Inhibition of Chromatin-bound Wild-type p53 by Mdm2

By

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Abstract

Inhibition of Chromatin-bound Wild-type p53 by Mdm2

By Nicoleta Catalina Arva

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In cancer cells the function of the tumor suppressor protein p53 is usually blocked. In tumor cells homozygous for a *Single Nucleotide Polymorphism* (SNP) in the *mdm2* gene at position 309 Mdm2 is over-expressed, resulting in impairment of the p53 pathway. In such tumor cells, we found that a chromatin associated Mdm2-p53 complex blocked the p53 transcriptional activity and p53 was not subjected to Mdm2-induced degradation. p53 protein accumulated in the nucleus of *mdm2* SNP309 cells after camptothecin, etoposide or mitomycin C treatment, with the p53 protein phosphorylated at Ser-15. Although the p53 protein was able to bind to DNA, quantitative PCR showed compromised transcription of endogenous target genes. Additionally, exogenously introduced p53 was incapable of activating transcription from p53 responsive elements in SNP309 cells, confirming the *trans*-acting nature of the inhibitor. Chromatin immuno-precipitation experiments demonstrated that p53 and Mdm2 bound to p53 responsive elements. Down-regulation of Mdm2 by siRNA or disruption of the p53-Mdm2 complex with the Mdm2 antagonist Nutlin resulted in transcriptional activation of p53 targets.

Although DNA damage did not activate p53 transcriptional activity in cells homozygous for *mdm2* SNP309, p53 target genes were up-regulated when Mdm2 over-

expression came from gene amplification, suggesting that over-expression from *mdm2* SNP309 gene confers a different phenotype than over-expression from amplified wild-type *mdm2* gene. Mass-spectroscopy/mass-spectrometry identified, in SNP309 cells, other interacting proteins (nucleolin, heat shock proteins) that might be part of the inhibitory p53-Mdm2 complex found on chromatin. Sequencing of the *mdm2* cDNAs from homozygous cell lines also discovered new *mdm2* isoforms that might explain this difference.

p53 is inhibited from activating transcription of its target genes in cells homozygous for *mdm2* SNP309 through a chromatin-bound inhibitory protein complex.

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Table of Contents

Table of Figures

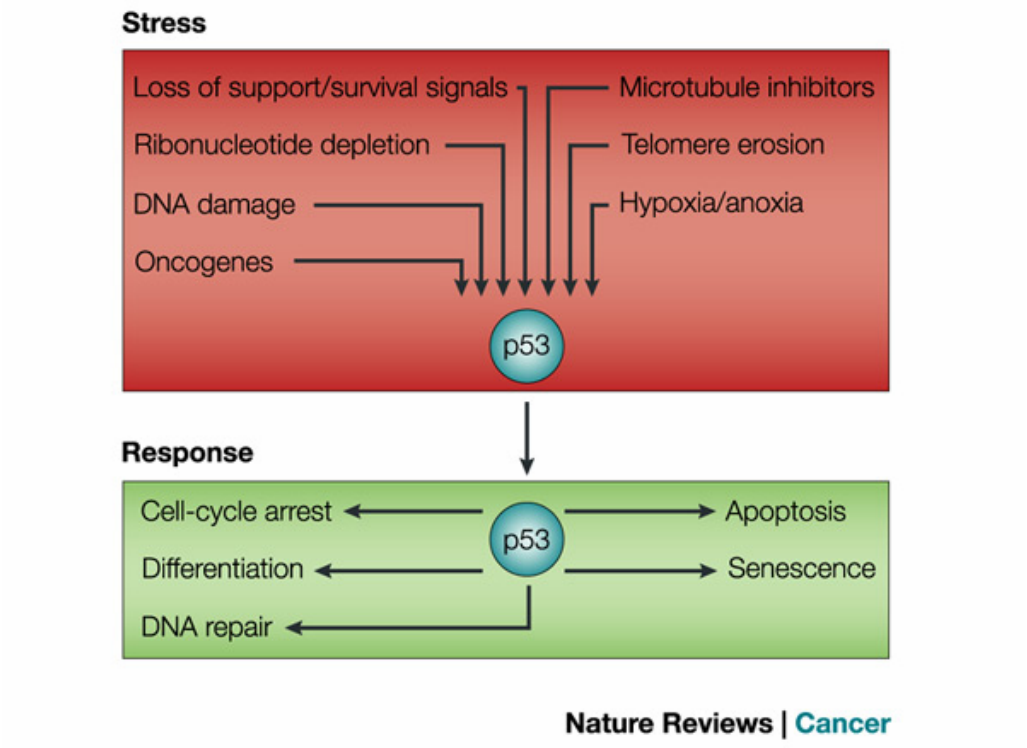
Chapter 1: Introduction

1.1 The p53 Tumor Suppressor Protein

The p53 tumor suppressor protein plays an important role in preventing cancer development, by arresting or killing potential tumor cells. p53 responds to the types of stress signals that can cause oncogenic alterations, such as DNA damage, or conditions that arise in developing tumor cells, such as abnormal proliferation or hypoxia. Activation of p53 transcriptional activity by these stress signals rapidly inhibits cell growth, by arresting proliferation or inducing apoptosis, thereby preventing the propagation of cells that may be undergoing malignant transformation.

Figure 1: p53 Responds to Different Stressors by Inducing Various Cellular Responses that Stop Cell Proliferation

(Vousden and Lu, 2002)



1.2 p53 Fate in Normal Cells

As p53 is anextremely efficient inhibitor of cell growth, its regulation is critical for both tumor suppression and control of normal cell division. p53 function must be dampened sufficiently in healthy cells to allow normal growth and development, while retaining the capacity for rapid induction in response to stress associated with tumorigenesis (Vousden, 2002). p53 is a short-lived protein that is maintained at low, often undetectable levels in normally proliferating cells due to rapid protein degradation (Levine, 1997).

One key component regulating p53 stability is Mdm2, which functions as an E3 ubiquitin ligase for p53 (Kubbutat et al., 1997). Mdm2 is a transcriptional target of p53 and the importance of this feedback loop was demonstrated by the generation of mice null for *mdm2* (Montes de Oca Luna et al., 1995), (Jones et al., 1995). Loss of Mdm2 induces embryonic lethality but it is rescued by simultaneous loss of p53 (Parant et al., 2001). Recent studies suggest that Mdm2 mono-ubiquitinates p53 and then the ubiquitin chain is extended by the histone acetylase p300 that also exhibits E4 ubiquitin ligase activity (Grossman et al., 2003). Even more, the control of p53 fate by Mdm2 can be dictated by the Mdm2 intracellular concentration (Li et al., 2003). A low level of Mdm2 seems to mono-ubiquitinate p53, followed by cytoplasmic shuttling of p53, where the ubiquitin chain is extended to allow for proteasomal recognition and degradation. However, when Mdm2 levels are high, Mdm2 signals for poly-ubiquitination of p53. Poly-ubiquitinated p53 is retained in the nucleus and is degraded by the nuclear proteasomes. Mdm2 does not only target p53 for degradation, but also has the ability to

inhibit p53 transcriptional activity by binding and blocking p53 trans-activation domain (Chen et al., 1995).

Recently, a protein related to Mdm2, Mdm4 (also called MdmX) has been identified. Mdm4 is another important regulator of p53 activity and although Mdm4 does not target p53 for degradation (Jackson and Berberich, 2000), (Stad et al., 2001), it can inhibit p53 ability to activate transcription of target genes (Shvarts et al., 1996).

Lately, another p53 target, Pirh2, with ubiquitin-ligase activity towards p53 has been identified. Like Mdm2, Pirh2 also participates in an auto-regulatory feedback loop that serves to control p53 function (Leng et al., 2003).

p53 also undergoes ubiquitin-independent degradation by the 20S proteasomes and this process is regulated by NAD(P)H quinone oxidoreductase 1 (NQO1) together with NADH. Inhibition of NQO1 activity induces p53 proteasomal degradation. Unlike Mdm2-mediated degradation, the NQO1-regulated p53 degradation pathway is not associated with accumulation of ubiquitin-conjugated p53 (Asher et al., 2002).

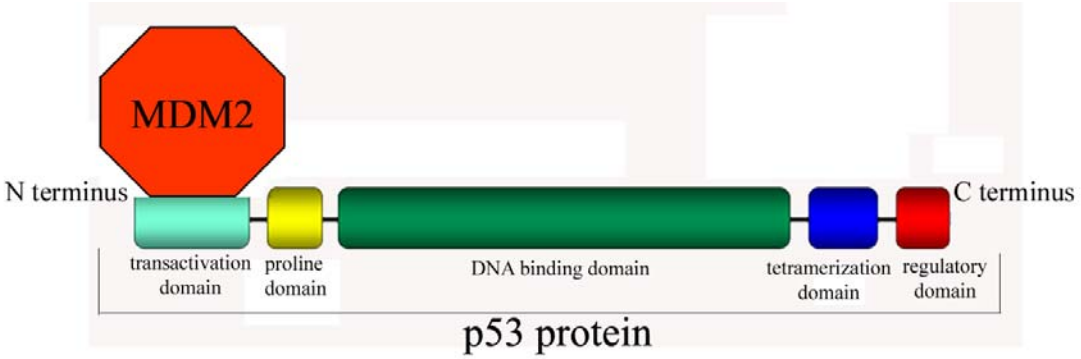
1.3 p53 Stabilization

The importance of Mdm2 in the negative regulation of p53 is underscored by the variety of mechanisms used by the cell to disrupt p53-Mdm2 feedback loop in response to stress. Post-translational modifications to p53 and Mdm2, sub-cellular redistribution, inhibition of Mdm2 activity and direct repression of Mdm2 transcription are all mechanisms exploited by the cell to promote the rapid accumulation of p53.

1.3.1 Post-translational Modifications

The p53 protein has been shown to be post-translationally modified on a variety of residues in response to cellular stress. The p53 region with which Mdm2 interacts (residues 17-27) is also located within the p53 transcriptional activation domain (Figure 2) and multiple phosphorylation sites have been identified in this region. Phosphorylation at the amino terminus in response to ionizing radiation, principally at Ser-15 (by ATM, ATR, and DNA-PK), Ser 20 (by CHK1, CHK2) and Thr 18 (by casein like kinase) have been demonstrated to be important in abrogating the p53-Mdm2 interaction (Shieh et al., 1997), (Shieh et al., 1999), (Sakaguchi et al., 2000). In addition, phosphorylation of Thr 81 by Jun amino terminal kinase can lead to p53 stabilization (Buschmann et al., 2001).

Figure 2: p53-Mdm2 Interaction



Mdm2 binds to the p53 trans-activation domain, located at the N terminus. Post-translational modifications in this area disrupt the complex.

Similarly, Mdm2 can be post-translationally modified to prevent the p53-Mdm2 interaction. ATM dependent phosphorylation of Mdm2 increases p53 levels, probably by

disrupting p53-Mdm2 interactions (Maya et al., 2001). Phosphorylation of mouse Mdm2 by CycA-CDK2 in a region outside the p53 binding domain has been shown to weaken the interaction with p53 (Zhang and Prives, 2001).

1.3.2 Proteins that Block p53 Ubiquitination

Upon DNA damage p53 levels need to increase rapidly. The cell has developed mechanisms to halt the ubiquitination pathway quickly and allow for the immediate accumulation of p53. Recently, a herpesvirus-associated ubiquitin specific protease, HAUSP, was discovered (Li et al., 2002). HAUSP is a direct antagonist of Mdm2 activity and acts by removing ubiquitin molecules specifically from p53 when stimulated by DNA damage.

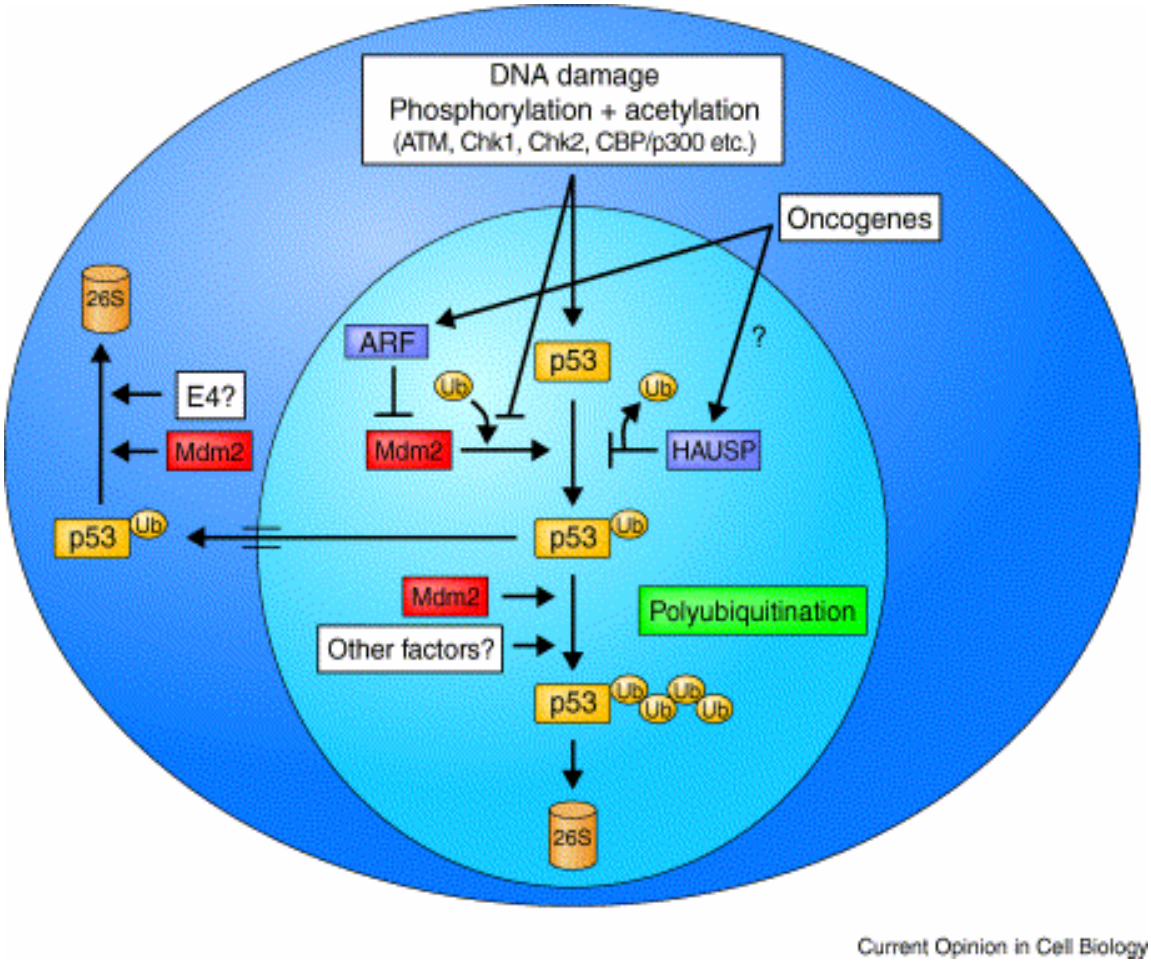
1.3.3 Proteins that Block p53-Mdm2 Interaction

Several proteins thwart the p53-Mdm2 interaction by binding to Mdm2 (e.g. pRb, ARF). The predominant regulator of Mdm2 is p19ARF, a protein derived from the *INK4a/ARF* locus. The *INK4A/ARF* locus codes for two, overlapping transcripts, $p16^{INK4A}$ and $p19^{ARF}$, both of which are frequently mutated in human cancer. The two proteins are created by the use of different first exons and reading frames. p16 is a cyclin-dependent kinase inhibitor, which targets cyclin-D–CDK4/6 and prevents phosphorylation of pRb; ARF, a known tumour suppressor, stabilizes p53 through its interaction with Mdm2. ARF is induced by oncogenic signals such as *Myc* (Zindy et al., 1998), adenovirus E1A (de Stanchina et al., 1998), mutated *Ras* (Palmero et al., 1998), *v-Abl* (Radfar et al., 1998) and is predominantly located within the nucleoli, where it sequesters Mdm2, promoting p53 accumulation.

Recently, other nucleolar proteins (L5, L11, L23) that might substitute for ARF have been shown to associate with Mdm2 (Dai and Lu, 2004), (Jin et al., 2004; Lohrum et al., 2003). For instance, when over-expressed, L23 inhibits Mdm2-induced p53 poly-ubiquitination and degradation and causes p53-dependent cell cycle arrest.

Figure 3: p53 Stabilization

(Brooks and Gu, 2003)



Several mechanisms for regulating p53 protein levels are depicted here. p53 is ubiquitinated within the nucleus by Mdm2 and possibly other factors, which leads to its degradation by the 26S proteosome either in or out of the nucleus. DNA damage-induced upstream factors can block this cascade of events by targeting Mdm2 and p53. ARF and HAUSP represent antagonists of Mdm2 in this process.

1.3.4 Regulation of p53 at Transcriptional/Translational Level

p53 mRNA levels are cell cycle regulated; in particular, transcription of *p53* gene is known to be induced upon mitogen stimulation of resting cells, with peak levels of mRNA occurring prior to S phase and maximal levels of p53 protein occurring in mid S phase (Reich and Levine, 1984; Reed *et al*, 1986;). A *trans*-acting factor, necessary for maximal *p53* promoter activity in exponentially growing cells has been shown to bind within the -972/-953 region of the *p53* gene and was identified as a member of the C/EBP family of transcription factors (Boggs and Reisman, 2005).

Agents in the environment are often associated with tumor formation. One such compound, phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) can promote cancer by preventing DNA damage-induced up-regulation of p53 protein. Suppression of p53 protein expression after TPA treatment is caused by the inhibition of *p53* gene transcription and is mediated through down-regulation of PKC δ (Abbas et al., 2004).

p53 accumulation in irradiated cells has been shown to be controlled also at translational level by nucleolar proteins. Ribosomal protein L26 (RPL26) and nucleolin were found to bind to the 5′ un-translated region (UTR) of *p53* mRNA. RPL26 over-expression enhances association of *p53* mRNA with heavier polysomes, increases the translation rate, induces G1 cell-cycle arrest, and augments irradiation-induced apoptosis. In contrast, nucleolin over-expression suppresses *p53* mRNA translation and p53 protein induction after DNA damage, whereas nucleolin down-regulation promotes p53 expression. These findings demonstrate the importance of increased translation of *p53* in DNA-damage responses and suggest critical roles for RPL26 and nucleolin in affecting p53 induction (Takagi et al., 2005)

1.4 p53 Activation

In addition to stabilization, p53 transcriptional activity is critical for initiating an early response to genotoxic stress. Post-translational modifications are responsible for p53 activation and also for p53 selectivity towards a particular cell fate. Although phosphorylation of p53 at its amino terminus leads to increased transcriptional activity caused by enhanced protein stability, it has also been demonstrated that phosphorylation at Ser-15 stimulates p53 trans-activation without directly influencing p53 interaction with Mdm2 (Dumaz and Meek, 1999). Phosphorylation at the carboxyl terminus stimulates the activity of the p53 protein by a distinct mechanism, involving enhanced DNA binding. Phosphorylation of p53 by CDK at Ser 315 was shown to up-regulate the transcriptional activity of p53 (Blaydes et al., 2001). A similar effect was caused by phosphorylation by CKII at Ser 392 (Hupp et al., 1992). A phosphorylation site has been identified at Ser 46 (Oda et al., 2000). This phosphorylation event seems to be specifically important in UV induced apoptosis through the activation of the gene *p53AIP1*. Recently, a potential mechanism that links post-translational modifications with p53 transcriptional activation was described. Pin1, a prolyl isomerase, binds to phosphorylated p53 and stimulates its transcriptional activity (Wulf et al., 2002). The interaction is strictly dependent on p53 phosphorylation and requires Ser 33, Thr 81 and Ser 315. On binding, Pin1 generates conformational changes in p53, enhancing its trans-activation activity (Zacchi et al., 2002).

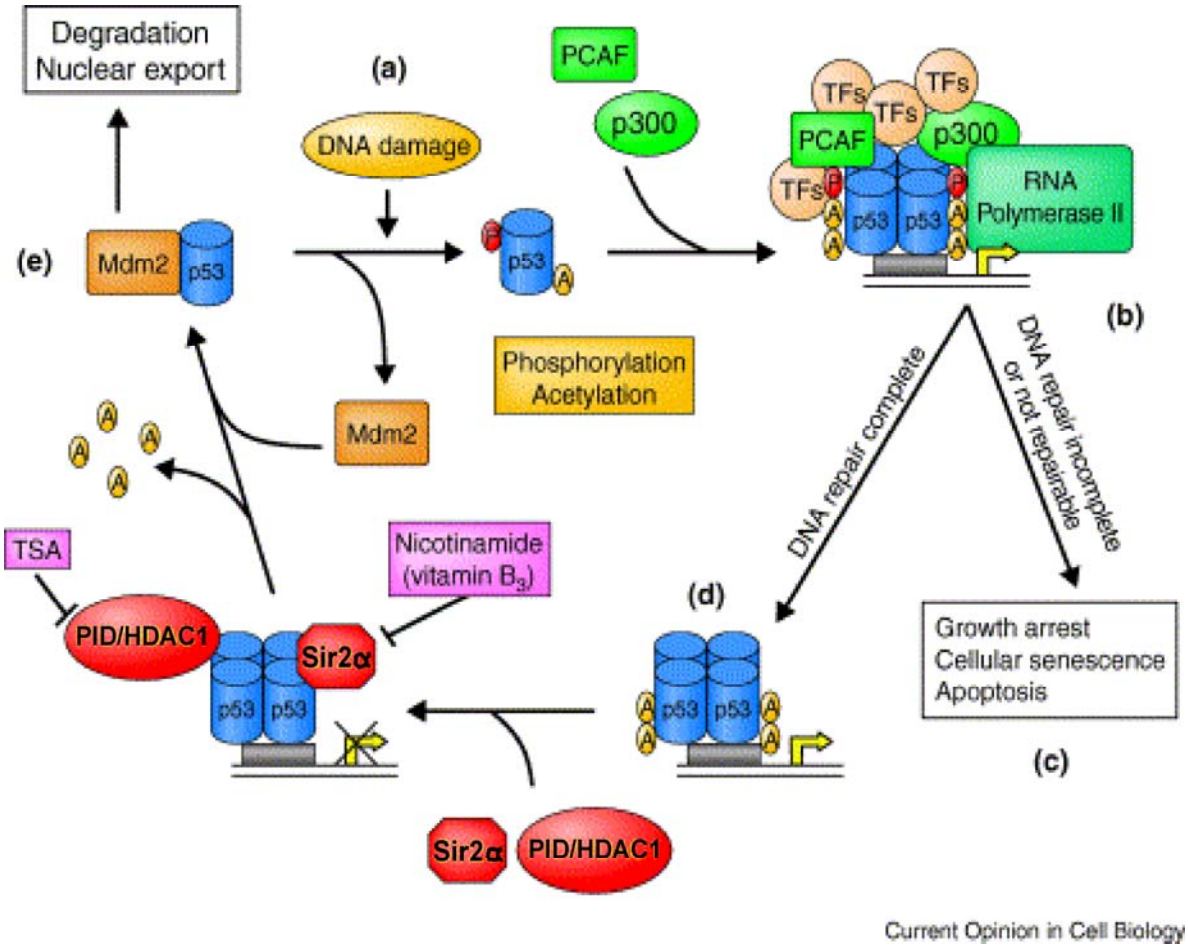
Acetylation is well known to be an important modification of histones that correlates with increased transcriptional activity. CBP/p300, a protein possessing histone acetyl transferase (HAT) activity is also a co-activator of p53 and potentiates its

transcriptional activity (Gu et al., 1997), (Lill et al., 1997). p53 is specifically acetylated at multiple Lys residues (Lys 370, Lys 371, Lys 372, Lys 381 and Lys 382) of the carboxy-terminal regulatory domain by CBP/p300 and to a lesser extent at Lys 320 by CBP/p300 associated factor PCAF. The acetylation levels of p53 are significantly increased *in vivo* in response to almost every type of stress. The sequence-specific DNA binding activity of p53 in electrophoretic mobility-shift assays (EMSA) was shown to be dramatically stimulated by acetylation, possibly as a result of an acetylation-induced conformational change (Gu and Roeder, 1997). By using highly purified and fully acetylated p53 proteins obtained from cells, Luo et all (Luo et al., 2004) has also shown that acetylation of the C-terminal domain can dramatically enhance site-specific DNA binding on both short oligonucleotide probes and long DNA fragments. Acetylation of endogenous p53 significantly augmented its ability to bind *p21/waf1* target gene in chromatin immuno-precipitation assay and p53 acetylation levels correlated well with p53-mediated transcriptional activation *in vivo* (Luo et al., 2004).

p53 activity has been recently shown to be regulated through Lys methylation. The reaction is catalysed by Set9 methyltransferase, which targets Lys 4 of histone H3. Set9 specifically methylates p53 *in vivo* at Lys 372 within the carboxyl-terminus regulatory region in response to DNA damage. This modification positively affects p53 stability. Methylated p53 is restricted to the nucleus and localizes to p53 responsive elements in ChIP assays. A stimulatory effect of Set9-mediated p53 methylation on *p21/waf1*, *bax* and *mdm2* gene expression was observed (Chuikov et al., 2004).

Figure 4: A Mechanism for p53 Transcriptional Activation

(Brooks and Gu, 2003)



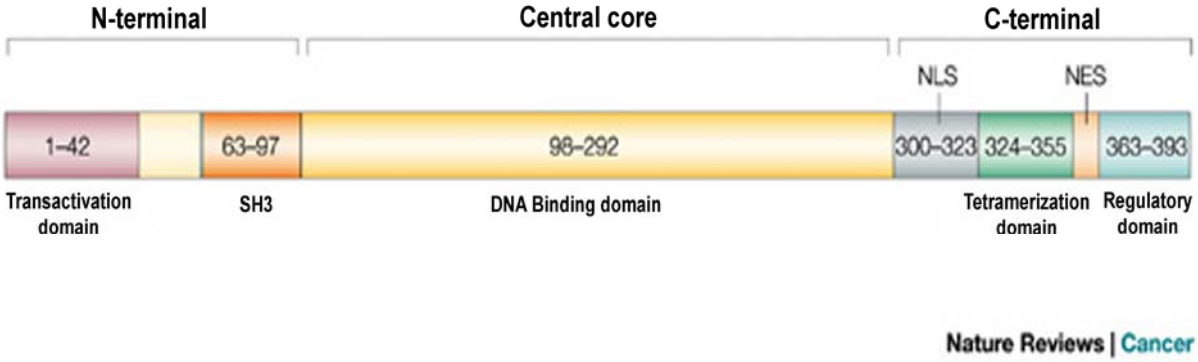
(a) DNA damage induces phosphorylation and acetylation events, p53 tetramerization and transcriptional activation. (b) Through unknown mechanisms, the cell may choose a particular fate on the basis of its DNA repair status. (c) When DNA repair is not complete or DNA damage is not repairable, the cell may choose growth arrest, apoptosis, or cellular senescence. (d) In contrast, if DNA repair is complete, deacetylases such as Sir2α and PID/HDAC1 may provide crucial p53 deacetylation activity to shut off p53-dependent transcription. (e) Mdm2 regulates p53 levels until the next DNA damage signal is received.

1.5 Signaling from p53

The p53 protein is a transcription factor and can activate transcription of genes which contain p53 sequence specific binding sites. The p53 consensus sequence has been determined and it contains two consecutive copies of 5'-PuPuPuCA/TT/AGPyPyPy-3' (el-Deiry et al., 1992). p53 consists of 393 amino acids protein, defining three main domains: the amino terminus of the protein contains the transcriptional activation domain, the central core region contains the site specific DNA binding domain and the carboxy terminus is responsible for oligomerization. Chromatin immuno-precipitation has shown that p53 promotes transcription by co-localizing with numerous components of the transcription initiation complex (Espinosa et al., 2003), the histone acetyltranserases (HAT) GCN5, p300 (Zacchi et al., 2002) and the HAT complex scaffolding protein TRRAP (Ard et al., 2002). Analysis of the p53 regulated gene expression patterns has shown that the nature of the p53 response is variable depending on the levels of p53 protein in a cell, the type of inducing agent or event and the cell type exposed to the stimulating event . The genes activated by p53 have different cellular functions, including apoptosis, growth arrest, DNA repair, cytoskeleton functions, regulation of adhesion genes and extra-cellular matrix.

Figure 5: Organization of p53 Structural Domains

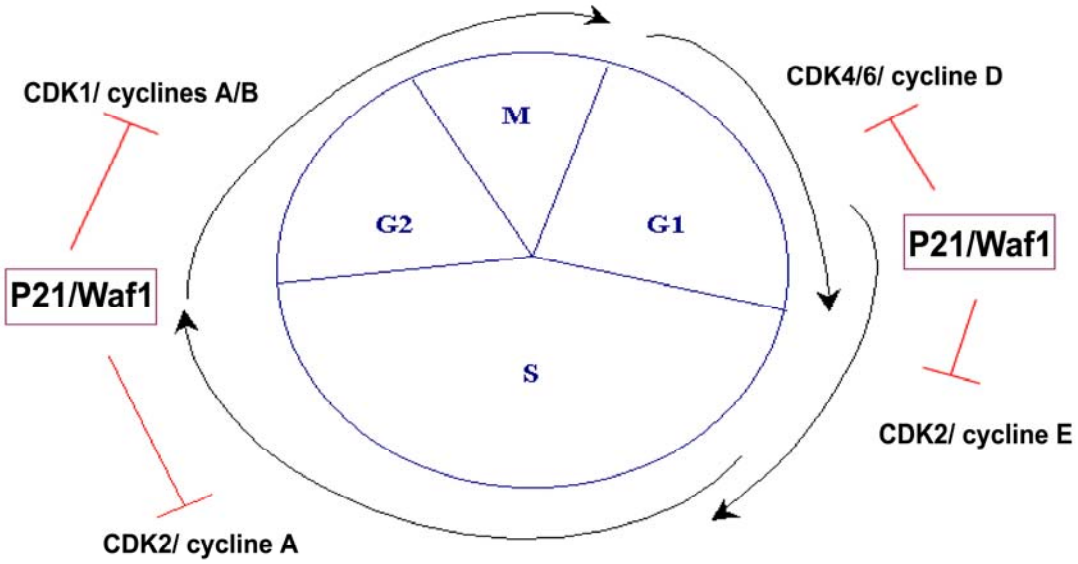
(Bode and Dong, 2004)



p21/Waf1 is the central p53 downstream target gene that controls G1/S cell cycle arrest and p21/Waf1 in conjunction with 14-3-3σ plays complementary roles in the G2/M checkpoint. The p21/Waf1 protein binds to CDKs and disrupts CDKs-cyclin complexes. pRb remains in the hypophosphorylated state, sequestering E2F1 transcription factor and arresting the cells in the G1 phase of the cell cycle (el-Deiry et al., 1993).

Figure 6: Role of p21/Waf1 in Cell Cycle Regulation

(el-Deiry et al., 1993)



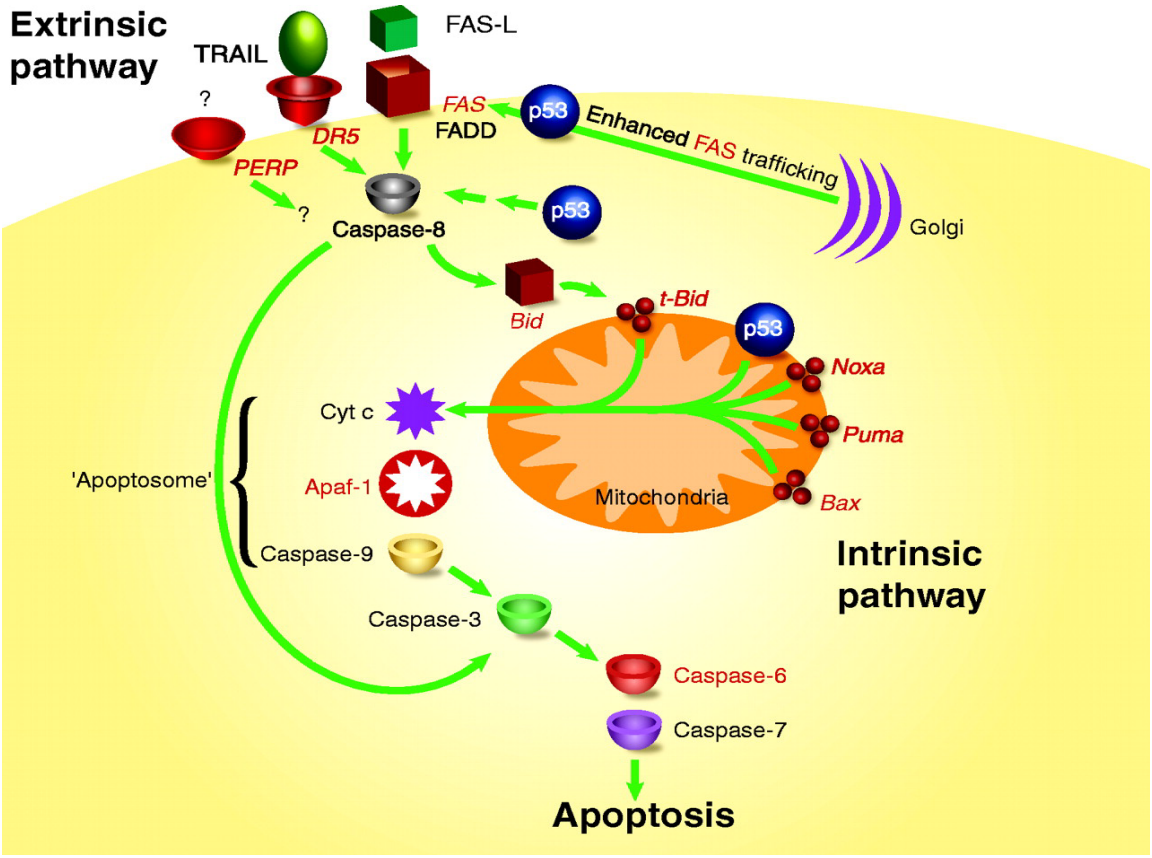
p53 can also activate numerous target genes that are involved in programmed cell death. In response to apoptotic stimuli, Bax protein, a Bcl family member that is up-regulated by p53, is translocated from the cytoplasm to the mitochondria where it mediates cytochrome c release (Knudson et al., 1995). PUMA and NOXA, two mitochondrial proteins that are also p53 targets, facilitate the apoptotic activity of the pro-apoptotic Bcl family factors (Nakano and Vousden, 2001). These proteins, through oligomerization, create pores in the mitochondrial membrane, followed by cytochrome c release into the cytoplasm. Here, cytochrome c in conjunction with APAF1, forms the apoptosome complex, which activates caspase 9. *Apaf 1* has also been shown to be a

direct transcriptional target of p53 (Fortin et al., 2001). P53AIP1, another protein expressed in a p53 dependent manner, is also localized to the mitochondria and appears to affect mitochondrial membrane potential, leading to cell death (Oda et al., 2000).

p53 is also involved in the apoptosis process initiated by cell surface receptor activation (extrinsic pathway). Death receptors, such as KILLER/DR5 (that respond to TNF like ligands) or Fas (that responds to Fas ligand) are activated in a p53 dependent manner (Takimoto and El-Deiry, 2000), (Owen-Schaub et al., 1995). Upon interaction between ligand and receptor, adaptor proteins (FADD, TRADD) are recruited to the complex and lead to caspase 8 activation. A newly identified death domain containing adaptor protein, PIDD, has been shown to be a direct p53 target as well (Lin et al., 2000). Both intrinsic (mitochondrial) and extrinsic (membrane-regulated) apoptotic pathways conclude with activation of caspase 3, considered to be the effector caspase.

Figure 7: p53 Involvement in Apoptotic Pathways

(Haupt et al., 2003)



p53 can trigger apoptosis independent of its transcriptional activity. In response to a broad spectrum of apoptotic stimuli, a fraction of wild-type p53 translocates to mitochondria in primary cells and *in vivo* in mice (Marchenko et al., 2000), (Sansome et al., 2001), (Mihara et al., 2003). p53 mitochondrial translocation occurs very rapidly, and precedes cytochrome c release, the collapse of the mitochondrial membrane potential and caspase 3 activation. The majority of mitochondrial p53 localizes to the outer membrane. Endogenous mitochondrial p53 physically interacts with the Bcl-2 family member

proteins Bcl-xL and Bcl-2 and antagonizes their anti-apoptotic function (Chipuk et al., 2004). Pro-apoptotic Bcl-2 family members Bid and Bax that have been sequestered by Bcl-xL in a preformed complex are liberated by recombinant p53 protein *in vitro*. Mitochondrial p53 also directly promotes the pro-apoptotic activities of Bak (Leu et al., 2004) and directly induces Bak oligomerization. After camptothecin treatment or ultraviolet irradiation stress, mitochondrial p53 physically interacts with the outer membrane resident protein Bak, thereby liberating Bak from its complex with the anti-apoptotic Bcl-2 family member Mcl-1.

The p53 protein also appears to have a direct influence on DNA repair. Ribonucleotide reductase gene (*p53R2*) is up-regulated in a p53 dependent manner and mediates the production of deoxyribonucleotides that are important for both DNA replication and repair (Tanaka et al., 2000). p53 can activate transcription of the mismatch repair gene *Msh2* (Scherer et al., 2000). Wild-type p53 facilitates also base excision repair and stabilizes the interaction of DNA polymerase with abasic DNA (Zhou et al., 2001). *Gadd45* (growth arrest and DNA damage 45) is another p53 target that was first identified as a gene that was rapidly induced after IR of lymphoblasts and fibroblasts. Gadd45 protein can interact directly with the essential replication factor PCNA (Proliferating Cell Nuclear Antigen), may block DNA replication, and may coordinately enhance nucleotide excision repair of damaged DNA (Xiao et al., 2000).

While most studies investigating the action of p53 have focused on its trans-activation functions, p53 also has trans-repression capabilities that may contribute to apoptosis (Mack et al., 1993), (Zhang et al., 1999). How p53 represses transcription is not fully established, but appears to involve its ability to recruit histone deacetylases to certain genes through the mSin3a co-repressor (Murphy et al., 1999). One of the targets

of p53-mediated repression is *Survivin*, which encodes the IAP protein (Inhibitor of Apoptosis Protein), capable of inhibiting apoptosis when over-expressed (Ambrosini et al., 1997). In principle, *p53* mutations might contribute to the high frequency of *Survivin* over-expression observed in human tumors (Hoffman et al., 2002). It has been suggested in some studies that the ability of p53 to trans-repress may be more important for inducing apoptosis than its trans-activation function (Koumenis et al., 2001).

1.6 p53 is Inactivated in Cancers

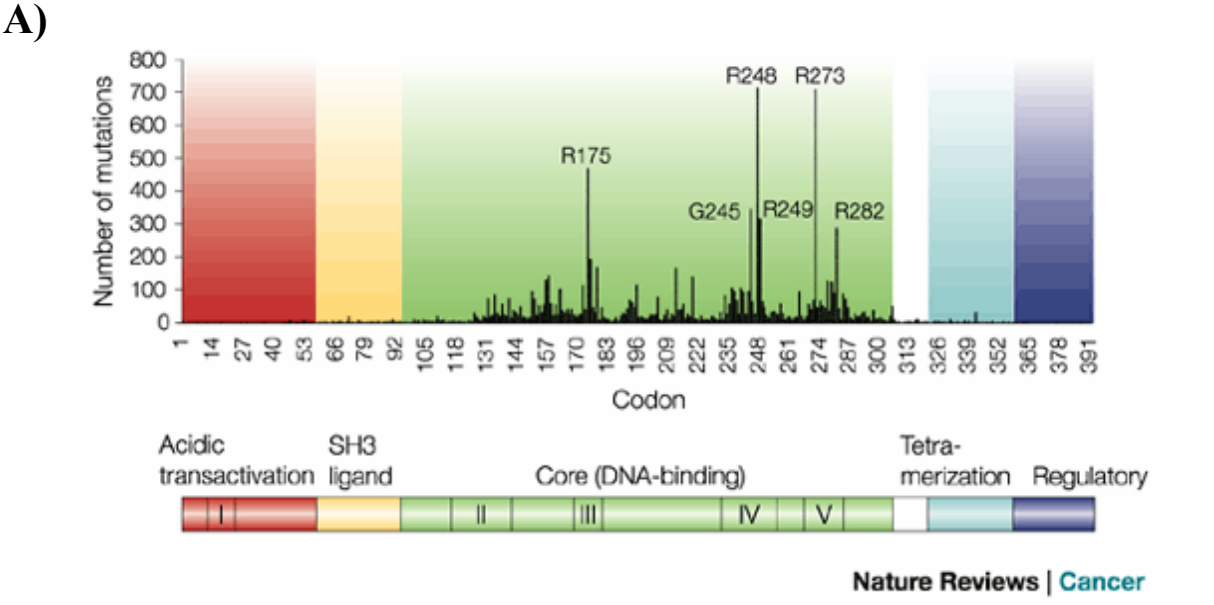
Malignant progression is dependent on loss of p53 function, either through mutations in the *p53* gene itself or by defects in the signaling pathways that are downstream or upstream of p53. Inactivating lesions in the p53 pathway that are critical for tumorigenesis fall into three classes. The most frequent way in which p53 is inactivated are intragenic mutations that perturb the structure of the sequence-specific DNA binding domain of p53, thereby incapacitating its trans-activation ability of target genes that mediate its tumor suppressor functions. From the data available, it would seem that only 5% of *p53* mutations are found in the regulatory domains whereas 95% of the mutations occur in the central region of p53, which is responsible for sequence specific DNA binding. Tumor associated mutations in *p53* are predominantly point mutations (93.6%) that result in single amino acid substitution. Six hot spot mutations cluster to the DNA-binding surface: two contact DNA directly — R248 and R273— and four stabilize the surrounding structure — R175, G245, R249 and R282 (Figure 8, panel A). The mutant protein can act as a dominant negative inhibitor of wild-type p53 (de Vries et al., 2002). A germ-line mutation in just one of the two *p53* alleles abrogates p53 function and

predisposes to cancer (Li–Fraumeni syndrome). The dominant negative effect of the mutant consists of formation of functionally inactive hetero-oligomers between mutant p53 and wild-type protein. The oncogenic potential of some mutant p53 proteins was shown to be the result of their ability to block binding of wild-type p53 to DNA (Bargonetti et al., 1992). Co-translation of wild-type and structural mutants of p53 *in vitro* produces mixed tetramers in which all subunits become misfolded (Milner and Medcalf, 1991). The crystal structures of human core domains show close packing contacts that are thought to mimic inter-subunit contacts in the tetramer (Cho et al., 1994). Misfolded mutants will expose an extended hydrophobic surface that is likely to aggregate with associated wild-type subunits and perturb their structure.

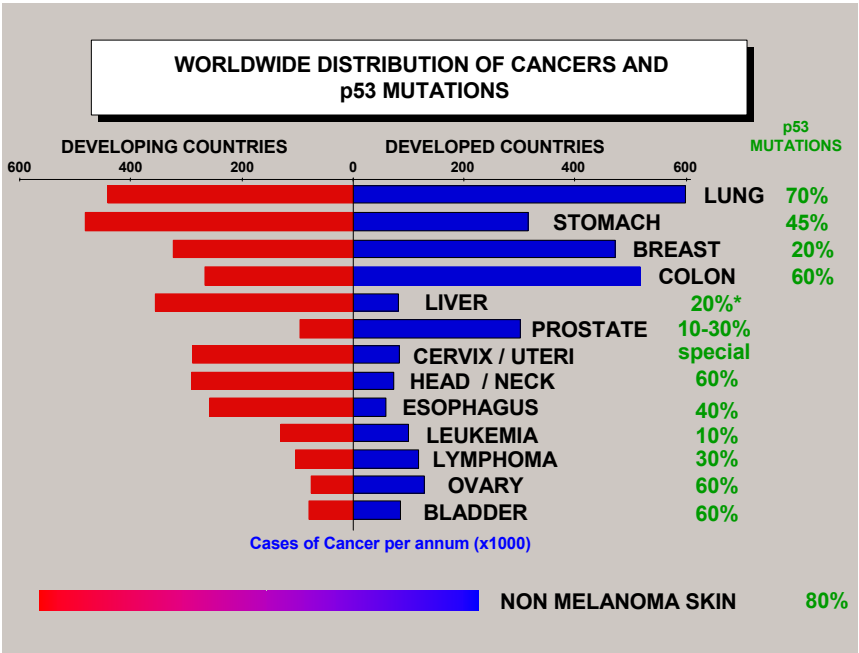
p53 mutations occur in more than 50% of human cancers. A distribution of organ-specific tumors and percentage of *p53* gene mutations is depicted in Figure 8, panel B.

Figure 8: Analysis of p53 Mutations

A)



B)



Panel A): Histogram of p53 mis-sense mutations shows that 95% of mutations occur in the core domain; six labeled residues are hot spots for mutation.
Panel B): Histogram depicting the p53 mutation frequency in different types of cancers. (Bullock and Fersht, 2001)

A second mechanism for wild-type p53 inactivation is its inhibition by viral oncoproteins. Several well-studied DNA tumor viruses encode proteins that target and inactivate p53. In adenovirus transformed cells, the E1B oncoprotein binds to p53 and inhibits p53 mediated transcriptional trans-activation, by specifically blocking p53 acetylation by PCAF (Zhao and Liao, 2003), (Liu et al., 2000). The E6 protein of the oncogenic human papillomavirus types 16 and 18 facilitates the rapid degradation of the tumor-suppressor protein p53 via the ubiquitin-dependent proteolytic pathway. The E6 protein binds to a cellular protein termed E6-AP. The complex of E6 and E6-AP specifically interacts with p53 and induces the ubiquitination of p53 (Scheffner et al., 1993), (Huibregtse et al., 1993).

A third class of wild-type p53 inactivating lesions comprises extragenic lesions affecting proteins that regulate the activity of the p53 protein, such as Mdm2 and ARF. Loss of *INK4A* locus through deletions, mutations or DNA methylation is frequently seen in cancers (Sherr, 2001) and is accompanied by an unrestrained Mdm2 activity, contributing indirectly to p53 inactivation.

Mdm2 over-expression through gene duplication (Momand et al., 1998), enhanced transcription (Phelps et al., 2003) or translation (Landers et al., 1994) might inactivate wild-type p53 and is frequently observed in those tumor cells that retain wild-type p53. Mdm2 is amplified and over-expressed in appreciatively one-third of human

sarcomas, including those of soft tissue and bone. Combined analysis of data obtained from 3889 tumor samples from 28 tumor types revealed an overall Mdm2 amplification frequency of 7% (Momand et all, 1998). Mdm2 inactivates p53 by tagging p53 for proteasomal degradation.

However, hypomorphic *mdm2* mice that express reduced levels of Mdm2 have enhanced p53 activity without increased p53 protein levels, demonstrating that degradation of p53 by Mdm2 is not the only way Mdm2 inactivates p53 (Mendrysa et al., 2003). Mdm2 can inhibit the transcriptional activity of p53 by blocking p53 association with the transcriptional machinery. The ubiquitination of p53 Lys residues at the C terminus impedes the acetylation of these sites, turning p53 inactive. Mdm2 also regulates p53 nuclear export; p53 mono-ubiquitination by Mdm2 constitutes the signal for p53 cytoplasmic shuttling and possible sequestration (Tao and Levine, 1999), (Geyer et al., 2000).

Mdm2 facilitates the interaction between p53 and various repressors of transcription. One such regulator is Daxx protein; the association of p53 with Daxx inhibits both p53 transcriptional and apoptotic activities (Zhao et al., 2004). Daxx has been previously characterized to act as a transcription regulator (Hollenbach et al., 1999) *in vivo* (Zhao et al., 2004). Mdm2 interacts also with the nuclear co-repressor KAP1 and this complex inhibits p53 acetylation by stimulating the association of p53 with HDAC1 (Wang et al., 2005). Mdm2 also promotes conjugation of Nedd8 to p53 (Xirodimas et al., 2004). Nedd8 belongs to the ubiquitin-like protein family and Nedd8 conjugation is mechanistically similar to ubiquitination. Mdm2 protein, by promoting Nedd8 modification, negatively regulates the p53 trans-activation function (Xirodimas et al., 2004).

Recently it has been seen that Mdm2 protein is present at p53 binding sites in chromatin in the Mdm2 over-expressing cell line SJSA-1 (Minsky and Oren, 2004). This Mdm2-chromatin association is p53-mediated and causes gene silencing due to H2A and H2B histone ubiquitination. Gene expression regulation through histone ubiquitination is a well documented phenomena in yeast (Sun and Allis, 2002; Wang et al., 2004), but is still poorly understood in mammalian cells. Acetylation of p53 at its C terminal Lys residues following DNA damage has been shown to stabilize p53 by preventing ubiquitination by Mdm2 at these sites (Zacchi et al., 2002). In order for chromatin-bound p53 to be ubiquitinated by Mdm2, deacetylases have to be recruited to these enhancer elements (Figure 4). The histone deacetylases HDAC1 and Sir2α have been shown to associate with p53 and repress transcription (Smith, 2002), (Ito et al., 2002). Additionally, HDAC1 has recently been found to associate with Mdm2 (Ito et al., 2002). Recruitment of histone deacetylases by chromatin-bound Mdm2 might not only facilitate p53 ubiquitination but they could also regulate p53 transcriptional activity by remodeling and silencing the chromatin at p53 responsive elements. In conclusion, the mechanism by which Mdm2 mediates repression of chromatin-bound p53 may be multifaceted and involve both recruitment of histone deacetylases and ubiquitination of histone proteins at enhancer sites.

Chapter 2: Material and Methods

Reagents: Camptothecin (CPT), propidium iodide, calpain inhibitor, N-Acetyl-leu- leu-norleu-al (LLnL/ALLN), etoposide (ETOP) and anti-actin antibody were purchased from Sigma. Bristol-Myers Squibb Co. provided mitomycin C (MC). The p53 phoshpo-specific antibody Serine-15 (Ser-15) was purchased from Cell Signaling Technology. The monoclonal PARP antibody was purchased from Pharmingen. The p53 antibodies 240, 1801, 421 and the Mdm2 antibody 2A10 were from monoclonal supernatants as described previously (Chen et al., 1993). The p53 Antibody 6 (Ab6) was purchased from Calbiochem and the p53 polyclonal antibody from Santa Cruz. The Mdm2 antibodies SMP14 and D7, Sp1 antibody PEP2 and antibody against Lamin A were also from Santa Cruz. HSP70 antibody was a generous gift from Dr. Maria Pereira laboratory. Nutlin was purchased from Calbiochem.

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, SJSA-1 and H460 cells were purchased from American Type Culture Collection. A875 and CCF-STTG-1 cells were a generous gift from the Levine laboratory. H1299 cells were a generous gift from Carol Prives laboratory and HCT116+/+ cell line was donated by Dr. Bert Vogelstein laboratory. All cells were maintained in 10% fetal bovine serum (FBS, Gemini), RPMI 1640 medium (Mediatech) and 5% CO2. The media was supplemented with 100µg of penicillin per milliliter and 100µg of streptomycin per milliliter. Cells were seeded at $2.5x10^5$ and exponentially growing cells were used in all experiments.

Drug Treatment: DNA damaging agents, the proteosome inhibitor LLnL and Nutlin 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: camptothecin 0.5μM, etoposide 8μM, mitomycin C 5μM, Decarbamoil-mitomycin C 5μM, LLnL 20μM, Nutlin 10μM.

Flow Cytometry: FACS analysis was carried out on a BD Biosciences FACS scan. Cells were spun down at 2000rpm for 7 minutes, washed twice with phosphate buffered saline (PBS) 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^{o}C for 30 minutes prior to analysis.

Nuclear Extracts: Cells were pelleted at 2300rpm for 7 minutes at 4^{o}C and washed 2X with ice-cold 1XPBS (pH=7.3). Cells were then washed 1X in 5 packed cell volumes with Buffer A (Hepes pH=7.9 10mM, MgCl2 1.5mM, KCl 10mM, PMSF 0.5mM, DTT 0.5mM, Leupeptin 2μg/mL, and phosphatase inhibitor cocktail I (Sigma)). After washing, cells were resuspended in 2 packed cell volumes of Buffer A and incubated on ice for 10 minutes. After centrifugation (10 minutes at 12,000rpm at 4^{o}C) the supernatant was removed to give the cytoplasmic fraction. The pellet was then resuspended in Buffer C (Hepes pH=7.9 20mM, glycerol 25%, NaCl 420mM, MgCl2 1.5mM, EDTA 0.2mM, PMSF 0.5mM, DTT 0.5mM, Leupeptin 2μg/mL, and phosphatase inhibitor cocktail I (Sigma). Cells were resuspended with a 20-gauge needle to a concentration of 40 million cells/120μl. 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 was the nuclear fraction, which was stored at –80°C.

Western Blotting Analysis: Nuclear extracts, whole cell extracts or immuno-precipitated samples were separated on a 10% SDS-PAGE followed by electro-transfer to a nitrocellulose membrane. The following p53 antibodies were used: 421, 1801 and 240 (supernatant antibodies), Serine-15 and the p53 polyclonal antibody. The following Mdm2 antibodies were used: 2A10 (supernatant antibody), SMP14 and D7. For p21/Waf1 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 the antibody MS-3 from Santa Cruz. For actin detection we used a rabbit polyclonal purchased from Sigma. For Sp1 detection we used PEP2 antidody from Santa Cruz and for HSP70 we used an antibody purchased from StressGen. The signals were visualized after incubation with goat anti-mouse or goat anti-rabbit secondary antibody (Sigma) using ECL solutions.

Immuno-fluorescence: Cells were grown on glass coverslips (for adherent cell lines) or plated onto glass coverslips using poly-L-Lys (Sigma). Coverslips were coated with an excess of poly-L-Lys, allowed to dry for 10 minutes at room temperature and then covered with cell suspension (the cell pellet from 20mls of growth media, resuspended in 1XPBS was used). Coverslips were incubated with the cell suspension for 45 minutes at room temperature, then cells were fixed with 4% paraformaldehyde for 15 minutes at room temperature and then permeabilized with Triton X-100 1% for 5 minutes at – 20° C. After being washed 3 times with PBS-FBS 1%, fixed cells were incubated for

1 hour at room temperature with mouse anti-human p53 Ab6 from Calbiochem (ML-1 cells) or rabbit anti-human polyclonal p53 antibody from Santa Cruz (MANCA and A875 cells) 1/200 dilution in PBS-FBS 1%. Cells were washed 3 times with PBS-FBS 1% and then incubated for 30 minutes with secondary Texas-Red -conjugated donkey anti-mouse antibody (Santa Cruz) or FITC-conjugated goat anti-rabbit antibody (Santa Cruz) 1/400 dilution in PBS-FBS 1%.

For Mdm2 staining, the coverslips were incubated for 1 hour at room temperature with mouse anti-human Mdm2 SMP14 antibody (from Santa Cruz), 1/200 dilution in PBS-FBS 1%. After 3 washings with PBS-FBS 1%, coverslips were incubated with Cy5 -conjugated donkey anti-mouse antibody (Jackson Immuno-research), 1/200 dilution in PBS-FBS 1%.

Coverslips were then mounted using Vectashield mounting medium with DAPI (Vector Laboratories) and observed under a fluorescence microscope.

Quantitative PCR: Reverse transcription (RT): For each sample, 5µg of RNA was obtained using the Qiagen RNeasy Kit, as per the manufacturer's protocol. Each sample was then diluted up to 50µl 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^{o}C for 2 hours. cDNA was stored at –20^{o}C. For PCR, the program was as follows: one cycle of 50^{o}C UNG incubation for 2 minutes and one cycle of 95^{o}C priming for 10 minutes, followed by 40 cycles of 95^{o}C denaturation for 15 seconds and 60^{o}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 *mdm2-P2* (order #: HS00242813_M1), *fas* (order #: HS00538709_M1), *p21/waf1* (order #: HS00355782_M1), *gadd45* (order #: HS00169255_M1), *pig3* (order #: HS00153280_M1), *mdm2-P1* (order #: HS00234753_M1), *puma* (order #: HS00248075_M1), *noxa* (order #: HS00560402_M1), *p53* (order #: HS00153340_1) 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. Samples were normalized to the GAPDH values.

Transient Transfection and Luciferase Activity Assay: The plasmids used in this study were the SN3 plasmid containing the wild-type p53 gene (Baker et al., 1990), and a plasmid containing the *mdm2* (Bond et al., 2004) or the *p21/waf1* (Datto et al., 1995) promoter with p53 responsive elements adjacent to a luciferase reporter (Bond et al., 2004). $3x10^6$ cells were transferred from RPMI media to OptiMEM media (Invitrogen) and co-transfected with 2μg of a plasmid containing the *mdm2* or *p21/waf1* p53 binding site adjacent to a luciferase reporter and increasing amounts (50 to 400 ng) of SN3 plasmid, containing the wild-type p53 gene. The total amount of DNA transfected into each sample was normalized with a carrier plasmid, pGL2. The transient transfections were performed using the Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer's instructions. After 24 hours, cells were harvested and analyzed using the Luciferase Assay System (Promega) according to the manufacturer's indications. Luciferase activity was read using a Luminoskan reader and the results were normalized to total protein values.

Chromatin immuno-precipitation: Cells from 100 mls of cell culture were cross-linked with 1% formaldehyde (Sigma) (diluted in Solution 1 (0.1 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Hepes (pH 8.0)) at 37 °C for 30 min, then quenched with glycine to 125 mM. The cells were washed with phosphate-buffered saline and collected into 100 mM Tris·Cl (pH 9.4), 10 mM dithiothreitol. The cell pellet was resuspended to a total volume of 5ml in Solution 2 (10 mM Tris·Cl (pH 8.0), 0.25% Triton X-100, 0.5% Nonidet P-40, 10 mM EDTA, 0.5 mM EGTA, 1 mM PMSF) and incubated on ice for 10 minutes. Nuclei were collected by centrifugation, washed in 2ml of Solution 3 (10 mM Tris·Cl (pH 8.0), 0.2 M NaCl, 1 mM EDTA, 0.5 mM EGTA, 1 mM PMSF) and resuspended in 1ml of the same buffer without NaCl. Samples were sonicated (10 X, 10 s each, with 1 minute break in between sonication), centrifuged, and 0.10 volume of 10X precipitation buffer (10% Triton X-100, 1% sodium deoxycholate, 1.4 M NaCl) was added to the supernatants. 1/5 of each sample was saved and designated as INPUT. Immuno-precipitations were carried out overnight with 2 μg of Mdm2 specific antibody (SMP14), 2 μg of p53 specific antibody Ab6 or 1/200 dilution of p53-Ser15 phospho-specific antibody. The next day 50 μl Protein A Plus G Sepharose beads (Amersham) were added for 2 hours with rocking at 4^{o}C. The subsequent washes were as described (Burakov et al., 2002). The washed resin was resuspended in 100 μl of elution buffer (1% SDS, 0.1 M NaHCO3) and reverse-cross-linked at 65 °C overnight. DNA fragments were purified (QIAquick Spin Kit, Qiagen, as per manufacturer indications) and PCR-amplified using primers designed to amplify the p53 binding sites in the *mdm2* gene (forward primer: 5'-CGGGAGTTCAGGGTAAAGGT-3', reverse primer: 5'-AGCAAGTCGGTGCTTACCTG-3') or *p21/waf1* gene (forward primer: 5'-

GTGGCTCTGATTGGCTTTCTG-3', reverse primer: 5'-CTGAAAACAGGCAGCCCAAG-3'). 1 µl ^{32}P dCTP (Perkin-Elmer) was added to the PCR reaction. Amplified products were analyzed by acrylamide gel electrophoresis. Gels were dried for 1 hour at 55^{o}C and autoradiography was performed. Quantitative PCR using ChIP samples was carried out on a PE5700 PCR machine using a TaqMan Master Mix (Perkin–Elmer). The PCR cycles were: 50°C for 2 min, 95°C for 10 min, then cycles of 95°C for 15 seconds and 60°C for 1 min repeated 40 times. The fold change in the specific binding was normalized to GAPDH and Mock IP values. The probe and primer sequences are given below. Primers are in italics, TaqMan probe is in bold, and p53-binding site is underlined: *p21/waf1*:*GTGGCTCTGATTGGCTTTCTG*GCCATCAGGAACATGTCCCAACATGTTGAGCTC**TGGCATAGAAGAGGCTGGTGGCTATTTTG**TC*CTTGGGCTGCCTGTTTTCAG.*

Electrophoretic Mobility Shift Assay: Custom oligonucleotides for DNA binding analysis were ordered from Operon Technologies and for SCS and RGC mutant as described (Molina et al., 2003). 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'. Labeling of the oligo was performed using the large fragment of DNA polymerase and ^{32}P dCTP. Electrophoretic mobility shift assays were carried out in reaction mixtures with 150pmol of ^{32}P oligonucleotide. 10µg 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, 1μg sheared salmon sperm DNA, and 10% glycerol. In the case of competition, unlabeled competitor in 50X-100X fold excess was added into the incubation. *mdm2*, *fas*, and a mutant oligo were used for competition studies. The sequences of the oligos are as follows:

mdm2:Top:5'CCGGGCTGGTCAAGTTCAGACACGTTCCGAAACTGCAGTAAAAG GAGTTAAGTCCTGACTTGTCTCCC-3', and Bottom:5'CCGGGGGAGACAAGTCAGGACTTAACTCCTTTTACTGCAGTTTCGG AACGTGTCTGAACTTGACCAGC-3',

fas:Top:5'CCGGGCTCCTGGACAAGCCCTGACAAGCCAAGCCAC-3', and Bottom:5'-CCGGGTGGCTTGGCTTGTCAGGGCTTGTCCAGGAGC-3',

mutant:Top:5'TCGAGTTTAATGGACTTTAATGGCCTTTAATTTTC-3', and Bottom: 5'-TCGAGAAAATTAAAGGCCATTAAAGTCCATTAAAC-3'.

Reactions were carried out in the presence or absence of 421 antibody as indicated. Samples were separated by 4% polyacrylamide gel electrophoresis (gels were pre-run at 100V for 15 minutes at 4^{o}C) at 200V for 3-3.5 hours. Gels were dried for 1 hour at 55^{o}C and autoradiography was performed.

Immuno-precipitations: Reactions were carried out using 250μg of nuclear extracts. Nuclear protein was incubated with Buffer C (Hepes pH=7.9, 20mM; glycerol 25%; NaCl 420mM, MgCl2 1.5mM, EDTA 0.2mM, PMSF 0.5mM, DTT 0.5mM, Leupeptin 2μg/mL, Aprotinin (Sigma) and phosphatase inhibitor cocktail I (Sigma)) and 2 μg of purified antibodies overnight at 4^{o}C while rocking. The next day Protein A Plus G Sepharose beads (Amersham) were added for 2 hours with rocking at 4^{o}C. Samples were then washed 3X in BC buffer (Hepes 20mM, pH=7.9, glycerol 10%, PMSF 0.5mM,

KCl 400mM, Leupeptin 2μg/mL, Aprotinin (Sigma), and NP40 0.05%). 6X protein sample buffer was then added and the samples were boiled and loaded onto a 10% gel to be separated by electrophoresis as previously described.

siRNA: Mdm2 expression was lowered using *mdm2* siRNA (Darmacon, SmartPool- a mixture of 4 different *mdm2* siRNA, catalog # M-003279-02); p53 expression was lowered using *p53* siRNA (Darmacon, SmartPool- a mixture of 4 different *p53* siRNA, catalog # M-003329-01); Sp1 expression was lowered using *Sp1* siRNA (Darmacon, SmartPool- a mixture of 4 different *Sp1* siRNA, catalog # M-026959-00). 200 pmoles siRNA was transfected into cells at 70%–80% confluencey using Oligofectamine reagent (Invitrogen) according to the manufacturer's instructions. Control siRNA (Darmacon, SmartPool, catalog # D-001206-13) has no known target in mammalian genomes. Cells were lysed 48 hours after transfection and protein levels of Mdm2, p53, p21/Waf1 and Actin were analyzed. Total RNA was also extracted from transfected cells using the Qiagen RNeasy Kit, as per the manufacturer’s protocol and analyzed by quantitative PCR as described above. Cells were also retransfected with 2μg of the reporter constructs mentioned above 24 hours after siRNA transfection and grown for an additional 24 hours. Lysates were prepared for luciferase analysis as described previously.

Cloning:

Creating cDNA clones

Cloning was done in cells *mdm2* wild-type (ML-1), *mdm2* SNP309 heterozygous cells (K562) and in *mdm2* SNP309 homozygous cells (MANCA and A875) cells. mRNA was isolated from these cells using a Quiagen kit, according to the manufacturer instructions. cDNAs are created using the *reverse transcriptase* enzyme isolated from a retrovirus. 1, 12 and 2, 12 transcripts were then selected and amplified via *Polymerase Chain Reaction* (PCR) using primer sets 1-12 and 2-12, respectively: primer 1 anneals in exon 1, primer 2 anneals in exon 2 and primer 12 anneals in exon 12 (see primer sequences in the table below). The PCR product is created in a manner called "TA cloning" developed by Invitrogen®. "A" overhangs are created such that the PCR target products are then easily ligated into the pCR®II- TOPO® (4kb) vector (see Figure 28, panel B), using DNA *ligase. E. coli* cells were then transformed with the plasmid.

Table 1: Primers Used to Amplify cDNA Clones of mdm2 mRNA Product

Primer 1	5'- CCTGTGTGGCCCTGTGTGTC -3'
Primer 2	5'- TGTGTTCAGTGGCGATTGGAG -3'
Primer 12	5'- GGGGGATTCATTTCATTGCATG -3'

DNA Isolation, restriction and analysis

E. coli cells are mutant for the *lacZ* α-peptide gene, which codes for *ß*-galactosidase, and are sensitive to the antibiotic ampicillin. The vector, on the other hand, contains the ampicillin resistance gene, Amp^R, and the *lacZ* gene. Integration of the target sequence into the vector disrupts the *lacZ* gene. The *E. coli* cells were plated onto

Amp^+, X-gal plates. The existence of the Amp^R gene in the plasmid allowed only successful transformants to grow on the Amp^+ plates. Furthermore, those transformants containing the entire plasmid (including insert) have a disrupted *lacZ* gene and appeared white, whereas those transformants containing the vector alone could produce *ß*-galactosidase, which enabled them to maintain a blue colour in the presence of X-gal via hydrolytic cleavage. The white colonies were re-picked and allowed to grow to confluence on secondary plates. Following over-night growth in LB broth, bacterial cells were pelleted, lysed and the DNA isolated in supernatant. The nucleic acids were then precipitated with ethanol, pelleted, dried and dissolved in buffer containing RNase (to eliminate any remaining ribonucleic acids). This "miniprep" technique resulted in pure DNA isolated in buffers. The isolated plasmid was then restricted with *Eco*RI restriction enzyme and run on an agarose electrophoretic gel alongside a molecular weight ladder (250bp DNA ladder) to visualise the size of the inserts in comparison to the known sizes of the DNA ladder.

Sequencing

The "miniprep" DNA was sent for sequencing at GeneWiz Company, New Jersey. The obtained sequence was then compared with the full length *mdm2* cDNA, and clean alignments for these sequences were generated using the CLUSTAL W® (version 1.81) software.

Chapter 3: p53 is Inhibited in *mdm2* SNP309 Homozygous Cells by Association with Mdm2 in Chromatin

3.1 Introduction

Many cancer cells have high levels of the oncogenic Mdm2 protein due to either increased expression (Landers et al., 1994) or amplification of the *mdm2* gene (Momand et al., 1998). Mdm2 over-expression also results when a *Single Nucleotide Polymorphism* (SNP) replacing thymidine with guanine is present at position 309 in the first intron of the *mdm2* gene (Bond et al., 2004). SNP309 was identified by sequencing 300 base pairs from genomic DNA isolated from 50 healthy volunteers and was found at relatively high frequency both in the heterozygous state (T/G, 40%) and in the homozygous state (G/G, 12%).

The presence of *mdm2* SNP309 debilitates the p53 pathway in both Li-Fraumeni family members (a rare autosomal dominant syndrome in which patients are predisposed to cancer due to inherited mutations of 1 of the 2 copies of the *p53* tumor suppressor gene) and in individuals with wild-type p53. SNP309 correlates with an earlier age of tumor onset. Eighty-eight individuals from Li-Fraumeni families having germ line mutations in one allele of *p53* were studied. Sixty-six were diagnosed with at least one cancer. Soft tissue sarcomas (STS) (20 individuals), breast cancer (17 individuals), and osteo-sarcomas (13 individuals) were the most prevalent types of tumors found. For individuals with SNP309, the median age of tumor onset was 18 years old, while in wild-type individuals the median age of tumor onset was 27 years old. Interestingly, the presence of SNP309 also correlated with the occurrence of multiple tumors in those individuals first diagnosed with soft tissue sarcomas. Specifically, two individuals homozygous for SNP309 (G/G) developed a second and a third cancer, and one developed a fourth and a fifth cancer. Of the nine people heterozygous for SNP309

(T/G), seven developed a second cancer, four a third, and one a fourth and a fifth cancer. In contrast, of the nine people wild-type for SNP309 (T/T), only two developed a second cancer and none a third, a fourth, or a fifth cancer. These data indicated an association with the presence of SNP309 and the occurrence of independent subsequent cancers (Bond et al., 2004).

SNP309 acts also upon sporadic cancers. From a group of patients who develop sporadic adult soft tissue sarcomas and had no known hereditary cancer predisposition, those individuals homozygous for SNP309 were diagnosed on average 12 years earlier than those individuals without SNP309.

Analyzing the molecular pathways in which SNP309 leads to an accelerated tumor onset, it has been shown that wild-type 53 protein cannot be stabilized in SNP309 homozygous cells after DNA damaging agents due to Mdm2 over-expression and p53 rapid turn-over (Bond et al., 2004).

In an effort to closely examine the compromised signaling from wild-type p53 in cells homozygous for *mdm2* SNP309, DNA damage was used to activate the checkpoint pathways and p53 functional activities were monitored. Extending the time-points of drug treatment, we found that p53 in cell lines homozygous for SNP309 could be significantly stabilized after 6 hours of DNA damage treatment with different drugs. This p53 remained associated with the cellular Mdm2 protein in the nucleus and was not sequestered to the nucleolus. Importantly, this p53 bound to chromatin in conjunction with Mdm2 and was unable to activate transcription of downstream target genes. As discussed herein, we re-addressed the inhibitory actions of Mdm2 upon p53 by showing that a DNA-bound form of p53 is blocked for trans-activation activity in the presence of high levels of chromatin-associated Mdm2.

The cells lines used in this study were:

Table 2: Cells Lines Used in the Study

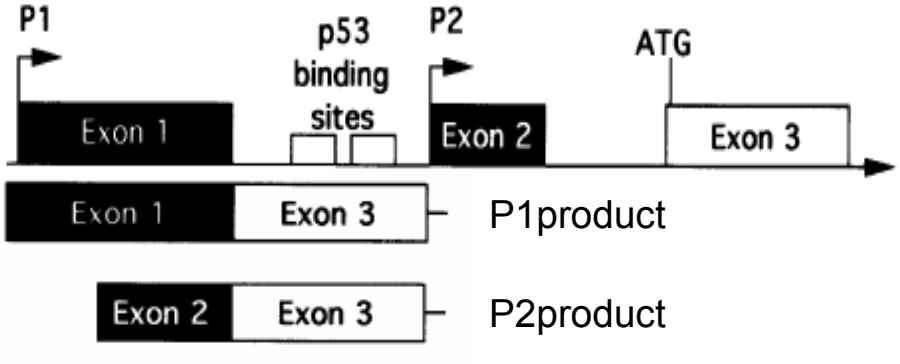
Cell Line Name	***p53* status**	***mdm2* SNP309 status**	**Cell Type**
ML-1	wt	wt (T/T)	chronic myeloid leukemia
K562	null	Heterozygous (T/G)	chronic myeloid leukemia cell line (erythro-leukemic type)
MANCA	wt	Homozygous (G/G)	Burkitt lymphoma
A875	wt	Homozygous (G/G)	melanoma
H460	wt	wt (T/T)	lung carcinoma
H1299	null	heterozygous (T/G)	lung carcinoma
HCT116+/+	wt	wt (T/T)	colon cancer cells
CCF-STTG-1	wt	homozygous (G/G)	astrocytoma
SJSA-1	wt	wt *mdm2* gene amplification	osteosarcoma

3.2 Results

3.2.1 SNP309 Increases the Binding Affinity of Sp1 Transcription Factor to *mdm2* Promoter2

Mdm2 gene transcription is driven by two promoters-P1 and P2. The P1 promoter (located in front of the first exon) is responsible for *mdm2* basal transcription; transcription from P2 promoter (located in the first intron, in front of exon 2) is induced and driven mainly by p53, which binds to the two p53 responsive elements in this area. This "induced" transcription is important for the p53-Mdm2 feed-back loop- it lowers the p53 protein levels after cellular stress has been removed and the cell is ready to continue proliferation. Promoter usage leads to two different transcripts: the P1 product, where exon 2 is spliced out and the P2 product, where exon 1 is not transcribed. The translation-initiation ATG site is located in front of exon 3 and thus it has been thought that the same Mdm2 protein is synthesized, regardless of the promoter that has been used for transcription.

Figure 9: Schematic Illustration of the 5' Portion of the Human mdm2 Gene and the Two Different Transcripts

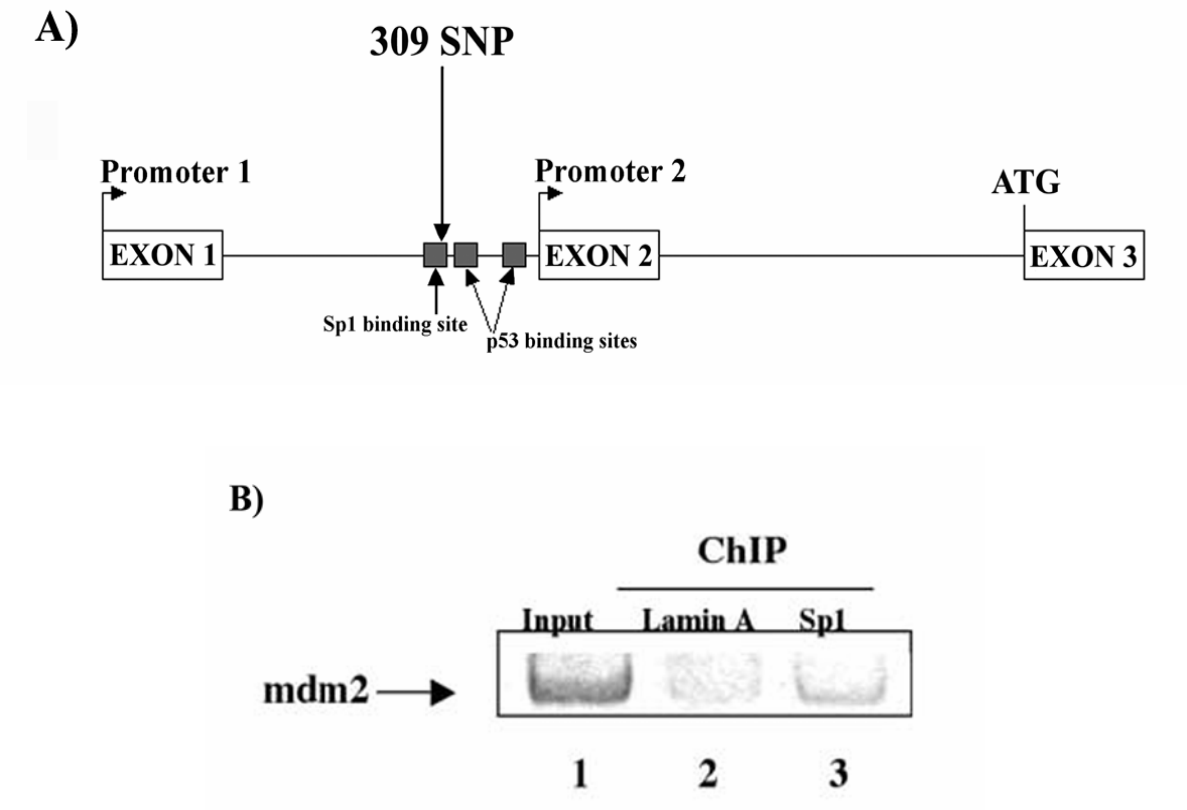


SNP309 replaces a thymidine with a guanine at position 309 in the first intron of the *mdm2* gene, up-stream of the p53 binding sites (Figure 10, panel A). This change creates a more CG rich region in the proximity of the gene's P2 promoter. Analysis of this region of the *mdm2* promoter using a computer algorithm revealed several putative binding sites for the Sp1 ubiquitous transcription factor, known to bind to very rich GC DNA. The presence of SNP309 extends the length of one of the putative Sp1 DNA binding sites, suggesting that SNP309 could increase the binding affinity of Sp1 to this region of the *mdm2* promoter. In fact, electro-mobility shift assays have shown that Sp1 binds to its putative consensus site in the *mdm2* promoter and that SNP309 greatly enhances its binding affinity to this site (Bond et al., 2004).

To verify the presence of Sp1 on the *mdm2* promoter *in vivo*, chromatin immuno-precipitation (ChIP) was performed. Lysates were prepared from growing MANCA cells, homozygous for SNP309 (G/G), and immuno-precipitations were carried out using antibodies against either Sp1 or lamin A. After extensive washing and elution, the DNA was purified and the presence of the *mdm2* promoter was assayed for using PCR (amplification primers encompassed both the Sp1 and p53 binding sites). As seen in Figure 10 panel B, the *mdm2* promoter was detected in the ChIP using the Sp1 antibody (lane 3) but not using the lamin A antibody (lane 2). These data suggest that Sp1 binds this region of the *mdm2* SNP309 promoter *in vivo*. The same experiments were carried out in the ML-1 cell line, which is *mdm2* wild-type (T/T). We also detected Sp1 binding to the *mdm2* promoter in ML-1 cells (data not shown). In the absence of absolute quantification, it was difficult to determine if more binding was present in the SNP309

homozygous versus wild-type cells. These results support the hypothesis that Sp1 binds to *mdm2* SNP309 promoter *in vivo*.

Figure 10: SP1 Binds to mdm2 SNP309 Promoter in Vivo



Panel A): SNP309 is located at position 309 in the first intron of the mdm2 gene, upstream of the p53 binding sites and increases the Sp1 binding affinity to the promoter 2 region in vivo.

Panel B): Sp1 has binding affinity for SNP 309 in vivo- Chromatin immuno-precipitation assay in MANCA cells using Sp1 or Lamin A antibody (mock IP). Lane 1: Input; lane 2: Lamin A ChIP; lane 3: Sp1 ChIP.

3.2.2 Sp1 Binding to SNP309 Region Leads to Increased *mdm2* Transcription

Further investigation into the possible role of Sp1 in the trans-activation of the *mdm2* promoter was carried out in luciferase reporter assays. These experiments suggested that Sp1 can bind to the *mdm2* promoter and activate transcription. Interestingly, the presence of SNP309 in the reporter plasmid consistently showed higher Sp1-induced luciferase expression (~50%) over the presence of wild-type sequence in the reporter plasmid, as the increased binding affinity of Sp1 to SNP309 region would have predicted (Bond et al., 2004).

Quantitative PCR was performed to compare the levels of *mdm2* transcription in cells either *mdm2* wild-type (H460 and ML-1), SNP309 heterozygous (K562) or SNP309 homozygous (MANCA and A875). In addition, higher levels of *mdm2* mRNA were detected in homozygous versus wild-type or heterozygous cells (data not shown).

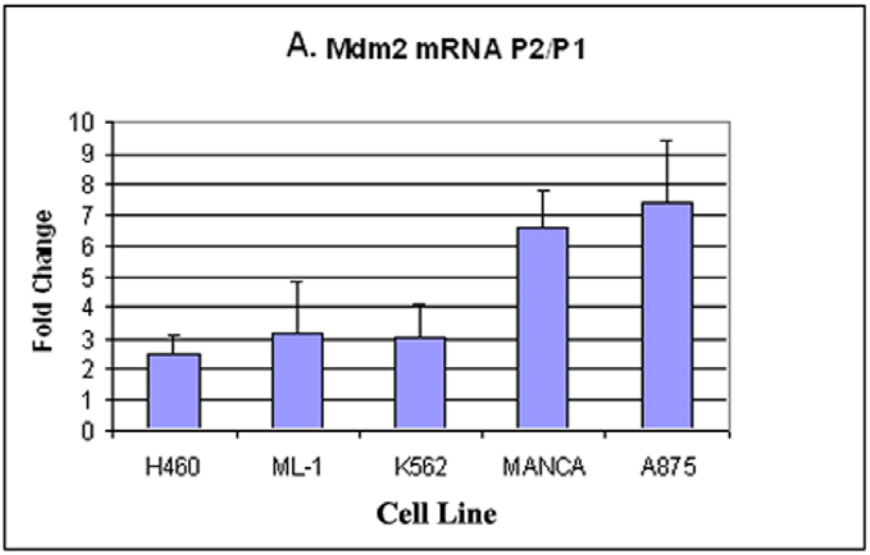
To determine if the *mdm2* transcript was produced from the P2 promoter, we quantified the ratio between the *mdm2* mRNA made from the promoter P2 and the *mdm2* mRNA produced from P1 promoter by real-time quantitative PCR. In the PCR, two different primer sets were used: the first one encompassed the exon 1-exon 3 region and thus detected only the P1 product (P2 product is missing exon 1); the second primer set amplified the exon 5-exon 6 region and thus detected *mdm2* mRNA from both P1 and P2 promoters. The ratio between the two PCR reactions was calculated and is depicted in Figure 11, panel A. Cells wild-type (ML-1, H460) or heterozygous for SNP309 (K562) produced on average 2-3 times more *mdm2* from P2 as compared to P1. In homozygous

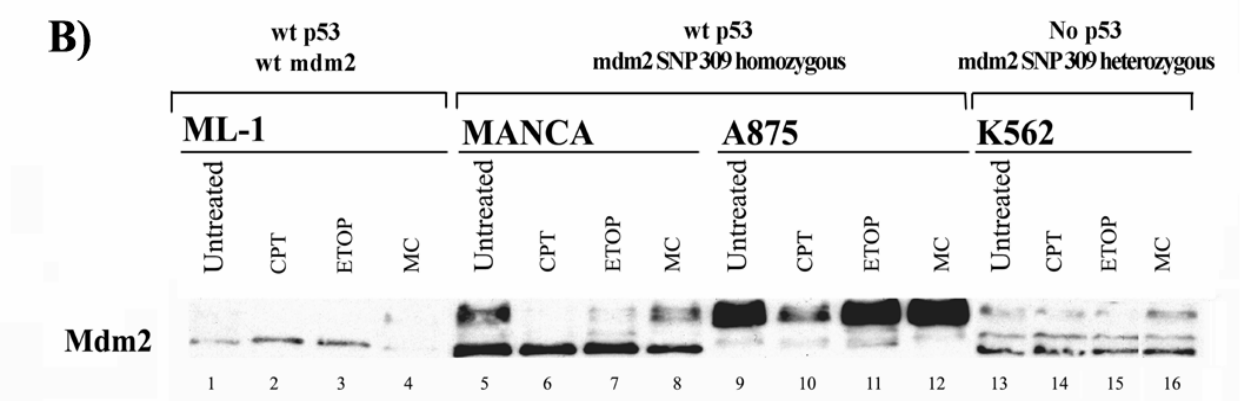
SNP309 cells (MANCA, A875), the *mdm2* P2 product exceeded P1 product 7-9 times on average.

Increased *mdm2* transcription was associated also with increased Mdm2 protein levels in SNP309 homozygous cells. Western-blot analysis demonstrated high amounts of Mdm2 protein in MANCA and A875 (Figure 11 panel B, lanes 5 and 9) as compared to ML-1 or K562 cells (Figure 11 panel B, lanes 1 and 13). Treatment for 6 hours with some of the DNA damaging agents slightly reduced the Mdm2 levels in MANCA and A875 cells. We repeatedly observed this phenomenon in our laboratory, but we do not have an explanation at this time. Mdm2 Western-blot revealed bands of multiple sizes in cells homozygous (MANCA and A875) or heterozygous (K562) for *mdm2* SNP309. These findings suggest that different Mdm2 isoforms could be produced from the SNP309 or Mdm2 protein is post-translationally modified in cells with SNP309.

These results suggest that cells homozygous for SNP309 over-express Mdm2 at both mRNA and protein level due to increased binding affinity for the Sp1 transcription factor. Homozygosity is important for this phenomenon, as cells SNP309 heterozygous (K562) have Mdm2 amounts comparable to wild-type cells.

Figure 11: Homozygosity for mdm2 SNP309 is Associated with Enhanced mdm2 Transcription, Leading to Mdm2 Protein Over-expression





Panel A): Ratio between the mdm2 mRNA made from P2 and mRNA produced from P1. Panel B): Western blot analysis of ML-1, MANCA, A875 and K562 samples. Cells were either left untreated (lanes 1,5,9 and 13) or were treated with 0.5μM camptothecin (lanes 2,6, 10 and 14), 8μM etoposide (lanes 3,7, 11 and 15), or 5μM mitomycin C (lanes 4,8, 12 and 16) for 6 hours and nuclear extracts were then prepared from these samples.

50μg of nuclear protein was subjected to SDS-PAGE (10%). The nitrocellulose membrane was probed with the Mdm2 specific monoclonal antibody SMP14.

3.2.3 Endogenous Over-Expression of Mdm2 via a Naturally Occurring SNP Inhibits Apoptosis Following Chemotherapeutic Drug Treatment

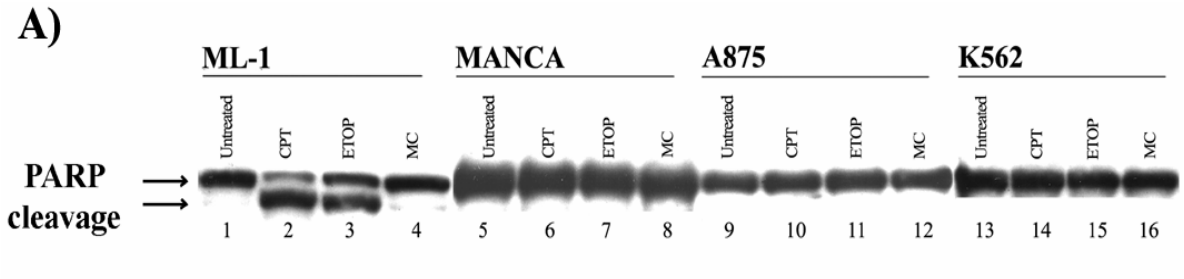
A naturally occurring SNP at position 309 in the promoter 2 region of the *mdm2* gene results in over-expression of Mdm2 protein and debilitation of the wild-type p53 pathway. Inhibition of apoptosis occurs in homozygous *mdm2* SNP309 containing cell lines MANCA and A875 after 1μM and 5μM etoposide treatment (Bond et al., 2004).

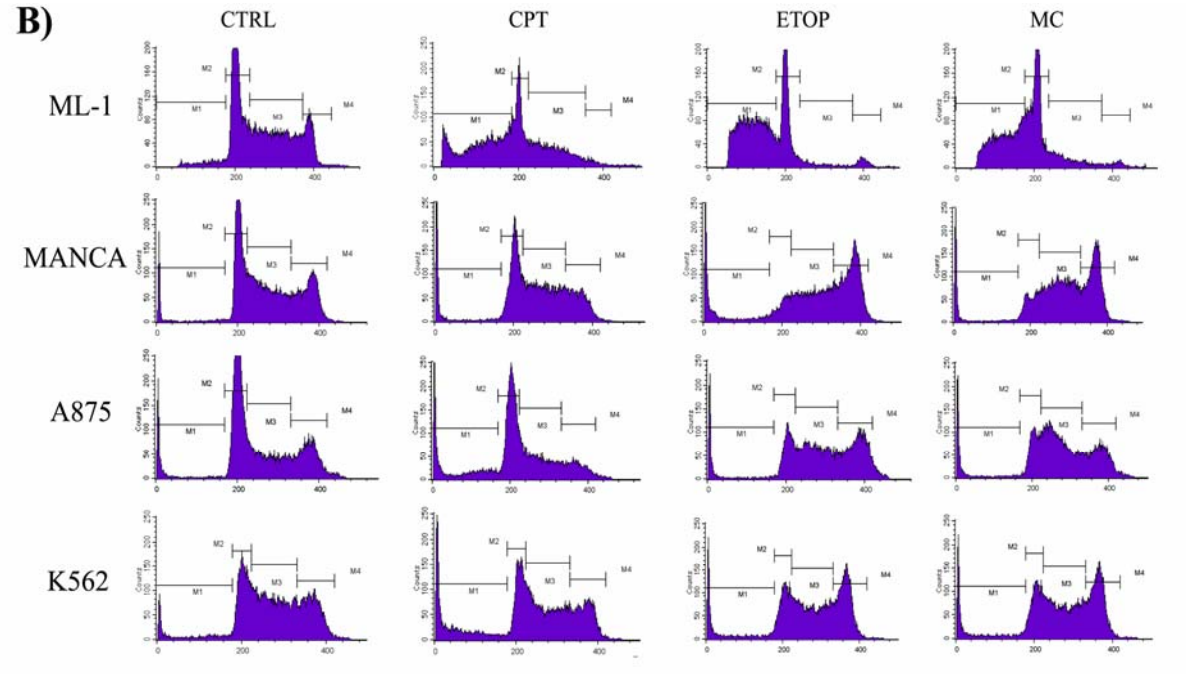
We extended this finding by treating cells with a higher concentration of etoposide (8μM) and also with other DNA damaging agents (camptothecin and mitomycin C). An examination of apoptosis indicators (PARP cleavage and FACS analysis with propidium iodide staining to score sub-G1 DNA content) was used to compare drug-induced apoptosis. Western-blot analysis demonstrated PARP cleavage in ML-1 cells after 6 hours of camptothecin (CPT) and etoposide (ETOP) treatment and slight cleavage of PARP after 6 hours of mitomycin C (MC) treatment (MC has been previously shown to induce apoptosis in ML-1 cells after 24 hours drug treatment (Abbas et al., 2004)). No PARP cleavage was detected in the other cell lines when they were treated with the drugs (Figure 12, panel A). FACS analysis demonstrated an increased sub-G1 population (indicated by the M1 gate) up to 55% only in the ML-1 cells treated with drugs and not in any of the other drug treated cell lines (Figure 12, panels B and C).

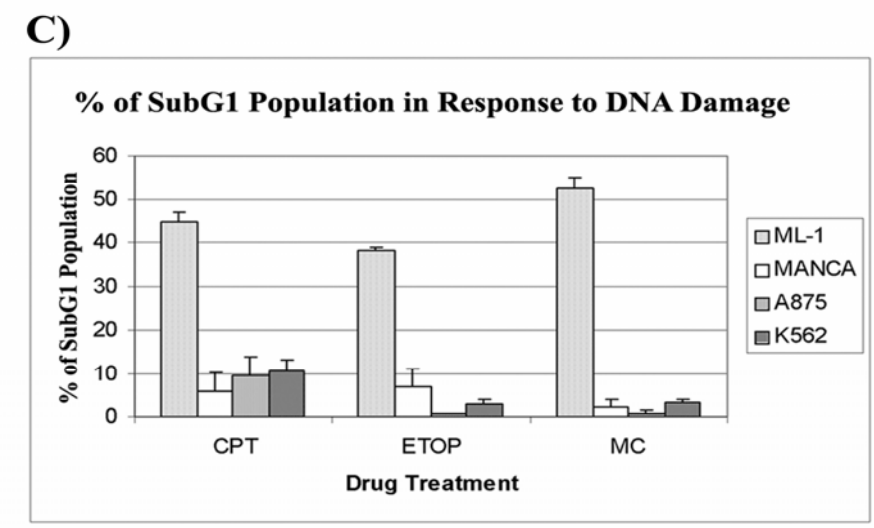
In MANCA and A875 cells no sub-G1 population was detected after DNA damage; instead a G2 arrest was visible after etoposide and mitomycin C treatment. The effect seems to be p53 independent, as it is present also in the p53- null cell line K562. These findings suggest that the wild-type p53 is not functioning correctly in SNP309 homozygous cells.

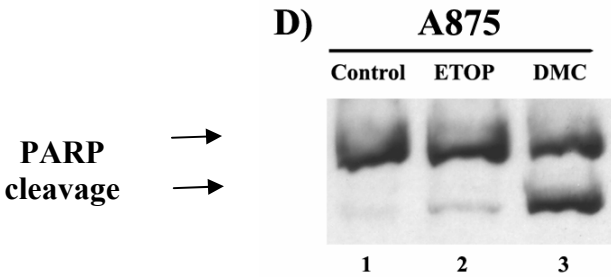
To ensure that the absence of an apoptotic response was not due to a deficient apoptotic pathway, we took advantage of the mitomycin C derivative – 10-decarbamoyl mitomycin C (DMC). This DNA damaging agent has been shown to induce cell death in a p53 independent manner (Abbas et al., 2004). Treatment with 5μM DMC for 24 hours induced PARP cleavage in A875 cells (Figure 12, panel D), suggesting that the cell death pathway was functional in this SNP309 homozygous cell line.

Figure 12: SNP309 Homozygous Cell Lines Do Not Undergo p53-Dependent Apoptosis after DNA Damage




B)
CTRL
CPT
ETOP
MC
ML-1
MANCA
A875
K562


C)
% of SubG1 Population in Response to DNA Damage
% of SubG1 Population
60
50
40
30
20
10
0
CPT
ETOP
MC
Drug Treatment
ML-1
MANCA
A875
K562


D)
A875
Control
ETOP
DMC
PARP
cleavage
1
2
3

Panel A): Western blot analysis of ML-1, MANCA, A875 and K562 samples. Cells were either left untreated (lanes 1,5,9 and 13) or were treated with 0.5μM CPT (lanes 2,6, 10 and 14), 8μM ETOP (lanes 3,7, 11 and 15), or 5μM MC (lanes 4,8, 12 and 16) for 6 hours and nuclear extracts were then prepared from these samples. 50μg of nuclear protein was subjected to SDS-PAGE (10%) and Western blot analysis. The nitrocellulose membrane was probed with the PARP specific antibody.

Panels B) and C): FACS analysis of ML-1, MANCA, A875 and K562 cells. Exponentially growing cells were either left untreated or were treated with 0.5μM camptothecin (CPT), 8μM etoposide (ETOP) or 5μM mitomycin C (MC) for 24 hours. Cells were harvested and fixed in 30% ethanol. 30 minutes prior to FACS analysis cellular DNA was stained with propidium iodide. Cells were analyzed by flow cytometry.

In panel C) the percentages of sub-G1 population after drug treatment were compared.

Panel D): Western blot analysis of A875 samples. Cells were either left untreated (lane 1, or were treated with 8μM ETOP (lane 2), or 5μM DMC (lanes 3) for 24 hours and nuclear extracts were then prepared from these samples. 50μg of nuclear protein was subjected to SDS-PAGE (10%) and Western blot analysis. The nitrocellulose membrane was probed with the PARP specific antibody.

3.2.4 p53 Protein is Stabilized and Phosphorylated at Ser-15 in Cells with *mdm2* SNP309

The previous experiments indicated inhibition of drug induced apoptosis in homozygous *mdm2* SNP309 containing cell lines MANCA and A875. Both cell lines over- express Mdm2 at the protein and RNA levels, which results in unstable p53 after 3

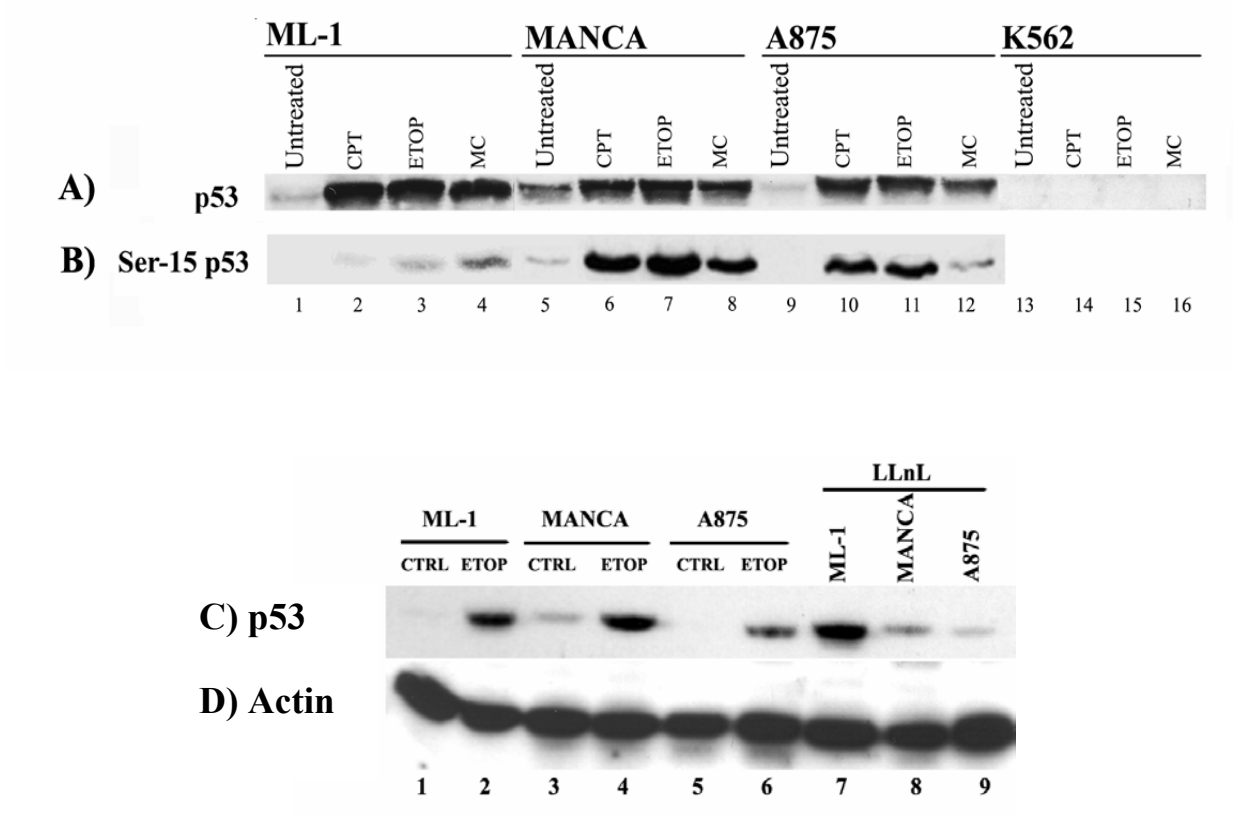
hours of etoposide drug treatment (Bond et al., 2004). We previously documented induced p53 stabilization and subsequent apoptosis in ML-1 cells treated for 6 hours with camptothecin, etoposide or mitomycin C and no apoptosis induction in the p53 deficient human cell line K562 treated with the same drugs (Abbas et al., 2002). To closely examine the compromised molecular signaling in SNP309 homozygous cells, we extended the treatment with DNA damaging agents for 6 hours. We analyzed by Western-blot the p53 protein levels in ML-1, MANCA and A875 cell lines after 6 hours of drug treatment, with expression of the proteins in the documented wild-type p53 expressing cell line ML-1 (which is homozygous wild-type for *mdm2*). Cells were treated with chemotherapeutic DNA damaging drugs or were left untreated and extracts were analyzed by SDS-PAGE (Figure 13, panel A). The drugs CPT, ETOP and MC increased the level of p53 in ML-1 cells as well as in MANCA and A875 cells (Figure 13, panel A, lanes 1-12), while no p53 was expressed in the p53 negative control cell line K562 (Figure 13, panel A, lanes 13-16). The p53 in MANCA and A875 is wild-type, as was seen by DNA sequence analysis of p53 exons 1- 11 (Arva et al., 2005). These results suggest that wild-type p53 protein could be stabilized in SNP309 homozygous cells at later time-points, without massive degradation by the over-expressed Mdm2 protein.

To further explore the p53 degradation pathway in SNP309 homozygous cells, we treated cells with the proteasomal inhibitor LLnL. This treatment produced an evident increase in p53 levels in ML-1 cells (Figure 13, panel C, compare lanes 1 and 7) but did not lead to significant stabilization of p53 in MANCA and A875 (Figure 13, panel C, compare lane 3 versus 8 and lane 5 versus lane 9). These data suggest that wild-type p53 is not excessively degraded by over-expressed Mdm2 in SNP309 cells. In fact almost no

p53 increase was detected after LLnL in SNP309 homozygous cells. This was a striking finding that indicates that other p53 degradation pathways are in place in these cells.

The signal transduction pathway towards p53 involves a critical phosphorylation event at Serine-15 (Ser-15) which helps not only to stabilize the p53 but also to activate the protein's transcriptional activity (Dumaz and Meek, 1999). To examine kinase signaling to the p53 protein in the cell lines examined, Western-blot analysis with antibody specifically recognizing Ser-15 phosphorylated p53 was carried out. Phosphorylation of p53 at Ser-15 in the MANCA and A875 cells was reproducibly detected (Figure 13, panel B). We examined the ability of the p53 protein to be phosphorylated in MANCA and A875 cells after 6 hours of chemotherapeutic treatment as we knew p53 activation in ML-1 cells occurred at this time point (Abbas et al., 2002). Interestingly, we observed significant p53 phosphorylation in cell lines homozygous for the *mdm2* SNP309 after CPT, ETOP and MC treatment (Figure 13, panel B, lanes 5-12). In response to drug treatments the level of p53 phosphorylation in the ML-1 cell line increased as previously described (Abbas et al., 2002) and MANCA and A875 cells demonstrated p53 phosphorylation greater than ML-1 cells (Figure 13, panel B, compare 1-4 to lanes 5-12). The p53 stabilization was not greater in MANCA and A875 cells, as shown in panel A. We reproducibly noted that the level of p53 protein in MANCA cells before drug treatment was high relative to other cell lines examined. We do not have an explanation for this increased basal p53 in MANCA cells at this time.

Figure 13: p53 is Stabilized After DNA Damage and the Kinase Signaling Cascade to p53 is Intact in mdm2 SNP309 Homozygous Cells



Panel A): Western blot analysis of ML-1, MANCA, A875 and K562 samples. Cells were either left untreated (lanes 1,5,9 and 13) or were treated with 0.5μM CPT (lanes 2,6,10 and 14), 8μM ETOP (lanes 3,7, 11 and 15), or 5μM MC (lanes 4,8,12 and 16) for 6 hours and nuclear extracts were then prepared from these samples. 50μg of nuclear protein was subjected to SDS-PAGE (10%) and Western analysis. The nitrocellulose membranes were probed with A) total p53 antibody.

Panel B): the p53 Serine-15 phospho-specific antibody

Panel C): Western-blot analysis of ML-1, MANCA, A875 samples. Cells were either left untreated (lanes 1,3 and 5) or were treated with 8μM ETOP (lanes 2, 4 and 6) or with

20μM LLnL (lanes 7, 8 and 9) for 6 hours and nuclear extracts were then prepared from these samples. 50μg of nuclear protein was subjected to SDS-PAGE (10%) and Western analysis. The nitrocellulose membranes were probed with C) total p53 antibody and Panel D): anti-Actin.

3.2.5 p53 is Localized into Nucleoplasm in Both *mdm2* Wild-type and SNP309 Homozygous Cells

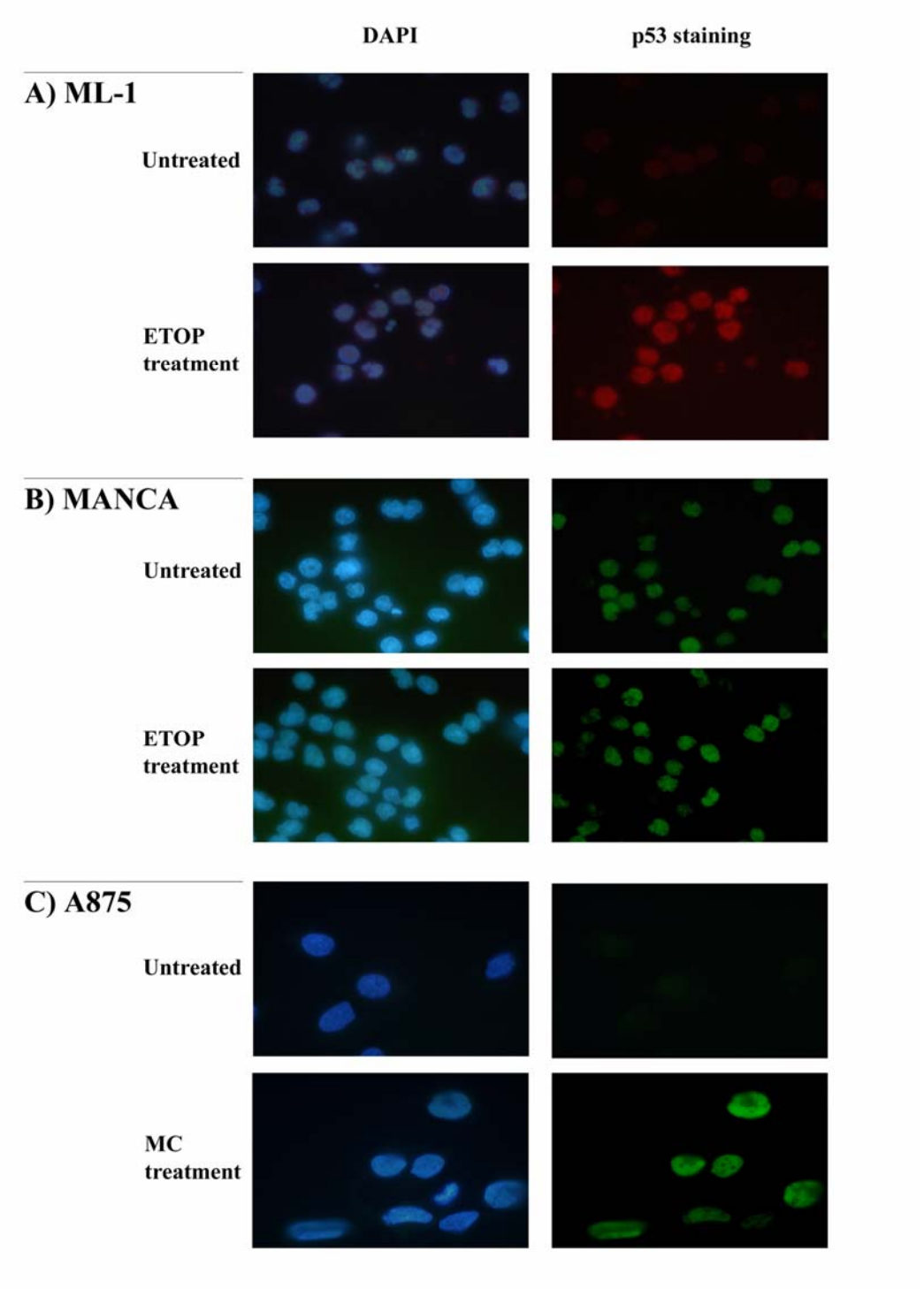
The regulation of p53 by nuclear transport has only recently been well studied and characterized. Because p53 function depends on its nuclear localization, the nuclear-cytoplasmic transport of p53 is normally tightly regulated. p53 contains three nuclear localization signals (NLS) and one nuclear export signal (NES) in the carboxyl terminus, as well as an additional NES in the trans-activation domain (Shaulsky et al., 1990), (Liang et al., 1998). The precise molecular mechanism by which p53 is exported is still unclear, although the dependence on Mdm2 in export has been determined. Mdm2 regulates p53 nuclear export through its carboxy-terminal NES, and ubiquitination of p53 by Mdm2 is necessary for p53 nuclear export (Tao and Levine, 1999), (Geyer et al., 2000).

p53 inactivation can occur from sequestration of p53 in the cytoplasm. The mechanism by which p53 is sequestered in the cytoplasm of certain tumour cells is unknown, but recent studies have begun to shed light on this subject. Cytoplasmic p53 might be a result of increased export, due to expression of a hyperactive form of Mdm2 (Li et al., 2002). Nuclear exclusion could also result from binding to a cytoplasmic

anchor protein, such as the glucocorticoid receptor or the parkin-like ubiquitin ligase (PARC) (Sengupta et al., 2000), (Nikolaev et al., 2003).

Although we detected p53 in nuclear extracts by Western-blotting, we further explored p53 sub-cellular localization in immuno-fluorescence experiments to detect any p53 cytoplasmic or nucleolar sequestration that might have occurred due to Mdm2 over-expression. We detected p53 localization to the nucleus in both wild-type and SNP309 homozygous cells (Figure 14). Additionally, immuno-fluorescence experiments indicated that the nuclear p53 levels increased after drug treatment of ML-1, MANCA and A875 cells.

Figure 14: p53 Protein is Localized in the Nucleoplasm in Both mdm2 Wild-type and SNP309 Homozygous Cells



Immuno-fluorescence was carried out in A) ML-1, B) MANCA and C) A875 cells. Cells were left untreated or treated with 8μM ETOP (ML-1 and MANCA cells) or 5μM MC (A875 cells) to induce the p53 protein.

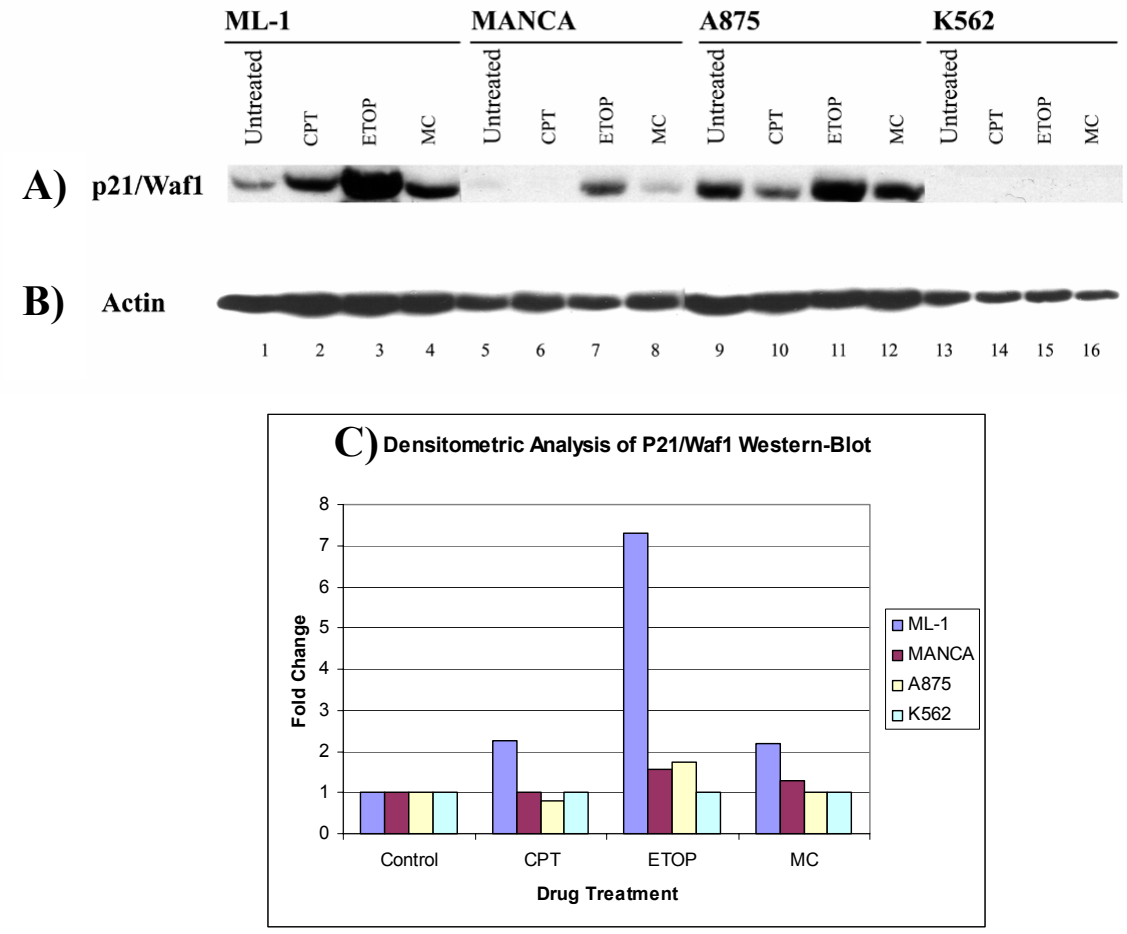
3.2.6 The p53 Protein is Compromised for Activating Downstream Target Genes in the MANCA or A875 Cells

The p53 protein induces the cyclin dependent protein kinase inhibitor p21/Waf1 (el-Deiry et al., 1993) and its increase is part of the cell cycle checkpoint (Waldman et al., 1995). As one indicator of checkpoint activation after DNA damage, we examined the levels of p21/Waf1 protein. Treatment of the ML-1 cells with CPT, ETOP and MC resulted in an increase in p21/Waf1 protein, while in K562 cells the absence of p53 resulted in no p21/Waf1 increase (Figure 15, panel A, lanes 1-4 and lanes 13-16). p21/Waf1 protein levels in MANCA and A875 cells showed great variation with severely attenuated DNA damage induction by CPT, ETOP and MC treatment. Etoposide was the most efficient drug to induce p21/Waf1 protein in MANCA and A875. However, densitometric analysis revealed seven fold induction in the amount of p21/Waf1 protein level in ML-1 cell line in response to etoposide and only 2 fold induction in SNP309 homozygous cell lines.

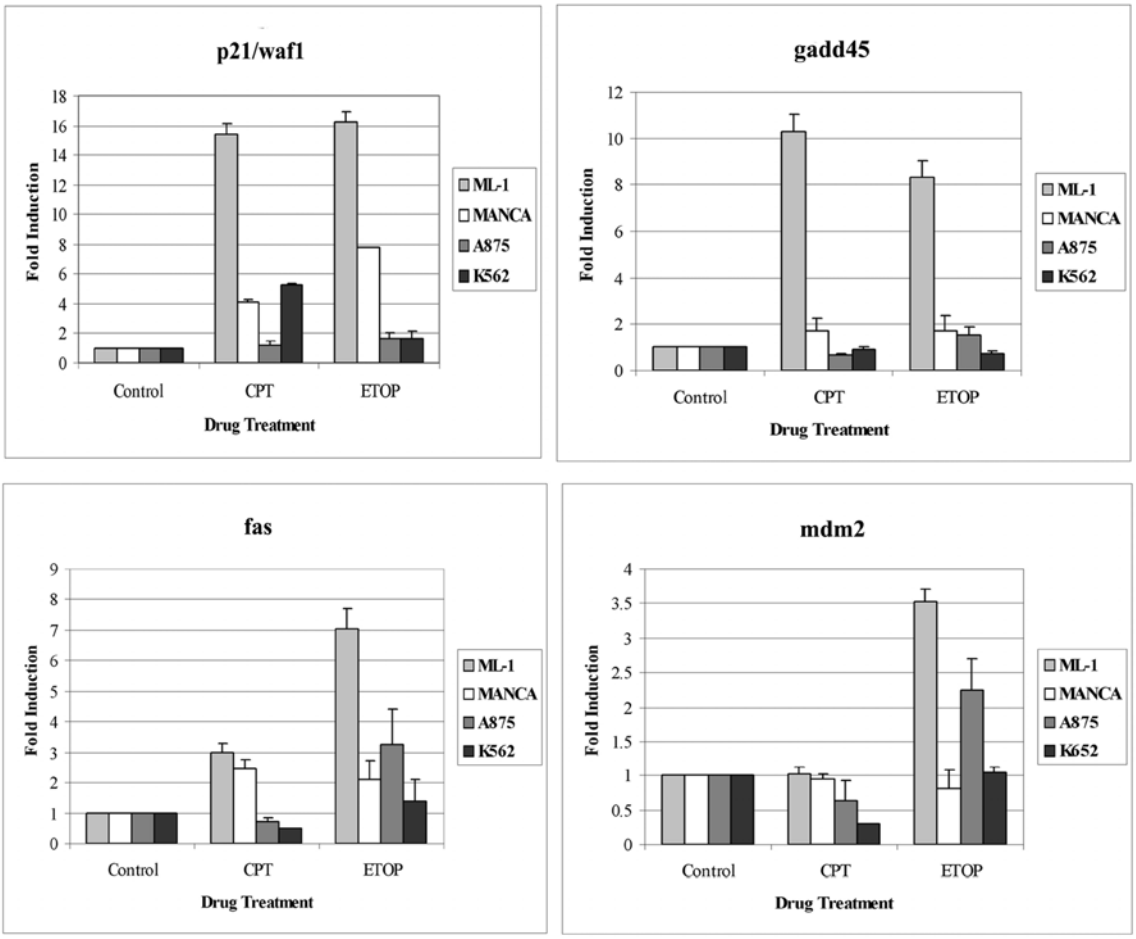
We examined the ability of p53 target genes to be activated after the DNA damage treatments that resulted in p53 stabilization. Examination of p53 down-stream target genes activation by real-time quantitative PCR revealed compromised activation in the MANCA and A875 cells (Figure 15, panel D), even though p53 stabilization was achieved. While the stabilized p53 in ML-1 cells activated *p21/waf1*, *gadd45*, *fas* and

mdm2, we saw compromised activation of these genes in MANCA and A875 cells (Figure 15, panel D). Comparison of activation of these p53 targets in the *mdm2* SNP309 cell lines to the activation in the p53 deficient cell line K562 demonstrated transcription slightly higher indicating some, albeit limited, p53 activity. Additionally, in the absence of p53 in the K562 cells, slight activation of *p21/waf1* transcription occurred. Overall the down-stream target genes activation in MANCA and A875 cells resembled the results seen in the cell line that did not express p53 (K562) more than those seen in the ML-1 cell line (Figure 15, panel D) indicating that the stabilized p53 protein in the two homozygous *mdm2* SNP309 cell lines was not functioning correctly.

Figure 15: The MANCA and A875 p53 Protein is Transcriptionally Compromised



D)



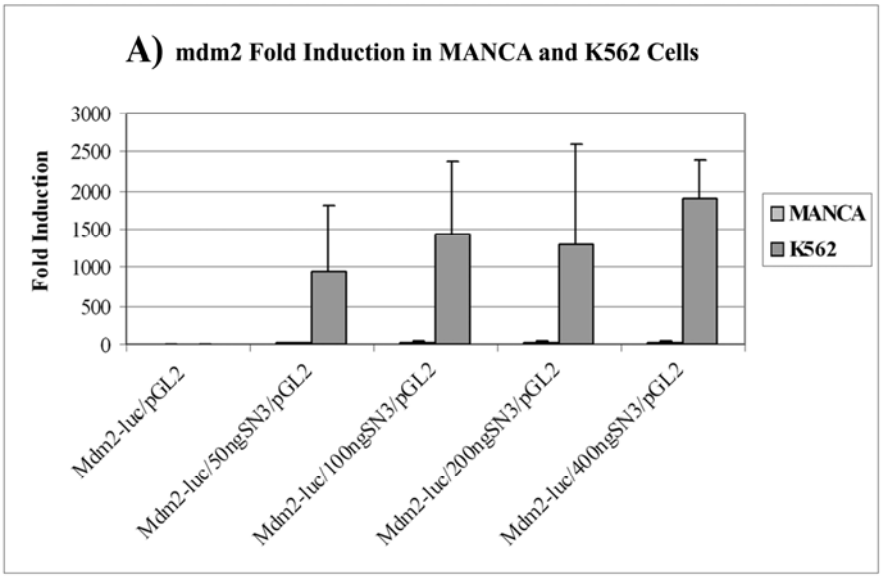
Panel A): Western blot analysis of ML-1, MANCA, A875 and K562 samples. Cells were either left untreated (lanes 1,5,9 and 13) or were treated with 0.5μM CPT (lanes 2,6,10 and 14), 8μM ETOP (lanes 3,7, 11 and 15), or 5μM MC (lanes 4,8,12 and 16) for 6 hours and nuclear extracts were then prepared from these samples. 50μg of nuclear protein was subjected to SDS-PAGE (10%) and Western analysis. The nitrocellulose membranes were probed with: panel A): Waf1 and panel B): Actin antibody. Panel C): Densitometric analysis of western-blot from panel A).

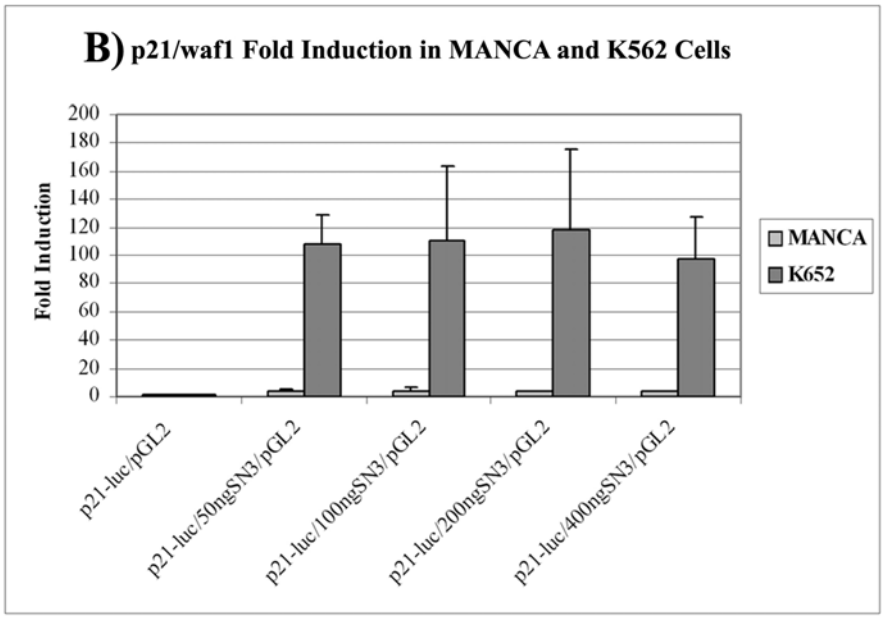
Panel D): Quantitative PCR was used to detect fold induction of the p21/waf1, gadd45, fas and mdm2 transcripts in ML-1, MANCA, A875 and K562 cells after DNA damage.

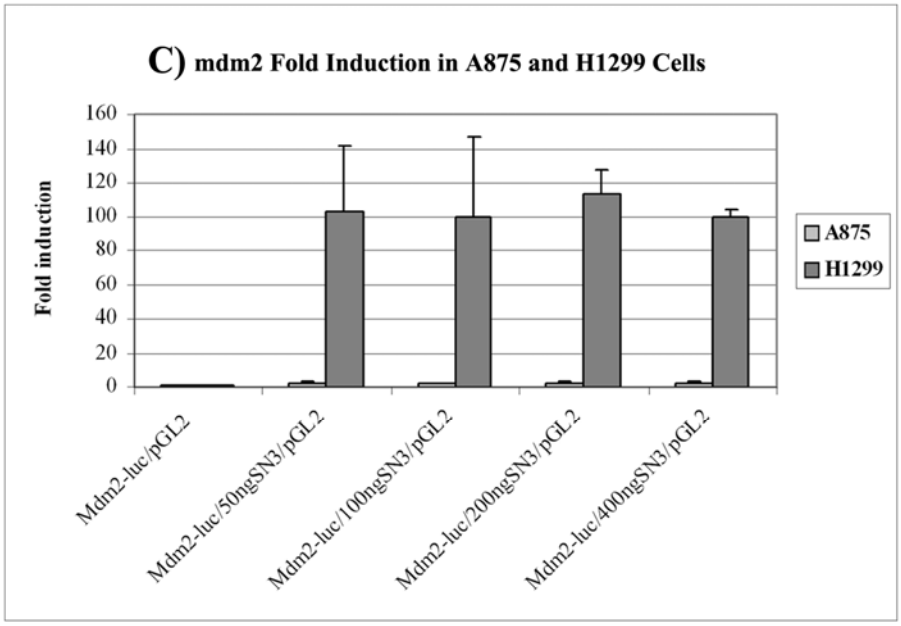
Cells were either left untreated or were treated with 0.5μM CPT or 8μM ETOP for 3 hours. RNA was extracted from samples and used in RT-PCR and then real time quantitative PCR. All results were normalized to untreated samples and glyceraldehyde-3-phosphate dehydrogenase values. Standard deviations represent results from two independent experiments.

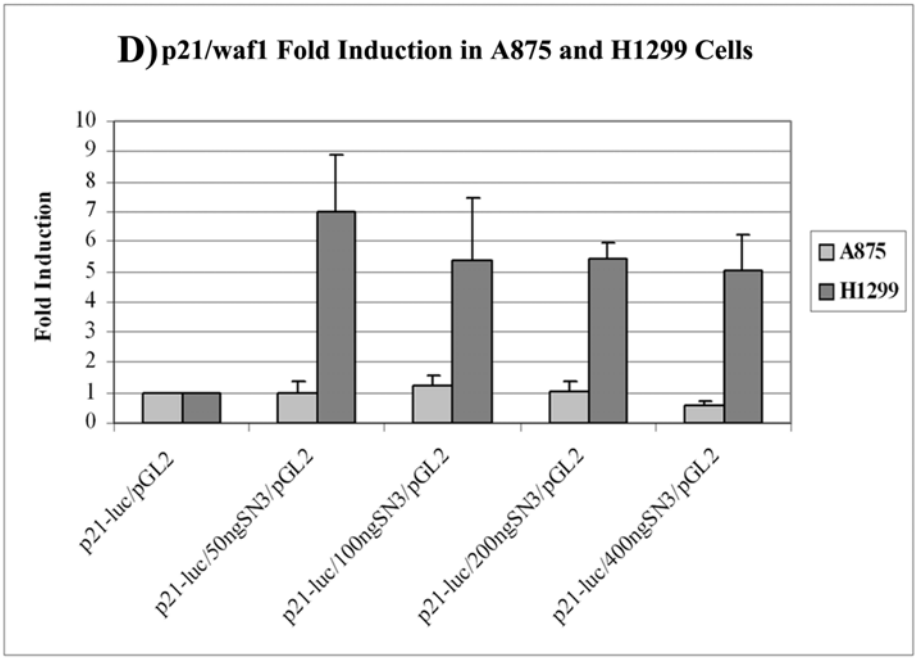
To closely examine the ability of wild-type p53 to be inhibited in the SNP309 cell lines, we asked if exogenously introduced p53 was capable of activating transcription from constructs with either wild-type *mdm2* or *p21/waf1* p53 responsive elements in the MANCA and A875 cells. Transient transfection experiments were carried out with p53 protein provided from the p53 expressing wild-type plasmid SN3 (a generous gift from Bert Vogelstein). Both reporters were activated by the exogenously introduced p53 in the K562 and H1299 cells, neither of which is homozygous for *mdm2* SNP309. These two cell lines do not express endogenous p53. The H1299 adherent cells were compared to A875 while the K562 suspension cells were compared to MANCA. Activation from both reporter constructs occurred in the presence of p53 plasmid in the H1299 and K562 cell lines (Figure 16, panels A-D), while severely compromised activation was evident in both MANCA and A875 cell lines (Figure 16, panels A-D). Western blot analysis indicated that this compromised activation occurred in the presence of a significant increase in exogenous p53 expression in the transient transfected cells (Figure 16, panels E and G). The ability of SNP309 homozygous cells to express high levels of p53 after transient transfection, demonstrate again that p53 is not excessively degradated by over-expressed Mdm2 in these cell lines. Our results point toward the presence of a *trans*-acting inhibitor of the p53 function.

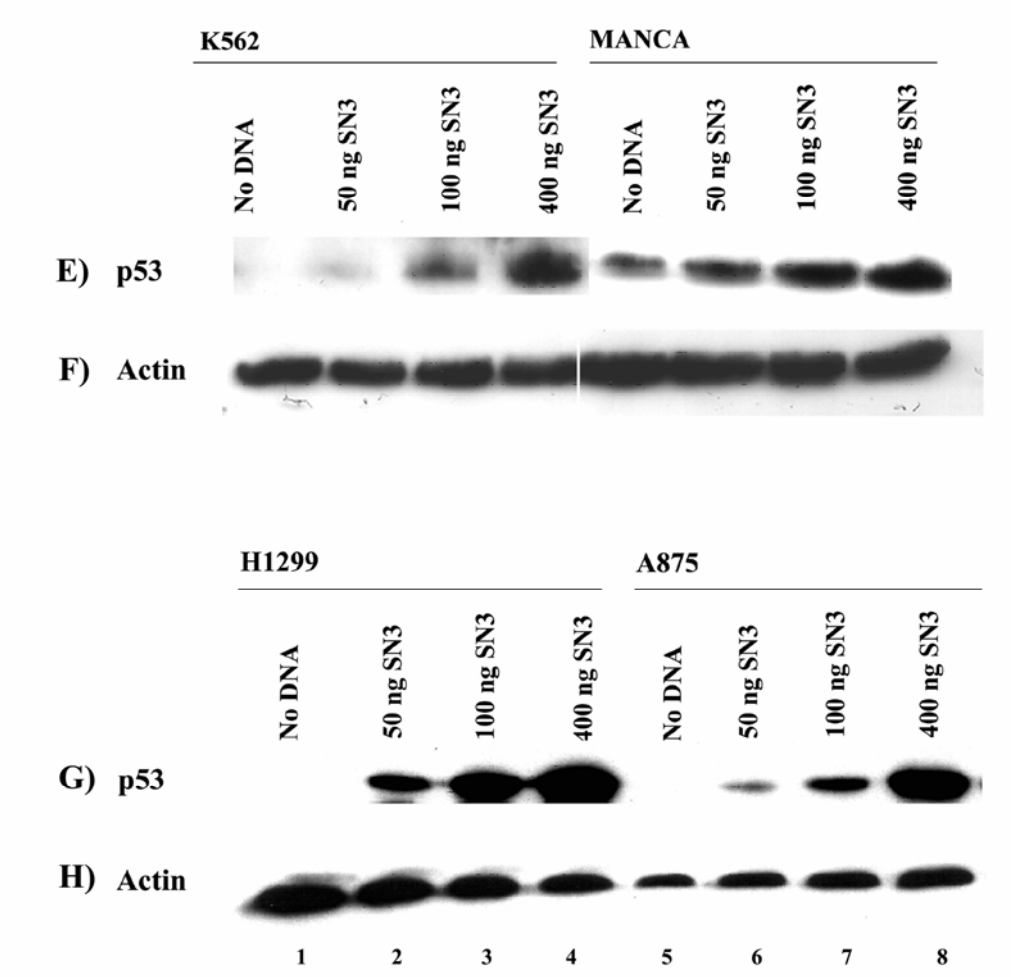
Figure 16: The p53 Protein Provided "in trans" is Transcriptionally Inactive in the mdm2 SNP309 Homozygous Cells






C) mdm2 Fold Induction in A875 and H1299 Cells
Fold induction
160
140
120
100
80
60
40
20
0
A875
H1299
Mdm2-luc/pGL2
Mdm2-luc/50ngSN3/pGL2
Mdm2-luc/100ngSN3/pGL2
Mdm2-luc/200ngSN3/pGL2
Mdm2-luc/400ngSN3/pGL2


D) p21/waf1 Fold Induction in A875 and H1299 Cells
Fold Induction
10
9
8
7
6
5
4
3
2
1
0
A875
H1299
p21-luc/pGL2
p21-luc/50ngSN3/pGL2
p21-luc/100ngSN3/pGL2
p21-luc/200ngSN3/pGL2
p21-luc/400ngSN3/pGL2



Panels A-D): Transient transfection in K562 and MANCA cell lines (A and B) or H1299 and A875 cells (C and D) was carried out with increasing amounts (50 ng to 400 ng) of a plasmid for expression of the wild-type p53 cDNA (SN3) and 2 µg of the plasmid containing the human mdm2 (A and C) or p21/waf1 (B and D) p53 binding site adjacent to a luciferase reporter. The amount of DNA co-transfected in each sample was normalized using a carrier plasmid, pGL2. Fold induction in luciferase activity was measured and normalized to total protein concentration. Results are representative of three independent experiments. Panels E-H): Western blot analysis was performed to assess the transfection efficiency and p53 expression in MANCA, K562, H1299 and A875

cells. Whole cell protein extract was prepared from the co-transfected cells and 50μg of extract was subjected to SDS-PAGE (10%) and Western blot analysis. The nitrocellulose membrane was probed with E and G) a mixture of the p53-specific monoclonal antibodies (240, 421, 1801) or F and H) anti-Actin. Lanes 1 and 5 represent non-transfected samples; lanes 2 and 6 are protein extracts from 50ng SN3 transfection, lanes 3 and 7 from 100ng SN3 transfection and lanes 4 and 8 from 400ng SN3 transfection.

3.2.7 Stabilized p53 in MANCA and A875 Cells Binds to DNA

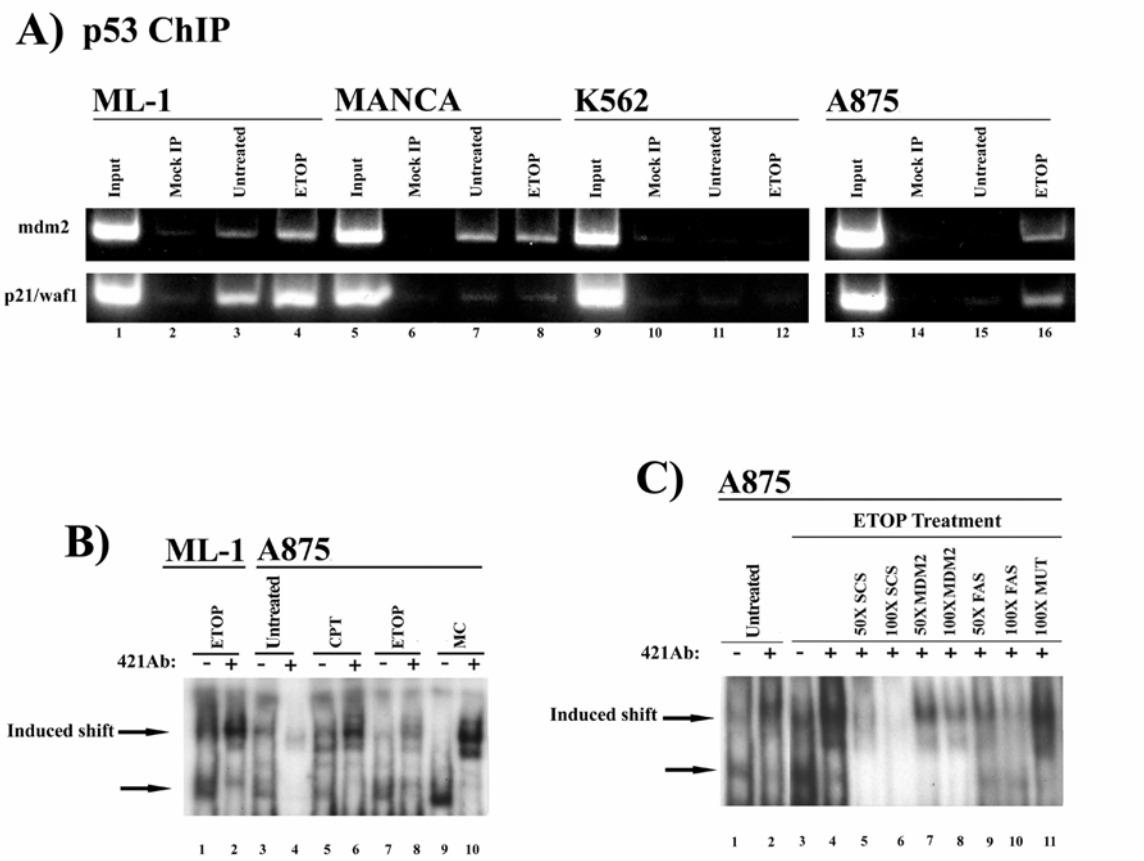
Inhibition of the DNA binding ability is one way in which Mdm2 inhibits p53 (Zauberman et al., 1993). Immuno-fluorescence images demonstrated that p53 in SNP309 cell lines was present in the nucleoplasm (Figure 14) and therefore we examined if this p53 was associated with the DNA. To determine if the p53 protein that was compromised for activating transcription was able to bind to p53 responsive elements in chromatin, we compared p53 chromatin immuno-precipitation (ChIP) in ML-1, MANCA, K562 and A875 cells (Figure 17, panel A). Increased *mdm2* and *p21/waf1* chromatin was immuno-precipitated with a p53 specific antibody in ML-1 cells treated with ETOP (Figure 17, panel A, lane 4) and some p53 localized on the chromatin of these genes prior to DNA damage (Figure 17, panel A, lane 3). Studies using chromatin immuno-precipitation have found p53 bound to its responsive elements prior to gene activation. Furthermore, increasing levels of p53 localize to these specific DNA binding sites following activation by stress (Espinosa et al., 2003). A DNA damage signal however is not required for p53 to associate with DNA (Espinosa and Emerson, 2001). We also carried out immuno-precipitation experiments in MANCA, A875 and K562 cells.

Comparison of the input chromatin and Mock immuno-precipitation for all samples demonstrated that the sets were normalized and gave barely detectable background (Figure 17, panel A, lanes 1, 2, 5, 6, 9, 10, 13 and 14). In MANCA cells p53 protein was associated with the *mdm2* gene with no evident increase after DNA damage (Figure 17, panel A, lanes 7 and 8, *mdm2* gene). Barely detectable *p21/waf1* chromatin was immuno-precipiated in MANCA cells using an antibody recognizing the N-terminus of p53 and was only evident by phosphor-imager analysis (Figure 17, panel A, lanes 7 and 8, *p21/waf1* gene and data not shown). It appears that this epitope was masked in the MANCA cell chromatin-associated p53, as an antibody to phosphorylated p53 indicated the association of the protein with *p21/waf1* chromatin (Figure 19, panel B, compare lanes 7 and 8, *p21/waf1* gene). Increased *mdm2* and *p21/waf1* chromatin was immuno-precipitated with the p53 specific antibody from A875 cells after ETOP treatment (Figure 17, panel A, compare lanes 15 and 16). In the p53 null cell line K562, p53 antibody did not precipitate any chromatin containing p53 responsive elements before or after ETOP treatment (Figure 17, panel A, lanes 11 and 12).

We also examined p53 DNA binding ability using an electrophoretic mobility shift assay. Interestingly, while the p53 binding activity was activated in the ML-1 cells after drug treatment, p53 binding activity was present in the MANCA nuclear extract prior to drug treatment and no further increase was evident in EMSA after drug treatment (data not shown). These data correlate with the Western blot (Figure 13, panel A) and immuno-fluorescence data showing high basal levels of p53 (Figure 14), as well as with ChIP results (Figure 17, panel A). We also compared the ability of A875 p53 protein in nuclear extracts to bind to the super-consensus sequence (SCS) by using the same assay (EMSA). The p53 DNA binding activity in the presence of the p53 specific antibody 421

is known to be activated in EMSA (Hupp et al., 1992) and such activated binding was assayed using nuclear extracts from CPT, ETOP and MC treated cells. In the presence of the p53 antibody 421, we observed an induced p53 shift in the A875 nuclear extract samples (Figure 17, panel B, compare odd lanes which contain no 421 antibody to even lanes where the 421 antibody is present and Figure 17, panel C, compare lanes 1-2 and 3-4). ML-1 nuclear extract (after ETOP treatment) was used as a positive control in these experiments (Figure 17, panel B, lanes 1-2). The p53 binding activity in A875 was activated after DNA damage and this binding was also specific as demonstrated by competition with oligonucleotides containing p53 responsive element sequences (50 and 100 fold unlabelled SCS or *mdm2*, *fas oligos*) but not with a mutant oligonucleotide sequence (Figure 17, panel C, compare lane 4 to lanes 5-11). This DNA binding specificity for p53 in EMSA was also seen previously for MANCA cell p53.

Figure 17: DNA Damage-Induced p53 Protein Has DNA Binding Ability in MANCA and A875 Cells



Panel A): p53 chromatin immuno-precipitation in ML-1 (lanes 1-4), MANCA (lanes 5-8), K562 (lanes 9-12) and A875 cells (lanes 13-16). Cells were either left untreated (lanes 3, 7, 11 and 15) or treated with 8μM ETOP (lanes 4, 8, 12 and 16) for 3 hours. PCR was carried out using primers specific for the p53 responsive elements in the mdm2 and p21/waf1 genes. In Mock IP samples (lanes 2, 6, 10 and 14) no antibody was added to the IP reaction. One fifth of the input DNA from each sample was also amplified and designated as Input (lanes 1, 5, 9 and 13).

Panel B): Electrophoretic mobility shift assay (EMSA) with the ^{32}P labeled super consensus sequence (SCS) containing three p53 binding sites and 10μg of nuclear extracts from ML-1 ETOP treated sample and A875 untreated sample (lanes 3-4) or samples treated with 0.5μM CPT (lanes 5-6), 8μM ETOP (lanes 7-8), or 5μM MC (lanes 9-10) for 6 hours. Reactions contained the p53-specific monoclonal antibody 421 where indicated.

Panel C): Electrophoretic mobility shift assay (EMSA) with the ^{32}P labeled super consensus sequence (SCS) containing three p53 binding sites and 10μg of nuclear extracts from A875 untreated samples (lanes 1-2) or treated with 8μM ETOP (lanes 3-11). Competition of the 421-induced gel shift from ETOP treated A875 nuclear samples was carried out using 50X or 100X fold excess unlabeled oligonucleotide corresponding to the unlabelled SCS (lanes 5-6), mdm2 (lanes 7-8) or fas (lanes 9-10) p53-binding sites, as well as 100X non-specific mutant oligonucleotide (lane 11).

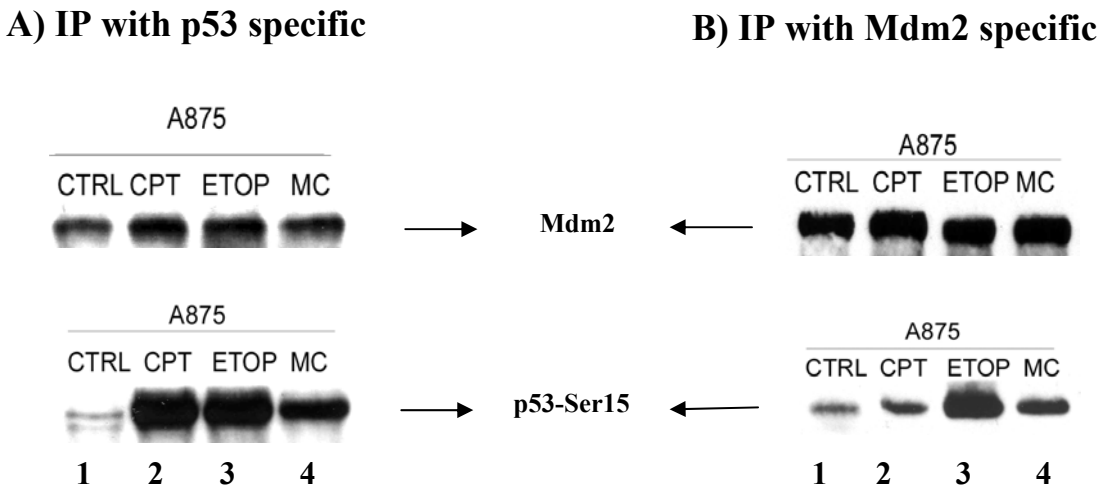
3.2.8 p53 Forms a Stable Complex with Mdm2 in SNP309 Homozygous Cells

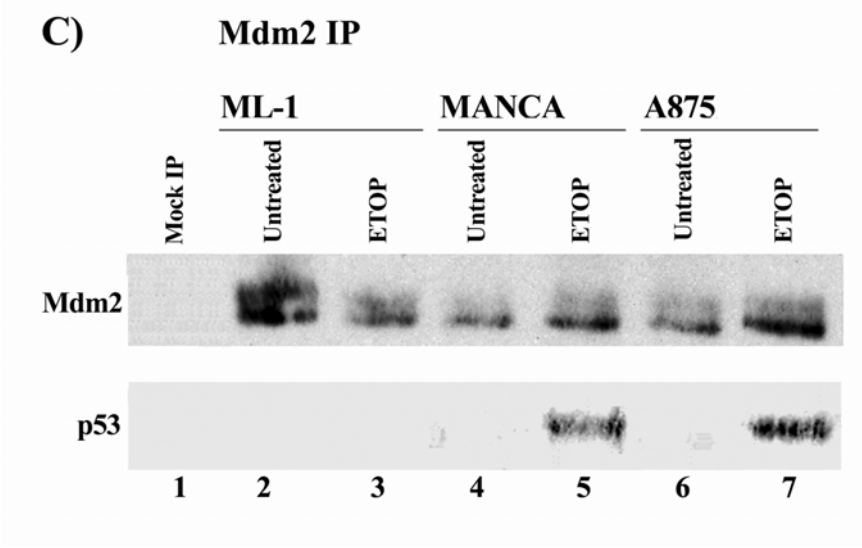
Our data so far point toward a model in which wild-type p53 protein can be stabilized in SNP309 homozygous cells and binds to DNA but such p53 is unable to activate transcription. We began a search for inhibitory factors that might hinder p53 transcriptional activity. Mdm2 has been shown in numerous studies to inhibit p53 trans-activation function. We examined the soluble p53-Mdm2 complex present in the nucleus of the tested cell lines before and after DNA damage. Previous work in our laboratory demonstrated the presence of a stable p53-Mdm2 complex in MANCA cells. We

extended the co-immuno-precipitation studies in A875 cells. Co-immuno-precipitation experiments were carried out using nuclear extracts derived from the cells treated with the DNA damaging agents to confirm that Mdm2 was associated with the stabilized p53 that had attenuated function. A875 nuclear extracts were compared for p53-Mdm2 complex formation using p53 and Mdm2 specific antibodies (421 antibody for p53 and D7 for Mdm2) to pull down the complex and the blot was analyzed for Mdm2 and p53-Ser15 proteins (Figure 18, panels A and B). Untreated and ETOP, CPT or MC treated samples have been analyzed. In the untreated sample very little p53 was pulled-down in the p53 IP (Figure 18, panel A, lane 1) whereas p53 levels increased, as expected, after DNA damage. Mdm2 was also pulled down together with p53 (less in the untreated and MC treated samples, probably due to lower p53 amounts). The reciprocal co-immuno-precipitation with D7 antibody specific for Mdm2 pulled down relatively the same amount of Mdm2 in all samples (Figure 18, panel B). p53 co-immuno-precipitated with Mdm2, with the largest levels in ETOP sample (Figure 18, panel B, lane 3).

We extended the immuno-precipitation experiments using a different Mdm2 antibody, 2A10. This approach ensured the specifity of the p53-Mdm2 complex formation. In ML-1 cells no p53 was detected when Mdm2 was immuno-precipitated, before or after ETOP treatment. However, in MANCA and A875 cells, p53 was pulled down in complex with Mdm2 when cells were treated with ETOP but not before treatment (Figure 18, panel C, lanes 4-7).

Figure 18: p53 is Stably Associated with Mdm2 in SNP309 Homozygous Cells





Panel A): Co-immuno-precipitation using the p53 specific antibody 421. Lane 1 represents untreated sample; lane2: treatment with 0.5μM CPT, lane 3: treatment with 8 μM ETOP and lane 4: treatment with 5μM MC. Mdm2 antibody 2A10. Upper panel was probed with D7 antibodies; lower panel with the p53-Ser15 antibodies.

Panel B): Co-immuno-precipitation using the Mdm2 specific antibody D7. Lane 1 represents untreated sample; lane2: treatment with 0.5μM CPT, lane 3: treatment with 8μM ETOP and lane 4: treatment with 5μM MC. Upper panel was probed with D7 antibodies; lower panel with p53-Ser15 antibodies.

Panel C): Co-immuno-precipitation using the Mdm2 specific antibody 2A10. Lane1: mock IP using MANCA cell extract, no antibody was added to the IP reaction; lanes 2, 4, 6: IP in untreated ML-1, MANCA and A875 samples; lane 3, 5, 7: IP in ETOP treated ML-1, MANCA and A875 samples. Upper panel was probed with Mdm2 specific antibody 2A10, bottom panel was probed with a mix of p53 specific antibodies (421, 1801 and 240).

3.2.9 Increased Mdm2 Protein Binds to Chromatin with p53 Responsive Elements in MANCA and A875 Cells

Although a bimodal mechanism for the inhibition of p53 by Mdm2 has been described, the capacity of Mdm2 to target p53 for degradation often overshadows the capacity of Mdm2 to inhibit p53 transcription activity. Recently, however, it has been seen that Mdm2 protein is present at p53 binding sites in chromatin in the Mdm2 over-expressing cell line SJSA-1 (Minsky and Oren, 2004). This Mdm2-chromatin association was p53-mediated and caused gene silencing due to histone ubiquitination. Our transient transfection experiments pointed towards a *trans*-acting inhibitory factor of the DNA bound p53. Co-immuno-precipitation experiments indicated Mdm2 as a possible p53 inhibitor. We wanted to determine if the stable p53-Mdm2 complex was chromatin-

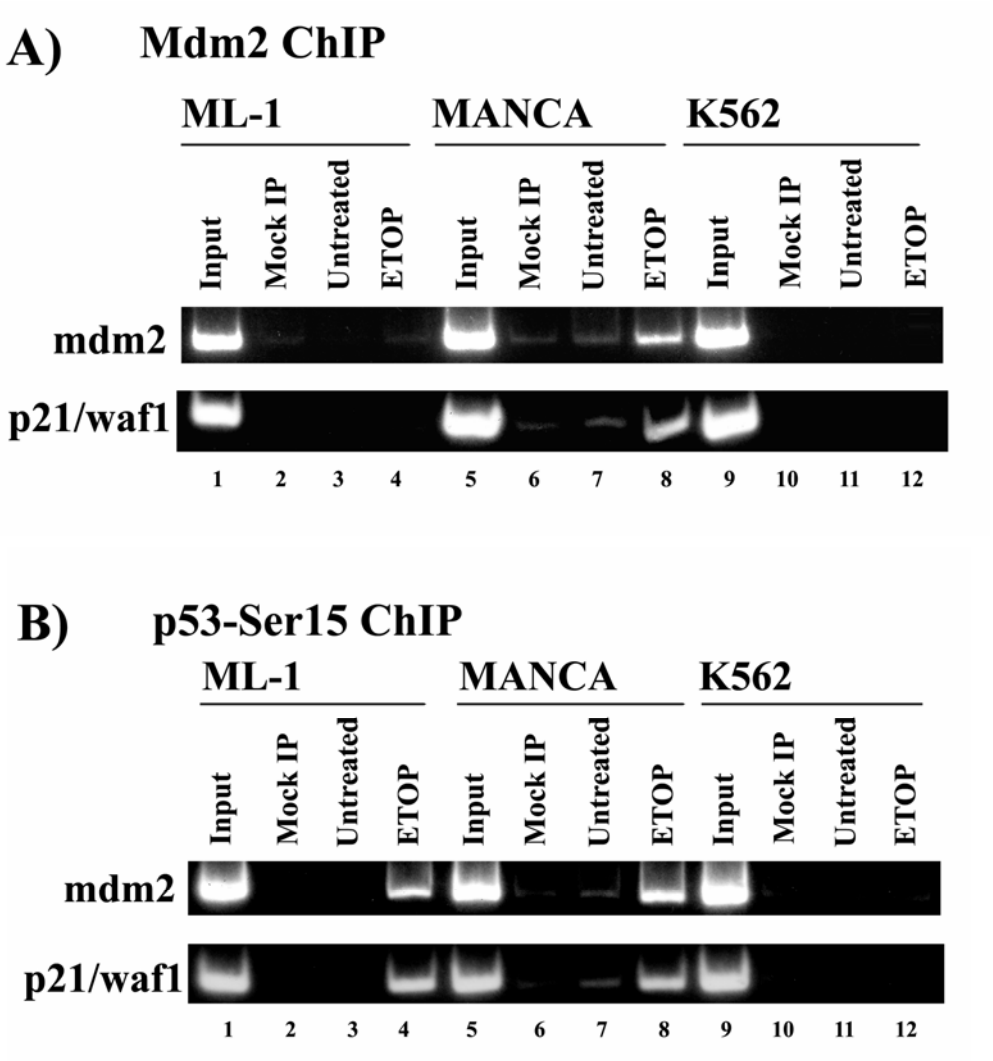
bound as well, given the fact that p53 was demonstrated to retain its DNA binding ability.

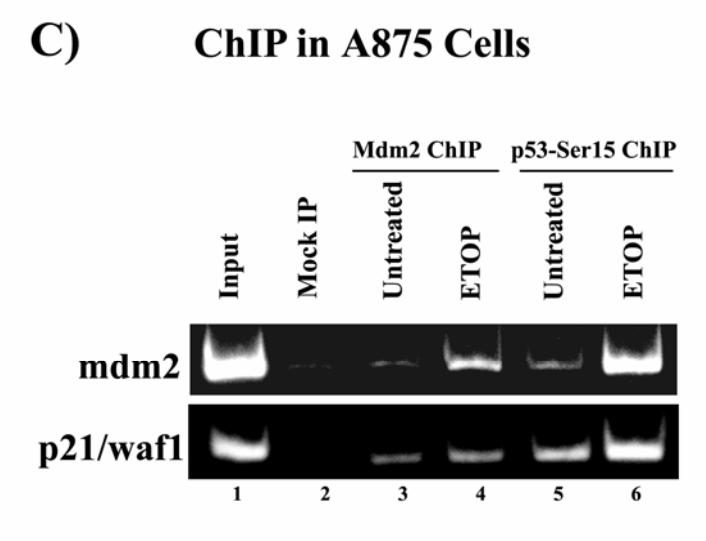
We carried out ChIP studies in cells wild-type or *mdm2* SNP309 homozygous to examine if Mdm2 was associated with the chromatin-bound p53 in MANCA and A875 cells but not in ML-1 cells. In ML-1 cells we did not identify any Mdm2 bound to p53 responsive elements of the *mdm2* or *p21/waf1* genes in both untreated samples or after 3 hours of ETOP treatment (Figure 19, panel A, lanes 3 and 4). We chose the ETOP treatment because it seemed to give the best stabilization of the Mdm2-p53 Ser15 complex. In MANCA and A875 cells there was a small amount of Mdm2 bound to *mdm2* and *p21/waf1* genes chromatin in untreated cells (Figure 19, panel A lane 7 and Figure 19, panel C lane 3) and a striking increase in Mdm2 localized at these sites after ETOP treatment (Figure 19, panel A lane 8 and Figure 19, panel C lane 4). Mdm2 protein was absent at the *mdm2* or *p21/waf1* responsive elements in the p53 null cell line K562 (Figure 19, panel A, lanes 11 and 12). Quantitative PCR using the precipitated *p21/waf1* chromatin showed a 2.5 fold increase above background in MANCA untreated samples and went up to an 8 fold increase in MANCA ETOP treated cells. Very little *p21/waf1* chromatin was immuno-precipitated with Mdm2 protein in ML-1 and K562 before and after DNA damage (Figure 19, panel D; fold increase represents the average of two independent experiments).

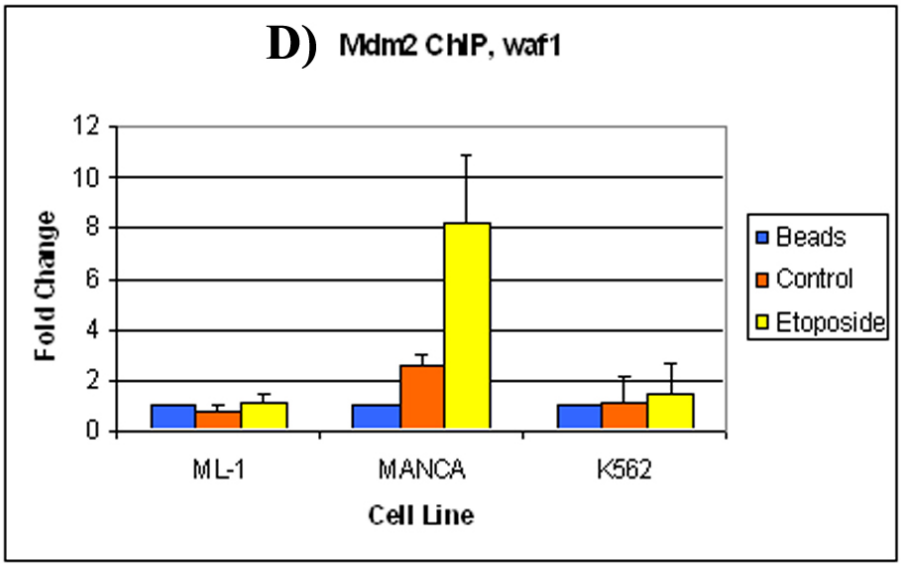
We next examined if the recruitment of Mdm2 to the p53 responsive elements was mediated by the p53 protein. ChIP with p53-Ser 15 phospho-specific antibody showed enhanced binding of p53 protein after DNA damage in ML-1 cells (Figure 19, panel B, compare lanes 3 and 4) and also in MANCA and A875 cells (Figure 19, panel B, compare lanes 7 and 8 and Figure 19, panel C, compare lanes 5 and 6). As expected, p53-

Ser 15 ChIP did not demonstrate any signal in the K562 cell line (Figure 19, panel B, lanes 11 and 12). The ChIP data argue that Mdm2 can localize to p53 responsive elements in the MANCA and A875 cells and Mdm2 localization increases with increased p53 binding.

Figure 19: Increased Mdm2 Protein Binds to Chromatin with p53 Responsive Elements in mdm2 SNP309 Homozygous Cells







Panel A): Mdm2 chromatin immuno-precipitation in ML-1 (lanes 1-4), MANCA (lanes 5-8) and K562 cells (lanes 9-12). Cells were either left untreated (lanes 3, 7 and 11) or treated with 8μM ETOP (lanes 4, 8 and 12) for 3 hours. PCR was carried out using primers specific for the p53 responsive elements in the mdm2 and p21/waf1 genes. In Mock IP samples (lanes 2, 6 and 10) no antibody was added to the IP reaction. One fifth

of the input DNA from each sample was also amplified and designated as Input (lanes 1, 5 and 9).

Panel B): p53-Ser15 chromatin immuno-precipitation in ML-1 (lanes 1-4), MANCA (lanes 5-8) and K562 cells (lanes 9-12). Cells were either left untreated (lanes 3, 7 and 11) or treated with 8μM ETOP (lanes 4, 8 and 12) for 3 hours. PCR was carried out using primers specific for the p53 responsive elements in the mdm2 and p21/waf1 genes. In Mock IP samples (lanes 2, 6 and 10) no antibody was added to the IP reaction. One fifth of the input DNA from each sample was also amplified and designated as Input (lanes 1, 5 and 9).

Panel C): Mdm2 (lanes 3-4) or p53- Ser15 (lanes 5-6) chromatin immuno-precipitation in A875 cells. Cells were either left untreated (lanes 3 and 5) or treated with 8μM ETOP (lanes 4 and 6) for 3 hours. PCR was carried out using primers specific for the p53 responsive elements in the mdm2 and p21/waf1 genes. In Mock IP sample (lane 2) no antibody was added to the IP reaction. One fifth of the input DNA from each sample was also amplified and designated as Input (lane 1).

Panel D): Quantification of Mdm2 ChIP, waf1 gene in ML-1, MANCA and K562 Cells.

3.2.10 Mdm2 Down-Regulation Activates p53

To ensure that the Mdm2 elevated protein levels were required to attenuate the p53 mediated transcriptional activation in cells homozygous for SNP309, we performed *mdm2* small interference RNA (siRNA) experiments. Down-regulation of Mdm2 (Figure 20, panel A, lane 3, Mdm2 Western blot) did not lead to any increase in the p53 protein levels (Figure 20, panel A, lane 3, p53 Western blot). This finding suggests once again

that p53 in not excessively degradated in SNP309 homozygous cells. However, p21/Waf1 protein levels were increased when Mdm2 was down-regulated (Figure 20, panel A, lane 3, p21/Waf1 Western blot). Mdm2 has been shown to be a p21/Waf1 negative regulator independently of p53, by facilitating the interaction of p21/Waf1 with the proteasomal C8-subunit (Zhang et al., 2004). To make certain that the increase in p21/Waf1 protein was due to reactivation of the p53 transcriptional activity, we looked at *p21/waf1* mRNA levels after *mdm2* siRNA treatment. Mdm2 down-regulation led on average to a 3 fold increase in *p21/waf1* mRNA levels (Figure 20, panel B), illustrating reactivation of the p53 transcription factor ability. *mdm2* mRNA were profoundly down-regulated (consistent with the Mdm2 protein levels in Western-blot), demonstrating a very good functionality of the *mdm2* specific siRNA. Transcription of a different p53 dependent reporter construct after *mdm2* siRNA has been reported previously to increase 3-4 folds, consistent with our results (Brady et al., 2005).

We also assessed the induction of other p53 targets (*fas*, *pig3*, *gadd45*) after Mdm2 down-regulation (Figure 20, panel C). Transcription of these genes was elevated 2-5 folds after the addition of *mdm2* siRNA. However, *Gadd45* was not induced since its up-regulation requires a DNA damage signal, which siRNA does not provide (Xiao et al., 2000). When cells were treated with etoposide after *mdm2* down-regulation, further increase in p53 targets was observed (Figure 20, panel C).

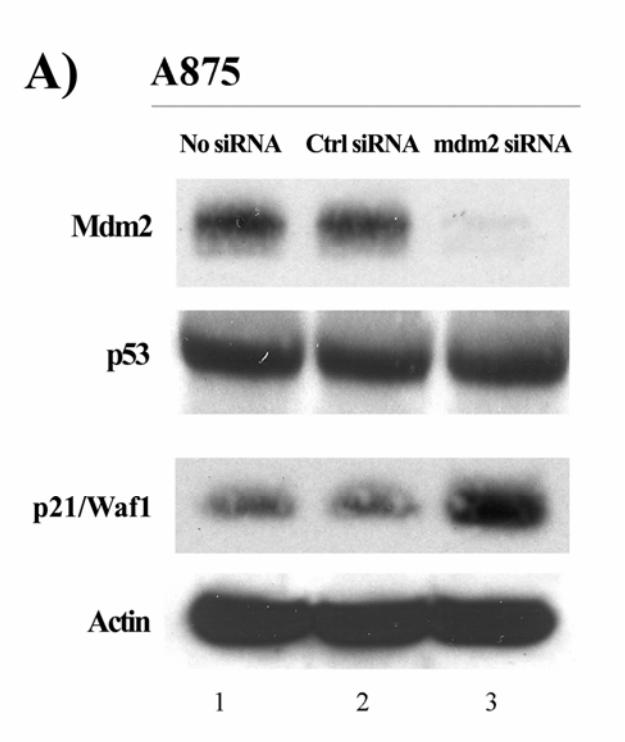
Re-transfection of cells with two p53 dependent reporter constructs after *mdm2* siRNA treatment also showed enhanced p53 function in the absence of Mdm2, as illustrated by the fold change in the luciferase readings (Figure 20, panel D).

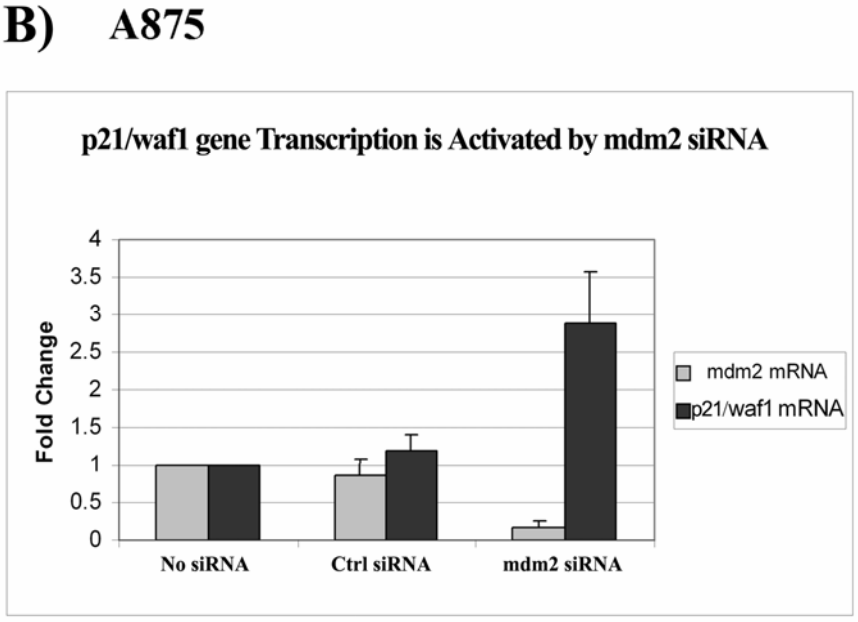
siRNA experiments failed to down-regulate Mdm2 levels in MANCA (data not shown). We tested the transfection efficiency in these cells by using a fluorochrome

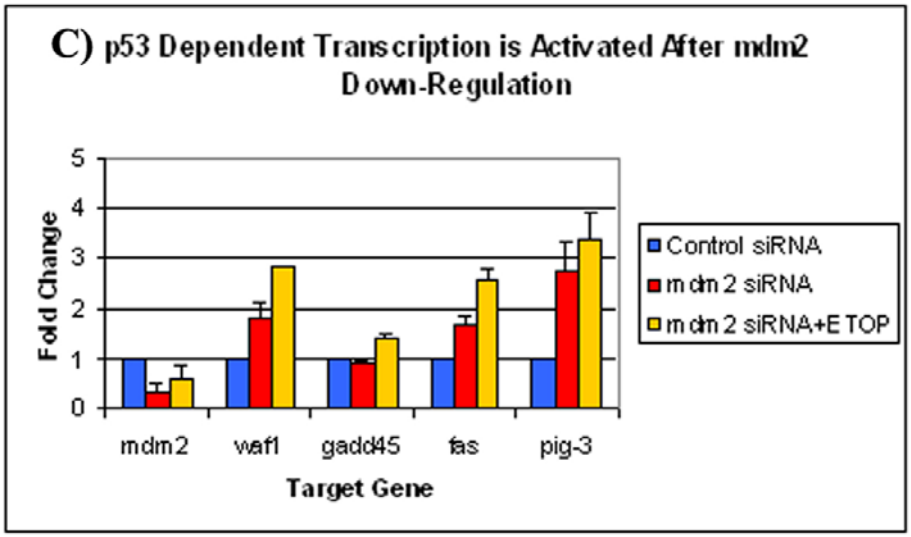
labeled siRNA and detected transfected particles inside the cells, suggesting another reason for malfunction of siRNA in this cell line. However, reactivation of p53 after *mdm2* siRNA is not specific for A875 cells, as p21/Waf1 protein was induced after Mdm2 down-regulation by siRNA in another SNP309 homozygous cell line, CCF-STTG-1 (Bond et al., 2004).

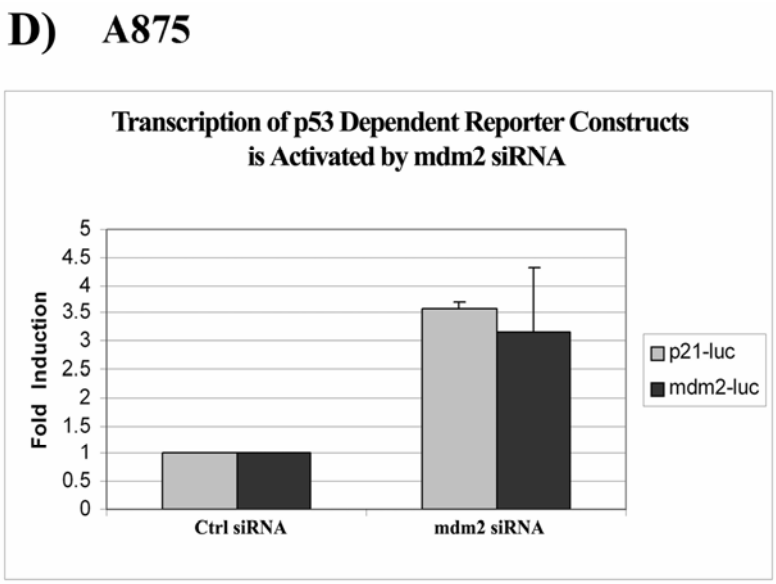
siRNA experiments point toward Mdm2 as the inhibitor of the p53 function in cells homozygous for SNP309.

Figure 20: Mdm2 Down-Regulation Reactivates p53 Transcriptional Activity









Panel A): Western blot analysis of A875 cells after mdm2 siRNA treatment. Cells were transfected with mdm2 or control siRNA and harvested 24 hours later. 50µg of total protein was subjected to SDS-PAGE (10%) and Western blot analysis. The nitrocellulose membranes were probed with the Mdm2 specific antibody SMP14, the p53-specific polyclonal antibody, the p21/Waf1-specific monoclonal antibody (Ab-1), and anti-Actin. Panel B): Quantification of mdm2 and p21/waf1 transcripts following mdm2 siRNA

treatment. RNA was extracted 24 hours following siRNA transfection in A875 cells. Quantitative PCR was used to detect fold change of the mdm2 and p21/waf1 transcripts. All results were normalized to untreated samples and glyceraldehyde-3-phosphate dehydrogenase values. Standard deviations represent results from three independent experiments.

Panel C): Quantification of mdm2, p21/waf1, gadd45, fas and pig3 transcripts after mdm2 siRNA treatment followed by 3 hours of 8μM ETOP treatment. All results were normalized to untreated samples and glyceraldehyde-3-phosphate dehydrogenase values. Standard deviations represent results from three independent experiments.

Panel D): Transient transfection in A875 cells. Cells were transfected with mdm2 or control siRNA and re-transfected 24 hours later with a plasmid containing the p21/waf1 or wild-type mdm2 p53 binding site adjacent to a luciferase reporter. Protein extracts were prepared and fold induction in luciferase activity was measured and normalized to total protein concentration.

3.2.11 Disruption of the Mdm2-p53 Complex Reactivates p53

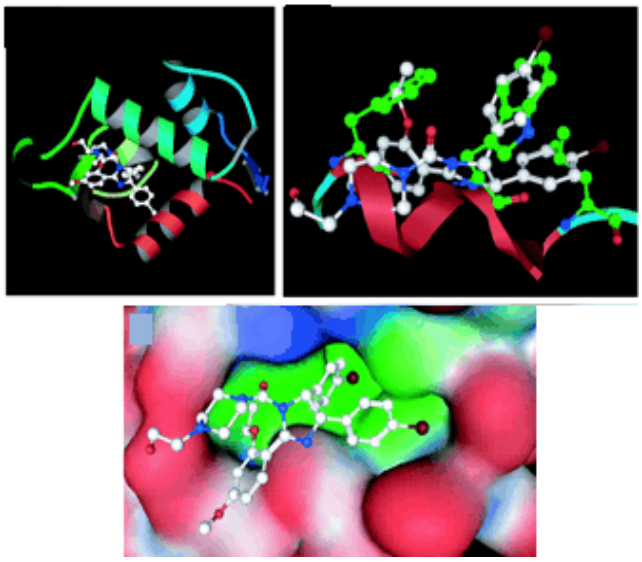
There have been efforts to identify potent and selective small-molecule inhibitors of the Mdm2-p53 interaction with *in vitro* and *in vivo* anti-tumor activity.

The crystal structure of Mdm2 bound to a peptide from the trans-activation domain of p53 has revealed that Mdm2 possesses a relatively deep hydrophobic pocket that is filled primarily by three side chains from the helical region of the peptide (Figure 21). The existence of such a well-defined pocket on the Mdm2 molecule raised the

expectation that compounds with low molecular weight could be found that would block the interaction of Mdm2 with p53.

One such class was a series of cis-imidazoline analogs that were named Nutlins. These compounds displaced recombinant p53 protein from its complex with Mdm2, with median inhibitory concentration (IC_{50}) values in the 100 to 300 nM range (Vassilev et al., 2004). The imidazoline compounds were synthesized as racemic mixtures from which the enantiomers could be separated with the use of chiral columns. Nutlin-3a possessed potent binding activity, whereas the other enantiomer, Nutlin 3b, was 150 times less active.

Figure 21: p53-Mdm2 Interaction and Nutlin Binding to the Mdm2 Hydrophobic Pocket

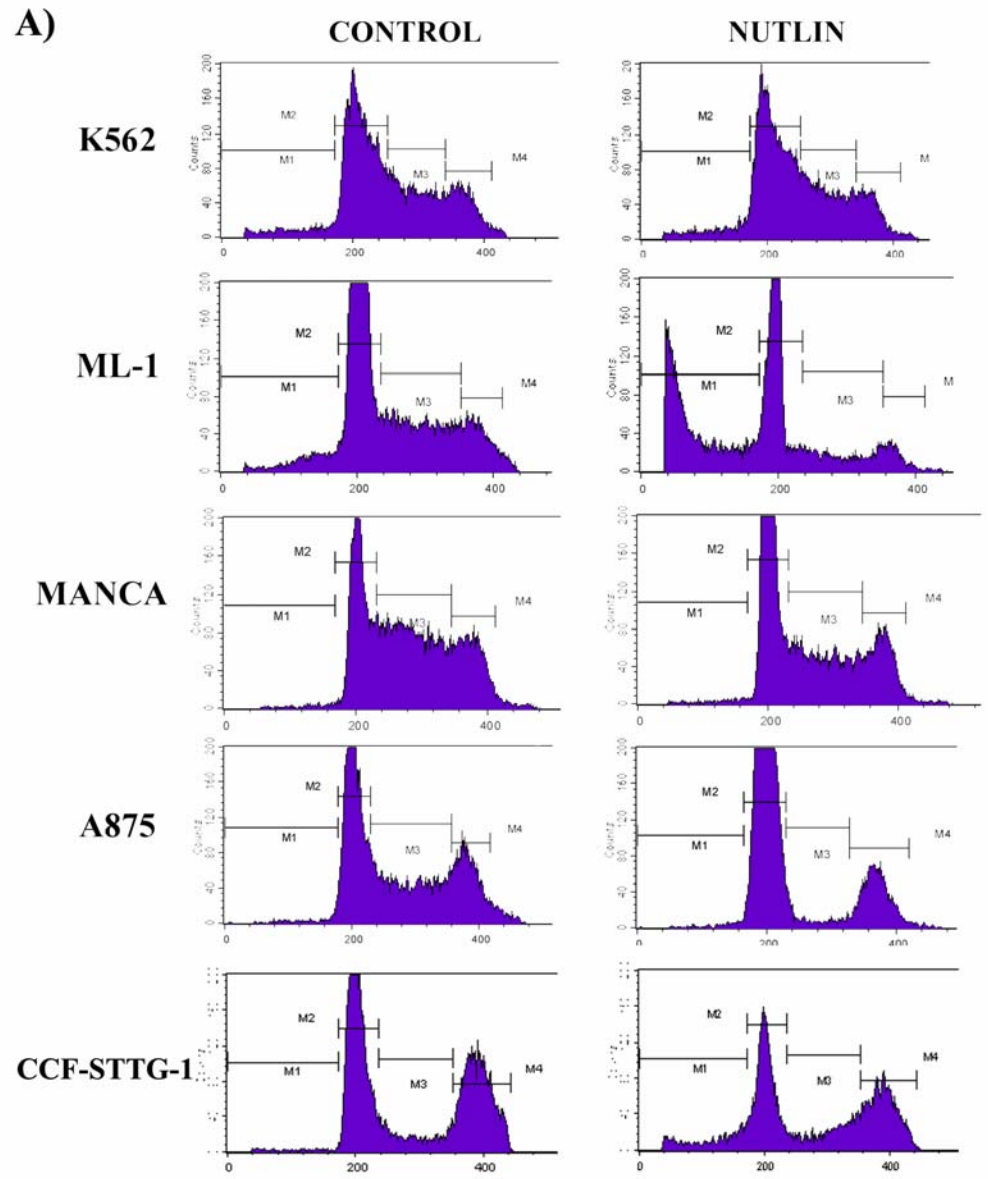


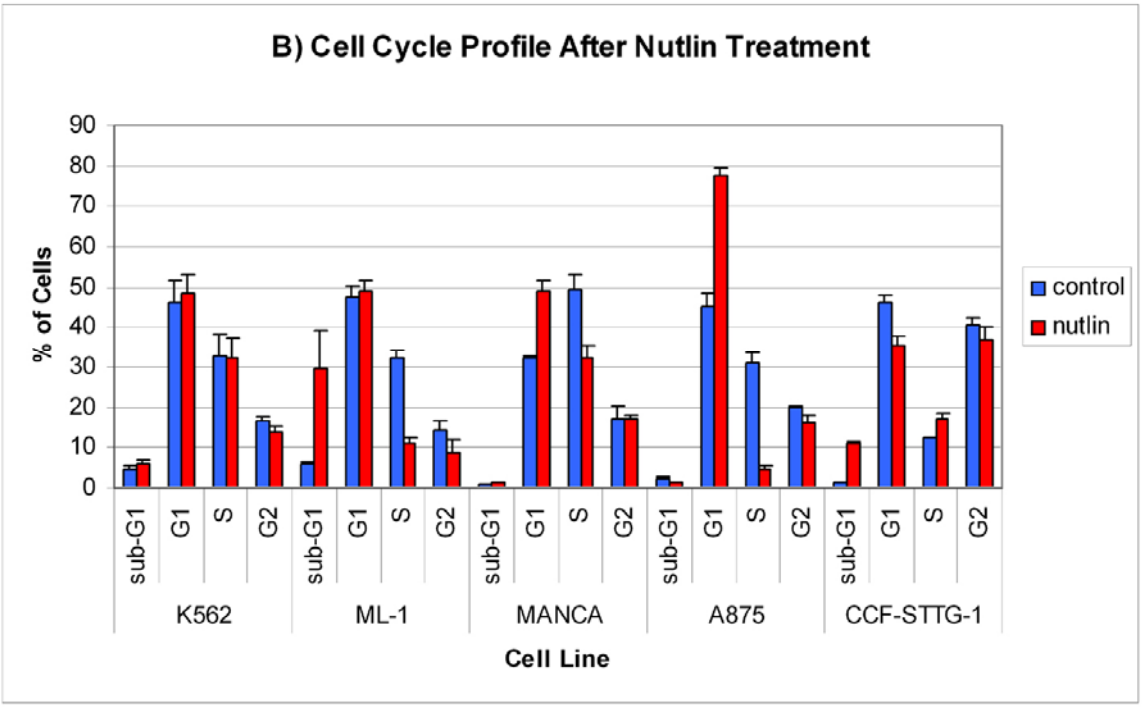
Nutlin was tested *in vivo* on tumor cells lines (SJSA-1 osteosarcoma cells) with wild-type p53 and Mdm2 over-expression from *mdm2* amplified genotype (Vassilev et al., 2004). Disruption of the p53-Mdm2 interaction stabilized p53 due to inhibition of p53 proteasomal degradation. Although phosphorylation events have not been detected after Nutlin treatment, p53 displayed transcriptional activity (Thompson et al., 2004). p53 target genes were turned up to the same extent after both DNA damage and Nutlin, suggesting that phosphorylation of p53 on key serine residues is dispensable for transcriptional activation and apoptosis.

Although Mdm2 over-expression does not lead to excessive p53 degradation in SNP309 homozygous cells, disruption of the p53-Mdm2 complex could also reactivate p53 function in these cells, by releasing the chromatin-bound p53 from its inhibitor. 10μM Nutlin3 racemic mixture was added to the growth media for 48 hours and cell

cycle profile was subsequently analyzed using FACS. Nutlin had no effect in K562 cells. ML-1 cells responded to Nutlin by mostly undergoing apoptosis (30% sub-G1 population). Substantial G1 arrest was induced by Nutlin in A875 and MANCA cells (from 46% to 79% in A875 and from 32% to 50% in MANCA) and some apoptosis (10.5 % sub-G1 population) was detected in the *mdm2* SNP309 homozygous cell line CCF-STTG-1. Remarkably, in all cell lines a decrease in S phase was observed after Nutlin treatment, except in CCF-STTG-1, which had a very low percentage of cells in S phase to start with (10% before addition of the drug) (Figure 22, panels A and B).

Figure 22: FACS Analysis of ML-1, MANCA, A875 and K562 Cells after Treatment with Nutlin





Exponentially growing cells were either left untreated or were treated with10μM Nutlin for 48 hours. Cells were harvested and fixed in 30% ethanol. 30 minutes prior to FACS analysis cellular DNA was stained with propidium iodide. Cells were analyzed by flow cytometry. M1 gate represents subG1 phase, M2 gate represents G1 phase, M3 gate represents S phase, M4 gate is G2 phase.

Panel A) represents cell cycle profile in untreated or Nutlin treated cells.

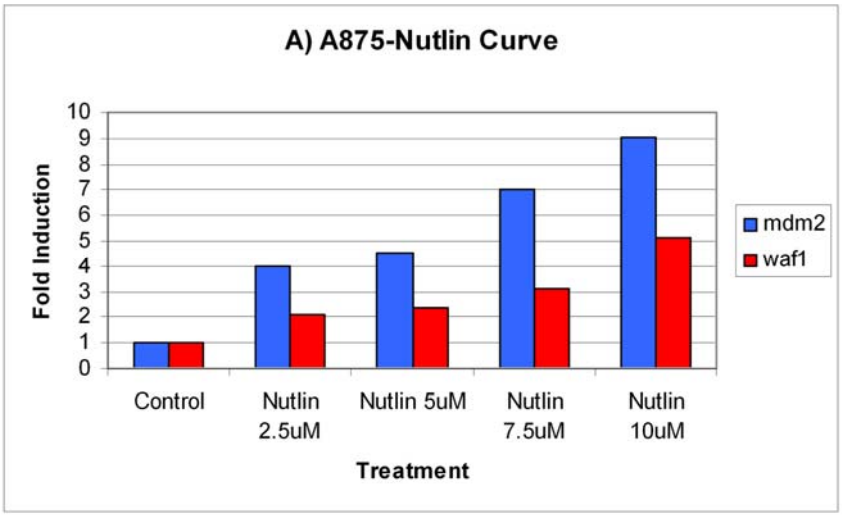
Panel B) is a graphic representation of the percentages of cells in each cell cycle phase before and after treatment with Nutlin.

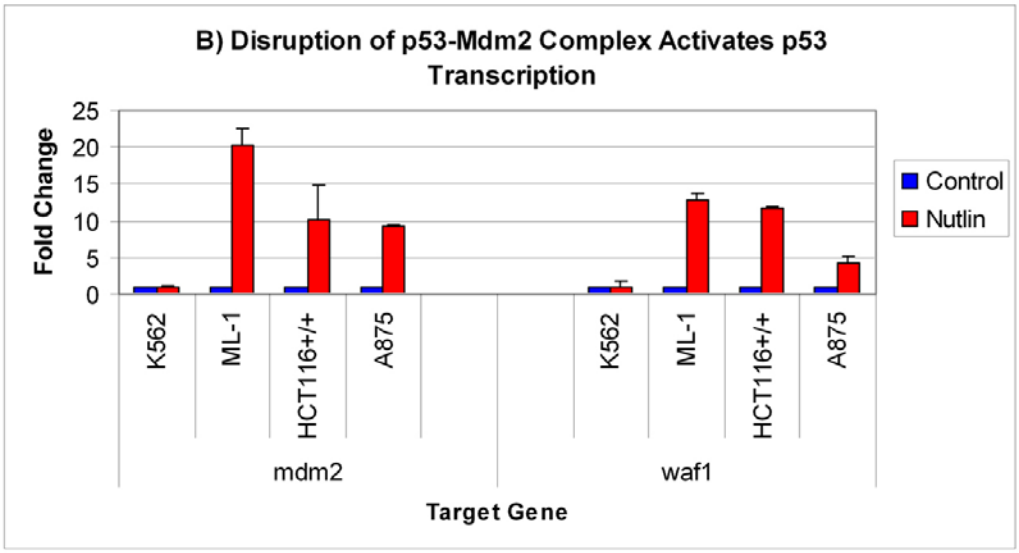
We also tested activation of the p53 targets in response to Nutlin by using real-time quantitative PCR. In a dose curve experiment (Figure 23, panel A) we established 10μM Nutlin as being the most effective concentration in terms of p53 target genes activation.

The same concentration has been used in all previous studies involving Nutlins (Thompson et al., 2004). In these experiments we also used an additional cell line (HCT116+/+) that expresses wild-type *p53* and *mdm2*. HCT116+/+ has been shown to exhibit robust p53 target genes activation in response to Nutlin (Thompson et al., 2004). In K562 cells, as expected, we did not observe any change in p53 target genes transcription after drug treatment (Figure 23, panel B). In ML-1 and HCT116+/+ cells (expressing wild-type *mdm2*), Nutlin produced substantial activation of *mdm2* and *p21/waf1* targets. In A875 cell line, *mdm2* transcription was substantially activated by Nutlin to the same level as in HCT116+/+ cells. *p21/waf1* transcription increased to a lesser extent in A875 cells in response to Nutlin when compared with *mdm2* wild-type cells (13 fold activation was achieved in ML-1 and HCT116+/+ cells and only 5 fold up-regulation in A875 cells).

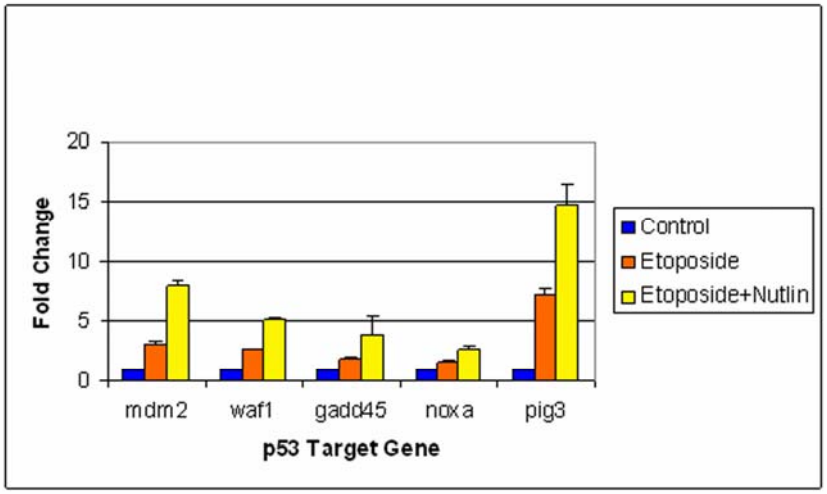
We also treated A875 cells with a combination of drugs: after p53 induction with etoposide for 3 hours, cells were re-treated with Nutlin for additional 24 hours to release p53 from the inhibitory p53-Mdm2-chromatin complex. As shown in Figure 23, panel C, the sequential treatment activated p53 dependent transcription more than etoposide did by itself.

Figure 23: Disruption of the Mdm2-p53 Complex Reactivates p53





C) Combined Therapy (Etoposide+Nutlin) in A875 Cells



Panel A): Nutlin dose curve in A875 cells. Cells were left untreated or treated with 2.5 μM, 5 μM, or 7.5 μM or 10μM Nutlin. RNA was extracted after 24 hours of drug treatment. Quantitative PCR was used to detect fold change of the mdm2 and p21/waf1 transcripts. All results were normalized to untreated samples and glyceraldehyde-3-phosphate dehydrogenase values.

Panel B): Disruption of the p53-Mdm2 complex activates p53 transcription in both mdm2 wild-type and SNP309 homozygous cells. K562, ML-1, HCT116+/+ and A875 cells were left untreated or treated with 10μM Nutlin. RNA was extracted after 24 hours of drug treatment. Quantitative PCR was used to detect fold change of the mdm2 and p21/waf1 transcripts. All results were normalized to untreated samples and glyceraldehyde-3-phosphate dehydrogenase values.

Panel C): Combination therapy (etoposide+Nutlin) in A875 cells. Cells were left untreated or treated with 8μM etoposide. 3 hours later, cells were re-treated with 10μM Nutlin for an additional 24 hours. RNA was extracted and quantitative PCR was used to detect fold change in different p53 target genes. All results were normalized to untreated samples and glyceraldehyde-3-phosphate dehydrogenase values.

These data suggest that disruption of the p53-Mdm2 complex activates p53 function in cells with normal Mdm2 expression (ML-1) but also in cells with Mdm2 over-expression from *mdm2* SNP309 (MANCA, A875 and CCF-STTG-1 cells). These results strengthen the hypothesis that p53 activity is blocked through an inhibitory association with Mdm2 protein in SNP309 homozygous cells.

3.3 Discussion

The interaction between soluble Mdm2 and p53 has been well described in experimental systems with forced over-expression of both proteins. However, the chromatin-bound association between p53 and Mdm2 proteins has only begun to be addressed (Minsky and Oren, 2004). It has been shown that Mdm2 regulates p53 by at least two mechanisms. The interaction of Mdm2 with p53 blocks the trans-activation domain of p53 and inhibits this protein's transcriptional activity (Thut et al., 1997). Additionally, Mdm2 is an E3 ubiquitin ligase for p53, helping to target p53 for degradation by the ubiquitin proteolysis pathway (Fang et al., 2000), (Grossman et al., 2003).

Mdm2-mediated inhibition of p53 trans-activation activity contributes to the impairment of the p53 pathway in cancer cell lines endogenously over-expressing Mdm2. Here we report that endogenous over- expression of Mdm2 via a naturally occurring SNP results in the formation of an inactive chromatin-bound complex.

We determined that two cancer cell lines (MANCA and A875) expressing wild-type p53 have stabilized and phosphorylated p53 after DNA damage, demonstrating limited clearance of p53 protein by degradation. Treatment with a proteasomal inhibitor LLnL and also Mdm2 down-regulation did not lead to any increase in the p53 protein levels, suggesting as well that p53 is not excessively degraded in SNP309 homozygous cells. It is possibly that a different form of Mdm2 protein that is deficient in its ability to target p53 for degradation is synthesized in cells homozygous for SNP309. In fact, Mdm2 Western-blot revealed bands of multiple sizes in cells homozygous (MANCA and A875) or heterozygous (K562) for *mdm2* SNP309, suggesting that different Mdm2 isoforms could be produced from the SNP309 (Figure 11, panel B).

p53 bound site-specifically to DNA and chromatin. Although the amount of p53 bound to the *mdm2* gene in MANCA cells remained unchanged after treatment in the total p53 antibody ChIP (Figure 17, panel A, lanes 7 and 8, *mdm2* gene) and very little p53 was detected at the *p21/waf1* gene (Figure 17, panel A, lanes 7 and 8, *p21/waf1* gene), we reasoned that the antibody epitope (which is located at the N terminus) was masked by p53 phosphorylation and by association with its Mdm2 partner.

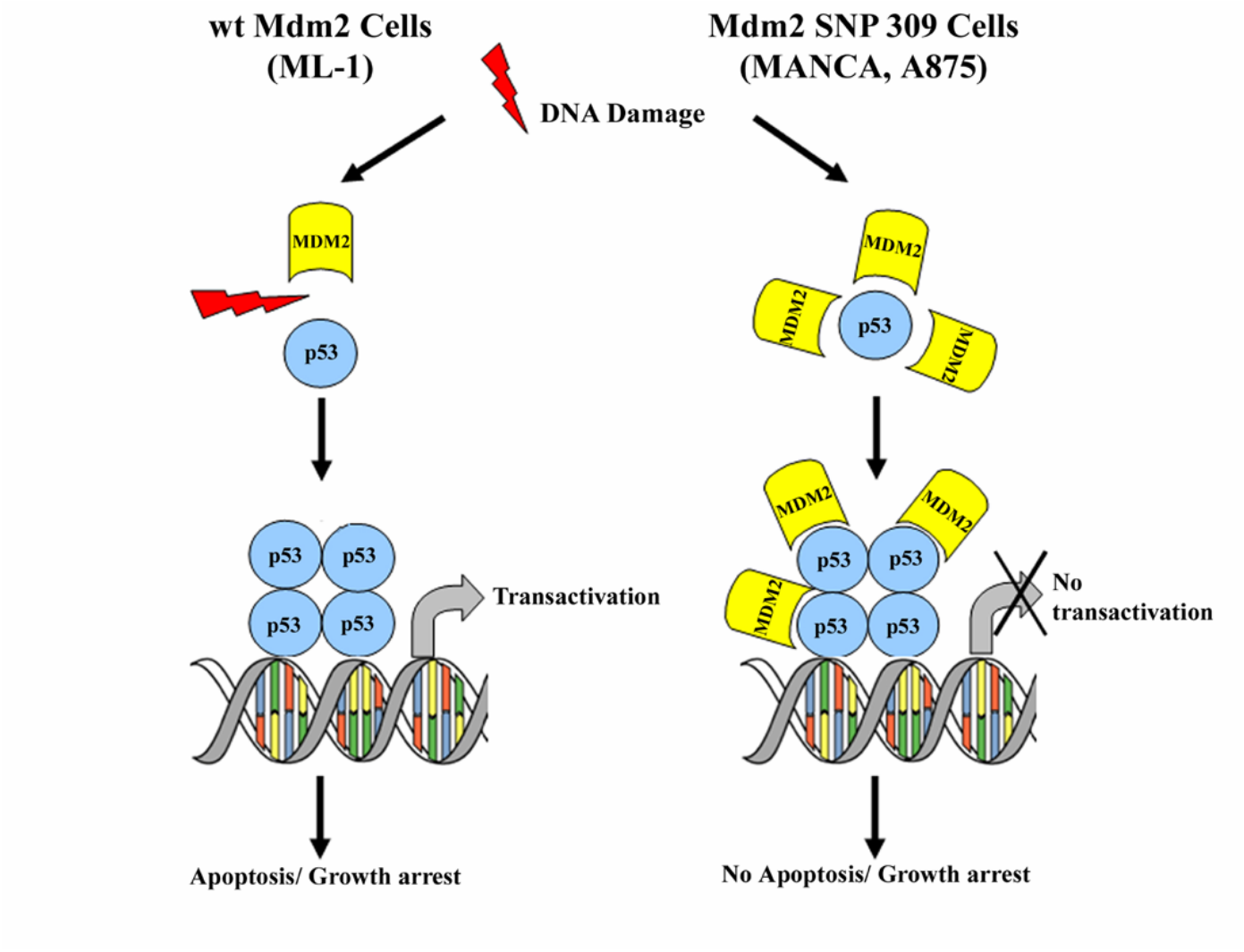
We also provide evidence that Mdm2 localized with p53 at the p53 responsive elements, suggesting that Mdm2 blocked the trans-activation activity of DNA-bound p53. This finding directly agrees with the recent work by Moshe Oren's group indicating that Mdm2 can be recruited to chromatin resulting in histone ubiquitination and repression of transcription. Both p53 and Mdm2 bound to chromatin in MANCA and A875 cells after DNA damaging drug treatment and Mdm2 protein recruitment to the chromatin was increased after such drug treatment (Figure 19, panels A and C). Our data support the findings that stabilized p53 can, at times, maintain an interaction with Mdm2 (Dumaz and Meek, 1999). The co-localization of Mdm2 with p53 on chromatin correlates with attenuated p53 function. This mechanism for inhibition of p53 transcriptional activity most likely applies in all cell types that over-express Mdm2 and would result in a latent form of p53 that could be reactivated in the absence of Mdm2 protein. Down-regulation of Mdm2 by siRNA or disruption of the p53-Mdm2 complex resulted in reactivation of p53 transcriptional activity (Figure 20, panels A-D and Figure 23, panels A-C).

We saw that exogenously introduced p53 was incapable of activating transcription from p53 response element reporter constructs in either MANCA or A875 cells, while exogenously introduced p53 was able to activate transcription from the reporter constructs in two other cell lines not homozygous for *mdm2* SNP309 (H1299 and K562).

These data further support the argument that a *trans*-acting factor, i.e. Mdm2, inhibited the activity of the exogenously introduced p53 in the naturally occurring *mdm2* SNP309 cell lines. The inactivation of *mdm2* by siRNA or disruption of the p53-Mdm2 complex reactivated the endogenous p53. Although the level of p53 activation induced by Nutlin was lower in cells *mdm2* SNP309 homozygous as compared with *mdm2* wild-type cells (as shown by a smaller percentage of cells undergoing apoptosis or a lower level of p53 target genes trans-activation), we postulated that Mdm2 over-expression does not allow for all the p53-Mdm2 complexes on chromatin to be disrupted by Nutlin and thus p53 function could not be fully restored. p53-Mdm2 association on chromatin could also lead to epigenetic changes that are very stable and difficult to reverse.

We present a model for how Mdm2 blocks the ability of p53 to activate target genes (Figure 24). In cells that have wild-type p53 we see stabilization of p53 protein after DNA damage. In the ML-1 cell line, which does not have a SNP at position 309 of the *mdm2* gene, we see dissociation of p53 Ser-15 from Mdm2. This allows for the activated p53 protein to behave as a viable transcription factor and turn on downstream target genes. In cells over-expressing Mdm2, such as cells homozygous for *mdm2* SNP309 (MANCA and A875), there is increased association of the p53 Ser-15-Mdm2 complex on chromatin after DNA damage. This p53, in the presence of Mdm2 on chromatin, does not act as a viable transcription factor.

Figure 24: A model illustrating that in cells homozygous for the mdm2 SNP309, p53 that is induced after DNA damage does not dissociate from the excessively expressed Mdm2 protein. This association does not interfere with the ability of p53 to bind to DNA but does impair the p53 transcriptional activity.



3.4 Conclusions

1. ***mdm2* SNP309 homozygous cells express high Mdm2 levels**

2. **Mdm2 forms a stable complex with p53 despite DNA damage**

3. **p53-Mdm2 complex is bound to chromatin at p53 responsive elements**

4. **p53 trapped in this complex is transcriptionally inactive**

5. **Inactivation of the p53 pathway renders *mdm2* SNP309 homozygous cells unable to undergo apoptosis/growth arrest**

6. **Mdm2 down-regulation or disruption of the p53-Mdm2 complex reactivates the transcriptional activity of p53 in *mdm2* SNP309 homozygous cells**

Chapter 4: Cells Over-expressing Mdm2 from Various Causes Have Different Phenotypes

4.1 Introduction

Mdm2 over-expression is a well documented system for inactivating wild-type p53 pathway in tumors. In the previous chapter, we provided evidence that Mdm2 can block the p53 trans-activation function by localizing with p53 in chromatin, at the p53 responsive elements. Mdm2-chromatin association was also recently reported in another Mdm2 over-expressing cell line, SJSA-1 (Minsky and Oren, 2004), leading to histone ubiquitination and epigenetic changes consistent with gene silencing. SJSA-1 is an osteosarcoma cell line with high Mdm2 protein levels due to *mdm2* gene amplification (Oliner et al., 1992). Although SJSA-1 cells have a high Mdm2 protein amount, they have been shown to respond well to both DNA damage signals (doxorubicin) and Mdm2 antagonists (Nutlin3) (Tovar et al., 2006).

Our transcription studies suggested that DNA damage was not able to activate p53-dependent trans-activation in cells with Mdm2 over-expression from *mdm2* SNP309, but Nutlin successfully reactivated p53 function in these cells. The different behavior of cells homozygous for *mdm2* SNP309 versus cells with *mdm2* gene amplification would suggest that SNP309 might confer a special phenotype, besides Mdm2 over-expression.

We have begun to investigate this hypothesis and started to search for other interacting proteins that could be part of the p53-Mdm2 chromatin-bound inhibitory complex. Mdm2 Western-blot also detected multiple bands in both cells hetereozygous and homozygous for SNP309 (Figure 11, panel B, compare lane 1 with lanes 5, 9 and 13) pointing that various *mdm2* isoforms might be produced from SNP309.

4.2 Results

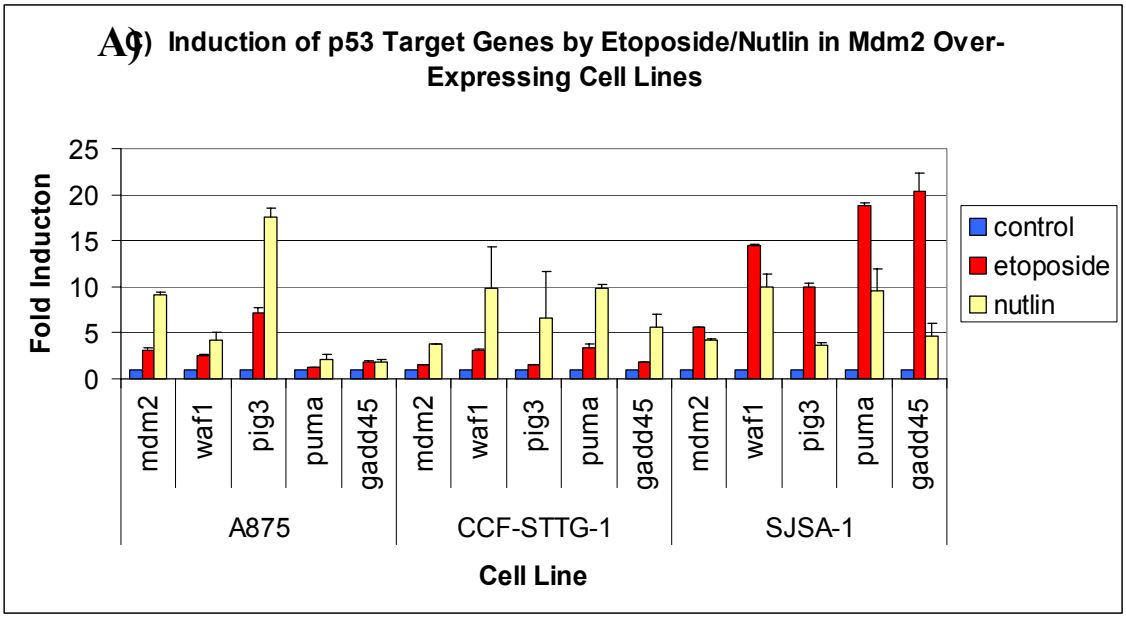
4.2.1 Cells Over-expressing Mdm2 from Various Causes Have Different Phenotypes

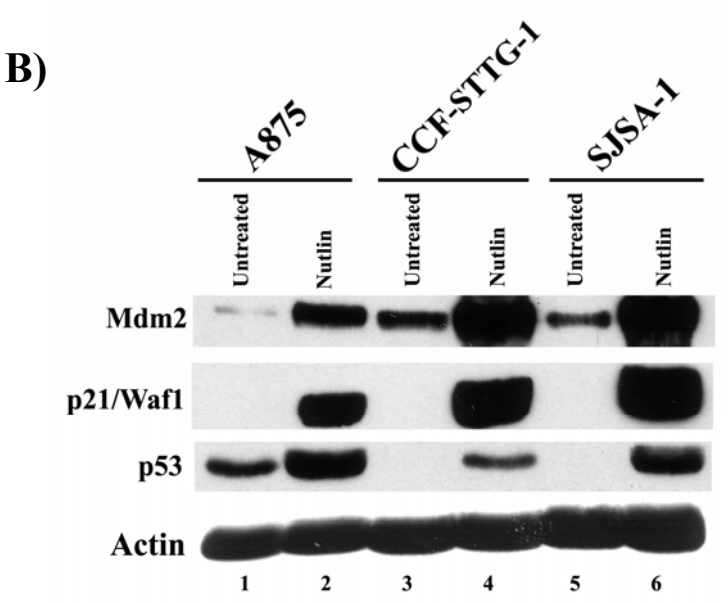
We compared the level of p53 target gene activation in cell lines with Mdm2 over-expression from either *mdm2* SNP309 (A875 and CCF-STTG-1) or amplified genotype (SJSA-1 cells). The cellular response after treatment with 8μM etoposide or 10μM Nutlin for 24 hours was investigated. Although Nutlin was less efficient in inducing p53-depndent transcription in cells homozygous for *mdm2* SNP309 when compared with *mdm2* wild-type cells (Figure 23, panel B), p53 target genes were induced to the same extent in all Mdm2 over-expressing cell lines (Figure 25, panels A, compare yellow bars). The level of p53 target gene activation following treatment with Nutlin was evidently higher than that achieved after etoposide in A875 and CCF-STTG-1 cells. In SJSA-1 cells, surprisingly, etoposide activated p53-dependent transcription at a greater level than Nutlin (Figure 25, panel A, compare red and yellow bars for SJSA-1 cells).

Western-blot analysis confirmed the reactivation of p53 transcriptional activity, as indicated by the increase in Mdm2 and p21/Waf1 protein levels after addition of Nutlin (Figure 25, panel B, compare odd lanes (untreated samples) with even lanes (Nutlin treated samples)). p53 protein was also stabilized, suggesting some Mdm2-dependent proteasomal degradation even in cells homozygous for *mdm2* SNP309.

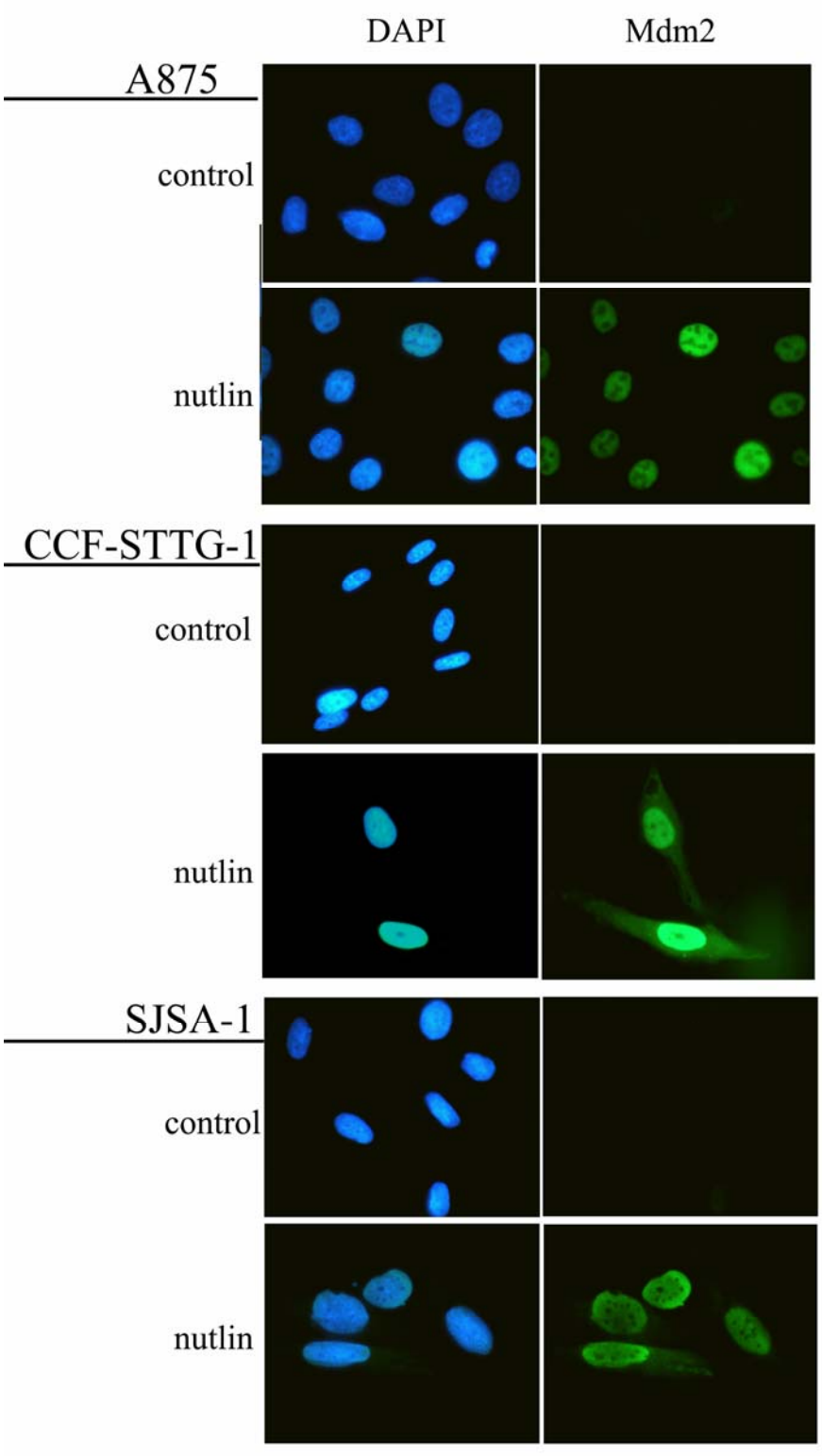
Additional immunofluorescence experiments testing Mdm2 localization in response to Nutlin confirmed Mdm2 induction and also revealed Mdm2 in the nucleus, despite disruption of its interaction with the nuclear p53 protein (Figure 25, panel C).

Figure 25: Disruption of the Mdm2-p53 Complex Reactivates p53 in Mdm2 Over-expressing Cells





C)


DAPI
Mdm2
A875
control
nutlin
CCF-STTG-1
control
nutlin
SJSA-1
control
nutlin

Panel A): Comparison of activation of p53 dependent transcription achieved after treatment of Mdm2 over-expressing cell lines with etoposide or Nutlin. A875, CCF-STTG-1 and SJSA-1 cells were left untreated or treated with 8μM etoposide or 10μM Nutlin. RNA was extracted after 24 hours of treatment. Quantitative PCR was used to detect fold change of the mdm2 p21/waf1, pig-3, puma and gadd45 transcripts. All results were normalized to untreated samples and glyceraldehyde-3-phosphate dehydrogenase values.

Panel B): Western blot analysis of A875, CCF-STTG-1 and SJSA-1 samples. Cells were either left untreated (lanes 1,3,5) or were treated with 10μM Nutlin (lanes 2,4,6) for 24 hours and nuclear extracts were then prepared from these samples. 50μg of nuclear protein was subjected to SDS-PAGE (10%) and Western analysis. The nitrocellulose membranes were probed with Mdm2 specific antibody SMP14, with p21/Waf1 specific antibody, with a total p53 antibody and anti-actin antibody.

Panel C): Immuno-fluorescence in A875, CCF-STTG-1 and SJSA-1 cells. Cells were left untreated or treated with 10μM Nutlin for 24 hours.

4.2.2 Other Proteins Might be Part of the p53-Mdm2 Complex in SNP309 Homozygous Cells

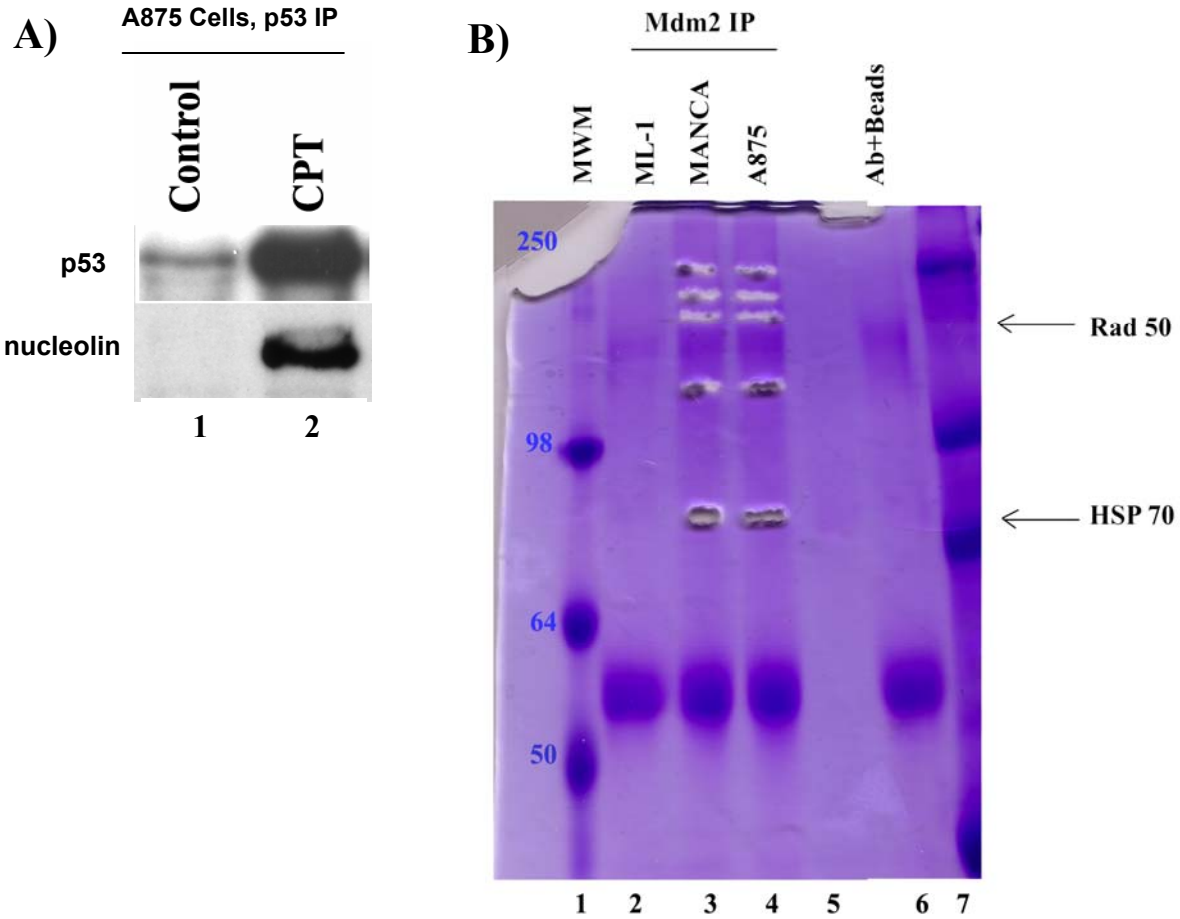
We have begun to look for other proteins that might be part of the p53-Mdm2 chromatin-associated complex. *In vivo* footprinting results have suggested that a large complex protects the p53 responsive element regions (Xiao et al., 1998). Co-immuno-precipitation with the p53 antibody 421 and subsequent Commassie Blue Staining demonstrated that a number of high mobility bands were specifically co-immuno-

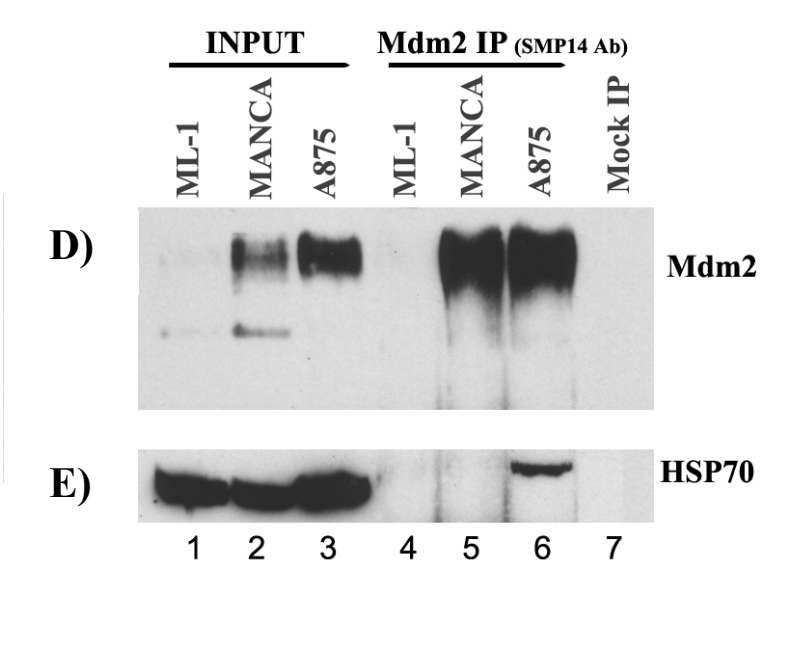
precipitated in SNP309 cells. Mass spectrometry/ mass spectroscopy analysis revealed that the nucleolin protein was one of the interacting proteins in MANCA cells, while this same band was not detected in the ML-1 samples (data not shown). Western-blot analysis of co-immuno-precipitated samples to compare the interaction between p53 and nucleolin indicated an interaction in the MANCA and A875 cells and none in the ML-1 cells (Figure 26, panel A and data not shown). Co-immuno-precipitation with the Mdm2 specific antibody D7 followed by Western-blot analysis demonstrated also a specific interaction between Mdm2 protein and nucleolin (data not shown).

Following the same logic, we also performed co-immuno-precipitation with the Mdm2 specific antibody D7. Half of the immuno-precipitation reaction was run on SDS-PAGE gel that was subsequently Commassie Blue Stained (Figure 26, panel B) and half of the immuno-precipitation reaction was run on SDS-PAGE gel, transfer to the nitrocellulose membrane and probed in Western-blot for Mdm2 protein using D7 specific antibody. Western-blot analysis confirmed that Mdm2 protein was pulled-down in all samples (Figure 26, panel C). In ML-1 cells, Mdm2 specific antibody D7 detected an Mdm2 isoform of approx.68kDa. In MANCA sample, three bands were evident (68KDa, 98kDa and a very high mobility band, below 250kDa marker, around 200kDa). A875 cells presented only the 68kDa and 200kDa variants. Commassie Blue staining demonstrated a number of bands that were specifically co-immuno-precipiated only in SNP309 cells. Mass spectrometry/ mass spectroscopy analysis of several bands revealed that the HSP70, HSP90 and Rad50 proteins were some of the interacting proteins in MANCA and A875 cells (HSP90 co-immuno-precipitated only in A875), while the same bands were not detected in the ML-1 samples (Figure 26, panel B).

Co-immuno-precipitation with a different Mdm2 antibody (SMP14) and subsequent Western-blot analysis demonstrated the interaction between Mdm2 and HSP70 in A875 cells (Figure 26, panel E, lane 6, HSP70 Western-blot). HSP70 was not co-immuno-precipitated from ML-1 and MANCA cells (Figure 26, panel E, lanes 4 and 5, HSP70 Western-blot). Mass spectrometry/ mass spectroscopy studies, which have a higher sensitivity than Western-blotting, were able to detect HSP70 protein in MANCA cells. However, the quantification of the amount of HSP70 protein interacting with Mdm2 showed that a substantially lower amount of HSP70 was co-immuno-precipitated in MANCA cells as compared to A875 cells (the score in MANCA was 1.1×10^{-2}; in A875 the score was 1.1×10^{-16}; the lower the score the higher the probability the protein is in the sample). This might explain why Western-blotting failed to detect co-immuno-precipitation of HSP70 and Mdm2 in MANCA cells.

Figure 26: Other Proteins Might be Part of the p53-Mdm2 Complex in SNP309 Homozygous Cells





Panel A): Co-immuno-precipitation in A875 cells using the p53 specific antibodies 421. Lane 1 represents untreated sample; lane2: treatment with 0.5μM CT. Upper panel was probed with p53 specific polyclonal antibodies; lower panel with nucleolin specific antibodies.

Panel B): Immuno-precipitation in untreated ML-1, MANCA and A875 nuclear samples using theMdm2 specific antibodies D7. Gel was Commassie Blue stained and several bands were cut out of the gel for mass-spectrometry/spectroscopy. Lanes 1 and 7 represent molecular weight marker; lane2: ML-1 sample; lane 3: MANCA sample; lane 4: A875 sample; lane 6: mock IP.

Panel C): Immuno-precipitation in untreated ML-1, MANCA and A875 nuclear samples using theMdm2 specific antibodies D7 (same IP samples from panel B were used). The blot was probed with the Mdm2 antibody D7. Lane 1: ML-1 sample; lane 2: MANCA sample; lane 3: A875 sample; lane 4: mock IP.

Panels D and E): Co-immuno-precipitation with untreated ML-1, MANCA and A875 nuclear samples, using the Mdm2 specific antibody SMP14. The blot was probed with the

Mdm2 antibody SMP14 (panel D) or the HSP70 specific antibody (panel E). Lane 1: input ML-1 sample; lane 2: input MANCA sample; lane 3: input A875 sample; lane 4: ML-1 IP sample; lane 5: MANCA IP sample; lane 6: A875 IP sample; lane 7: mock IP.

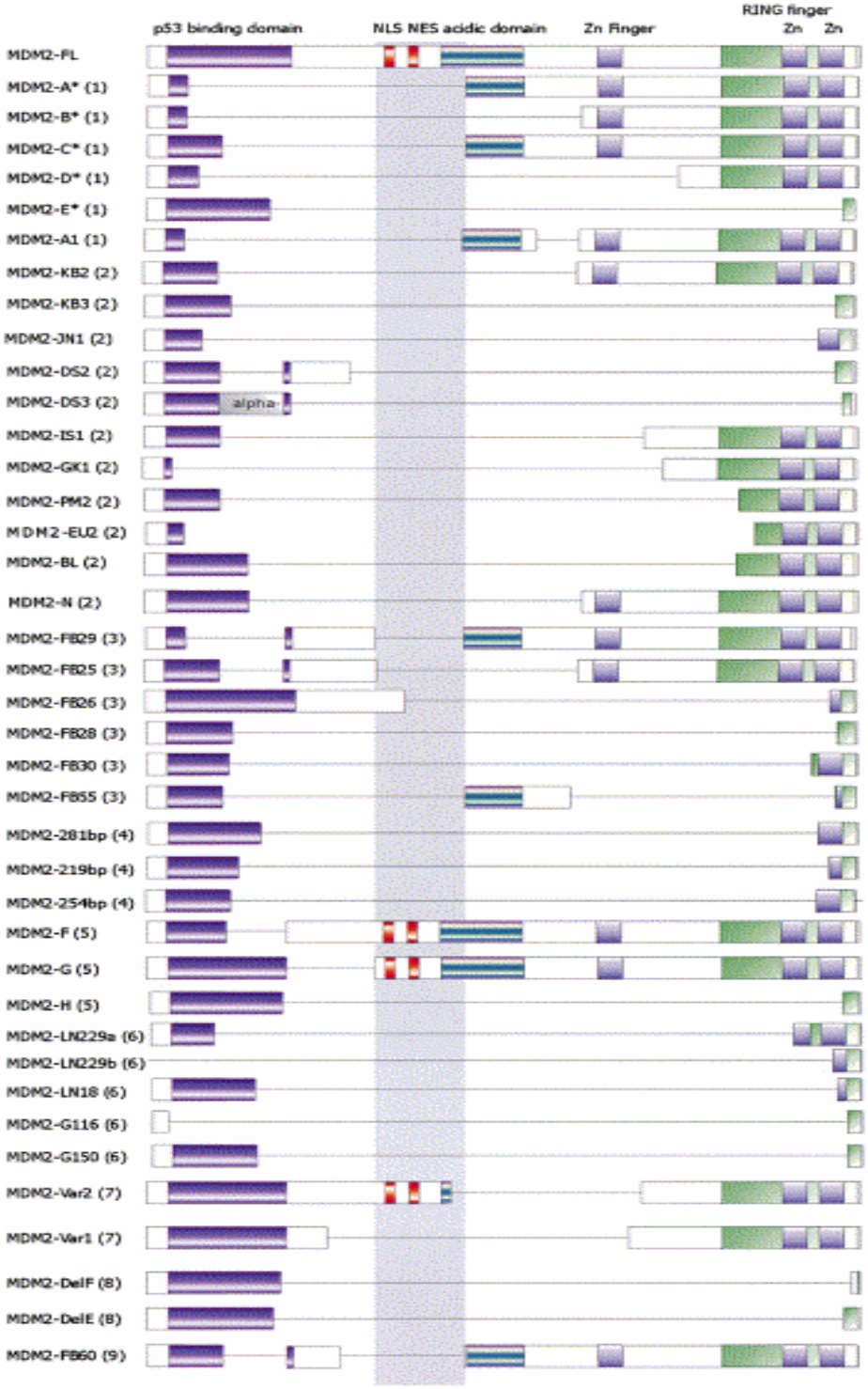
4.2.3 Mdm2 Alternative/Aberrant Splice Variants Might Be Present in SNP309 Homozygous Cells

mdm2 alternative splice variants have been discovered in tumors and have been reported to exhibit more oncogenic properties than the wild-type variants. Expression of *mdm2* oncogenic splice variants occurs more frequently in high grade than low grade tumors (Bartel et al., 2002).

More than 40 such isoforms have been identified (depicted in Figure 27).

Figure 27: Summary of All Known mdm2 mRNA Splice Variants and the Domains that They Encode

(Bartel et al., 2002)

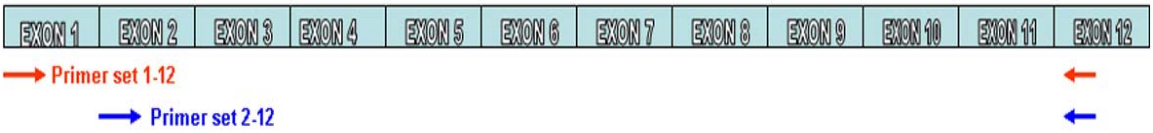


Mdm2 Western-blot analysis detected multiple bands in both cells heterozygous (K562) and homozygous for *mdm2* SNP309 (MANCA and A875) (Figure 11, panel B and Figure 26, panel C), pointing that various *mdm2* alternative/aberrant splice isoforms might be produced from SNP309.

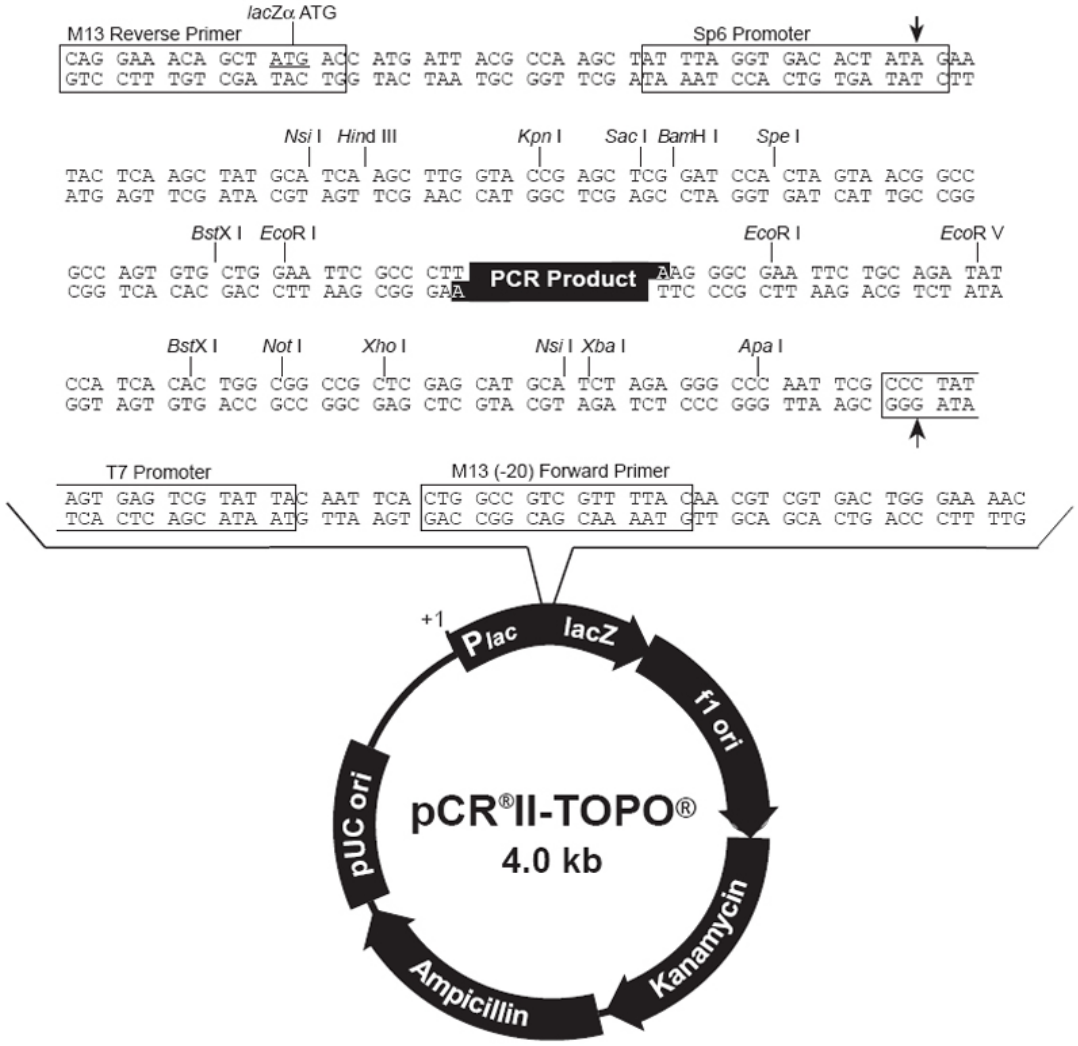
We have begun to investigate this hypothesis. *mdm2* cDNAs from K562, ML-1, MANCA and A875 cells were amplified in a PCR reaction using two different primer sets: primer set 1 amplified the product generated from promoter 1; primer set 2 amplified the product transcribed from promoter 2 (Figure 28, panel A). The PCR products were then cloned in *E. Coli* bacteria using pCRII-TOPO vector (Figure 28, panel B). After restriction, clones of different sizes were detected: 400bp (from ML-1, MANCA), 900bp (K562, ML-1, MANCA, A875), 1.1kb (present in all cell lines) (Figure 28, panel C). Sequencing of the clones generated with primer set 2 (2, 12) indicated that the 1.1kb product was the *mdm2* full length. Some 900 bp clones (from K562, ML-1, MANCA and A875 cells) were identical to the previously described *mdm2*-F isoform, missing exon 5 (Tamborini et al., 2001). A different 900bp product was recognized in ML-1 and K562 cells- it is an aberrant isoform, missing part of exon 10. One 900bp clone from MANCA was identified as missing exons 7 and 8 - this isoform has not been described in the literature so far. In addition, MANCA generated another new isoform of 400pb, missing exons 5, 6, 7, 8, 9, and 11 (Figure 28, panel D).

Figure 28: Alternative/Aberrant Splice Variants Might Be Present in mdm2 SNP309 Homozygous Cells

A)



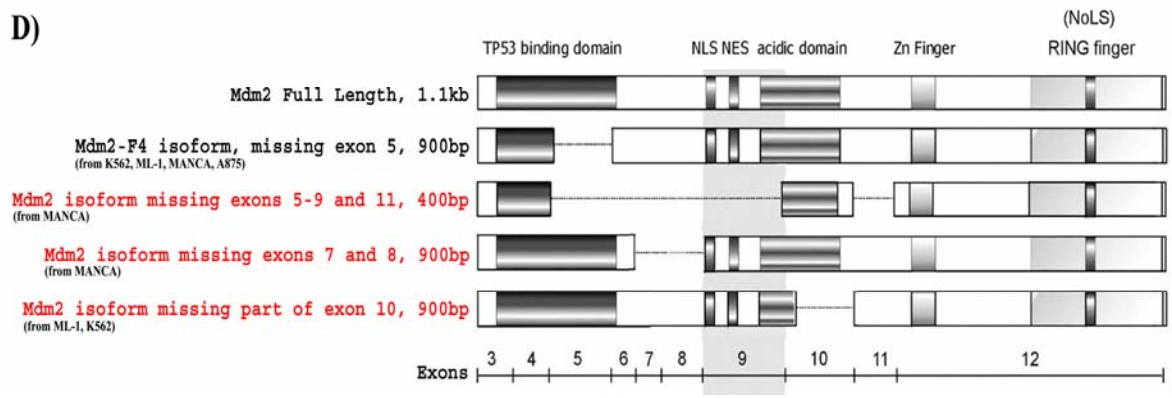
B)



C)

Cell Line	Sizes of the isolated clones
ML-1	400bp, 900bp, 1.1kb
K562	900bp, 1.1kb
MANCA	400bp, 900bp, 1.1kb
A875	900bp, 1.1kb

D)



Panel A): mdm2 gene and the primer pairs (1, 12 and 2, 12) used to amplify mdm2 cDNA

Panel B): pCR-TOPOII vector, in which mdm2 cDNA clones were legated.

Panel C): Table presenting sizes of the clones isolated from cells mdm2 wild-type (ML-1), heterozygous (K562) or homozygous for mdm2 SNP309 (MANCA, A875).

Panel D): mdm2 cDNA isoforms identified in ML-1, K562, MANCA and A875 cell lines.

4.2.4 p53 Could Cooperate with Sp1 to Enhance *mdm2* Transcription in Cells SNP309 Homozygous

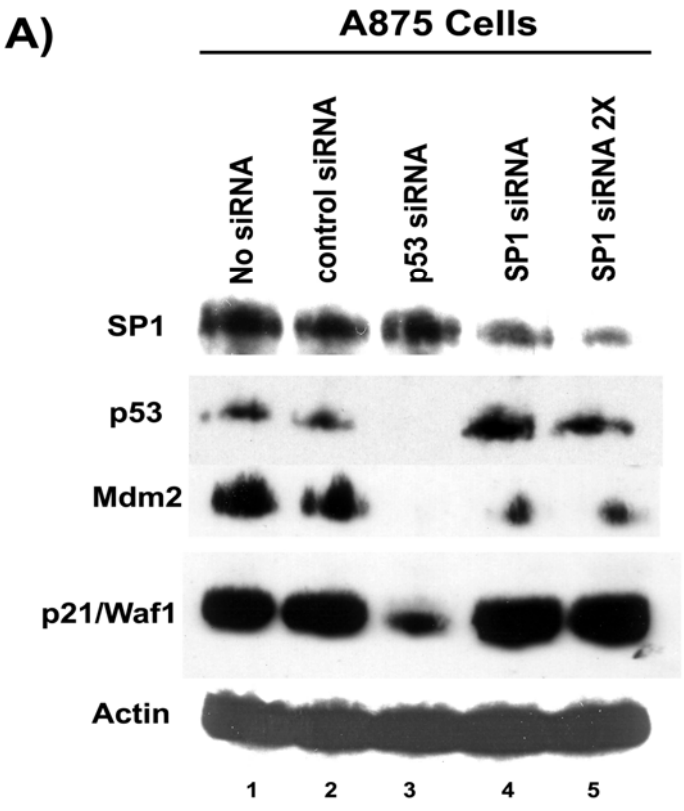
It has been reported previously that both wild-type and mutant p53 interacts with Sp1 and this association translates into a functional cooperation of the two proteins with synergistic trans-activation of those genes containing binding sites for both classes of transcription factors in their promoters (*p21/waf1*, *puma genes*, *HIV-LTR*) (Koutsodontis et al., 2005), (Chicas et al., 2000).

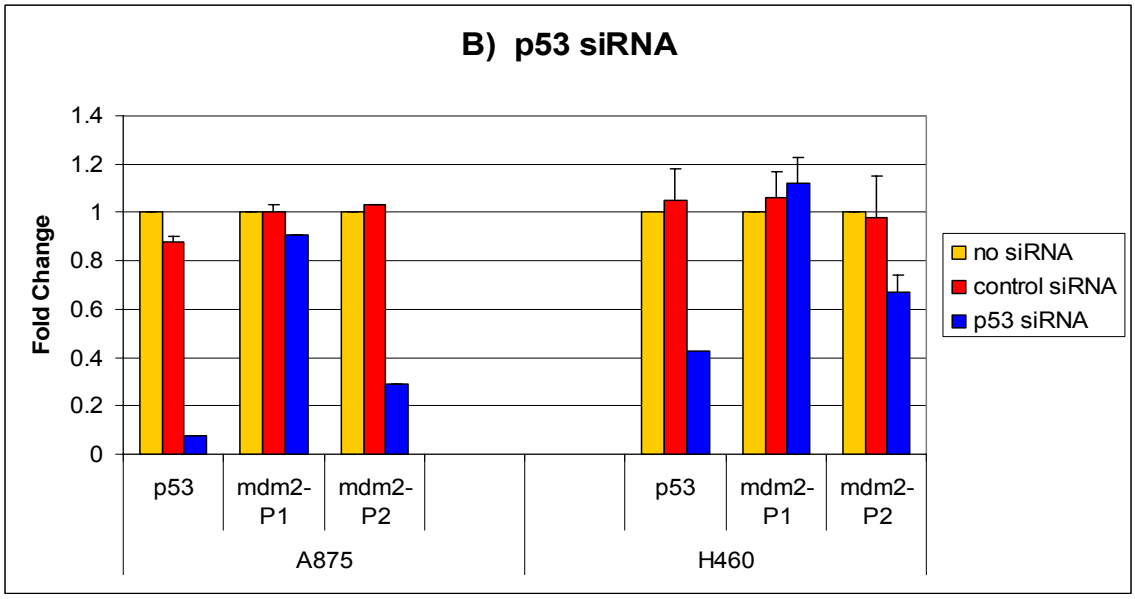
Enhanced *mdm2* transcription occurs in cells homozygous for *mdm2* SNP309 due to increased affinity for the ubiquitous transcription factor Sp1. SNP309 is located up-stream, very close to the p53 responsive elements. We wanted to determine if p53 cooperates with Sp1 to promote higher firing from the *mdm2* SNP309 P2 promoter. Bond et al. (2004) showed about 70% decrease in Mdm2 protein levels in A875 cells after Sp1 down-regulation with siRNA. We performed p53 siRNA and observed striking reduction (more accentuated than after Sp1 siRNA) in the Mdm2 protein levels (Figure 27 panel A, Mdm2 western-blot, compare lane 3 with lanes 4 and 5). *mdm2* mRNA transcribed from P2 promoter (*mdm2*-P2) went down to about 30% after p53 siRNA in the *mdm2* wild-type cell line H460 and the decrease was more evident in the *mdm2* homozygous cell line A875 (about 70% reduction) (Figure 29, panel B). *mdm2* transcript made from promoter 1 (*mdm2*-P1) was not affected by p53 siRNA. Transcription of other p53 target genes also decreased in A875 cells, suggesting that basal p53 is active in this cell line, but cannot be activated after DNA damage (data not shown).

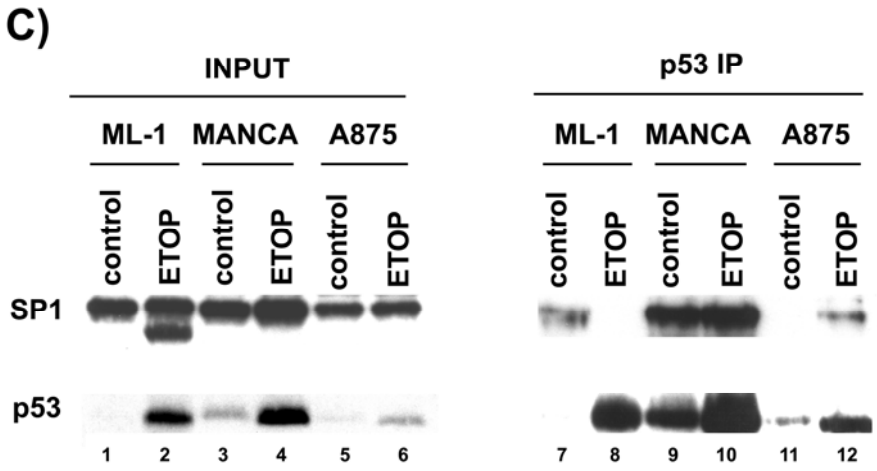
Mdm2 down-regulation after p53 siRNA indicated a role for p53 in basal *mdm2* transcription. Both Sp1 and p53 binding sites are very close in the *mdm2* P2 promoter

region, which suggest a possible physical association between these two proteins. Co-immuno-precipitation experiments using the p53 specific antibody Ab6 pulled down Sp1 protein in MANCA and A875 cells before and after ETOP treatment (Figure 29, panel C, lanes 9, 10 and 11, 12). In ML-1 cells, some Sp1 precipitated with p53 before DNA damage but this association was abolished after ETOP treatment (Figure 29, panel C, compare lanes 7 and 8).

Figure 29: p53 and Sp1 Might Cooperate to Enhance mdm2 Transcription in mdm2 SNP309 Homozygous Cells







Panel A): Western blot analysis of A875 cells after p53 or Sp1 siRNA treatment. Cells were transfected with p53, Sp1 (at two different concentrations) or control siRNA and harvested 24 hours later. 50µg of total protein was subjected to SDS-PAGE (10%) and Western blot analysis. The nitrocellulose membranes were probed with the Sp1 specific antibody PEP1, the p53-specific polyclonal antibody, Mdm2 specific antibody SMP14, the p21/Waf1-specific monoclonal antibody (Ab-1), and anti-Actin.

Panel B): Quantification of p53 and mdm2 (P1 and P2) transcripts following p53 siRNA treatment in A875 and H460 cells. RNA was extracted 24 hours following siRNA transfection. Quantitative PCR was used to detect fold change of the p53 and mdm2 transcripts. All results were normalized to untreated samples and glyceraldehyde-3-phosphate dehydrogenase values. Standard deviations represent results from two independent experiments.

Panel C): Co-immuno-precipitation in ML-1, MANCA and A875 cells using the p53 specific antibodies 421. The left side of the panel represents Inputs, the right side represents the co-immuno-precipitated proteins. Lanes 1,3,5,7,9, and 11 represents untreated sample; lanes 2,4,6,8, and 10: treatment with 8μM ETOP. Upper panel was probed with Sp1 specific antibody PEP2; lower panel with p53 specific polyclonal antibody.

4.3 Discussion/ Further Directions

Transcription studies suggested that DNA damage was not able to activate p53-dependent trans-activation in cells with Mdm2 over-expression from *mdm2* SNP309, but Nutlin successfully reactivated p53 function in these cells (Figure 25, panel A). However, in SJSA-1 cell line harboring *mdm2* gene amplification, both therapeutic strategies reactivated p53 and strikingly, etoposide induced up-regulation of p53 targets to a greater extent than Nutlin (Figure 25, panel A). These data indicate that SNP309 might confer a special phenotype that associates with Mdm2 over-expression. The phenotype manifests at the molecular level with no release of Mdm2 from chromatin-bound p53 following DNA damage.

In an effort to discover what distinguishes cells homozygous for *mdm2* SNP309 from other Mdm2 over-expressing cell lines, we started to look for other partners of the p53-Mdm2 chromatin-bound inhibitory complex. Mass spectrometry/ mass spectroscopy studies identified three other possible components: nucleolin, Rad50 and heat shock proteins HSP70 and HSP90.

We demonstrated no p53 nucleolar sequestration despite its association with the nucleolar protein. Nucleolin has previously been described to interact with p53 and translocate into the nucleoplasm after DNA damage (Daniely et al., 2002). It is possible that nucleolin is part of the chromatin-associated complex. Nucleolin plays a role in transcription, being a functional B-cell specific transcription factor (Tuteja and Tuteja, 1998) and may have a yet to be determined role in the p53 function.

Association of Mdm2 with Mre11-Nbs1-Rad50, a DNA double strand break repair complex, has been previously reported (Alt et al., 2005). Mdm2 binds to the Mre11-Nbs1-Rad50 complex in primary cells and in cells containing inactivated p53 or

p19ARF. Mdm2 directly associates with Nbs1 but not to Mre11 or Rad50 and amino acids 198-314 of Mdm2 are required for Mdm2/Nbs1 association. Mdm2 co-localizes with Nbs1 to sites of DNA damage following gamma-irradiation. Notably, Mdm2 over-expression inhibits DNA double strand break repair, and this is independent of p53 and ARF. The delay in DNA repair imposed by Mdm2 requires the Nbs1 binding domain of Mdm2. Although we did not identify other components of the Mre11-Nbs1-Rad50 complex in our co-immuno-precipitation experiments, this might be explained by a much lower Mdm2 input (we precipitated Mdm2 from 500 μg nuclear protein versus 10mg used by Alt and all) in a similar assay. Alt et all (Alt et al., 2005) also showed Rad50 as being the most abundant factor co-immuno-precipitating with Mdm2, although the interaction was not direct.

In previous studies (Peng et al., 2001) Mdm2 immuno-precipitation and Western-blot analysis showed that both HSP90 and HSP70 were co-precipitated with Mdm2 in cells expressing mutant p53 but not in cells with wild-type p53 or null for p53. The interaction is indirect and bridged by mutant p53. Conformational changes that result with mutant p53 often cause an association with chaperones such as HSP70 and HSP90 and it has been hypothesized that HSP may play a role in its stabilization (Hinds et al., 1987), (Selkirk et al., 1994). Inhibition of p53-HSP90 can lead to enhanced ubiquitination and degradation of mutant p53 (Whitesell et al., 1998), (Nagata et al., 1999). Therefore, mutant p53 may be resistant to degradation in part due to binding of heat sock proteins. We immuno-precipitated both HSP70 and HSP90 together with Mdm2 in SNP309 homozygous cells, results suggesting that molecular chaperones might be part of the p53-Mdm2 stable complex. It is possible that p53, although wild-type, to be in a mutant conformation that would impair its transcriptional activity and would also

facilitate its interaction with heat shock proteins and subsequent stabilization (as we detected high levels of basal p53 in MANCA cells).

The involvement of the newly identified partners (nucleolin, heat shock proteins, Rad50) in the regulation of p53 function will be studied. These proteins might be part of the chromatin-bound complex and they could also contribute to the inactivation of p53 transcriptional activity. We will perform chromatin immuno-precipitation experiments to detect if these proteins are also present in chromatin at the p53 responsive elements following DNA damage. p53 trans-activation activity will also be studied after down-regulation of these proteins using specific siRNA. This approach will allow us to detect if they play any inhibitory role in p53 function.

Mdm2 Western-blot also detected multiple bands in cells heterozygous and homozygous for SNP309 (Figure 11, panel B and Figure 26, panel C) suggesting that various *mdm2* isoforms might be produced from SNP309. Mdm2 splice variants have been reported to exhibit more oncogenic properties than the wild-type variants and expression of *mdm2* oncogenic splice variants occurs more frequently in high grade than low grade tumors (Bartel et al., 2002).

Although more than 40 *mdm2* isoforms have been identified, little is known about their functions. Initial studies by Sigalas et al. (Sigalas et al., 1996) demonstrated that the expression of *mdm2* splice forms in transfected NIH3T3 cells could grow as colonies in soft agar. These findings are supported by data from Steinman et al. who demonstrated that *mdm2-B*, which is the most prevalent isoform identified in tumors, can cause tumors independently of p53 (Steinman et al., 2004). *Mdm2-B* has been previously detected in high grade bladder and uterine cancers, lacks the p53-binding region present in full-length Mdm2, and is incapable of complexing with the p53 protein. *Mdm2-B* increases the

proliferation of transduced NIH3T3 cells without altering p53 stability, and also increases the proliferation of p53-null MEFs, suggesting that *mdm2-B* could cause cell proliferation via a p53-independent mechanism. In addition, *mdm2-B* interferes with cell death, and is able to induce foci formation in cultured cells, indicating that *mdm2-B* is oncogenic. Expression of *mdm2-B* in transgenic mice leads to spontaneous tumor formation in myeloid progenitor cells and B-lymphocytes.

Recent data from Fridman et al. show that the murine equivalents of the human *mdm2*-B, -D, and -E splice forms significantly accelerates lymphomagenesis in an Eμ-myc transgenic mouse model (Fridman et al., 2003). These data provide evidence that at least some *mdm2* isoforms can contribute to tumor development in an *in vivo* mouse model.

It has been suggested that *Mdm2* splice variants might have oncogenic properties due to their inability to bind to ARF.

Evans et al. (Evans et al., 2001) have shown that at least one splice variant (*mdm2*-B, Figure 25) encodes a protein that binds full-length Mdm2, resulting in sequestration of both proteins in the cytoplasm. This finding is important because alternatively and aberrantly spliced *mdm2* mRNAs are usually expressed together with full-length *mdm2* transcripts. Binding of Mdm2-B to full-length Mdm2 increases wild-type p53 activity (Evans et al., 2001). In soft tissue sarcomas, the expression of *mdm2* splice forms is also associated with an over-expression of mutant and wild-type p53 (Bartel et al., 2001). These findings suggest that p53 accumulation arises as a consequence of alternative and aberrant *mdm2* splicing independent of the mutational status of p53. Although wild-type p53 over-expression induced by Mdm2 isoforms is inconsistent with tumor progression, it is conceivable that the stabilization of mutant p53

might contribute to transformation and tumor growth. Although we detected high basal p53 protein levels in the MANCA cell line, p53 stabilization is unlikely to be caused by *mdm2* splice variants in *mdm2* SNP309 homozygous cells, since p53 was found to exist in a stable complex with Mdm2 protein. In addition, in the immuno-fluorescence experiments with Mdm2 staining, we detected Mdm2 protein in the nucleus, with no cytoplasmic shuttling.

(Brown et al., 1998) demonstrated that the full-length human oncoprotein Mdm2 expressed from its cDNA could arrest the cell-cycle progression of untransformed cells at the G0/G1 phase. Two growth inhibitory domains (ID1 and ID2) residing between amino acid residues 155 and 324 within the human Mdm2 protein and corresponding to exon 9 and 11 respectively, have been characterized. Elimination of this growth-inhibitory function converts the protein to a tumorigenic form. These domains are missing in a large number of Mdm2 isoforms. These variants could display an oncogenic function that overrides the growth-inhibiting phenotype and/or the apoptotic phenotype of full-length Mdm2 protein.

Sequencing of the *mdm2* variants expressed in *mdm2* wild-type cells (ML-1), *mdm2* SNP309 heterozygous (K562) or homozygous (MANCA and A875) cells, identified three new alternatively/aberrantly spliced isoforms, together with the previously described *mdm2*-F variant. *Mdm2*-F was found to be the most common *mdm2* form found in liposarcomas, alone or in association with other splice variants. This finding suggests an important functional role for this transcript in the transformation process. Even more, its oncogenic potential seems to be independent of the p53 pathway, since *in vitro* translation of the *mdm2*-F variant led to a protein that did not interact with p53 (Tamborini et al., 2001).

Although two of the newly discovered isoforms were present only in the *mdm2* SNP309 homozygous cell line MANCA, the relationship between alternative/aberrant *mdm2* slicing and homozygosity for SNP309 still needs further investigation. *In vivo* translation of the newly identified isoforms will also be carried out and the function of their protein products will be described. Splice variants that lack the ring domain (E3 ubiquitin ligase domain) are possible to be produced. This would interfere with p53 and Mdm2 proteasomal degradation and could explain the high Mdm2 and p53 protein levels in MANCA and A875 cells. Such splice variants, together with other proteins (nucleolin, HSP70), might stabilize p53 in a chromatin-bound transcriptionally inactive complex in *mdm2* SNP309 homozygous cells. The presence of these variants in chromatin, at the p53 responsive elements, will be studied using chromatin immuno-precipitation experiments. It is possible that only specific Mdm2 variants are able to bind *in vivo* to DNA, leading to epigenetic changes responsible for chromatin silencing. Such variants might lead to histone ubiquitination or could recruit histone deacetylases at the p53 dependent promoters. Sequential chromatin immuno-precipitation experiments will be performed to compare the level of histone ubiquitination in cell homozygous for *mdm2* SNP309 versus *mdm2* wild-type cells. It is also possible that the Mdm2 species present in chromatin have lost the domain responsible for interaction with p300 protein. This would interfere not only with the histone acetylation process but would also contribute to p53 stabilization.

Cells null for *mdm2* will be stably transfected with the *mdm2* isoforms that are expressed in SNP309 homozygous cells. The phenotype generated by different *mdm2* isoforms will be compared with the phenotype acquired after transfecion with the full-

length *mdm2* variant. This approach will allow the identification of those variants important for the process of tumor-genesis.

Knock-in gene targeting replacing the *mdm2* wild-type gene with *mdm2* SNP309 gene would be the ultimate experiment to prove that homozygosity for SNP309 plays a fundamental role in tumor formation. Using this genetic tool, isogenic cell lines that differ only at position 309 in the first intron of the *mdm2* gene will be created. It is expected that replacement of the wild-type gene with the SNP309 gene in cells expressing wild-type p53 protein would generate a phenotype identical to that observed in other SNP309 homozygous cells (eg. MANCA, A875 cells), in which p53 function is blocked through an inhibitory chromatin-bound complex. On the reverse side, substitution of both SNP309 alleles with the wild-type gene in cells homozygous for *mdm2* SNP309, should restore p53 transcriptional activity and sensitize them to DNA damage-induced apoptosis.

In our study we compared the wild-type p53 pathway in multiple tumor cell lines wild-type, heterozygous or homozygous for *mdm2* SNP309. In addition to their status for *mdm2* gene, in each of these cell lines different oncogenic pathways are activated. For instance, in MANCA cells a translocation t(8;14) occurs and places the *c-myc* proto-oncogene in front of the immunoglobulin heavy chain enhancer, leading to c-Myc over-expression (Saito et al., 1983). A875 cells express high levels of the nerve growth factor receptor (Fabricant et al., 1977) and SJSA1 cell line exhibit a 15 fold amplification of the *gli* proto-oncogene (Roberts et al., 1989). Although our data strongly suggest that Mdm2 is the inhibitor of the p53 function, as shown by restoration of its activity after Mdm2 down-regulation or disruption of the p53-Mdm2 complex, there is also a slight possibility that other pathways are responsible for the inability of these cells to respond to stress

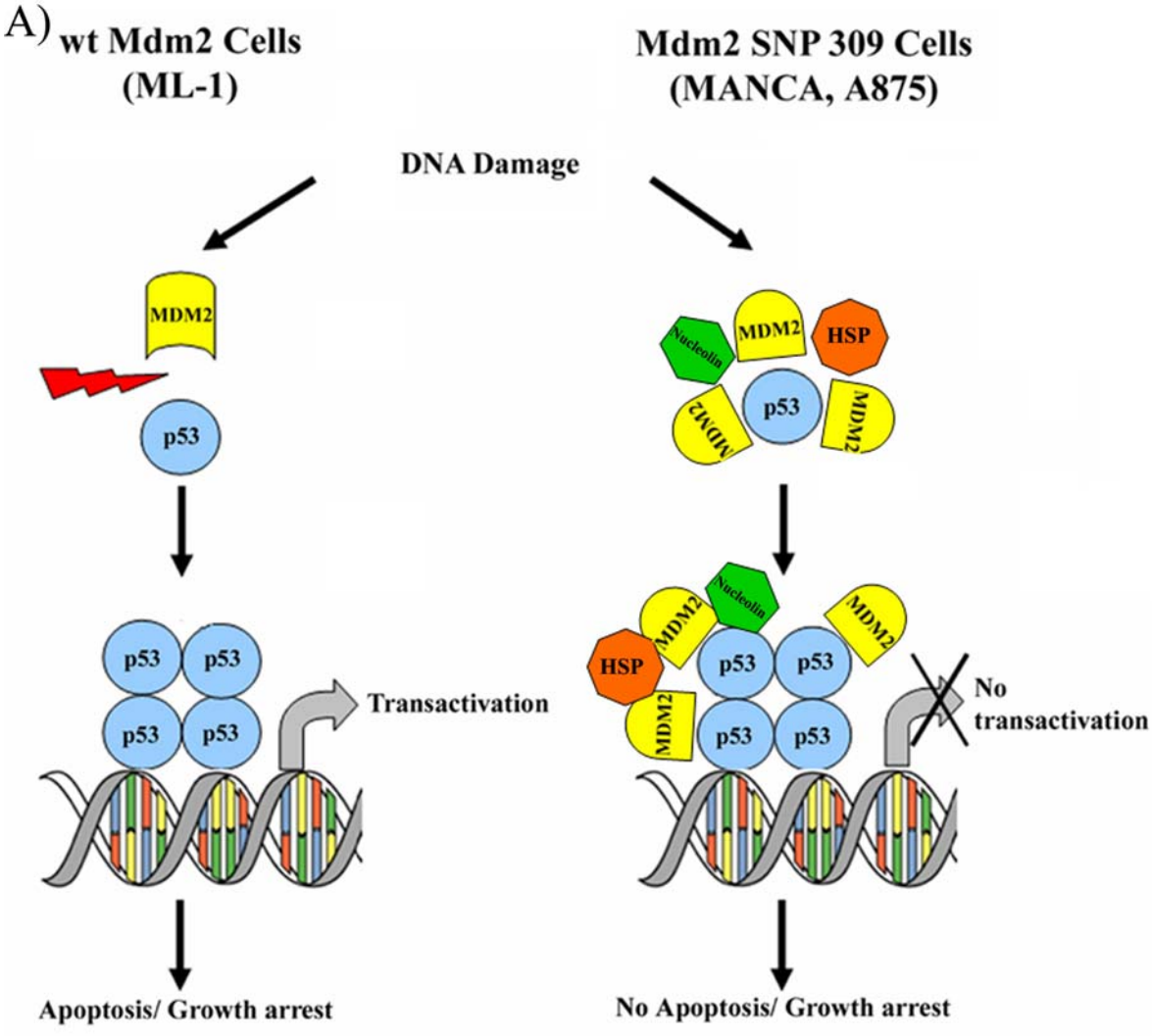
signals. Even if p53 was phosphorylated at Ser-15 residue in response to DNA damage, it is possible that other posttranslational modifications critical for p53 activity to be deficient in our cell lines. p53 acetylation by p300 protein has been shown to play a significant role in p53 activation, by increasing p53 DNA-binding ability, by inducing its stability, and enhancing its interactions with co-activators. The level of p53 acetylation and the interaction between p53 and p300 on chromatin at p53 responsive elements should also be investigated. The transcription factor Yin Yang 1 (YY1) has been reported to bind p53 and inhibit its transcriptional activity by interfering with the recruitment of p300 (Gronroos et al., 2004). In a similar manner, Mdm2 could also impede the p53-p300 interaction.

Activated oncogenes induce p53 pathway by up-regulating the ARF protein. Loss of ARF function through gene deletions, mutations or DNA methylation is frequently seen in cancers (Sherr, 2001) and is accompanied by an unrestrained Mdm2 activity, contributing indirectly to p53 inactivation. It would be interesting to analyze if our cell lines express ARF and if so, why ARF is not capable of disrupting the p53-Mdm2 interaction. If no expression is detected, would also be attractive to exogenously introduce ARF and study cell response in this new context.

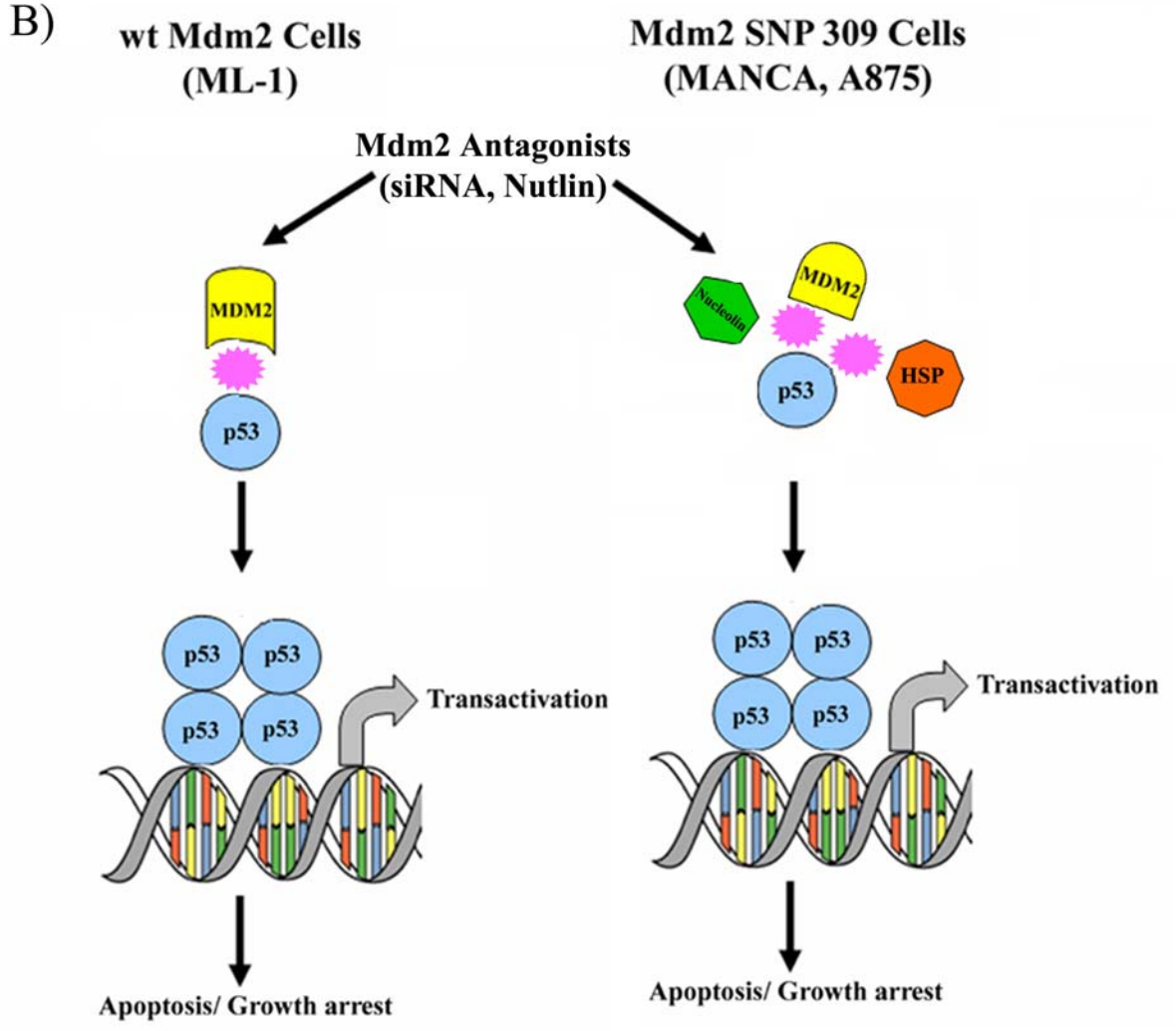
Our study concludes with a model where an inhibitory complex containing a particular Mdm2 protein isoform and also other partners (nucleolin, heat shock proteins) is present at the p53 responsive elements in cells homozygous for *mdm2* SNP309. This complex cannot be disrupted by DNA damage (Figure 30, panel A), but Mdm2 antagonists (Nutlin) are capable to dismantle the association, leading to p53 reactivation (Figure 30, panel B). If Nutlin completely removes Mdm2 from chromatin or Mdm2 is just transferred to a different molecule in chromatin will be investigated by carrying out

chromatin immuno-precipitation experiments following treatment of the cells with Nutlin. Regardless of how Nutlin reactivates p53, this compound proves to be a much more efficient therapeutic strategy for treating *mdm2* SNP309 homozygous patients.

Figure 30: A model illustrating that in cells homozygous for mdm2 SNP309 p53 transcriptional activity is blocked through a chromatin-bound inhibitory complex that might contain a particular Mdm2 isoform and also other partners (nucleolin, HSPs). DNA damage is not able to release p53 from this complex; however, Mdm2 antagonists (Nutlin) could reactivate p53 function.



B)


wt Mdm2 Cells
(ML-1)
Mdm2 SNP 309 Cells
(MANCA, A875)
Mdm2 Antagonists
(siRNA, Nutlin)
MDM2
p53
Nucleolin
MDM2
HSP
p53
p53
p53
p53
p53
Transactivation
Apoptosis/ Growth arrest
p53
p53
p53
p53
Transactivation
Apoptosis/ Growth arrest

Chapter 5: References

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Abbreviations

The abbreviations used are: SNP, single nucleotide polymorphism; CTP, camptothecin; ETOP, etoposide; MC mitomycin C; DMC 10-decarbamoyl-mitomycin C; Ab, antibody; FBS, fetal bovine serum; PBS phosphate-buffered saline; PARP, poly(ADP-ribose) polymerase; LLnL, *N*-acetyl-Leu-Leu-Nor-leu-al; RT, reverse transcriptase; AMV, avian myeloblastosis virus; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; EMSA, electrophoretic mobility shift assay; RGS, ribosomal gene cluster; ChIP, chromatin immunoprecipitation; FACS, fluorescence-activated cell sorter; PDAR, pre-developed assay reagents; siRNA, small interference RNA; SCS, superconsensus site; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HSP, heat shock protein; HAT, histone acetyl deacetylase; HDAC, histone deacetylase; Lys, lysine; STS, soft tissue sarcoma; CDK, cycline-dependent kinase; PCNA, proliferating cell nuclear antigen; PCAF, p300/CBP-associated factor; IAP, inhibitor of apoptosis protein.