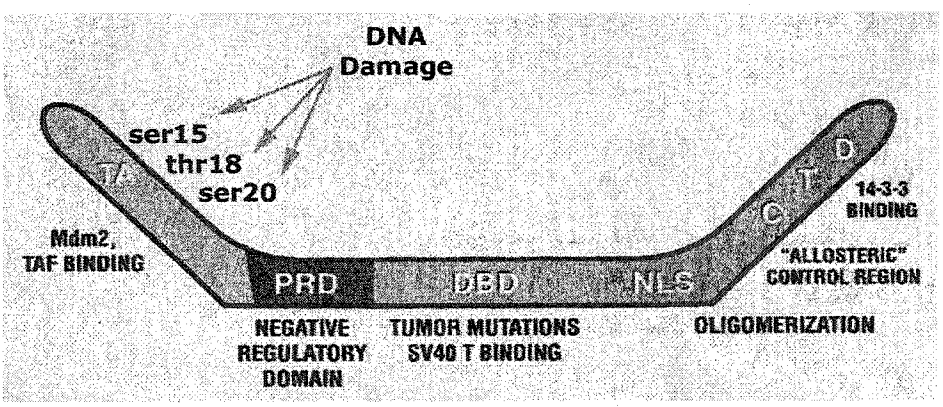


Figure 1.2 Post-translational modifications of the p53 protein. *(Giaccia and Kastan, 1998).*

p53 as a Transcription Factor

Once the p53 protein has been post-translationally modified, as described above, the stabilized protein can then go on to act as a transcription factor. The transcriptionally active form of the p53 protein will allow for the initiation of a growth arrest or apoptotic program dependent on the downstream genes that are targeted (Colman et al., 2000) **(Figure 1.3)**. The p53 protein can intergrate signals from many different types of stress in the cellular environment. Additionally, the complex regulation of the p53 protein will allow for differential regulation of downstream target genes containing p53 response elements (El-Deiry, 1998). One hypothesis states that p53's decision to activate apoptotic or growth arrest genes depends upon specific interactions between transcriptional co-factors (Vousden, 2002). p53 may also be driven to activate apoptosis or growth arrest due to its specific interaction with other proteins, suggesting the final decision may be independent of *de-novo* transcription (Vousden, 2002). The DNA binding activity of p53 has been extensively studied and at this point over 300 genes containing potential p53 response elements have been found which contain p53 elements (Nakamura, 2004).

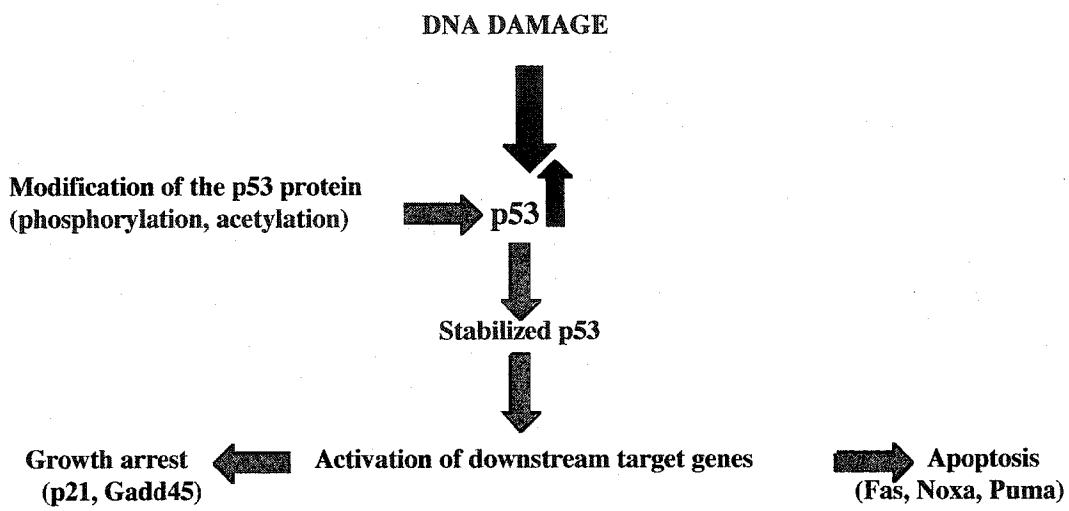


Figure 1.3 Activation of the p53 tumor suppressor protein. *In the presence of genotoxic stress, or DNA damage the p53 protein is activated and enabled to act as a transcription factor of downstream target genes allowing for cell cycle arrest, or apoptosis*

Downstream Targets of the p53 Protein

The p53 protein can regulate genes involved in growth arrest pathways. Specifically, *Gadd45*, and *waf1* are two genes involved in growth arrest, and both contain p53 responsive elements in their regulatory domains. *Gadd45* has been shown to play a role in the inhibition of cell cycle progression at the G2/M phase of the cell cycle by possibly interacting with the cyclin B/cdc2 complex (Zhan et al., 1998). The c-myc proto-oncogene has been to shown inhibit the activity of *gadd45* by binding to its promoter and preventing transcription (Marhin et al., 1997). Interestingly, c-myc can also prevent transcription of *waf1* in a similar manner (Dang, 1999). Waf1 is responsible for growth arrest at the G1/S phase border (El-Deiry et al., 1993). The Waf1 protein can

bind to and inhibit cyclin dependent kinase activity (Harper et al., 1993). Cyclin dependent kinases are then no longer able to complex with the cyclin proteins and the Rb tumor suppressor protein remains in a de-phosphorylated state. The Rb protein can then remain bound to the E2F transcription factor and the cell cycle remains arrested at the G1/S phase border (Harper et al., 1993).

Two well-characterized genes involved in DNA damage dependent apoptosis are the *noxa*, and *fas/apo1* genes. *Noxa,* a member of the BH3-only like family of pro-apoptotic proteins, is a p53 dependent pro-apoptotic target gene whose promoter contains a p53 response element. Increased Noxa levels allow for the initiation of an apoptotic program in the presence of a DNA damage signal (Oda et al., 2000). The *fas* receptor, a member of the TNF-_ family of death receptors, is another example of a p53 dependent pro-apoptotic target. In the presence of a damage signal the upregulation of the *fas* receptor allows for the cell to become sensitized to initiation of an apoptotic cascade. Although, it appears that the balance between the *fas* receptor and the *fas* ligand is the ultimate deciding factor in the regulation of initiating apoptosis (Muller et al., 1998).

Mdm2

In addition to growth arrest and apoptotic targets, p53 is involved in the regulation of growth promoting genes. One example is the *mdm2* gene, a gene that forms a feedback loop with the p53 protein (Barak et al, 1993; Wu et al., 1993). The p53 protein

can activate transcription of the *mdm2* gene by binding to the P2-p53 dependent promoter (Zauberman et al., 1995). The Mdm2 protein is a negative regulator of p53's transcriptional activity (Momand et al., 1992; Oliner et al., 1993; Thut et al., 1997). The Mdm2 protein has also been shown to play a critical role in the regulation of p53 during development (Jones et al., 1995). Mice deficient for Mdm2, die early on in development, whereas mice null for both p53 and Mdm2 develop normally (Jones et al., 1995).

The Mdm2 protein has been shown to interact directly with p53 and behave as a direct inhibitor of p53 activity, possibly by masking the p53 transactivation domain (Momand et al., 1992). More recent studies have found that not only does Mdm2 bind to p53, but the Mdm2 protein is capable of inhibiting the binding of basal transcription machinery to p53 (Thut et al., 1997). Mdm2's interaction with the 34K subunit of TFIIE may be the key target that allows for Mdm2's repressive activities. In some instances the interaction between p53 and Mdm2 leads to the inhibition of p53 DNA binding activity (Oliner et al., 1993).

The structure of the Mdm2 protein makes it a good candidate for a transcriptional regulator (Fakharzadeh et al., 1991). The Mdm2 protein contains a zinc finger domain and an acidic domain **(Figure 1.4)**. Previous work has shown that domains of this type may be involved in transcriptional regulation (Hope et al., 1988, Ptashne, 1988).

When there is no stress in the cell the Mdm2 protein serves to keep the p53 level low. Post-translational modifications of the Mdm2 protein via the Akt kinase, enhance entry to the nucleus where Mdm2 can then associate with p53, and target the protein for degradation via a ubiquitin proteolysis pathway (Mayo et al., 2001; Ogawara et al., 2002). The Akt kinase phosphorylates Mdm2 at serine residues 166 and 168 (Mayo et al., 2001). The phosphorylated form of Mdm2 also has increased E3 ligase activity due to its enhanced interaction with p300 (Ogawara et al., 2002). It has been previously shown that increased levels of Mdm2 can inhibit the growth arrest and apoptotic functions of the p53 protein (Capoulde et al., 1998; Dong et al., 2003).

In the presence of a DNA damage signal the association between p53 and Mdm2 is terminated **(Figure 1.5)**. The stress present in the cellular environment activates kinases, which lead to post-translational modifications of the amino terminal residues of p53 that interact with the Mdm2 protein. Specifically, there exists a phosphorylation cascade which leads to activation of the p53 protein. The first step being the phosphorylation of serine 15 and then subsequent phosphorylation of serine 20 and threonine 18 (Moll and Petrenko. 2003). These modifications of p53 prevent the tagging of the protein for degradation and allow for subsequent activation of p53 downstream targets (Sakaguchi et al., 2000). Additionally, Mdm2 is post-translationally modified to further inhibit its interaction with p53, and therefore enhancing the stability of the p53 protein (Khosravi et al., 1999). Phosphorylation of Mdm2 at serine 395 by ATM kinase leads to loss of p53-Mdm2 protein-protein complex formation, and therefore decreased degradation of the p53 protein (Maya et al., 2001). As an exception p53 can be

stabilized without post-translational modification. The Arf/p14 and L11 proteins have been shown to bind to the Mdm2 protein thereby removing the negative regulation imposed upon p53 (Weber et al., 1999, Tao and Levine, 1999, and Lohrum et al., 2003). The p14 and L11 pathways are activated solely in the presence of oncogenic stimulation and nucleotide depletion, respectively (Zindy et al., 1998, Zhang et al., 2003).

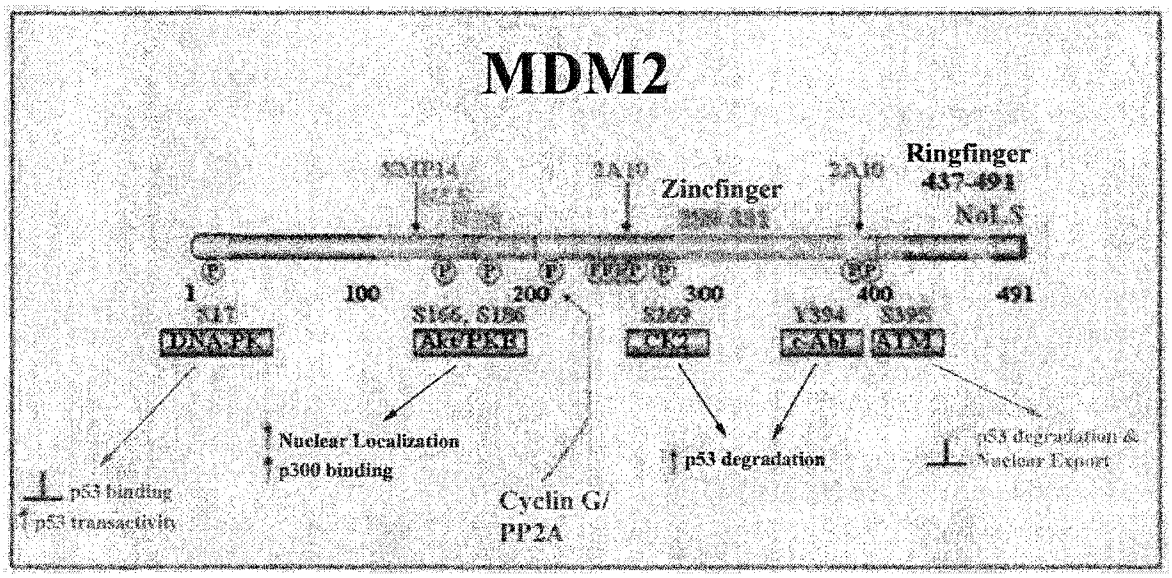


Figure 1.4 Structural domains and post-translational modifications of the Mdm2 protein. *(www.allgemeinchirurgie.uni)*

The Mdm2 protein has been shown to be amplified in 30% of all tumors (Evans et al., 2001). Overexpression of the *mdm2* gene has been demonstrated to lead to a transformed phenotype in NIH3T3 cells (Fakharzadeh et al., 1991). Altered *mdm2* expression has been associated with aggressive tumor progression (Ladanyi et al., 1993). Several studies have demonstrated that the presence of increased Mdm2 levels may lead

to loss of G1 arrest or apoptosis in the presence of DNA damage (Capoulade et al., 1998). Specifically, overexpression of Mdm2 in the presence of DNA damage can inhibit p53's transcriptional activity, despite induction of p53 protein levels (Dong et al., 2003). More recently, Bond et al have demonstrated a link between a single nucleotide polymorphism (SNP 309) in the *mdm2* gene and breast cancer. The SNP has also been shown to allow for the presence of increased Mdm2 levels. The SNP is located in the area of the p53 dependent P2 promoter of the *mdm2* gene (personal communication from G Bond, and AJ Levine).

Another mechanism which can effect p53 regulation is the presence of *mdm2* alternative splice variants (Fridman et al., 2003). Tumors which contain multiple *mdm2* splice variants have been shown to correlate with poor patient prognosis, possibly due to the inhibition of apoptosis (Steinman et al, 2004). In contrast several *mdm2* splice variants allow for increased p53 protein stability (Evans et al., 2001, Kraus et al., 1999). Splice variants of *mdm2* often have loss of the p53 binding domain, thereby these proteins resulting from such variants cannot interact with the p53 protein (Kraus et al., 1999). The different splice variant products may also bind and sequester full length Mdm2 leading to enhanced p53 activity (Evans et al., 2001). Lastly, via a p53 independent mechanism, mdm2 splice variant Mdm2-b has been shown to promote cell growth and inhibit apoptosis (Steinman et al., 2004).

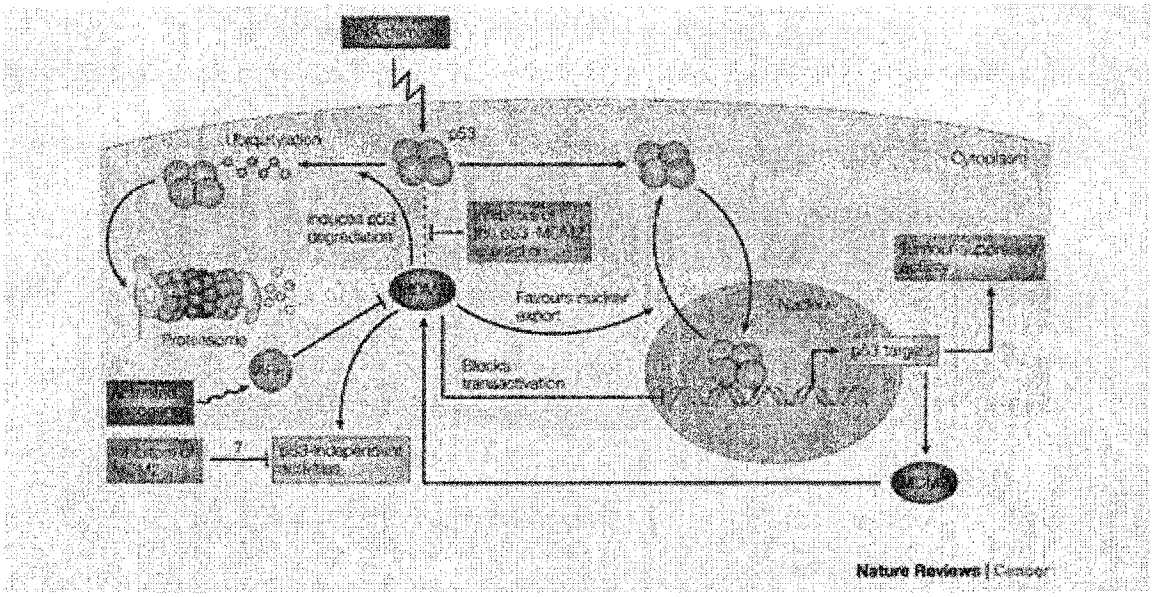


Figure 1.5 Representation of p53-Mdm2 protein-protein interaction in the presence of DNA damage. *In the absence of DNA damage the p53 protein is targeted for degradation by Mdm2's E3 ubiquitin ligase activity. In the presence of DNA damage the p53 protein is stabilized by post-translational modifications allowing for the activation of p53 dependent downstream checkpoint genes.(Nature Reviews Cancer 3 102-109. 2003)*

Ribosomal Proteins

Recent studies have focused on the importance of ribosomal proteins in the p53 response. Two such proteins are L11, and nucleolin. L11 has been shown to enhance the p53 response in the presence of cellular stress by further inhibiting the negative regulation of Mdm2 (Lohrum et al., 2003, Zhang et al., 2003). The ribosomal protein, L11, is released from the nucleolous during cellular stress enabling the interaction to occur between L11 and Mdm2 (Lohrum et al., 2003; Zhang et al., 2003). The p53 is then

readied to allow for the activation of downstream targets for the initiation of apoptosis and/or growth arrest. The mechanism of action of the L11 ribosomal protein works in a similar manner to the tumor suppressor protein, ARF. The ARF protein has also been shown to bind to the Mdm2 protein and sequester the Mdm2 thereby freeing the p53 from the negative regulation of Mdm2 (Tao and Levine, 1999; Weber et al., 1999). Although mechanistically very similar to the L11 protein, ARF is only induced in the presence of oncogenic stimuli such as de-regulated c-myc, and ras (de Stanchina et al., 1998; Zindy et al., 1998).

Recent literature has also shown a unique interaction between p53, and the ribosomal protein nucleolin. Nucleolin, in the presence of ionizing radiation, or treatment with camptothecin is released from the nucleolous into the nucleus where it can then interact with the p53 protein (Daniely et al., 2002). This interaction causes a temporary inhibition of replication and may allow for subsequent DNA repair. The p53 carboxy terminal regulatory domain is required for the release of nucleolin from the nucleus, and is necessary for formation of the p53-nucleolin complex. Interestingly, nucleolin levels have been shown to decrease in the presence of DNA damage mediated apoptosis (Kito et al., 2003).

Dissertation Hypothesis

In this study we investigated the p53-dependent DNA damage response pathway in a Burkitt's lymphoma cell line, MANCA. We initially sequenced the full-length *p53* gene to ensure its wild-type status. After determining the p53 gene in the MANCA cell line to be wild-type we looked at the DNA damage response pathway in the presence of several different types of damaging agents. We were able to conclude that in fact the p53 protein was induced and post-translationally modified at Serine-15 in the presence of DNA damage. Despite the induction and stabilization of p53 in the MANCA cell line we were not able to detect any significant level of apoptosis after treatment with the different drugs.

These results led us to investigate if the p53 downstream pathways were intact in the MANCA cell line. In the presence of DNA damage, MANCA p21 protein levels were not induced. Additionally, we looked at the Mdm2 protein and did not see a decrease in level after DNA damage. This suggested that in fact the MANCA p53 protein may not be capable of binding DNA and therefore is transcriptionally inactive.

The MANCA p53 protein was able to bind specifically to DNA as seen by using EMSA analysis. The MANCA nuclear protein showed DNA binding activity to the SCS oligonucleotide and the murine and human mdm2 oligonucleotides. Despite the MANCA p53 DNA binding activity, the p53 protein was comprised in the transcriptional activation of a number of p53 downstream targets (Waf1, Gadd45, Mdm2, fas, and

Noxa). These results suggested that the p53 protein was being inhibited from activating transcription possibly due to the presence of an inhibitor molecule.

Previous studies have shown that the Mdm2 protein is a negative regulator of p53's transactivation activity (Momand et al., 1993, Thut et al., 1997). Immunoprecipitation experiments showed that the Mdm2 and p53 remained in complex in the presence of DNA damage and that phosphorylation at Serine-15 of the p53 protein did not effect the protein-protein interaction. Preliminary data proposes that the Mdm2 protein may not be modified after DNA damage thereby not allowing for the release of p53. The lack of modification of the Mdm2 protein may also prevent its enhanced interaction with p300 and therefore there is no increase in the E3 ligase activity of Mdm2. Taken together these results suggest that the p53 protein does not become polyubiquitinated and therefore is not degraded. This study presents a novel mechanism for the inactivation of wild-type p53.

Chapter 2
Materials and Methods

2.1 Reagents: Camptothecin (CPT), propidium iodide, calpain inhibitor, N-Acetyl-leu-leu-Norleu-al (LLnL/ALLN), Nocodazole, anti-actin, and MTT (3-{4,5-dimethylthiazol-2-yl]-2,5-di [phenyl-tetrazolium bromide) were purchased from Sigma. Etoposide (ETOP) was purchased from Calbiochem. Zeocin (ZEO) was purchased from Invitrogen. Bristol-Myers Squibb Co. provided mitomycin C (MC). Trizol was purchased from Gibco. The p53 phospho-specific antibody, Serine-15, was purchased from Cell Signaling Technology. The monoclonal PARP antibody was purchased from Pharmingen. The Mdm2 monoclonal antibody (D7) was purchased from Santa Cruz as were the monoclonal nucleolin antibody, (MS-3), and the p53 polyclonal antibody (sc-6243). The 421, 240, and 1801 p53 antibodies are derived from hybridoma cells donated by Carol Prives.

2.2 p53 cDNA Sequencing: RNA was extracted by Trizol reagent as described in the manufacturer's protocol (Gibco). 5ug of RNA was then incubated with 250 pmol of oligo dT primers for 10 minutes at 65°C, and then subjected to a reverse transcriptase (RT) reaction. The RT reaction contained AMV, 5X buffer, DTT, dNTP (25mM), and RNase inhibitor. 2uL of cDNA was then used for PCR reactions. The PCR program carried out was as follows: 94°C for 2 minutes, 60°C for 1 minute, 72°C for 2 minutes and was done for 30 cycles. PCR products were then run on a 1% agarose gel and excised using a scalpel. Excised products were purified using the Qiagen qiaquick gel purification protocol as per manufacturer's instructions. The following primers were used for sequencing of the complete p53 gene: primer set #1: 5'-GGA ATT CCA CAC CCC CGC CCG- 3', 5' GGA ATT CAT GCC GCC CAT GCA- 3', primer set # 2: 5'-GGA

ATT CTG ACT GTA CCA CCA TCC –3', 5'-GGA ATT CTC CAT CCA GTG GTT TC- 3', primer set # 3: 5'-GGA ATT CCA CGA CGG TGA CAC G –3', 5'-GGA AT CGG TGT AGG AGC TGC TCG –3', primer set # 4: 5'-GGA ATT CCC AAC AAC ACC AGC TCC –3', 5'-GGA ATT CAA AAT GGC AGG GGA GGG –3' (Baker, S.J., et al 1989). Each set of sequencing was done twice, and compared to the wild type p53 gene within the SN3 plasmid (Baker, S.J., et al, 1990). Sequencing was done at the Rockefeller Protein and DNA Technology Center.

2.3 Cell Culture: ML-1 cells were a generous gift from Michael Kastan and the MANCA cell line was a generous gift from Andrew Koff. K562 cells were purchased from ATCC and do not contain p53. All cells were maintained in 10% fetal bovine serum, RPMI 1640 medium (Cellgro) and 5% CO2. The media was supplemented with 100ug of penicillin per milliliter and 100ug of streptomycin per milliliter. Cells were seeded at $2.5x10^5$ and exponentially growing cells were used in all experiments.

2.4 DNA Damaging Agents: DNA damaging agents, and the proteasome inhibitor, LLnL, were added to the media of exponentially growing cell cultures. The cells were treated at a concentration of $5x10^5$-$6x10^5$ cells per milliliter. The following concentrations were used throughout the study: CPT, 0.5uM, or 5uM, ETOP, 8uM, MC, 5uM, ZEO, 50ug/mL, Nocodazole, 200-2000ng/ml, and the proteasome inhibitor LLnL, 20uM.

2.5 Flow Cytometry: FACS analysis was carried out on a BD Biosciences scan. Cells were spun down at 2300rpm for 7 minutes, washed twice with phosphate buffered saline containing 2% bovine serum albumin, and 0.1% NaN3. Cells were then fixed in 30% ethanol. Propidium iodide staining and RNase treatment were carried out at 37°C for 30 minutes 24 hours prior to analysis.

2.6 MTT (3-{4,5-dimethylthiazol-2-yl]-2,5-di[phenyl-tetrazolium bromide Cytotoxicity Assay: Cells were grown in 1X RPMI supplemented with 10% FBS and Penn/Strep as described previously. Exponentially growing cells were seeded into 24 well plates at a final concentration of $2.5x10^5$ cells/ml. Cells were then either left untreated or were treated with CPT (0.5uM), ZEO (50ug/ml), ETOP (8uM), MC (5uM), or LLnL (20uM) for 3, 9 or 24 hours. Cells were spun down and re-suspended in 0.5ml of media supplemented with MTT (0.5mg/ml), and incubated for 1 hour at 37° C. Cells were then re-spun and re-suspended in 1ml of 0.04N HCl in isopropanol for 5 minutes at room temperature to allow for cell lysis. Samples were spun down and aliquots of 250ul were used for absorbance readings. Cell viability readings were calculated as the difference in the absorbance at 550 and 620nm. Data are expressed as a percentage of the control, which for this study was untreated cells.

2.7 Nuclear Extracts: Cells were pelleted at 2300rpm for 7 minutes at 4°C and washed 2X with ice-cold 1XPBS (pH=7.3). Cells were then washed 1X in 5 packed cell volumes, Buffer A (Hepes pH=7.9, 10mM, $MgCl_2$, 1.5mM, KCl, 10mM, PMSF, 0.5mM, DTT, 0.5mM, Leupeptin, 2ug/mL, and phosphatase inhibitor cocktail I (Sigma)). After

washing cells were re-suspended in 2 packed cell volumes of Buffer A and incubated on ice for 10 minutes. After centrifugation (10 minutes at 12,000rpm at 4°C) the supernatant was removed, this is the cytoplasmic fraction. The pellet is then re-suspended in Buffer C (Hepes pH=7.9, 20mM, glycerol, 25%, NaCl, 420mM, $MgCl_2$, 1.5mM, EDTA, 0.2mM, PMSF, 0.5mM, DTT, 0.5mM, Leupeptin, 2ug/mL, and phosphatase inhibitor cocktail I (Sigma)). Cells were re-suspended, with a 20-gauge needle, to a concentration of 120uL/40 million cells. The cell suspension was rocked for 30 minutes at 4°C and then centrifuged for 30 minutes at 13,000rpm at 4°C. The supernatant is the nuclear fraction, which is stored at –80°C.

2.8 Western Blotting Analysis: Nuclear extracts or immunoprecipitated samples were separated on a 10% SDS-PAGE followed by electro-transfer to a nitrocellulose membrane. The following p53 antibodies were used: 421, 240, 1801 (supernatant antibodies), Serine-15 (Cell Signaling Technology), polyclonal p53 (Santa Cruz). The following Mdm2 antibodies were used: 2A10 (supernatant antibody), D7 (Santa Cruz). For p21 detection we used Ab-1 from Oncogene Research Science. For PARP cleavage we used anti-mouse monoclonal antibody from Pharmingen. For nucleolin detection we used antibody, MS-3 from Santa Cruz. For actin detection we used a rabbit polyclonal purchased from Sigma. The signals were visualized after incubation with goat anti-mouse or goat anti-rabbit secondary antibody using ECL solutions.

2.9 Shrimp alkaline phosphatase treatment of nitrocellulose membranes: 100ug of nuclear extracts were separated on a 10% SDS-PAGE gel and electro-transferred to a

nitrocellulose membrane. The nitrocellulose membrane was then incubated with the following for 30 minutes with gentle shaking: 0.1M Tris/Cl (pH 8.5), 0.2mM EDTA (pH=8.0), and shrimp alkaline phosphatase (20U/ml) (Maya and Oren, 2000). Membranes were washed 3X with 1XPBS/0.1% Tween 20 and were then subjected to Western analysis as previously described in Materials and Methods.

2.10 Immunoprecipitation: Reactions were carried out using between 250 and 750ugs of nuclear extracts. Nuclear protein was incubated with phosphate buffered saline (pH=7.3) and 10ug of purified antibody overnight at 4°C while rocking. The next day Protein A Sepharose beads were added for 1 h with rocking at 4°C. Samples were then washed 5X in BC buffer (Hepes 20mM, pH=7.9, glycerol 10%, PMSF 0.5mM, DTT 3mM, KCl mM, Leupeptin 2ug/mL, phosphatase inhibitor cocktail I (Sigma), and NP40 0.05%). 3X protein sample buffer was then added to a final concentration of 1X and the samples were boiled and loaded onto a 10% gel to be separated by electrophoresis as previously described.

2.11 Immunoprecipitation using coupled beads: Reactions were carried out using between 250 and 750ugs of nuclear extracts. Nuclear proteins were incubated with phosphate buffered saline (pH=7.3) and purified antibody cross-linked to Protein A Sepharose beads overnight with rocking at 4° C. In order to cross-link the antibody to protein A Sepharose, antibody was first incubated with Protein A Sepharose beads for 1 hour with gentle rocking. The solution was then washed with 0.2M $Na_2B_4O_7$-$10H_2O$ (pH=9.0), and resuspended in 10 volumes of $Na_2B_4O_7$-$10H_2O$. Dimethyl pimilidate was

then added to a final concentration of 20mM, and rocked for 30 minutes at room temperature. Finally, the beads were washed 2X in 0.2M ethanolamine (pH=8.0) and incubated for 2 hours at room temperature in 0.2M ethanolamine with gentle mixing. Beads were resuspended in 1XPBS (pH=7.3).

2.12 Release of immunoprecipitated protein using 421 peptide: Experiments were carried out as previously described. After incubation of nuclear protein with antibody, and Protein A Sepharose beads, samples were incubated for 30 minutes on ice with a peptide specific for the p53 421 epitope. The 421 peptide was re-suspended in a 0.25M NaCl solution to a final concentration of 1mg/ul. The bumped supernatant proteins were then analyzed by SDS gel electrophoresis (10%) as previously described.

2.13 Mass Spectrometry Analysis: Immunoprecipitation experiments were performed as previously described, using 421 uncoupled beads. Samples were then separated by SDS-PAGE (10%). After separation gels were stained with 0.075% Coomassie Brilliant Blue R-250 (Bio-Rad), 40% methanol, and 10% acetic acid. Gels were then de-stained overnight. Bands of interest were cut form de-stained gels and sequenced by mass spectrometric analysis at Rockefeller University Protein and DNA Technology Center.

2.14 Electrophoretic Mobility Shift Assay: Custom oligonucleotides were ordered from Operon Technologies. Labeling of the oligo was performed using the large fragment of DNA polymerase and α^{32}P dCTP. Electrophoretic mobility shift assays were carried out in reaction mixtures (30uL) with 150pmol of ^{32}P oligonucleotide. 10ug of

nuclear extract was added and the reaction was incubated for 20 minutes at room temperature in a reaction buffer containing 20mM Hepes, pH 7.8, 100mM KCl, 1mM EDTA, pH=8.0, 1mM DTT, 1ug sheared salmon sperm DNA, and 10% glycerol. In the case of competition, unlabeled competitor in 50X-100X fold excess was added 5 minutes into the incubation. Mdm2, Fas, and a mutant oligos were used for competition studies. The superconsensus site (SCS) contained three adjacent p53 half sites. The sequence of the oligonucleotide was Top:5'TCGAGCCGGGCATGTCCGGGCATGTCCGGGCATGTC-3', and Bottom:5'-TCGAGACATGCCCGGACATGCCCGGACATGCCCGGC-3'. The sequences of the oligos used for competition studies are as follows: human Mdm2:Top:5'CCGGGCTGGTCAAGTTCAGACACGTTCCGAAACTGCAGTAAAAG GAGTTAAGTCCTGACTTGTCTCCC-3', and Bottom: 5'-CCGGGGGAGACAAGTCAGGACTTAACTCCTTTTACTGCAGTTTCGGAACGTG TCTGAACTTGACCAGC-3', Fas:Top:5'CCGGGCTCCTGGACAAGCCCTGACAAGCCAAGCCAC-3', and Bottom:5'-CCGGGTGGCTTGGCTTGTCAGGGCTTGTCCAGGAGC-3', and Mutant: Top: 5'-TCGAGTTTAATGGACTTTAATGGCCTTTAATTTTC, and Bottom: 5'-TCGAGAAAATTAAAGGCCATTAAAGTCCATTAAAC-3'. The murine Mdm2 sequence is as follows: 5'-GATCCCTGGTGAAGTTGGGACACGTCCGGCGTCGGCTGTCGGAGGAGCTAAG TCCTGACATGTCTCCG-3'. Reactions were carried out in the presence or absence of pAb421 as indicated. Samples were separated by 4% polyacrylamide gel electrophoresis

(gels were pre-run at 100V for 15 minutes at 4°C) at 200V for 3-3.5 hours. Gels were dried for 1 hour at 55°C and autoradiography was performed.

2.15 PCR using Epstein Barr virus primers: RNA was extracted by Trizol reagent as described in the manufacturer's protocol (Gibco). 5ug of RNA was then incubated with 250 pmol of oligo dT primers for 10 minutes at 65°C, and then subjected to a reverse transcriptase (RT) reaction. The RT reaction contained AMV, 5X buffer, DTT, dNTP (25mM), and RNase inhibitor. 2uL of cDNA was then used for PCR reactions. The PCR program was as follows: 94^0 C for 2 minutes, 94^0 C for 1 minute, 60^0 C for 1 minute, and 60^0 C for 7 minutes and was done for 30 cycles. PCR products were then run on a 1% agarose gel. The following primers were used: 5'-AAAACATGCGGACCACCA-3', and 5'-AGGACCTACGCTGCCCTA-3' (Orentas, 1998).

2.16 Quantitative PCR: Reverse transcription (RT): For each sample, 5ug of RNA was obtained using the Qiagen RNeasy Kit, as per the manufacturer's protocol. Each sample was then diluted up to 50ul with water. The samples were then incubated with reagents from the cDNA Archive Kit (Applied Biosystems). The mixture contained RT buffer, dNTP mix, random primers, and multiscribe RT. The 2X RT Master Mix, along with the cDNA was incubated at room temperature for 10 minutes, and then at 37°C for 2 hours. cDNA was stored at –20°C. For PCR, the program was as follow: one cycle of 50°C UNG incubation for 2 minutes, and one cycle of 95°C priming for 10 minutes, followed by 40 cycles of 95°C denaturation for 15 seconds and 60°C annealing for 1 minute. This

reaction was carried out in an Applied Biosystems 5700 prism spectrofluorometric thermal cycler. Fluorescence was measured during the annealing step and plotted automatically for each sample. Assays on demand for Mdm2 1,2 (order #: HS00234753_M1), Mdm2 5,6 (order #: HS00242813_M1) Fas (order #: HS00538709_M1), Waf1 (order #: HS00355782_M1), Noxa (order #: HS00560402_M1), and Gadd45 (order #: HS00169255_M1) were purchased from Applied Biosystems, as was the PDAR for GAPDH (order #: 4333764F). Sequences are copyright of the company and they are not offered upon request.

2.17 In-vivo labeling of total protein: Exponentially growing cells were resuspended in fresh complete media and incubated at 37° C for 6 hours in the presence of ^{32}P ortho-phosphate at a final concentration of 1mCi/ml. At the same time the ortho-phosphate was added, 5uM CPT was added to the cell suspension. Cells were then washed 2X with ice-cold 1XTBS, and nuclear extracts were prepared as previously described in Materials and Methods. Nuclear samples were then immunoprecipitated with 421-coupled beads as previously described in Materials and Methods. Immunoprecipitated proteins were then separated by SDS-PAGE (10%), and phosphorylated protein bands were visualized by autoradiography.

2.18 RNA Extraction and Northern Analysis: Total RNA was extracted using the Trizol reagent (Gibco) according to manufacturer's instructions. Total RNA (40ug) was resolved on a 1% denaturing formaldehyde gel, and electro-transferred to a nylon membrane. The membrane was then dried at 80°C for 2 hours, and the RNA was UV

cross-linked to the membrane. The nylon membrane was incubated with 10mL of pre-hybridization buffer (Amersham Biosciences) for 1.5 hours and radiolabeled probes were added directly to the pre-hybridization buffer for 24 hours. The membrane was then washed twice in 2X SSC buffer at 65° C with shaking for 5 minutes each, followed by another wash with 1XSSC buffer at 65° C with shaking for 15 minutes and twice in 0.1X SSC buffer at 65° C with shaking for 5 minutes each. The signal was visualized by autoradiography. The mdm2 probe is a 1.3kb cDNA fragment cloned into the EcoRI site of the Bluescript plasmid (Oliner, J.D., et al. 1992).

Chapter 3
Analysis of the Effects of DNA Damaging Agents in the Burkitt's Lymphoma Cell Line, MANCA

Introduction:

The tumor suppressor, p53, is a central player in preserving the integrity of the genome (Vousden, 2002). The p53 protein can sense stress in the cellular environment and act as a transcription factor to activate a growth arrest or apoptotic pathway (Vousden 2000). Topoisomerase poisons can allow for initiation of the apoptotic cascade (Kaufmann, 1998, Pommier, et al., 1998). The presence of DNA damage in the cellular environment will allow for induction of the p53 protein and subsequent post-translational modifications to allow for the p53 protein to be further stabilized (Tishler et al., 1995; Colman et al., 2000; Nelson and Kastan, 1994). There are several ways in which the p53 protein may become inactivated. Mutation of the p53 protein is a very common event and renders the organism susceptible to tumor formation. As seen in mice that lack p53, there is early onset of spontaneous tumor formation (Donehower et al., 1992). Additionally, the p53 protein may become inactivated due to the presence of viral proteins, such as the SV40 large T antigen, or the Epstein-Barr virus (Bargonetti et al., 1992; Fries et al., 1996; Szekely et al., 1993). Another mechanism which can allow for the functional inactivation of p53 is the p53 interaction with cellular proteins, such as increased Mdm2 levels (Pykett et al., 1998).

In this study we carefully examined the apoptotic response of a Burkitt's lymphoma cell line, MANCA, in the presence of several different DNA damaging agents. Previous work on this cell line has been limited, and very little is know about the MANCA response to DNA damage. The MANCA cell line contains a translocation of

the c-myc gene placing the gene in between the J_H cluster and the C_u-coding segments of the immunoglobulin heavy chain locus (Saito et al., 1983) **(Figure 3.1).**

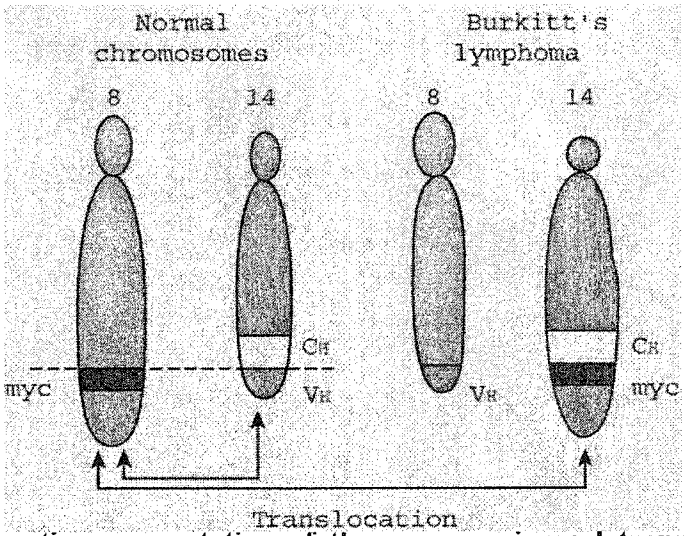


Figure 3.1 Schematic representation of the c-myc reciprocal translocation event in a Burkitt's lymphoma cell line. *(www.nature.com/.../fig_tab/nature01409_F3.html)*

Additionally, this cell line was used early on for numerous cell cycle studies due to its rapid growth profile (Soos et al., 1996). To begin this study we had the complete *p53* gene (exons 1-11) from the MANCA cell line sequenced at the Rockefeller Protein and DNA Technology Center. The MANCA cell line was found to contain wild-type *p53* (as described in Materials and Methods). Previous FACS data we collected showed that these cells could not undergo DNA damage-dependent apoptosis (data not shown).

Results:

DNA damage does not induce apoptosis in cells lacking functional p53 protein.

Treatment of the human ML-1 (myeloid leukemia) cell line with camptothecin (CPT), etoposide (ETOP), mitomycin C (MC), and the proteasome inhibitor, LLnL has been shown to induce apoptosis (Abbas et al., 2002, Lazebnik et al., 1994). The ML-1 cell line contains wild-type functional p53. In contrast similar treatment of the K562 cell line, which does not contain p53, did not show significant levels of apoptosis. To further investigate if the p53 protein from MANCA cells contained functional p53 that could induce an apoptotic program we treated these cells with the above agents for various time points. Using FACS analysis we were able to compare the level of sub G1 DNA content detected in the three cell lines, ML-1, MANCA, and K562 **(Figure 3.2).** FACS analysis showed that after 48 hours of drug treatment the amount of apoptosis, as determined by sub-G1 DNA, in the MANCA cell line never exceeded 7% **(Figure 3.2B)** whereas our control sample, ML-1, had as much as 70% sub G1 DNA **(Figure 3.2A).** The level of apoptosis detected in the untreated MANCA and ML-1 cell lines was equivalent. Both the K562 cell line and the MANCA cell line had more equivalent levels of apoptosis after drug treatment **(Figures 3.2B and 3.2C).** Similar results were seen in all three-cell lines after 24 hours treatment (data not shown). To look at apoptotic levels at an earlier time point we examined the PARP cleavage by Western blot analysis of nuclear protein **(Figure 3.2D).** In this experiment the MANCA, ML-1, and K562 samples were drug treated for a shorter time point, 6 hours, as PARP cleavage is an early indicator of

apoptosis (Duriez PJ, and Shah GM, 1997). In the absence of DNA damage there was no apoptosis detected in the MANCA, ML-1, or K562 cell lines, as evidenced by lack of PARP cleavage **(Figure 3.2D, lanes 1,7, and 13).** However, after 6 hours of drug treatment the PARP protein was cleaved in the ML-1 cell line in the presence of CPT and ETOP, while this same phenotype was not seen in the MANCA and K562 cell lines **(Figure 3.2D compare lanes 2 and 4 to lanes 8, 10, 14, and 16).** After 6 hours of treatment the other drugs did not induce apoptosis in any of the cell lines tested **(Figure 3.2D, lanes 3, 5, 6, 9, 11, 12, 15, 17, and 18).** The results from these experiments suggested that the p53 in the MANCA cell line was functionally inactive to induce apoptosis.

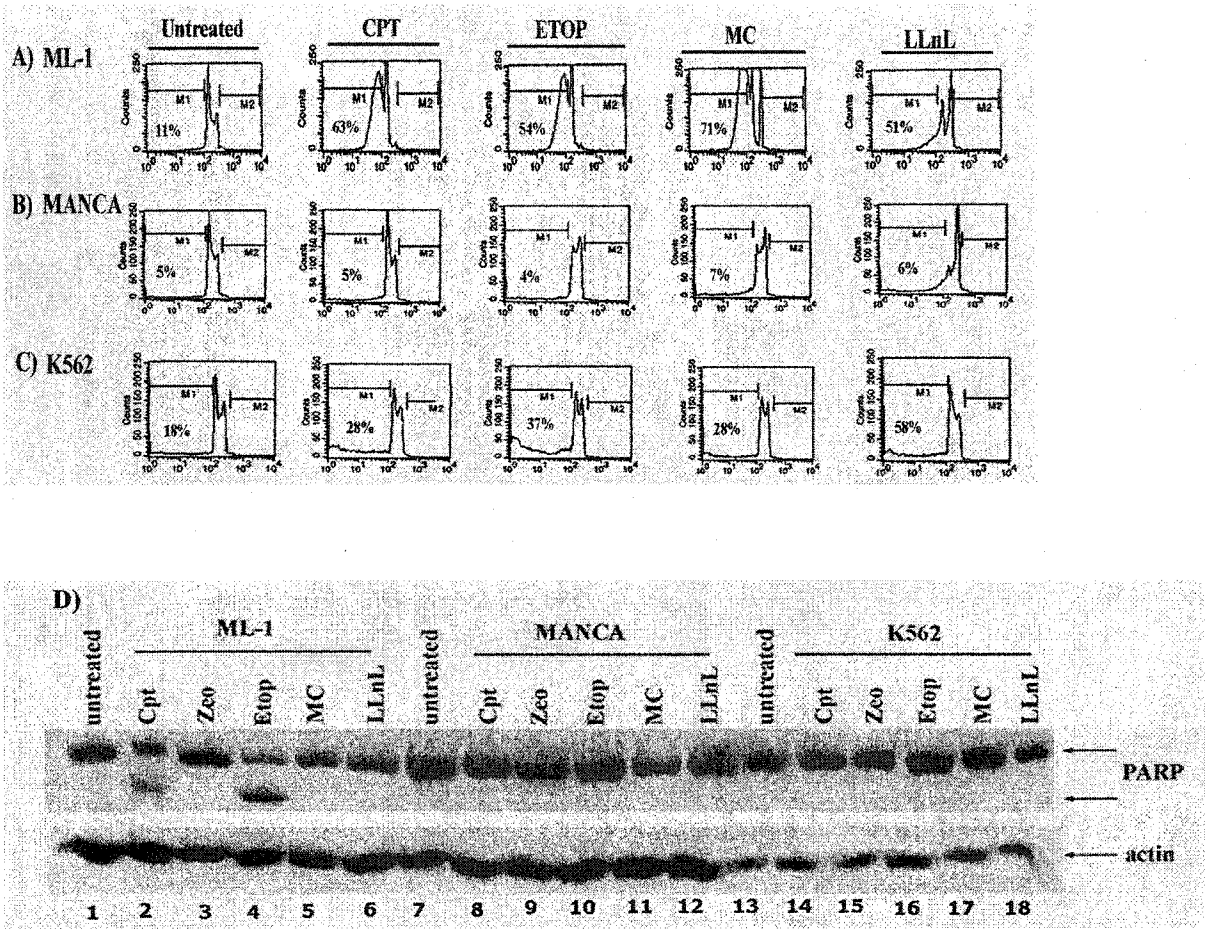


Figure 3.2 There is no apoptosis detected in the MANCA cell line after treatment with drugs. *FACS analysis of* ***(A)*** *ML-1,* ***(B)*** *MANCA, and* ***(C)*** *K562 cells. Exponentially growing cells were either left untreated or were treated with 0.5uM camptothecin (CPT), 8uM etoposide (ETOP), 5uM mitomycin C (MC), or 20uM LLnL, for 48 hours. Cells were harvested and fixed in 30% ethanol. 24 hours prior to FACS analysis cellular DNA was stained with propidium iodide. Cells were analyzed by flow cytometry cell sorting.* ***(D)*** *Western blot analysis of ML-1, MANCA,*

and K562 samples. ML-1, MANCA, or K562 cells were either left untreated (lanes 1, 7, and 13) or were treated with 0.5uM CPT (lanes 2, 8, and 14), 50mg/ml ZEO (lanes 3, 9, and 15), 8uM ETOP (lanes 4, 10, and 16), 5uM MC (lanes 5, 11, and 17), or 20uM LLnL (lanes 6, 12, and 18) for 6 hours. 100ug of nuclear protein was subjected to SDS-PAGE (10%) and Western analysis. The upper portion of the nitrocellulose membrane was probed with a PARP specific monoclonal antibody. The lower portion was probed with an actin polyclonal antibody. This was used as a normalizing agent.

MANCA cells arrest in the G2/M phase of the cell cycle after treatment with nocodazole.

To further study the ability of the MANCA cells to undergo growth arrest or apoptosis we carried out studies using nocodazole. Nocodazole, a microtubule inhibitor, has been found to cause cells to undergo a G2/M arrest (Tishler et al., 1995). MANCA and ML-1 cells were treated with nocodazole for 24 hours and then subjected to FACS analysis **(Figure 3.3)**. Not only did we see that more than 80% of the MANCA cells arrested in the G2/M phase of the cell cycle, but we also detected significantly more sub G1-DNA content than with the treatments previously used **(Figure 3.3B)**. In contrast, only 25% of the ML-1 cells arrested in G2 after nocodazole treatment **(Figure 3.3A)**. Previous studies have found that DNA damage in Burkitt's cell lines can induce apoptosis within the G1/S phase of the cell cycle in the presence of functional p53 protein (Allday et al., 1995). When p53 is functionally incompetent, apoptosis can only be detected during the G2/M phase of the cell cycle (Allday et al., 1995; Kastan et al., 1991).

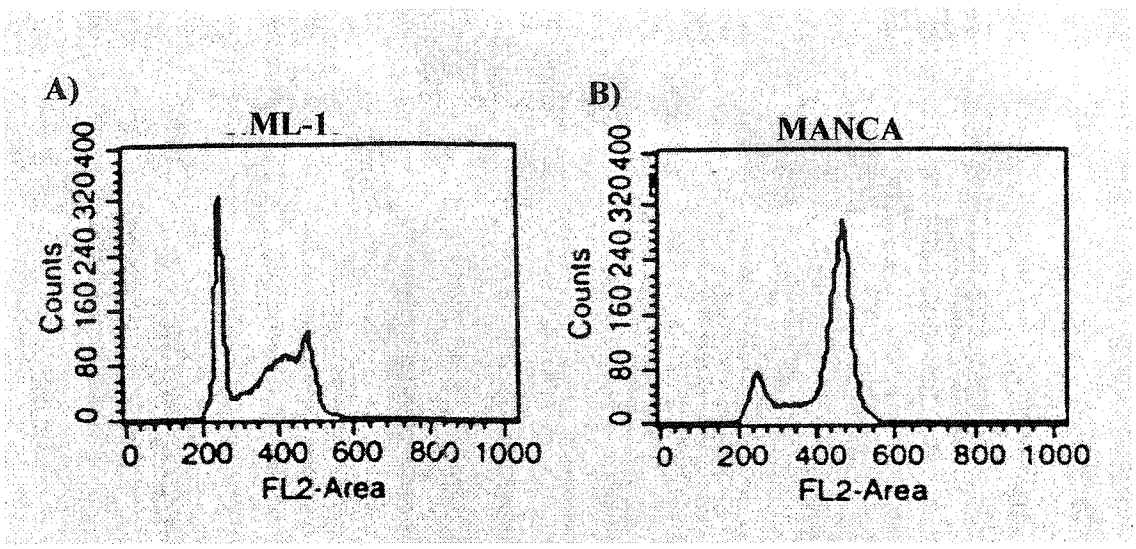


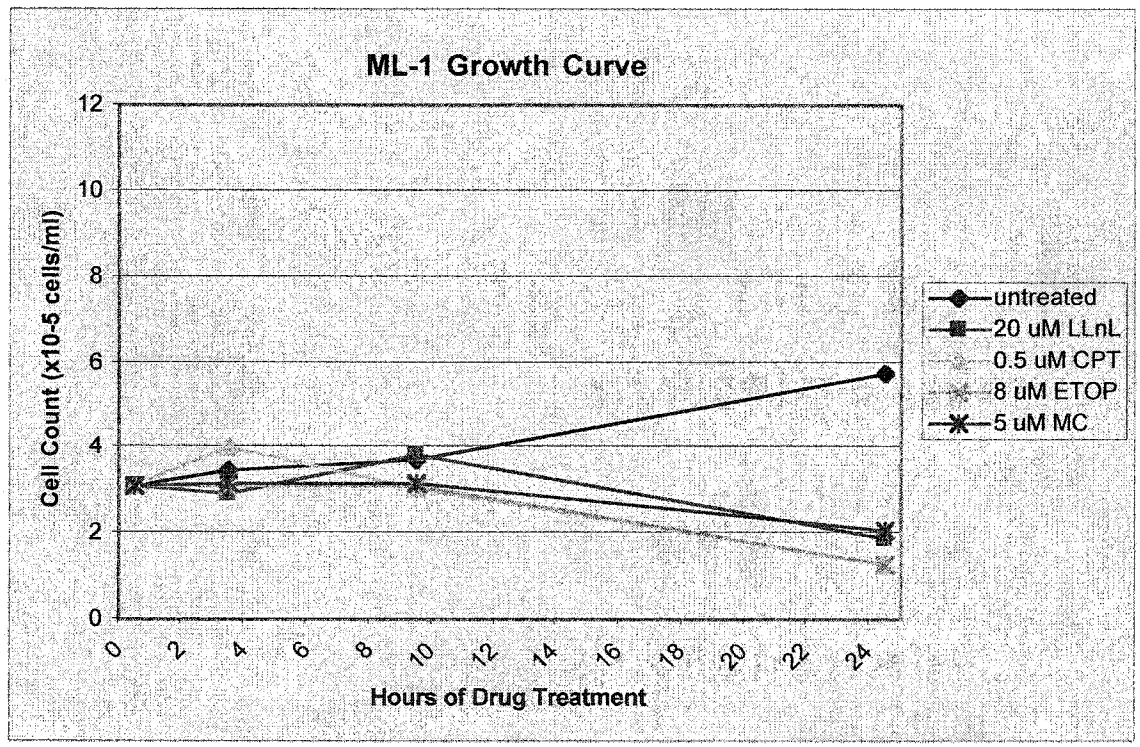
Figure 3.3 MANCA cells can arrest in the G2/M phase of the cell cycle. *FACS analysis of* ***(A)*** *ML-1 and* ***(B)*** *MANCA cells. Exponentially growing cells were treated with 25uM Nocodazole for 24 hours. Cells were harvested and fixed in 30% ethanol. 24 hours prior to FACS analysis cellular DNA was stained with propidium iodide. Cells were analyzed by flow cytometry cell sorting. Untreated FACS profiles were identical to those of untreated MANCA and ML-1 samples as seen in Figure 3.2A and B.*

DNA damage shows selective cytotoxicity in the MANCA cell line.

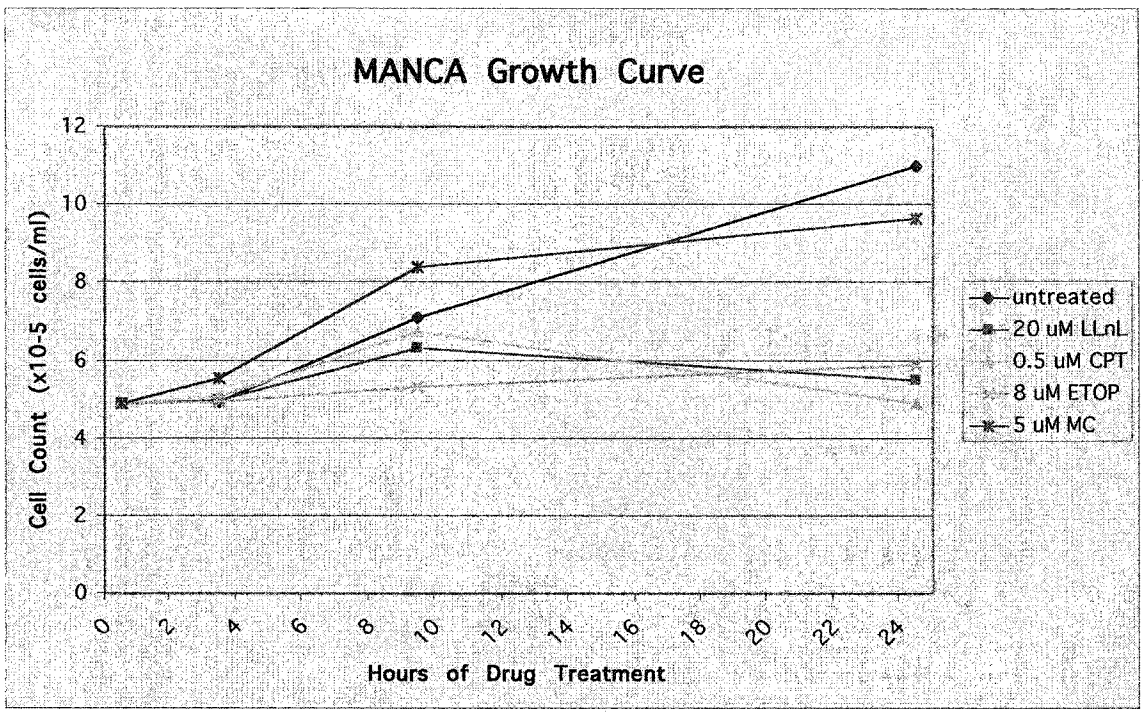
The MANCA, ML-1, and K562 cell lines were analyzed to examine the comparative cytotoxicity of the CPT, ETOP, MC, or LLnL drugs for 3, 9, or 24 hours. These experiments were only done one time but confirmed that the MANCA cell line was not sensitive to the drugs tested **(Figure 3.4)**. The p53 protein has been shown to influence cytoxicity in the presence of DNA damage (Lowe et al., 1993). In the presence of the different drug treatments, the MANCA cells exhibited selective signs of cytotoxicity, similar to the results obtained from the K562 treated cells **(Figure 3.4, compare panel B to panel C).** Additionally, longer drug treatments were needed to effect the growth rate in MANCA cells. In contrast, the ML-1 cells showed some degree of cytotoxicity to all of the different drugs used in this study, and results were seen as early as 9 hours **(Figure 3.4A).**

Figure 3.4 Several different DNA damaging agents show selective signs of cytotoxicity in the MANCA cells. *Growth curves of the* ***(A)*** *ML-1,* ***(B)*** *MANCA, and* ***(C)*** *K562 cell lines. ML-1, MANCA, and K562 cells were either left untreated, or were treated with 0.5uM CPT, 8uM ETOP, 5uM MC, or 20uM LLnL for 3, 9, or 24 hours. The MTT assay was performed as per the manufacturer's instructions. Briefly cells were spun down and re-suspended in 0.5ml of media supplemented with MTT (0.5mg/ml), and incubated for 1 hour at 37° C. Cells were then re-spun and re-suspended in 1ml of 0.04N HCl in isopropanol for 5 minutes at room temperature to allow for cell lysis. Samples were spun down and aliquots of 250ul were used for absorbance readings. Cell viability readings were calculated as the difference in the absorbance at 550 and 620nm.*

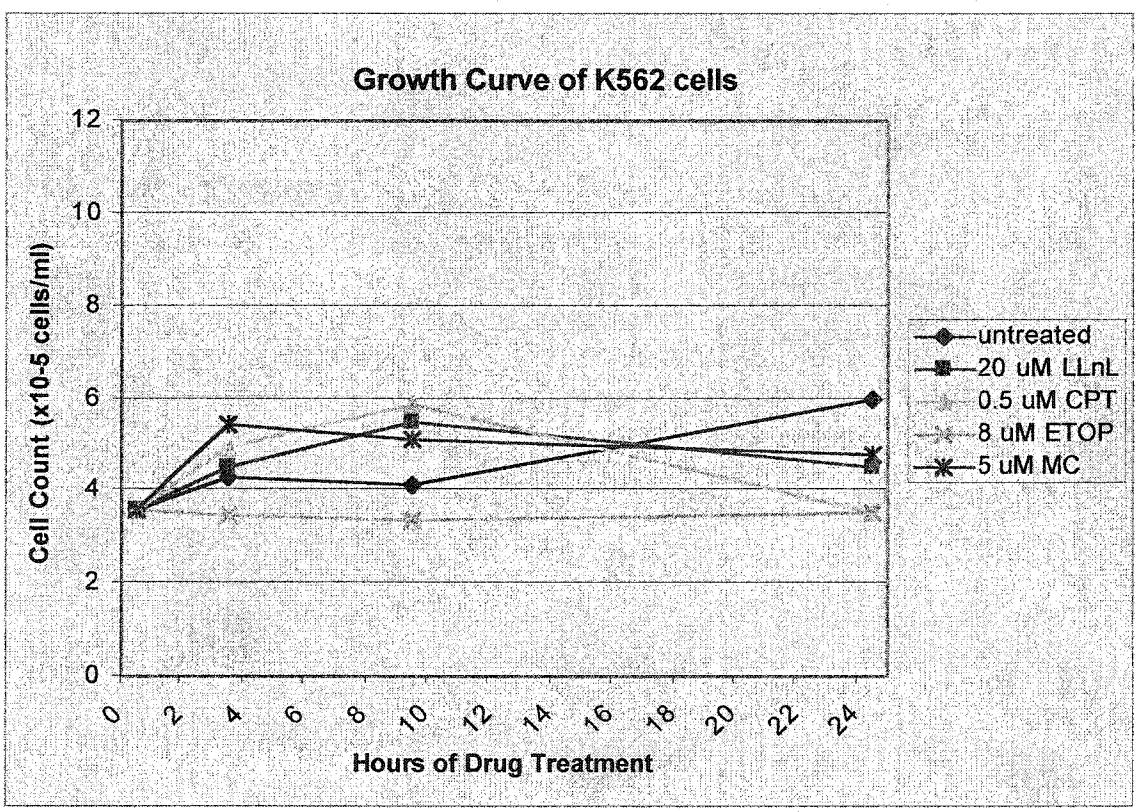
A)



B)



C)



The p53 protein from MANCA cells is phosphorylated and stabilized after DNA damage.

We next set out to determine if the cascade upstream from the p53 protein was active in the presence of drug treatment. In accordance with Abbas et al., we have shown that CPT, ETOP, and MC can induce p53 protein levels in the ML-1 cell line, as early as three hours after treatment **(Figure 3.5A, compare lane 1 to lanes 2-4)** (Abbas et al., 2002). It is important to note that the faster migrating species in the ML-1 cells may be due to high levels of apoptosis, and increased calpain activity (Kubbutat and Vousden, 1997) Western blot analysis showed that there was also induction of the p53 protein in the MANCA cells after treatment with the different DNA damaging agents **(Figure 3.5A, compare lane 5 to lanes 6-8)**. As expected there was no p53 present before or after DNA damage in the K562 nuclear extracts **(Figure 3.5A, lanes 9-12).** We next examined if the induced p53 protein was phosphorylated at serine-15. Several studies have shown that the phosphorylation of p53 at serine-15 allows for further stabilization by inhibiting the negative regulation of p53 by the Mdm2 protein (Shieh et al., 1997; Siliciano et al., 1997).Western blot analysis using an antibody specific for the serine-15 phosphorylated form of p53 showed a high level of phosphorylation at serine 15 in the MANCA cell line after drug treatment **(Figure 3.5B, lanes 6-8).** The level of phosphorylation of p53 detected in the ML-1 cell line was significantly less than the level detected in the MANCA cell line possibly as a consequence of the p53 cleavage seen in the ML-1 cell line **(Figure 3.5B, lanes 2-4).** Lanes 9-12 of **Figure 3.5B** contain the K562 cell extract which did not contain the p53 protein. Despite the lack of apoptosis detected in the MANCA cell line, the p53 protein was induced and post-translationally

modified. These data suggests that the p53 protein was activated and therefore might be capable of binding to DNA and activating transcription.

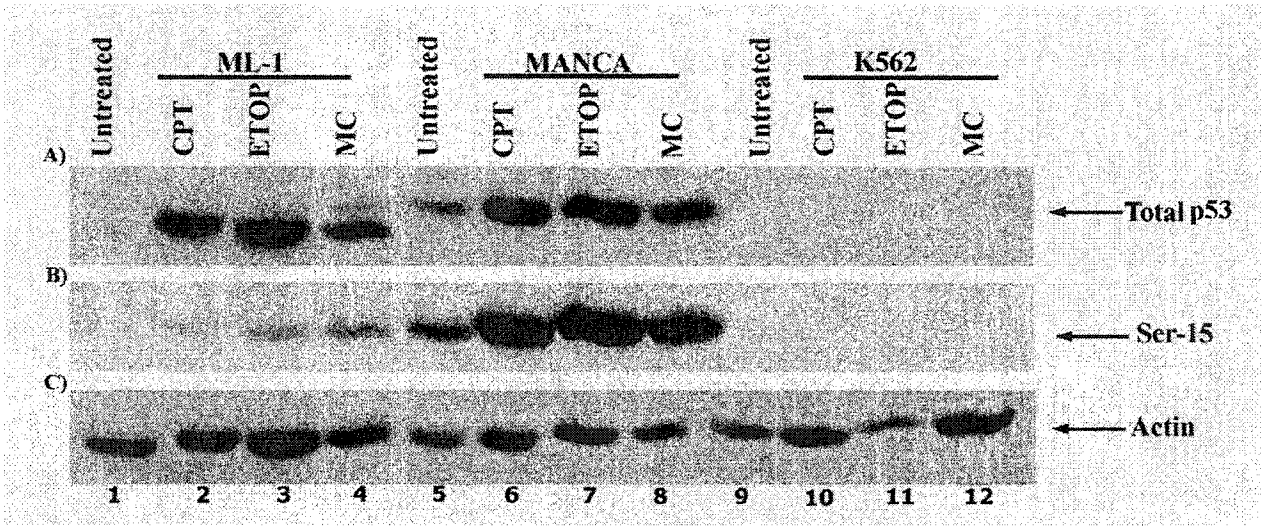


Figure 3.5 The p53 protein in MANCA cells is induced and phosphorylated after DNA damage. *Western blot analysis of ML-1, MANCA, and K562 nuclear proteins. ML-1, MANCA, and K562 cells were either left untreated (lanes 1,5, and 9), or were treated with 0.5uM CPT (lanes 2, 6, and 10), 8uM ETOP (lanes 3, 7, and 11), or 5uM MC (lanes 4, 8, and 12) for 6 hours and nuclear extracts were then prepared from these samples. 100ug of nuclear protein was then subjected to SDS-PAGE (10%) and Western analysis. The nitrocellulose membranes were probed with* ***A)*** *a mixture of the p53 –specific monoclonal antibodies, 421, 240, and 1801,* ***B)*** *the serine-15 p53 phospho-specific polyclonal antibody, or* ***C)*** *anti-actin polyclonal antibody.*

DNA damage has no effect on the level of the p21 or Mdm2 proteins in the MANCA cell line.

Stabilization of the p53 protein in the presence of DNA damaging agents allows for the p53 protein to act as a transcription factor. In particular, p53 can activate the expression of the cyclin dependent kinase inhibitor, p21 (El-Deiry et al., 1993). The p21 protein is essential for cell cycle arrest at the G1/S phase border (El-Deiry et al., 1993). Using Western analysis we looked at the level of induction of the p21 protein after treatment with DNA damaging agents **(Figure 3.6A).** Treatment of the ML-1 cells with CPT, ETOP, and MC showed an increase in p21 protein levels **(Figure 3.6A, compare lane 1 to lanes 2-4).** Interestingly, MANCA cells treated with the same battery of DNA damaging agents showed no induction of the p21 protein level **(Figure 3.6A, compare lane 5 to lanes 6-8)**; exhibiting the same pattern as seen in the K562 cell line **(Figure 3.6A, lanes 9-12).** In contrast to the lack of p21 protein detected in the MANCA cell line, we were able to detect high levels of the Mdm2 protein before and after treatment with the different DNA damaging agents **(Figure 3.6B).** Previous work has shown that in the presence of CPT and ETOP *mdm2* RNA levels decrease (Arriola et al., 1999; Xiao et al., 2000). However there are some recent papers that investigate Mdm2 protein levels after DNA damage (Cho et al., 2001). The level of Mdm2 protein in the MANCA cells remained unchanged after DNA damage **(Figure 3.6B, compare lane 5 to lanes 6-8).** Interestingly, in the presence of CPT and ETOP we saw a decrease in the Mdm2 protein level in the ML-1 cell line **(Figure 3.6B compare lane 1 to lanes 2 and 3).** As

previously noted the phenotype seen in the K562, p53 null line, was very similar to that of the MANCA cells, suggesting that the MANCA cells contain a transcriptionally inactive species of wild –type p53.

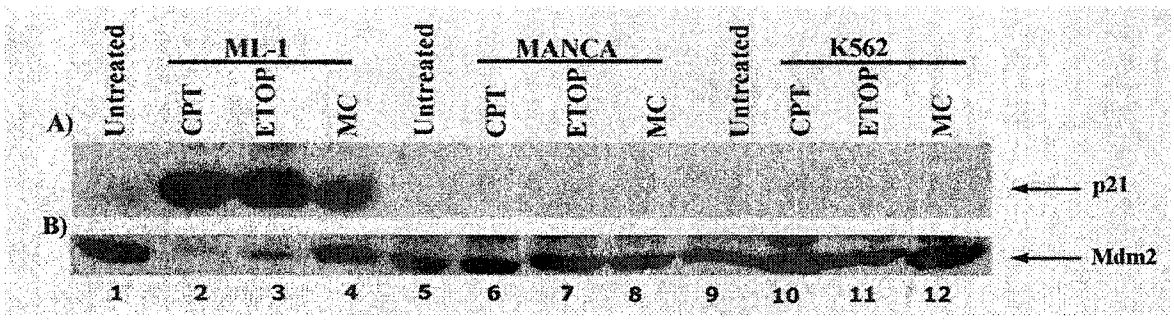


Figure 3.6 DNA damage does not effect the level of the p21 and Mdm2 proteins in the MANCA cell line. *Western blot analysis of ML-1, MANCA, and K562 nuclear proteins. Cells were either left untreated (lanes 1, 5, and 9), or were treated with 0.5uM CPT (lanes 2, 6, and 10), 8uM ETOP (lanes 3, 7, and 11), or 5uM MC (lanes 4, 8, and 12) for 6 hours. Nuclear extracts were prepared from the ML-1, MANCA and K562 cells, and proteins were separated by SDS-PAGE (10%). Subsequent Western analysis was preformed on nuclear samples. The nitrocellulose membrane was probed* ***A)*** *a p21-specific monoclonal antibody, and* ***B)*** *the Mdm2-specific monoclonal antibody, D7. These samples were shown to be normalized in Figure 3.5C. This experiment is representative of three different analyses.*

Discussion:

p53 DNA damage-dependent apoptosis is a crucial event in the prevention of malignant transformation. The loss of this specific type of apoptotic function in lymphoma is often due to the presence of mutant p53 (Fan et al., 1994). In this study we have found a situation in which the p53 dependent apoptotic pathway is inactivated in the presence of wild type p53. Similar situations have been described and often result from increased degradation of p53 due to high levels of Mdm2 or viral proteins (Capoulade et al., 1998). The *p53* gene from the MANCA cell line was found to have a *wild –type* sequence (data not shown). Additionally, the protein was induced and subsequently stabilized in the presence of different DNA damaging agents **(Figure 3.5).**

Our results suggested that the cascade upstream from the p53 protein was intact and therefore we expected to see activation of downstream target genes. Surprisingly, the p21 protein was not shown to be induced when p53 levels in the MANCA cell line were increased **(Figure 3.6)**. It is highly plausible that the high levels of the c-myc protein present in the MANCA cell line led to the lack of p21 induction. MANCA cells, are a type of Burkitt's lymphoma and Burkitt's lines have been shown to overexpress the c-myc protein due to a translocation of the *c-myc* gene (Klein, 1993). C-myc has been shown to inhibit transcription from the p21 promoter (Dang, 1999). Therefore, other p53 target genes will need to be examined to determine if the repression was correlated to c-myc mediated inhibition.

The loss of p21 protein did not help us to explain why these cells were not undergoing p53 dependent apoptosis **(Figure 3.2).** In the presence of DNA damaging agents the level of apoptosis detected in the MANCA cell line was negligible. But, in

contrast to undetectable levels of the p21 protein we found high levels of the Mdm2 protein in the presence of DNA damage **(Figure 3.6).** Unlike the ML-1 cell line the Mdm2 protein levels were not downregulated in the presence of a DNA damage signal. This allowed us to speculate that it is possibly the presence of the Mdm2 protein which is inhibiting the p53 protein from activating a DNA damage-dependent apoptotic pathway. Previous work has shown that cells with high Mdm2 levels are often capable of escaping p53 dependent regulation (Oliner et al., 1992; Finlay, 1993). The Mdm2 protein can interact directly with p53 and serve to inhibit p53's transactivation activity (Momand et al., 1992). The Mdm2 protein interacts with the amino terminus of the p53 protein and therefore can mask the p53 transcription domain. In addition to transcriptional inhibition the Mdm2 protein may target p53 for degradation via the E3 ubiquitin ligase pathway (Fang et al., 2000, Grossman et al., 2003). In the MANCA cells this is not the case as we see high levels of the p53 protein **(Figure 3.5)**. Our data raised another question, if there is no apoptosis detected in the MANCA cell line is this p53 protein able to bind DNA and act as a transcription factor? Or is the Mdm2 possibly always in complex with the p53 protein thereby inhibiting its transcription activity of downstream target genes which would allow for initiation of p53 dependent apoptosis? p53 protein that is transcriptionally inactive is not proficient in its ability to activate an apoptotic program.

These results led us to look further into the DNA binding and transcription activity of the p53 protein in the presence of DNA damaging agents which have been shown to induce p53 dependent apoptosis.

Chapter 4
The MANCA p53 Protein has DNA Binding Ability, yet Lacks Transcription Activity.

Introduction:

The p53 protein plays an essential role in inhibiting tumorigenesis by functioning as a transcription factor (Fields and Jang, 1990, Farmer et al., 1992). The central region of the p53 protein can bind specifically to DNA responsive elements, which contain the following sequence: 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3' (El-Deiry et al., 1992). The central region of the p53 protein remains free of post-translational modifications, and rather it is the modification of the amino and carboxy termini that allow for stabilization of the p53 protein (Giaccia and Kastan, 1998). The stabilized p53 protein is then able to activate transcription from a number of genes, all of which contain the consensus sequence (El-Deiry et al., 1992).

In the absence of a DNA damage signal the half-life of the p53 protein is short, measuring on the average between 5 and 20 minutes (Maltzman and Czyzyk., 1984). In the presence of DNA damage, the p53 protein is stabilized by a cascade of post-translational modifications (Sakaguchi et al., 2000). Following DNA damage or cellular stress the p53 protein is modified at serine 15, 20 and threonine 18 residues (Shieh et al., 1997, Bean and Stark, 2002). The modification of the amino terminus of the p53 protein leads to inhibition of the p53-Mdm2 protein-protein interaction complex formation and allows for subsequent activation of p53 transactivation (Dumaz and Meek, 1999).

Additionally, the transcription factor, p53, can then go on to activate a growth arrest or apoptotic pathway by targeting different downstream checkpoint genes. The

product of the Waf1 gene is involved in activating a G1/S phase cell cycle arrest (El-Deiry et al., 1993). Gadd45 has been shown to play a partial role in the activation of a G2/M cell cycle arrest (Zhan et al., 1998). p53 also has the ability to transactivate apoptotic genes which contain p53 responsive elements. An example of one such gene is the *Noxa* gene. Studies have shown that Noxa induced apoptosis is p53 dependent. Noxa has the ability to activate caspase-9, and interact with the anti-apoptotic protein Bcl-2 (Oda et al., 2000). In addition, DNA damage induced p53 is able to transactivate the *Fas-receptor*, which contains a p53-responsive element within the first intron (Muller et al., 1998).

We have found that MANCA cells do not undergo apoptosis in the presence of several different DNA damaging agents. We have also shown that there are high levels of the Mdm2 protein present after drug treatment. Previous works by Zauberman et al, and Thut et al, demonstrated that the Mdm2 protein can inhibit p53's ability to act as a transcription factor. The p53 transactivation activity can be inhibited by a direct masking of the amino terminal transactivation domain (Momand et al., 1992). This would prevent further post-translational modification of the p53 protein, therefore allowing the Mdm2 protein to remain in complex with p53. p53-Mdm2 complexes have been shown to lack binding activity to DNA containing p53 binding sites (Zauberman et al., 1993). Additionally, amino terminal binding of Mdm2 would prevent the p53 protein from interacting with transcription machinery (Thut et al., 1997). To test p53's ability to act as a transcription factor we first looked to see if the MANCA p53 protein was able to

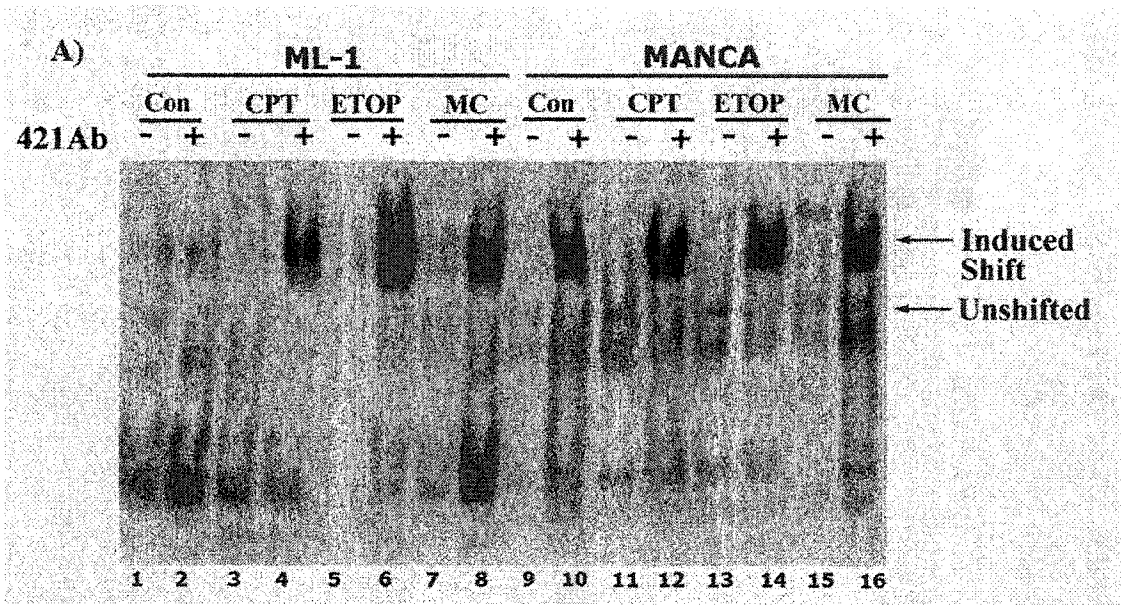
bind DNA specifically, we then looked to see if p53 had the ability to activate and induce transcription of the downstream checkpoint genes.

Results:

Stabilized p53 binds specifically to DNA

MANCA and ML-1 cells were treated with CPT, ETOP, MC, and LLnL to allow for p53 induction and post-translational modification **(Figure 3.5)**. We were then able to assess the p53 DNA binding ability in the MANCA cell line. We compared binding activity of p53 in nuclear extracts derived from MANCA cells to nuclear extracts derived from ML-1 cells **(Figure 4.1)**. ML-1 cells have wild-type *p53* and undergo apoptosis in the presence of DNA damage **(Figure 3.2).** Using the DNA super-consensus sequence (SCS) and employing the use of electrophoretic mobility shift assay (EMSA) we were able to determine that the p53 protein, from both the ML-1 and MANCA cells, had DNA binding ability **(Figure 4.1A).** The p53 DNA binding activity was activated in the presence of a p53 specific antibody, 421 (Hupp et al., 1992), and we were able to see an induced shift in the MANCA and ML-1 nuclear extract samples derived from cells treated with the various DNA damaging agents **(Figure 4.1A, compare odd lanes with no 421 antibody to even lanes where 421 antibody is present).** Published work has shown that the p53 specific, 421 antibody, can activate p53 DNA binding activity in an EMSA (Hupp et al., 1992). 421 antibody binds within the carboxy terminus of the p53 protein and allows for a conformational change to occur, which possibly serves to prevent masking of the amino terminal p53 transactivation domain (Hupp et al., 1992). Unlike with ML-1 cells we were able to see p53 induced DNA binding activity in the MANCA cell nuclear extract derived form untreated cells **(Figure 4.1A, lanes 11 and 12).**

Using EMSA competition assays with 50 to 100 fold excess unlabeled competitor oligonucleotide, we examined if the p53 DNA binding activity was specific (Figure 1B). The 421 induced shift was specifically competed in both the ML-1 and MANCA nuclear extracts. The best competition was seen in the presence of an oligonucleotide containing the sequence of the *mdm2* gene p53 DNA binding site **(Figure 4.1B, lanes 6, 7, 16, and 17)**. Furthermore, there was no competition in the presence of a *fas* oligonucleotide containing a p53 binding site, or in the presence of mutant oligonucleotide **(Figure 4.1B, lanes 8-10 and lanes 18-20).** We expected the fas oligonucleotide to compete, therefore we believe there may have been a problem with this sample. Perhaps the *fas* oligonucleotide was degraded. These data suggested that the p53 in the MANCA nuclear extracts was able to bind DNA specifically, and that this same p53 had a high affinity for the mdm2 oligonucleotide containing the p53 DNA binding site.



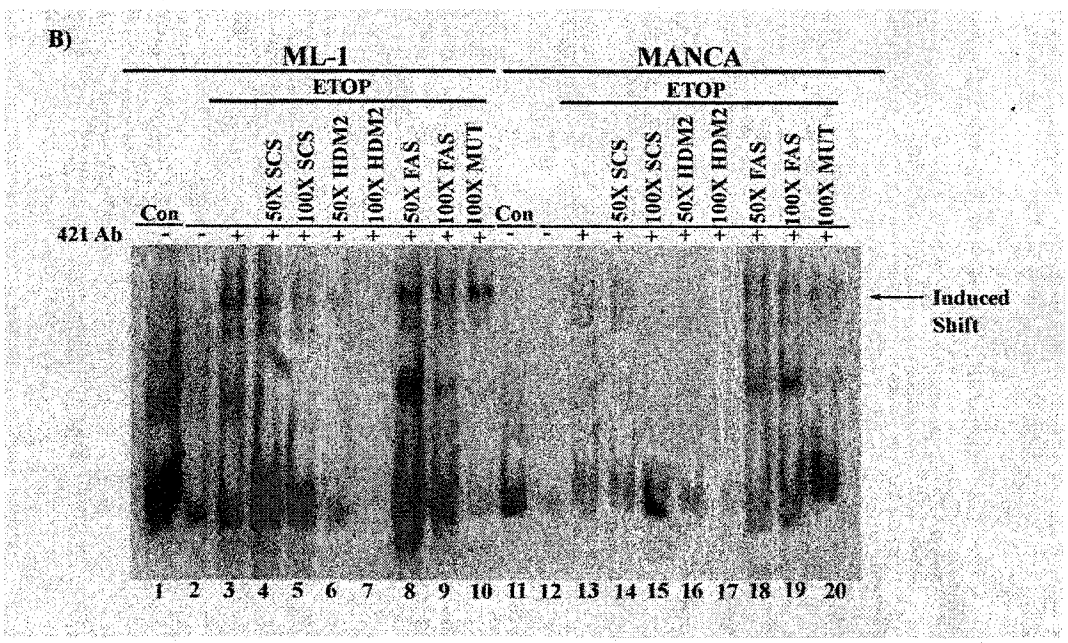


Figure 4.1 DNA damage induced MANCA p53 protein has DNA binding ability. A) *Electrophoretic mobility shift assay (EMSA) with the ^{32}P labeled superconsensus sequence (SCS), and 10ug of nuclear extracts from ML-1 and MANCA untreated samples (lanes 1-2, and 11-12) or samples treated with 0.5uM CPT (lanes 3-4, and 13-14), 8uM ETOP (lanes 5-6, and 15-16), 5uM MC (lanes 7-8, and 17-18), or 20uM LLnL (lanes 9-10, and 19-20) for 6 hours. Reactions*

contained the p53-specific monoclonal antibody, 421, where indicated. ***B)*** *Competition of the 421-induced gel shift from ETOP treated ML-1 (lanes 2-10), and MANCA (lanes 12-20) samples using 50X or 100X fold excess unlabeled oligonucleotide corresponding to the SCS (lanes 4, 5, 14, and 15), Mdm2 (lanes 6, 7, 16, and 17) and Fas (lanes 8, 9, 18, and 19) p53-binding site as well as 100X non-specific mutant oligonucleotide (lanes 10 and 20). Exponentially growing cells were treated with 8uM etoposide for 6 hours and then nuclear extracts were prepared from these samples, as previously described.*

In an effort to look at the ability of the MANCA and ML-1 nuclear extract to bind to the human *mdm2* oligonucleotide, containing the p53 binding site, we carried out several more EMSAs. Unfortunately, we were never able to get clean, definitive results using this oligonucleotide under the same conditions we used to demonstrate specific binding to the SCS oligonucleotide (data not shown). Although, with the use of a murine *mdm2* oligonucleotide we were able to acquire interesting data **(Figure 4.2).** In the absence of DNA damage the MANCA and ML-1 nuclear extract samples exhibit similar binding patterns. There is binding to the mouse oligonucleotide, and it is specific as it is only competed with 100 fold excess mdm2 oligonucleotide, and not the SCS, or mutant oligonucleotides **(Figure 4.2, lanes 1-4, and lanes 10-13).** Interestingly, the ML-1 CPT nuclear extracts showed a change in binding affinity. There was some binding to the oligonucleotide, but all of the binding was competed by either the mdm2, SCS, or mutant olgonucleotides **(Figure 4.2, lanes 5-8).** This suggested that the binding of the nuclear protein from ML-1, CPT treated cells was not specific after drug treatment. The presence of the DNA damage signal in the cellular environment altered the binding affinity to the mdm2 oligonucleotide. In contrast the nuclear extract samples from MANCA, CPT

treated cells showed no change in their ability to bind to the mdm2 oligonucleotide specifically **(Figure 4.2, lanes 14-17).**

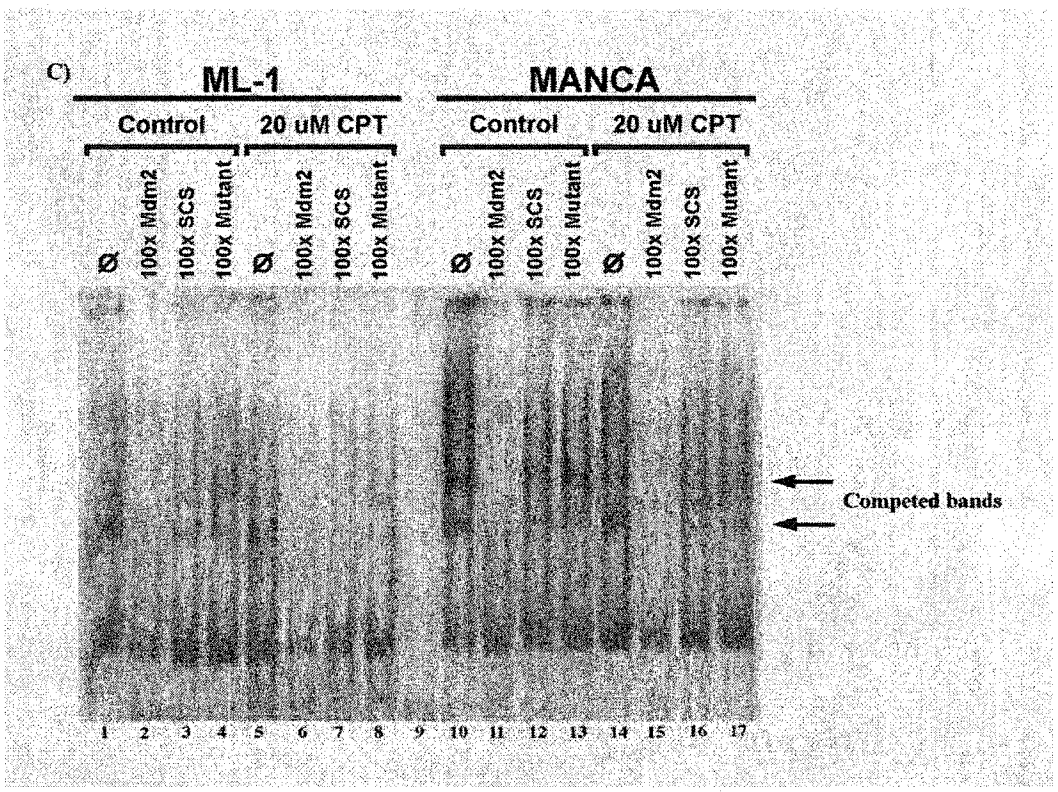


Figure 4.2 MANCA cells exhibit specific binding to a murine Mdm2 oligonucleotide in the presence and absence of DNA damage. *Electrophoretic mobility shift assay with the ^{32} P labeled murine Mdm2 oligo, and 10ug of nuclear extracts. ML-1 and MANCA samples were either left untreated (lanes 1-4, and 10-13) or were treated with 20uM CPT (lanes 5-8, and lanes 14-17) for 6 hours. Competition was carried out using 100X fold excess unlabeled oligonucleotide corresponding to the murine Mdm2 (lanes 2, 6, 11, and 15), SCS (lanes 3, 7, 12, and 16) p53-binding sites as well as 100X non-specific mutant oligonucleotide (lanes 4, 8, 13,*

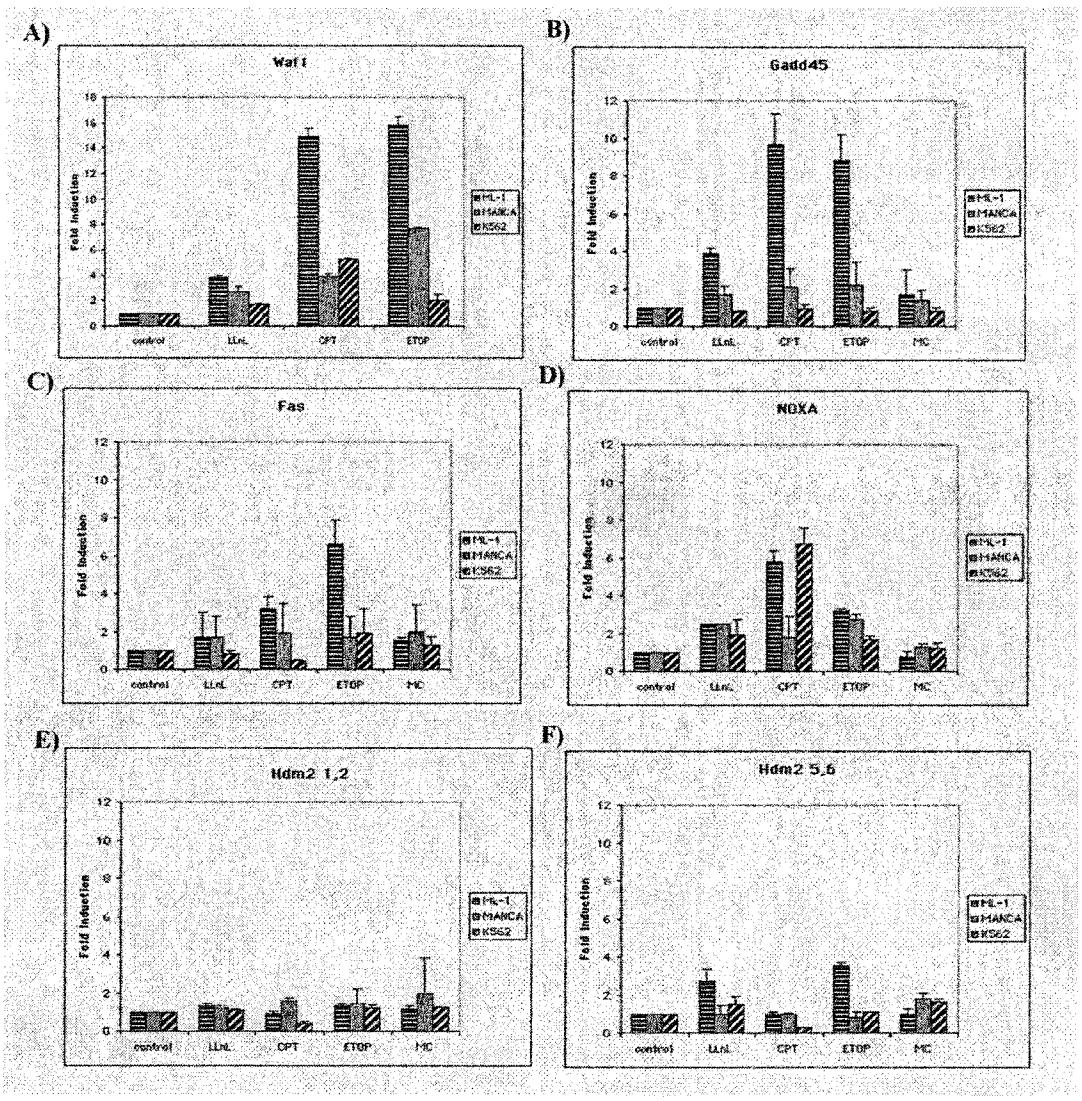
and 17). Lane 9 does not contain protein. All reactions were carried out in the presence of p53 specific 421 antibody.

The p53 protein in the MANCA cell line has compromised transactivation ability.

The DNA binding assays proved the p53 protein in the MANCA cells had DNA binding activity. Despite DNA binding activity we expected to see no induction of downstream target transcripts due to lack of p21 protein detection. We speculated that the amino terminal transactivation domain was inhibited from interacting with transcription machinery and therefore p53 activity was hindered (Thut et al., 1997). The inhibition was possibly due to the presence of increased Mdm2 protein levels, which we saw by Western analysis. Quantitative PCR and probes for different growth arrest and apoptotic target genes demonstrated that the MANCA p53 protein was compromised in its transactivation ability **(Figure 4.3).** MANCA cells treated with CPT, ETOP, MC, and LLnL for 3 hours showed no induction of the gadd45, fas, and mdm2 (p53 dependent, and independent) transcripts **(Figure 4.3, B, C, E, and F).** There was some activation of the waf1, and noxa transcripts in the presence of ETOP **(Figure 4.3, A, and D).** The waf1 data was confirmed via Northern analysis **(Figure 4.3, G).** The level of induction was still significantly less than the level detected in the treated ML-1 samples. This was surprising because earlier we saw that no p21 protein was present at 6 hours after drug

treatment. Perhaps protein production takes longer to become evident. In the presence of the different DNA damaging agents, the induced ML-1 p53, showed activation of all the p53 downstream targets **(Figure 4.3, A-F).** Therefore despite the stabilization, and DNA binding activity of the p53 protein in the MANCA cells, transactivation of the p53 target genes was compromised. Similarly, the K562 cells showed no induction of the gadd45, mdm2, and fas transcripts **(Figure 4.3, B, C, E and F).** Interestingly, there was significant induction of the noxa transcript in the camptothecin treated K562 sample **(Figure 4.3D)**. As shown previously there is some apoptosis detected in the K562 cell line after camptothecin treatment **(Figure 3.2C)** thereby possibly explaining the noxa transcript induction. And as seen in the MANCA cell line, the K562 cell line exhibited slight induction of the waf1 transcript **(Figure 4.3A).** This suggests that the slight induction of the waf1 transcript in the MANCA cell line might be p53 independent.

Figure 4.3 The MANCA p53 protein is transcriptionally compromised. *Quantitative PCR was used to detect fold induction of the* ***A)*** *Waf1,* ***B)*** *Gadd45,* ***C)*** *Fas,* ***D)*** *Noxa,* ***E)*** *Mdm2 1,2 and* ***F)*** *Mdm2 5,6 transcripts. All results were normalized to untreated samples, and glyceraldehydes-3-phosphate dehydrogenase values. Standard deviations represent the results from two separate experiments.* ***G)*** *Northern analysis confirmed the quantitative PCR data. ML-1, MANCA, and K562 samples were either left untreated (lanes 1, 5, and 9), or were treated with 0.5 CPT (lanes 2, 6, and 10), 8uM ETOP (lanes 3, 7, and 11), or 20uM LLnL (lanes 4, 8, and 12) for 3 hours. 40ug of total RNA was resolved on a denaturing formaldehyde gel, electro-transferred to a nylon membrane and probed for waf1 transcript.*



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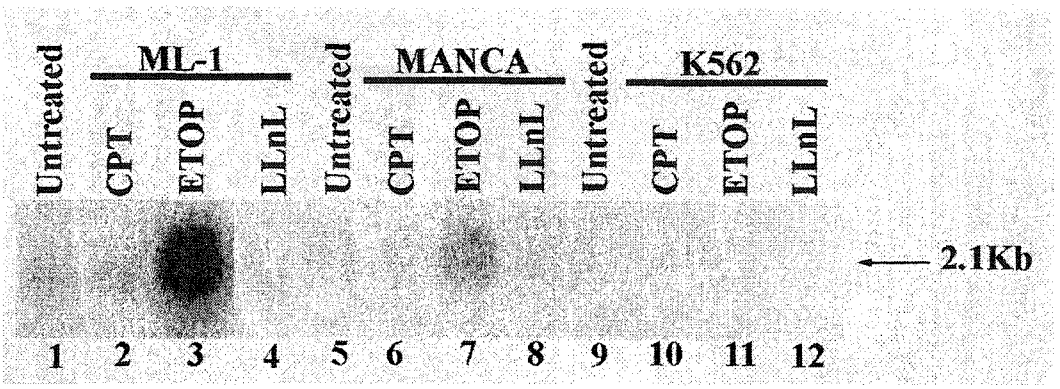

Discussion:

The p53 protein has the ability to function as a transcription factor (Farmer et al., 1992). Once the p53 protein is induced, and stabilized by post-translational modifications, it is capable of binding to and activating several genes which contain p53 response elements. Many of the p53 downstream targets are growth arrest and apoptotic activating genes (El-Deiry, 1998). The data in this study demonstrate that it is possible to have wild-type p53 that is stabilized in the presence of DNA damage **(Figure 3.5)**, has specific DNA binding activity **(Figure 4.1)**, and yet remains transcriptionally inactive **(Figure 4.3).**

The MANCA cell line has recently been found to contain a single nucleotide polymorphism (SNP) at position 309 of the *mdm2* gene. The location of the SNP is slightly upstream from the P2 p53 dependent promoter. The human *mdm2* gene contains two different promoters, one p53 dependent, and the other which is not under control of the p53 protein (Zauberman et al., 1995). Bond et al., have also found that the presence of the SNP in the *mdm2* gene correlates with increased Mdm2 overexpression, with early onset breast cancer, and is a possible indicator of decreased sensitivity to chemotherapeutic agents. Lastly, the presence of the 309 SNP has been shown to create a high affinity site for the Sp1 transcription factor, possibly allowing for increased transcription of the *mdm2* gene. We have also seen that the MANCA cells have increased *mdm2* transcript levels in the presence of CPT (data not shown).

The SNP causes a T to G change at position 309 of the *mdm2* gene. This creates a region of almost exclusively G and C residues which has been shown to cause an increased affinity for several different transcription factors. Specifically, the ETS transcription factor binds within this region of the *mdm2 gene*, and has been shown to have an affinity for GC rich residues (Kawai, et al, 2003). The increased transcript along with the high levels of Mdm2 protein in the presence of DNA damage may be the cause of the lack of transcription activity from the MANCA p53 protein (Figure 3.6). It is plausible that the Mdm2 protein binds to the p53 protein and acts as an inhibitor of basal and activated transcription. High levels of Mdm2 protein have been shown to inhibit p53 DNA damage-dependent apoptosis, as also seen in the MANCA cell line (Gao et al., 1999). Previous studies have shown that simply the downregulation of *mdm2* transcript will allow for the stabilization of the p53 protein in the presence of DNA damaging agents (Inoue et al., 2001).

Recent work from our laboratory supports the hypothesis that a trans-acting factor, such as Mdm2, is responsible for the inhibition of p53 transcription (unpublished data). Transient transfection assays have shown that exogenously introduced p53 was incapable of activating transcription from the p53 binding site in the MANCA cell line. If the p53-Mdm2 protein-protein interaction remained intact we might expect ubiquitination of the p53 protein by Mdm2 (Grossman et al., 2003). Normally, the presence of DNA damage leads to a decrease of the p53-Mdm2 interaction, therefore p53 activity is no longer inhibited and the half-life of the protein is greatly extended (Shieh et al., 1997). Several studies clearly show that the inhibition of p53 transcription and

ubiquitination are independent of each other. The amino terminus of Mdm2 interacts with the p53 protein, and it is the ring finger domain that is responsible for Mdm2's E3 ubiquitin ligase activity (Fang et al., 2000, Meek and Knippschild, 2003). Our data clearly showed compromised p53 dependent transcription activity in MANCA cells, despite the increase in p53 protein levels. This suggested that the p53–Mdm2 protein-protein interaction was not inhibited in the presence of DNA damage, and subsequently the p53 protein was rendered transcriptionally inactive. This led us to look closely at the p53-Mdm2 complex and the post-translational modifications of these two proteins.

Chapter 5
The p53-Mdm2 Protein-Protein Interaction is not Disrupted in the Presence of DNA Damage, Despite the Phosphorylation at Serine-15 of the MANCA p53 Protein.

Introduction:

The activity of the p53 tumor suppressor protein is dependent on its ability to activate downstream checkpoint targets which inevitably will allow for the initiation of an apoptotic or growth arrest program (El-Deiry, 1998; Bargonetti and Manfredi, 2002). In the presence of DNA damage it is a combination of post-translational modifications of the p53 and Mdm2 protein that allow for the increased p53 protein stability (Meek and Knippschild, 2003; Moll and Petrenko, 2003). It is the modification of the p53 phosphorylation sites within the amino terminal-Mdm2 binding site, which have been shown to be the most crucial in dissociating the p53-Mdm2 complex (Shieh et al., 1997; Bean and Stark, 2002). Additionally, phosphorylation of the Mdm2 protein has been shown to effect the dynamics of the p53-Mdm2 interaction (Khosravi et al., 1999).

The ATM kinase has been previously shown to be a key regulator in the phosphorylation cascade and activation of the p53 protein (Banin et al., 1998). In addition to p53, ATM kinase can phosphorylate the Mdm2 protein at Serine 395 and subsequently there is a reduction in the level of Mdm2 dependent p53 degradation (Maya et al., 2001). In contrast when Mdm2 is phosphorylated by Akt at serine 166 and 186, there is increased entry of Mdm2 into the nucleus and enhanced interaction with the p53 protein (Mayo et al., 2001). The phosphorylated Mdm2 can then go on to stimulate p53 degradation (Ogawara et al., 2002). Recent work has shown that only high levels of Mdm2 can allow for p53's polyubiquitination and nuclear degradation (Li et al., 2003). It has also been shown that Mdm2 by itself cannot cause polyubiquitination, p300 is required (Grossman et al., 2003). Interestingly, Akt dependent phosphorylation of

Mdm2 enhances its interaction with p300 (Mayo et al., 2001). Transcriptionally active p53 can directly result from a decrease in the level of ubiquitination, as seen in the presence of the synthetic molecule, CP-31398 (Wang et al., 2003). This compound does not lead to dissociation of the p53-mdm2 complex and yet still allows for preferential activation of some p53 downstream target genes (Wang et al., 2003).

There are several hypotheses as to how the Mdm2 protein inhibits p53's transactivation activity. One such theory proposes a dual role for Mdm2. In this model the Mdm2 protein binds to the p53 transactivation domain at which point the Mdm2 protein can interfere with basal transcription machinery at the promoter sites of p53 target genes (Thut et al., 1997). A second mechanism suggests that the presence of Mdm2 in association with p53 has the ability to inhibit p53's DNA binding ability (Zauberman et al, 1993).

Recent work by G. Bond and AJ Levine has shown that the presence of a homozygous single nucleotide polymorphism in the *mdm2* gene leads to early onset breast cancer and poor prognosis. Alternative splice variants of the *mdm2* gene have also demonstrated a similar phenotype (Lukas et al., 2001). The cell line used in this study, MANCA, contains a homozygous single nucleotide polymorphism at position 309 of the *mdm2* gene, directly upstream of the p53 dependent promoter.

Our work in this study demonstrates that the induced, stabilized p53 protein is in constant association with the Mdm2 protein. The presence of a DNA damage signal in

the cellular environment does not cause the dissolution of the p53-Mdm2 complex. This presents a unique situation, not only because the phosphorylated p53 remains in complex with Mdm2, but also because the p53 protein does not become degraded. The p53 remains bound to Mdm2 and is sequestered in the nucleus as an inactive complex.

Results:

DNA damage induced phosphorylation of p53 at serine-15, does not lead to the dissociation of the $p53^{ser\text{-}15}$-Mdm2 complex.

To examine if the induced p53 protein in the MANCA cell line may be inactivated by an interaction with Mdm2 we analyzed if the stabilized p53 protein was able to interact with the Mdm2 protein. To assess the presence of such a complex we did several immunoprecipitation experiments using nuclear extracts derived from cells treated with several different DNA damaging agents. We also employed various immunoprecipitation techniques to ensure that in fact the proteins that we saw in complex were p53 and Mdm2.

Immunoprecipitation experiments using the, 421 and 1801, p53 specific antibodies demonstrated that the p53 protein in the MANCA samples was in complex with the Mdm2 protein before and after the addition of CPT **(Figure 5.1A).** The p53 and Mdm2 proteins in the ML-1 cell line showed a different pattern of interaction **(Figure 5.1A).** The immunoprecipitation of the MANCA untreated samples showed an existing interaction between p53, and Mdm2 **(Figure 5.1A, lanes 1-3).** In the presence of DNA damage there was an enhancement of the p53-Mdm2 interaction, as was seen using the 421, or 1801 antibodies **(Figure 5.1A, lanes 4-6).** In contrast there was no p53 detected in the ML-1 cell line in the untreated samples, and therefore no Mdm2 was pulled down with the 421, or 1801 antibodies **(Figure 5.1A, lanes 7-9).** Additionally, in the presence of CPT, when there was induction of the ML-1 p53 protein, we still did not see formation of a p53-Mdm2 complex **(Figure 5.1A, 10-12).**

To confirm that the p53-Mdm2 interaction, seen in the MANCA cell line, was specific we employed the use of a peptide specific for the p53 421 epitope, found at the carboxy terminus of the p53 protein. Immunoprecipitation experiments were conducted using the p53 specific 421 antibody. After incubation of the nuclear extract samples with the 421 coupled beads, we used a peptide resuspended in a low salt concentration, to bump off p53 and associated Mdm2 bound to the 421 coupled beads. The MANCA p53 protein was found to be in complex with the Mdm2 protein **(Figure 5.1B).** And as seen previously, there was enhancement of the p53-Mdm2 interaction in the presence of DNA damage **(Figure 5.1B, compare lanes 4, and 8).** Interestingly, we detected many different species of Mdm2 protein when the immunoprecipitates were blotted with the 2A10 antibody. The 2A10 antibody is specific for the carboxy terminus of the Mdm2 protein. As a negative control, we showed that using just 0.25M NaCl (the same concentration as the peptide is resuspended in) did not bump anything off the beads **(Figure 5.1B, lanes 3, and 7).**

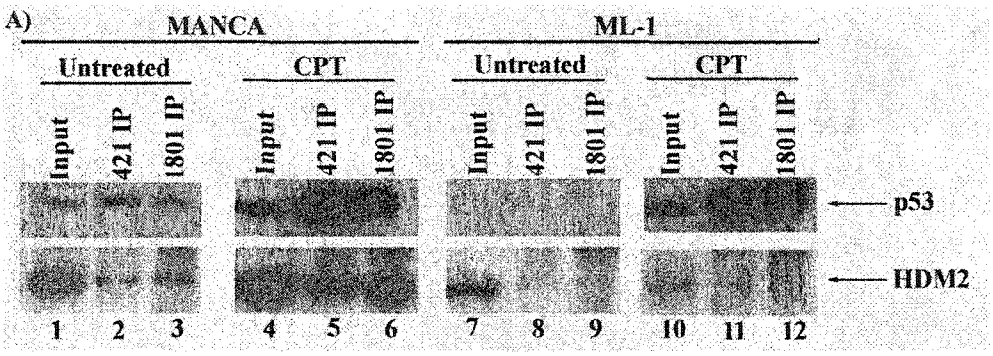

Figure 5.1 The MANCA p53 and Mdm2 proteins co-immunoprecipitate in the presence and absence of DNA damage. *Immunoprecipitation experiments using the p53-specific antibodies, 421, and 1801, coupled to Protein A Sepharose IgG beads. ML-1 and MANCA samples were either left untreated or were treated with 5uM CPT for 6 hours. Immunoprecipitation experiments were carried out and samples were subjected to SDS-PAGE (10%) and Western analysis.* ***A)*** *MANCA untreated samples immunoprecipitated with 421 (lane 2) and 1801 (lane 3) antibodies. ML-1 untreated samples immunoprecipitated with 421 (lane 8)*

and 1801 (lane 9) antibodies. MANCA CPT treated samples immunoprecipitated with 421(lane 5) and 1801 (lane 6) antibodies. ML-1 CPT treated samples immunoprecipitated with 421 (11) and 1801 (12) antibodies. The upper panel was probed with a mixture of the p53-specific antibodies, 421, 240, and 1801. The lower panel was probed with the Mdm2 antibody, 2A10. Lanes labeled input contain 100ug of nuclear protein (lanes 1, 4, 7, and 10). ***B)*** *MANCA untreated (lanes 1-4), and 5uM CPT treated (lanes 5-8) samples were immunoprecipitated with 421antibody and then incubated in the presence of a peptide specific for the 421 epitope (lanes 4, and 8). Lanes 1, and 5 contain 100ug of nuclear protein input. Lanes 2, and 6 are 421 immunoprecipitated samples. Lanes 3, and 7 show no proteins were eluded from 421 beads in the presence of 0.25M NaCl. The upper panel was probed with a mixture of the p53-specific antibodies, 421, 240, and 1801. The lower panel was probed with the Mdm2 antibody, 2A10. These experiments were each performed three times.*

We next investigated whether the serine-15 phosphorylated form of p53 could interact with the Mdm2 protein. In these experiments we compared ML-1, and MANCA nuclear extracts derived from DNA damage treated cells. Immunoprecipitation experiments were carried out using the p53 specific 421 antibody, and analyzed with both the p53 phospho-specific serine 15 antibody, and the Mdm2 monoclonal antibody, D7. Serine-15 phosphorylation of the p53 protein has been shown to cause dissociation of the p53-Mdm2 complex in the presence of DNA damage (Shieh et al., 1997). In the MANCA cell line the p53 and Mdm2 protein remained in complex despite the modification of the p53 protein at serine-15 **(Figure 5.2A)**. The MANCA samples treated with ETOP, or the proteasome inhibitor, LLnL, showed a constitutive p53-Mdm2 protein-protein interaction **(Figure 5.2A, lanes 4-6).** The ML-1 samples show a reduced

interaction of the p53 and Mdm2 proteins, although there is still some interaction **(Figure 5.2B, lanes 1-3).** This may be explained by the fact that p53 binds DNA as a tetramer (Mclure and Lee, 1998). The ML-1 and MANCA cells may have different levels of phosphorylation of the p53 tetramer at serine-15. This would allow for a different ratio of interaction of the phosphorylated p53 protein with the Mdm2 protein. Perhaps the ML-1 cells have a higher phosphorylated p53 to Mdm2 ratio than the MANCA cells. The reciprocal co-immunoprecipitation experiment was done using the Mdm2 specific antibody, D7, and this analysis demonstrated an increased interaction of Mdm2 with the serine-15 phosphorylated form of p53 in the DNA damage treated MANCA samples **(Figure 5.2B, lanes 1-3).** In comparison, the ML-1 cells show a decreased interaction of the p53 (Serine-15) and Mdm2 proteins after treatment with ETOP and LLnL **(Figure 5.2B, lanes 4-6)**. The presence of LLnL in this study allowed us to look at an induced form of p53 that was not cleaved. This demonstrated that the ML-1 p53-Mdm2 protein complex is not disrupted as a consequence of cleavage.

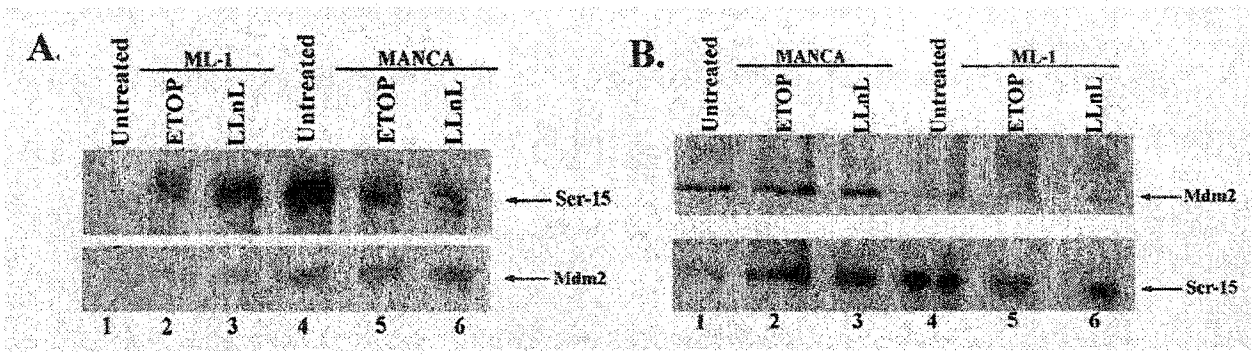

Figure 5.2 Serine-15 phosphorylated p53 can remain in complex with Mdm2. *Immunoprecipitation experiments using the uncoupled p53 antibody, 421 and the Mdm2 specific antibody, D7. ML-1 or MANCA samples were either left untreated or were treated with 8uM ETOP or 20uM LLnL for 6 hours. Immunoprecipitation experiments were carried out and samples were subjected to SDS-PAGE(10%) and Western analysis.* ***A)*** *Immunoprecipitation using the p53 antibody, 421. Lane 1, ML-1 untreated sample. Lanes 2 (ETOP) and 3(LLnL) treated ML-1 samples. Lane 4, MANCA untreated sample. Lanes 5 (ETOP) and 6 (LLnL) MANCA treated samples. The upper panel was probed with the p53 phospho-specific antibody serine-15, and the lower panel was probed with the Mdm2 specific antibody, D7.* ***B)*** *Immunoprecipitation using the Mdm2 specific antibody, D7. Lane 1, MANCA untreated sample. Lanes 2 (ETOP) and 3 (LLnL) MANCA treated samples. Lane 4, ML-1 untreated samples. Lanes 5 (ETOP) and 6 (LLnL) ML-1 treated samples. The upper panel was probed with D7, and the lower panel was probed with serine-15. This experiment was performed two times.*

The Mdm2 protein is not post-translationally modified in the MANCA cell line.

We have clearly shown that the serine-15 modified p53 protein can associate with the Mdm2 protein in the presence of DNA damage. We have also shown that the MANCA p53 protein is induced in the presence of DNA damage. The upstream signaling cascade leading to the p53 protein is intact, but the p53-Mdm2 interaction remains unchanged in the presence of DNA damage.

To assess the phosphorylation status of the Mdm2 protein, we phosphatase treated nitrocellulose membranes containing nuclear protein samples from ML-1 and MANCA drug treated cells. After phosphatase treatment the membranes were blotted with a mixture of the p53 monoclonal antibodies (421, 240, and 1801), and with the Mdm2 monoclonal antibody, 2A10 **(Figure 5.3A).** The 2A10 antibody cannot detect species of the Mdm2 protein that are post-translationally modified in carboxy terminal acidic domain (Khosravi et la., 1999; Maya and Oren, 2000). When the nitrocellulose was not phosphatase treated, the ML-1 samples showed decreased reactivity with the 2A10 antibody in the presence of DNA damage **(Figure 5.3A, lanes 1 and 2),** suggesting DNA damage leads to modification of Mdm2. In the MANCA samples there was an increase in reactivity with the 2A10 antibody after DNA damage, suggesting that the presence of DNA damage did not alter the 2A10 epitope of the Mdm2 protein **(Figure 5.3A, lanes 3 and 4).** When the membranes were probed after the nitrocellulose was treated with phosphatase we saw a different pattern emerge **(Figure 5.3B).** The 2A10 antibody regained reactivity in the ML-1 CPT treated samples demonstrating that the Mdm2

protein was present in the these samples but there was a modification blocking the 2A10 epitope **(Figure 5.3B, lanes 1,2)**. The pattern observed in the MANCA cell line before and after phosphatase treatment was identical, the 2A10 antibody was always reactive with the MANCA Mdm2 protein **(Figure 5.3B, lanes 3,4).** This technique showed some change in p53 levels, it is possible that the phosphatase treatment also unmasked an epitope for the p53 specific 421 antibody **(compare Figure 5.3A to Figure 5.3B).**

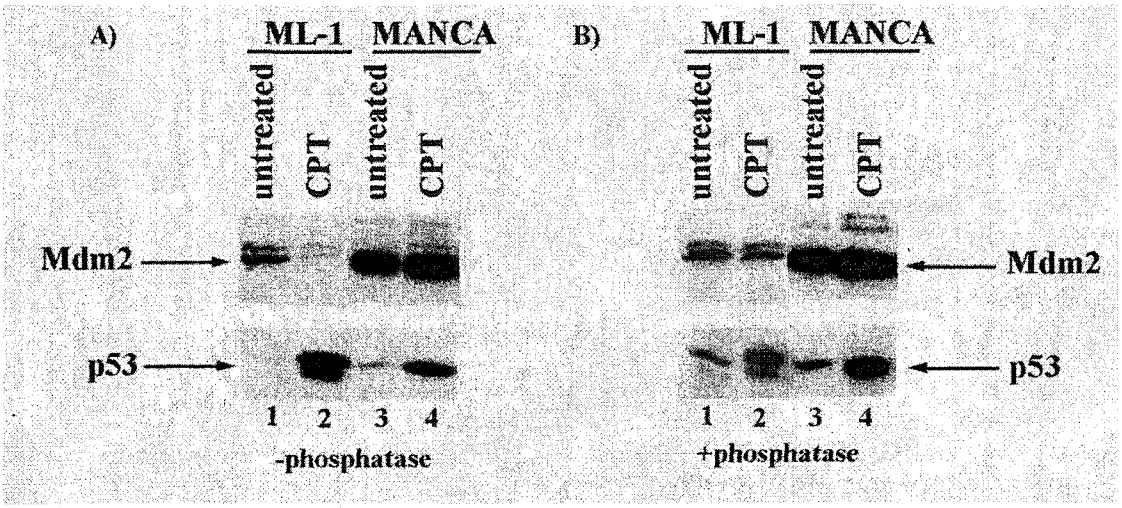

Figure 5.3 The MANCA Mdm2 protein is not modified by CPT treatment. *ML-1 and MANCA samples were either left untreated (lane 1 and 3) or were treated with 0.5uM CPT (lane 2 and 4) for 6 hours. Nuclear extracts were prepared and then subjected to SDS-PAGE and Western analysis. The nitrocellulose blot in panel B was then treated with shrimp alkaline phosphatase while the membrane in panel A was left untreated. The upper panels were probed with the Mdm2 specific antibody, 2A10. The lower panel was probed with a mixture of the p53-specific antibodies, 240, 1801, and 421. This experiment was performed two times.*

To confirm that there was in fact a difference in the post-translational modification states of the MANCA, and ML-1 Mdm2 proteins we *in-vivo* labeled the cells with ortho-phosphate. The untreated and CPT treated samples were then immunoprecipitated using the 421 antibody **(Figure 5.4)**. Our results show that there was a difference in the pattern of phosphorylated proteins in the ML-1 and MANCA samples but we cannot be sure that the high molecular band we detected was Mdm2. We did observe the increased phosphorylation of a high molecular weight band in the ML-1 cell line in the presence of DNA damage **(Figure 5.4, compare lane 3 to lane 4).** The phosphorylation pattern in the MANCA cell line remained the same in the presence of a DNA damaging agent **(Figure 5.4, lanes 1-2)**. This *in-vivo* labeling experiment was only done once, but the preliminary evidence suggested there was a lack of post-translational modification, possibly of the Mdm2 protein, in the MANCA cell line. The same conditions in the ML-1 cell line showed a modified pattern of phosphorylated proteins immunoprecipitated with the 421 Ab, and we predict that the major phosphorylated band was Mdm2. This is a key observable difference between two cell lines, one that undergoes apoptosis in the presence of DNA damage, ML-1, and one that does not, MANCA.

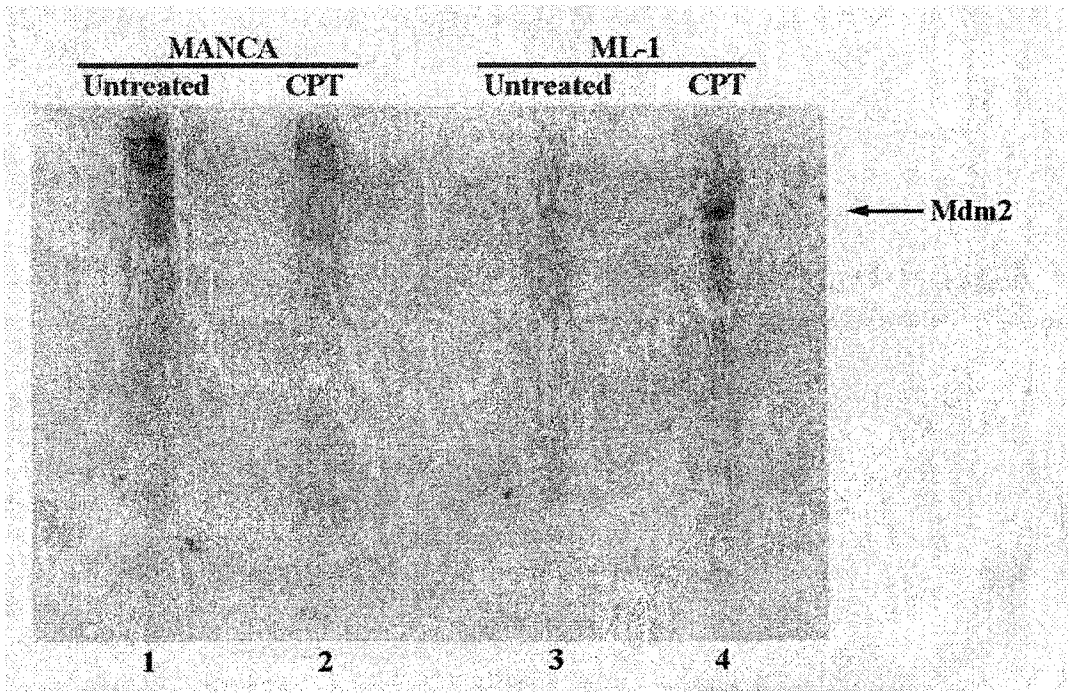

Figure 5.4 Figure 5.4 MANCA and ML-1 samples exhibit a different pattern of phosphorylated proteins immunoprecipitated with the 421 antibody. *MANCA and ML-1 untreated and treated samples were in-vivo labeled with ^{32} P ortho-phosphate. The untreated (lanes 1 and 3) and 0.5uM CPT treated (lanes 2 and 3) nuclear proteins were then immunoprecipitated with 421 antibody. Immunoprecipitated proteins were visualized using autoradiography.*

Discussion:

The p53-Mdm2 feedback loop allows for the existence of a unique system to keep p53 levels down in the absence of a stress signal (Barak et al., 1993; Picksley and Lane, 1993). In the presence of cellular stress the p53 protein is rapidly stabilized by many post-translational modifications (Moll and Petrenko, 2003). The post-translational modifications of the p53 protein allows for a dissolution of the p53-Mdm2 complex, thereby allowing p53's transcription ability to be unmasked (Meek, 1999). This study has looked at a unique interaction between the p53 and Mdm2 proteins in the Burkitt's lymphoma cell line, MANCA.

Immunoprecipitation experiments show a clear interaction between the p53 and Mdm2 protein in MANCA cells **(Figure 5.1).** Additionally, the p53-Mdm2 interaction remains intact despite the presence of a DNA damage signal and extensive phosphorylation of the p53 protein at Serine-15 **(Figure 5.2).** However, the ML-1 cell line exhibited disruption of the p53-Mdm2 interaction (in the presence of CPT and ETOP), and these cells also show a downregulation of the Mdm2 protein in the presence of DNA damage. The increased levels of the Mdm2 protein in the MANCA cell line that remain in contact with the p53 protein, led us to speculate that this Mdm2 protein may be the molecule responsible for the inhibition of p53 function. We have also shown that these cells do not undergo p53 mediated DNA damage dependent apoptosis, and they are compromised in their transcriptional ability **(Figure 3.2 and 4.3).** High levels of Mdm2 have been shown to inhibit p53 function, as increased Mdm2 protein levels are common in many types of human tumors (Reifenberger et al., 1993).

Additionally these cells have been found to have a single nucleotide polymorphism at position 309 of the *mdm2* gene. Position 309 lies directly upstream of the p53 dependent P2 promoter of the *mdm2* gene. ML-1 cells which, like the MANCA cells, express wild-type p53 have been found to be homozygous wild-type at position 309 of the *mdm2* gene. These results suggest that the increased levels of protein produced from the *mdm2* gene containing the homozygous SNP may serve to inhibit p53 transactivation function. This single nucleotide polymorphism causes a change from a T to a G residue creating a region that may have enhanced affinity for transcription factors (Kawai et al., 2003).

Preliminary data regarding the Mdm2 protein status in the MANCA cell line has shown that the post-translational modification is lacking when compared with the ML-1 cell line **(Figure 5.3).** The residues at the 2A10 epitope remain unchanged after treatment with DNA damaging agents. In the presence of DNA damage it is the phosphorylation of Mdm2, at serine 395, in concert with p53 amino terminal modifications that allow for activation of the p53 protein (Maya et al., 2001; Mayo et al., 1997). The modifications within the p53 and Mdm2 proteins allow for the allow for the Mdm2 negative regulation of p53 to be removed.

The situation in the MANCA cell line is also unique due to the fact that the p53 protein is never degraded despite its association with the Mdm2 protein. The p53-Mdm2 complex remains in the nucleus and the p53 remains transcriptionally inactive. The lack of p53 degradation in the MANCA cell line may be a direct consequence of the 309 SNP

present in the *mdm2* gene or it may be a mechanism we have not yet looked upon. Our model describes a possible hypothesis which relies on the fact that the p53 protein binds specifically to DNA in the form of a tetramer **(Figure 5.5)**. It may be that the MANCA p53 protein is not phosphorylated at serine-15 on all of the p53 tetramer subunits, and therefore the Mdm2 protein can remain in complex with p53 after DNA damage. This also means that the phosphorylated p53: Mdm2 ratio in the MANCA cell line may be lower than in the ML-1 cell line. Disruption of the p53-Mdm2 protein-protein interaction by small molecule inhibitors may shed some light on the situation (Chene, 2004; Vassilev et al., 2004). If these small molecule inhibitors were able to disrupt the $p53^{ser\text{-}15}$-Mdm2 complex, we may be able to regain p53 activity in the MANCA cell line. These possibilities remain to be tested.

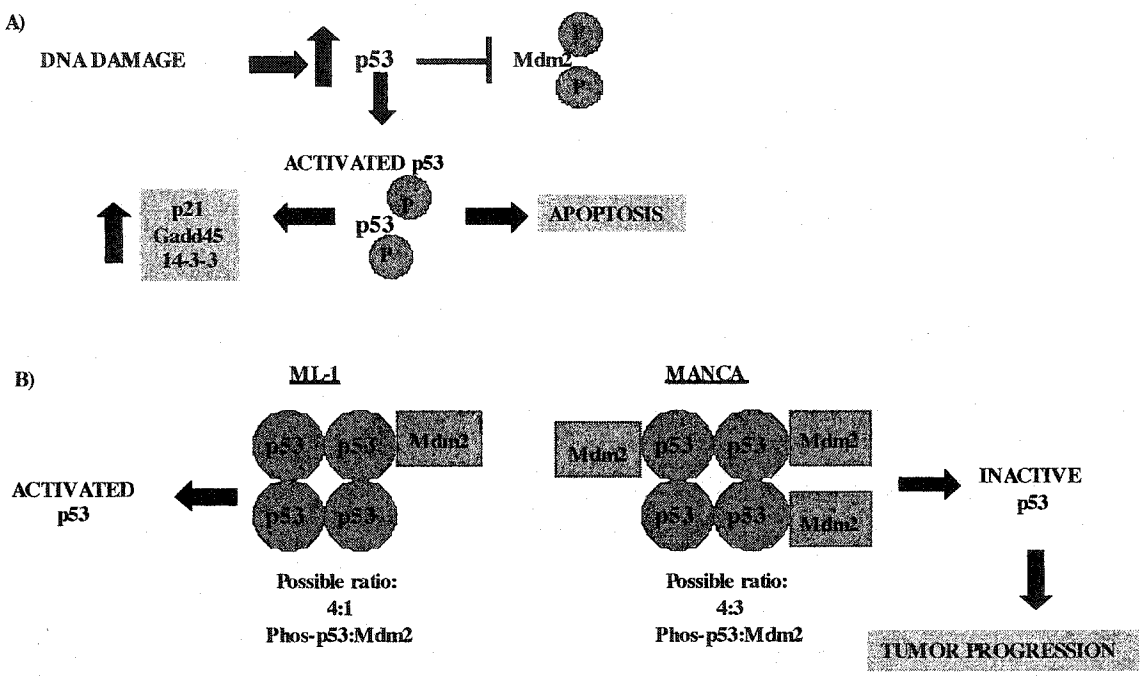

Figure 5.5 A model representing the inactivation of wild-type p53 in a Burkitt's lymphoma cell line. *A) Representation of how p53 is activated in the presence of DNA damage. B) A schematic representing how the MANCA p53 protein may be inactivated. We propose that the ML-1 phosphorylated- p53:Mdm2 ratio is higher therefore the p53 protein can become activated to turn on downstream targets after DNA damage. The MANCA cells may have a lower phosphorylated-p53:Mdm2 ratio and the p53 remains bound to Mdm2 and transcriptionally inactive.*

Chapter 6
Preliminary Data and Future Direction

The work in this study has shown a unique interaction between the p53 and Mdm2 proteins in the MANCA cell line. Our data began with the idea that the MANCA cells do not undergo p53 DNA damage dependent apoptosis. We then demonstrated that despite the presence of induced wild-type p53, that is post-translationally modified at serine-15, the transcriptional activity of the p53 protein is compromised for the activation of downstream checkpoint genes. In light of these results we hypothesized that a molecule may be inhibiting the p53 activity.

The Mdm2 protein levels were not downregulated in the presence of CPT and ETOP, and the p53-Mdm2 complex remained intact despite the presence of a DNA damage signal (Gao et al., 1999, Inoue et al., 2001). Interestingly the modification at serine-15 of the p53 protein did not disrupt Mdm2 protein binding to the amino terminus of p53. It may be that the phosphorylation of p53 at serine 15 was not sufficient to remove the negative regulation of Mdm2. Phosphorylation of serine 20 may be a more critical residue in allowing for the dissociation of the p53-Mdm2 complex (Knights et al., 2003). The study by Knights et al, showed that DNA bound p53-Mdm2 does not associate with p300. Therefore the p53 protein remains transcriptionally inactive and is not polyubiquitinated by p300. This may help to explain why the MANCA p53 protein remained as an inactive complex sequestered in the nucleus. Recent data from our laboratory by L. Campbell demonstrated that p53 exogenously introduced into the MANCA cell line did not show increased activation from a reporter plasmid containing the *mdm2*-binding site. The SN3 plasmid containing the wild-type *p53* gene was a generous gift from Bert Vogelstein, and the plasmid containing the p53, *mdm2* binding

site was a generous gift from G. Bond and AJ Levine. This data furthered the hypothesis that in fact an inhibitor molecule was present in the MANCA cell line.

All existing data eluded to the Mdm2 protein as the inhibitor molecule but to ensure that there weren't other players we carried out an additional immunoprecipitation experiment using several different methods to pull down the proteins (Figure 1). Immunoprecipitated samples were separated on a 10% denaturing acrylamide gel and the gel was Coomassie stained. In MANCA samples all three immunoprecipitations, 421, 2A10, and peptide bumping, showed the same high molecular weight bands **(Figure 6.1, lanes 2, 5, 8).** These bands were cut from the gel and sequenced by mass spectroscopy. Immunoprecipitation experiments were done in the presence of the p53 specific antibody, 421, or in the presence of the Mdm2 specific antibody, 2A10 **(Figure 6.1, lanes 1-3 and lanes 7-9).** Additionally, samples immunoprecipitated in the presence of 421 antibody were pulled down using a peptide specific for the 421 epitope **(Figure 6.1, lanes 4-6).** The high molecular weight bands were found to be the ribosomal protein, nucleolin. These same bands were not present in the ML-1 and K562 cell lines **(Figure 6.1, lanes 1-3 and 7-9).**

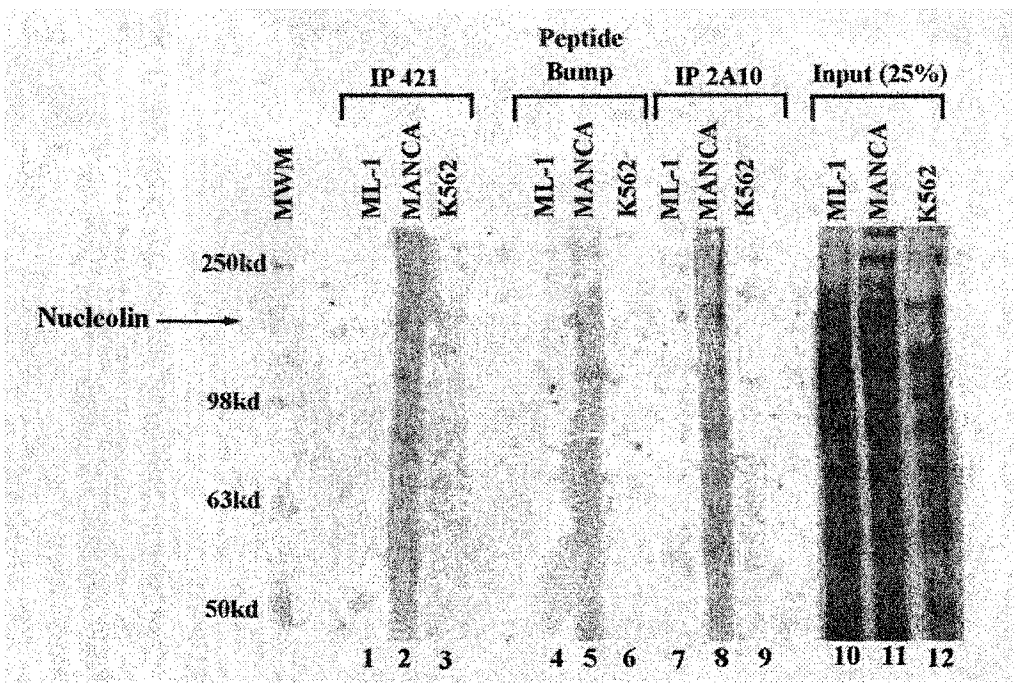

Figure 6.1 The nucleolin protein is immunoprecipitated in the MANCA cell line using p53 or Mdm2 specific antibodies. *ML-1, MANCA, and K562 nuclear extracts were immunoprecipitated with the p53-specific antibody, 421 (lanes 1-3), the p53 specific 421 antibody and a 421 peptide (lanes 4-6), and the Mdm2 specific antibody, 2A10 (lanes 7-9). Lanes 10-12 represent 25% of the input protein, these samples were not immunoprecipitated. Samples were separated by SDS-PAGE (10%) and the gel was then Coomassie stained, as described in Materials and Methods.*

Nucleolin has previously been described as a protein that interacts with p53 (Daniely et al., 2002). Recently, several ribosomal proteins have been implicated as players in response to cellular stress (Lohrum et al., 2003; Zhang et al., 2003). Nucleolin in particular, has been shown to be a functional component of a B cell specific transcription factor (Tuteja and Tuteja, 1998). Additionally, nucleolin relocalization and complex formation has been found to be independent of p53 transactivation ability (Daniely et al., 2002).

Western analysis of ML-1, MANCA, and K562 DNA damage treated cell lines shows nucleolin present in the nucleus and cytoplasm of all three lines before and after DNA damage **(Figure 6.2).** Interestingly, there is cleavage of the cytoplasmic nucleolin band in the CPT, and ETOP treated ML-1 samples **(Figure 6.2, lanes 2, and 4)**, which is not seen in the MANCA or K562 samples. This same treatment has been shown to induce p53 dependent apoptosis in the ML-1 cell line (as previously shown). Nucleolin cleavage has been demonstrated in cells undergoing apoptosis (Kito et al., 2002). This suggests that the presence of high levels of nucleolin may in some way inhibit p53 dependent apoptosis. Recent work from our laboratory by N. Arva, has shown that the wild-type p53 from the A875 cell line co-immunoprecipitates with the nucleolin protein. This cell line is similar to the MANCA cell line, in that DNA damage does not induce apoptosis, there is a constitutive interaction between the modified p53 protein and Mdm2, and the *mdm2* gene has a single nucleotide polymorphism at position 309.

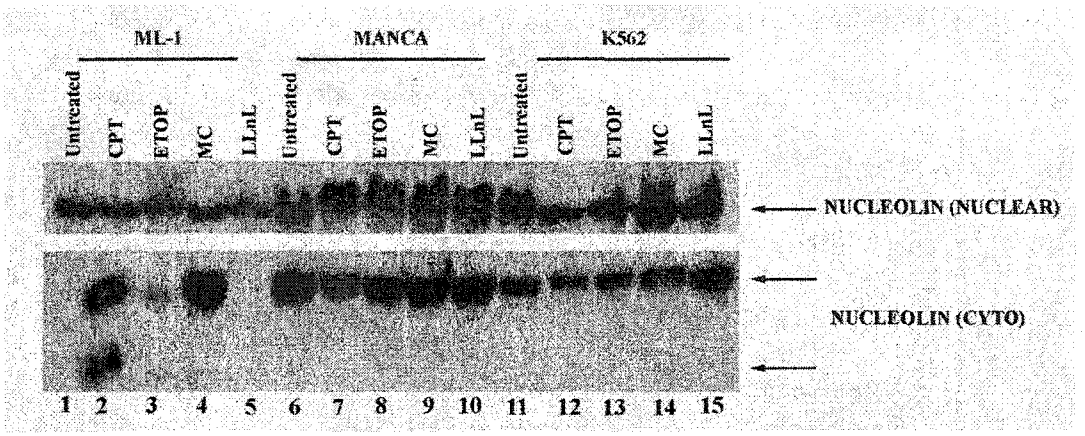

Figure 6.2 MANCA samples have an abundance of the nucleolin protein that is not cleaved in the presence of DNA damage. *Western blot analysis of ML-1, MANCA, and K562 samples. ML-1, MANCA, and K562 cells were either left untreated (lanes 1, 6, and 15), or were treated with 0.5uM CPT (lanes 2, 7, and 12), 8uM ETOP (lanes 3, 8, and 13), 5uM MC (lanes 4, 9, and 14), or 20uM LLnL (lanes 5, 10, and 15) for 6 hours. Nuclear and cytoplasmic extracts were then prepared from these samples. 100ug of protein was then subjected to SDS-PAGE (10%) and Western analysis. The upper panel are nuclear proteins and the lower panel is cytoplasmic proteins. Both panels were probed with a nucleolin specific antibody.*

Preliminary immunoprecipitation experiments using nucleolin antibodies have not led to conclusive results in the MANCA and ML-1 cell lines. The future of this project will include close study of how nucleolin interacts with the p53 and Mdm2 proteins in the presence of DNA damaging agents. It is possible that the presence of nucleolin in association with the p53-Mdm2 complex may serve to inhibit the activation of p53 checkpoint genes, thereby inhibiting p53's tumor suppressor function. Careful analysis of how ribosomal proteins may effect apoptosis will allow for a broader understanding of tumor progression in cell lines which contain increased levels of ribosomal proteins.

Chapter 7
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