Exploring Chromatin-bound MDM2 Functions in Compromised Transcriptional Regulation of p53 Target Genes

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Exploring chromatin-bound MDM2 functions in compromised transcriptional regulation of p53 target genes

By

Melissa Rosso

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

2015
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Abstract

Exploring chromatin-bound MDM2 functions in compromised transcriptional regulation of p53 target genes

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Melissa Rosso

Advisor: Dr. Jill Bargonetti

MDM2 overexpression is a common occurrence in many types of cancer. A single nucleotide polymorphism (T to G) near the mdm2 promoter, termed mdm2 SNP309, leads to MDM2 overexpression. This polymorphism is associated with accelerated tumor formation, decreased sensitivity to DNA damage treatment and compromised p53 transcriptional activity. Two G/G SNP309 cancer cell lines MANCA and A875, a Burkitt’s lymphoma and melanoma respectively, express a stable wild-type p53 protein. We previously reported these cells have DNA damage resistant MDM2-p53 chromatin complexes and hypothesized that MDM2 is the contributing factor for the compromised p53 transcriptional activity. We created constitutive mdm2 shRNA cell lines to address MDM2 function. MDM2 knockdown in MANCA and A875 cells moderately increased expression of subsets of p53 target genes in a cell-type specific manner; although no additive effect was seen with DNA damage. Additionally, MDM2 knockdown did not affect p53 protein stability. We explored the mechanism for compromised p53 transcriptional activity in G/G SNP309 MANCA and A875 cell lines using chromatin immunoprecipitation analysis. When compared to fully activated T/T SNP309 ML-1 cells (myeloid leukemia with functional wild-type p53) treated with etoposide, MANCA and A875 cell lines displayed comparable recruitment of total and initiated RNA polymerase II at transcription start sites (TSS) for p21 and puma genes. This indicated that G/G SNP309 cells had
functional transcription initiation at p53 target genes after DNA damage treatment. Next, we assessed transcriptional elongation using H3K36 trimethylation (H3K36me3) as a mark for active elongation. In ML-1 cells with etoposide treatment, we observed higher H3K36me3 at p21 and puma TSS than in either MANCA or A875 cells at these same regions. This suggested transcriptional elongation is compromised and also suggests that in G/G SNP309 cancer cells reactivation of p53 transcriptional activity is difficult. We sought to explain this phenomenon by examining the other well-known p53 negative regulator, MDMX, which is a homolog of MDM2. Interestingly, in MANCA cells the knockdown of MDM2 caused a substantial increase in MDMX protein levels. However, in A875 cells this was not observed. This suggests that for certain cell types, MDMX may function in some redundant roles for MDM2. Finally, we tested if inducing p53-independent cell death would be more effective than reactivating the wild-type p53 pathway. We treated the cells with 8-amino-adenosine, an inducer of p53-independent cell death. Indeed, treatment of MANCA and A875 cells with 8-amino-adenosine reduced cell viability more effectively than other chemotherapeutics.
Acknowledgements

I would like to thank my mentor Dr. Jill Bargonetti. She took a chance on me when I had little research experience. Her hard work and dedication to everything she does inspire me to always be better as a person and scientist. I am truly grateful for all her years of mentorship experience and look to her as a role model in many aspects of life.

I would like to thank my committee members for all of their valuable advice and feedback throughout the years on my graduate work.

I would like to thank all Bargonetti lab members who made going into work every day a pleasure. I am grateful for all of their support and friendship throughout my years in the laboratory. I am also grateful to the MBRS-RISE program for support as a graduate student at Hunter College. The office was a place of solace for me thanks to Janerie Rodriguez and Christina Medina-Ramirez.

I would also like to thank all of my friends and extended family, too many to name, who have been on the sidelines supporting me and cheering me on. I would not even be here without the unconditional love and support from my parents, Efrain and Maritza Rosso, who have helped me in ways I cannot even begin to describe. Finally, I would like to thank both of my grandmothers, Julia Gaudinot Santos and Minerva Torres, for taking care of me and inspiring me to pursue my goals.
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Chapter 1: Introduction
1.1 The Role of Tumor Suppressor p53 Protein

Since the p53 protein was discovered in 1979 it has become one of the most widely studied proteins (Linzer and Levine 1979). The p53 protein is well known for its important role as a tumor suppressor in cancer. This is highlighted by the fact that p53 is mutated in approximately 50% of all cancers and its pathway is inactivated in others (see reviews (Harris and Hollstein 1993; Vogelstein et al. 2000; Jin and Levine 2001)). Under non-stress conditions in cells, the p53 protein is kept at low basal levels due to rapid protein degradation (Haupt et al. 1997). Under stress conditions, such as DNA damage, oncogene activation, and hypoxia, the p53 protein becomes stabilized leading to varying cellular responses including cell-cycle arrest, DNA repair, apoptosis and senescence (Figure 1) (see reviews (Levine 1997; Jin and Levine 2001; Vousden and Lane 2007)). The tumor suppressive functions of p53 are generally the result of cellular responses that decrease cell proliferation and promote cell death. However, p53 has broader functions in development, metabolism and ageing (Vousden and Lane 2007).

**Figure 1: p53 is activated by varying stressors leading to numerous cellular responses**

From (Vousden and Lane 2007). Many different cell stressors (Blue) are able to activate p53 signaling leading to numerous cellular responses (Purple).
1.1.1) p53 Protein Structure

The p53 protein consists of 393 amino acids which can be divided into several functional domains (Figure 2) (see review (Levine 1997)). At the N-terminal region of p53 is a transactivation domain that interacts with the cellular basal transcription machinery (Fields and Jang 1990; Lu and Levine 1995; Thut et al. 1995; Farmer et al. 1996). This is followed by the proline-rich domain shown to have some involvement in mediation of apoptosis and anti-proliferative signals (Walker and Levine 1996; Venot et al. 1998). The central domain contains the DNA binding domain, which binds to sequence specific response elements (Bargonetti et al. 1991; Bargonetti et al. 1992). Most of the p53 mutations are found within in this domain (Harris 1993). The tetramerization domain allows p53 to form a tetramer thus increasing both the strength and conformation of p53-DNA complexes (Friedman et al. 1993; Wang et al. 1994; McLure and Lee 1998). Finally, the C-terminal regulatory domain is reported to regulate the sequence-specific DNA binding ability of p53 (Hupp and Lane 1994a; Hupp and Lane 1994b; Yakovleva et al. 2001; Weinberg et al. 2004).

**Figure 2: Schematic of p53 protein structure**

From (Bell et al. 2002). Represents a schematic of p53 protein structure with is given protein domains. The N-terminal region has the transactivation domain (TAD) followed by the proline-rich domain (Pro). The central region has the DNA binding domain (DBD). At the C-terminal region is the tetramerization domain (TD) followed by the regulation domain (RD).

1.1.2) Stabilizing p53 Protein via Cellular Stress

As described earlier, the p53 protein is kept at low basal levels under non–stress conditions, but becomes stabilized after cellular stress. Different kinds of cellular stress can lead
to stabilization of p53 for activation (Kastan et al. 1991; Graeber et al. 1996; Linke et al. 1996). The MDM2 protein is mainly involved in p53 stabilization via targeting p53 for ubiquitination and rapid degradation and will be discussed further in a later section (Haupt et al. 1997; Honda et al. 1997). The stress pathways usually regulate p53 through post-translational modifications with the kind of cellular stress determining which modifications are present (see reviews (Appella and Anderson 2001; Meek and Anderson 2009). The p53 protein can be post-translationally modified and hence regulated by many different proteins (Figure 3) (see review (Meek and Anderson 2009)).

**Figure 3- Post-translational modifications on p53 protein.**

Taken from (Meek and Anderson 2009). The figure shows the functional domains of p53 with the sites for post-translational modifications along modifying and de-modifying enzymes.

There are multiple residues on p53 that can be phosphorylated. In response to DNA damage, several important protein kinase cascades are activated that in turn are responsible for
phosphorylating p53 protein at the N-terminus including ATM, ATR, Chk2 and DNA-PK (see review (Meek and Anderson 2009)). In particular Serine 15 and 20 phosphorylation on p53, by the ATM and Chk2 pathway is reported for stabilization and transcriptional activation with conflicting reports as to whether it inhibits MDM2 binding (Siliciano et al. 1997; Canman et al. 1998; Dumaz and Meek 1999; Nakagawa et al. 1999; Unger et al. 1999; Hirao et al. 2000). The acetylation of p53 is also promoted as a mechanism for p53 stabilization with several key C-terminal residues that can either be acetylated or ubiquitinilated, but not both (Li et al. 2002). Oncogenic stress signaling can lead to cooperation of ARF and ATM/ATR signaling to p53 for apoptotic induction (Pauklin et al. 2005). Under hypoxic conditions, p53 can be stabilized by HIF-1α (An et al. 1998). Ribosomal stress is also reported to stabilize p53 through MDM2 binding to ribosomal proteins preventing p53 degradation (Linke et al. 1996; Choong et al. 2009; Holzel et al. 2010; van Leeuwen et al. 2011). Overall, the literature shows that p53 is able to respond to varying stressors through its interactions with numerous proteins.

1.1.3) p53 as a Transcription Factor

The major function for p53 is as a transcription factor of its numerous target genes that lead to tumor suppressive functions including cell cycle arrest and apoptosis (see reviews (Jin and Levine 2001; Vousden and Lane 2007; Kruse and Gu 2009; Beckerman and Prives 2010). The p53 protein binds to DNA at its target genes at sequence specific consensus sites, known as p53 response elements (p53RE), which consist of two copies of a 10 base pair (bp) motif 5'-PuPuPuC(A/T)(T/A)GPyPyPy-3’ with 0 to 13 bp separating this sequence (el-Deiry et al. 1992). This binding activates transcription via interactions with members of the basal transcription machinery (Lu and Levine 1995; Thut et al. 1995; Farmer et al. 1996). Interestingly, the p53RE sequence leads to differential transactivation with up to a 1,000 fold difference with the p21
5’p53 RE ranked highest and apoptotic gene p53RE’s having lower transactivation activity (Inga et al. 2002).

In response to stress conditions, the p53 protein is post-translationally modified and stabilized. The phosphorylation of Serine 15 at the N terminus of p53 is reported as a critical target for p53-dependent transactivation at p53 response elements (Dumaz and Meek 1999; Loughery et al. 2014). Acetylation on the C-terminus of p53, including lysines (K) 320,370,372,373 and 383 by p300/CBP and PCAF lead to enhancement of DNA binding activity (Gu and Roeder 1997; Liu et al. 1999). Additionally, site specific acetylation on p53 can regulate target gene expression to influence cellular response. For example, acetylation of K320 on p53 allows activation of genes with strong-affinity binding like p21, while p53 K373 acetylation enhances interaction with lower-affinity apoptotic genes (Knights et al. 2006).

1.1.4) Transcriptional Regulation of p53 Target Genes

The chromatin landscape of p53 target genes plays a fundamental role in their transcription. Analysis of the chromatin structure of promoter regions of p53 target genes p21,14-3-3α and KARP-1, by DNase I hypersensitivity assays, revealed that transcription start sites for these genes exist in an open conformation irrespective of p53 activation while the downstream elements are closed (Braastad et al. 2003). These genes all have p53 response elements localized near promoter regions. The P2 promoter region of the p53 target gene mdm2, which has p53 response elements localized closely upstream, exists in a nucleosome-free region (Xiao et al. 1998). Additionally, the chromatin structure of the p53 target gene gadd45 is not significantly altered upon transactivation (Graunke et al. 1999). This suggests that transactivation of p53 target genes does not massively change the chromatin structure at p53 response elements near promoter regions (Braastad et al. 2003).
Several histone modifying enzymes are known to interact with p53 protein. Histone acetyltransferases (HAT) including p300 and CBP along with co-activator TRRAP are recruited by p53 promoting acetylation of the surrounding histones at promoter regions of target genes (Lill et al. 1997; Barlev et al. 2001; Espinosa and Emerson 2001). Histone methyltransferases, PRMT1 and CARM1, interact with p53 and cooperate with p300 during transcription (An et al. 2004). Histone H3 and H4 acetylation increase at p53 target genes when p53 becomes activated by DNA damage (Kaeser and Iggo 2004). Therefore, p53 interaction with these histone modifying enzymes is implicated in transcriptional activation. p53 also recruits histone deacetylases, such as HDAC 1, for repressed transcription at specific genes (Juan et al. 2000).

Transcription initiation of p53 target genes varies depending upon the gene and the type of stress given for activation. The p53 protein assists in the recruitment several components of the pre-initiation complex including TFIID, TBP, TFIIA, and TFIIH (Lu and Levine 1995; Thut et al. 1995; Farmer et al. 1996; Ko and Prives 1996; Xing et al. 2001). The p21 gene has higher basal p53 recruitment and high levels of poised RNA pol II, while the fas promoter has low levels of poised RNA pol II before activation allowing these genes to respond differently upon DNA damage treatment (Espinosa et al. 2003). Therefore, specific genes can be more readily activated dependent upon whether there was p53 recruitment and initiated RNA pol II before addition of stress, which can directly contribute to the cellular response.

Factors involved in transcriptional elongation can also be directly linked to p53. The transcriptional elongation factor ELL, known to increase transcription rates of RNA pol II, interacts with p53 via binding to the C-terminal domain negatively regulating p53 transcriptional activity (Shinobu et al. 1999). The H3K36 methyltransferase SETD2, which trimethylates H3K36 for a transcriptional elongation mark, interacts with the p53 transactivation domain and
is implicated to selectively regulate the transcription of a subset of p53 target genes (Xie et al. 2008).

**Figure 4: p53 is involved in regulation of transcription initiation and elongation.**

![Diagram](image)

From (Beckerman and Prives 2010). The p53 protein is involved in regulation of transcription initiation and elongation since it interacts with general transcription factors (GTFs) in the pre-initiation complex, histone acetyltransferases (HATs), histone methyltransferases (HMTs) and elongation factors (EFs).

The p53 protein also directly interacts with HEXIM1 via the C-terminal domain to increase p53 stability by preventing MDM2-mediated degradation (Lew et al. 2012). HEXIM1 is a protein that negatively regulates the positive elongation factor-b (P-TEFb) to maintain it in an inactive state (Dulac et al. 2005). MDM2 ubiquitinates HEXIM1 leading to greater inhibition of P-TEFb transcriptional activity (Lau et al. 2009). There is an obvious interplay between p53, MDM2 and HEXIM1 for P-TEFb regulated transcription (see review (Lew et al. 2013)).
As seen in this preceding section, a fundamental understanding of eukaryotic transcription is necessary to see how p53 functions as a transcription factor. Additionally, p53 is involved in multiple steps of the transcription process (Figure 4). The following section addresses the basics of transcription for protein-coding genes and will provide important background information.

1.2 Eukaryotic Transcription of Protein-Coding Genes in Cells

1.2.1) The Stages of Transcription by RNA pol II

Transcription is an evolutionary conserved process that occurs in most known living organisms. At the most basic level, transcription is described as a process where a particular DNA sequence is complementarily made into RNA by the enzyme RNA polymerase, which for protein-coding genes in most eukaryotic cells starts with RNA polymerase II (RNA pol II). Transcription is divided up into several stages to include: pre-initiation complex (PIC) formation, promoter clearance, elongation and termination (Figure 5) (see reviews (Lemon and Tjian 2000; Peterlin 2006)).

Activation of transcription usually starts with binding of a transcription factor to a sequence-specific region of DNA that recruits general transcription factors (GTFs) and RNA pol II to form the pre-initiation complex in the promoter region of the given gene (see reviews (Sims et al. 2004; Geng et al. 2012)). A heptad repeat sequence on the C-terminal domain (CTD) of RNA pol II is post-translationally modified to signal for initiation and allow transcription elongation to occur (see reviews (Sims et al. 2004; Buratowski 2009)). One of the GTFs of the initiation complex, TFIIH, phosphorylates the CTD of RNA pol II on Serine-5 (S5) at this heptad repeat sequence (see reviews (Peterlin 2006; Buratowski 2009)). Due to being found primarily in the promoter regions of genes, the phosphorylated Serine-5 CTD of RNA pol II is linked to
transcription initiation (Komarnitsky et al. 2000). These post-translational modifications on RNA pol II leads to promoter clearance and a small amount of messenger RNA (mRNA) made near the transcription start site (see reviews (Peterlin 2006; Buratowski 2009)).

After promoter clearance, the RNA pol II is paused by the negative elongation factor (NELF) and DRB sensitivity inducing factor (DSIF) so that other elongation factors are recruited to form the transcription elongation complex (TEC). Additionally, mRNA capping enzymes are recruited to put a 5’ methylated cap on the RNA that was initially made (see reviews (Sims et al. 2004; Peterlin 2006)). The positive elongation factor complex-b, P-TEFb, transitions RNA pol II into its elongating form by phosphorylating DSIF and Serine-2 (S2) on the CTD of RNA pol II causing NELF to dissociate and allow for productive transcriptional elongation (see reviews (Sims et al. 2004; Peterlin 2006; Buratowski 2009)).

The transition from Serine-5 to Serine-2 phosphorylation on the CTD of RNA pol II represents signaling associated with early to later stages of transcriptional elongation because the Serine-2 modifications are found in coding regions of genes (Komarnitsky et al. 2000; Buratowski 2009). Phosphorylated serine-2 CTD RNA pol II increases with productive elongation of the transcript. Additionally, during this elongation phase there is recruitment of machinery necessary for splicing and polyadenylation of the resulting mRNA (see reviews (Sims et al. 2004; Peterlin 2006; Buratowski 2009)).
1.2.2) Regulation of the Chromatin Landscape during Transcription

Chromatin is a highly structured mixture of DNA and proteins found in the nucleus of cells. The basic subunit is the nucleosome composed of histones H2A, H2B, H3 and H4 formed in an octamer surrounded by approximately 150 bp of DNA (see reviews (Luger et al. 1997; Strahl and Allis 2000; Jenuwein and Allis 2001)). The amino-terminal region or “tail” of histones, and the DNA is subject to modifications associated with either gene activation or gene silencing (see review (Jenuwein and Allis 2001)). During the multiple stages of transcription, many changes occur to the chromatin landscape of the gene being transcribed.

When a transcription factor binds to a promoter region it signals for the recruitment of general transcription factors, histone modifying enzymes, and RNA pol II for transcription initiation (see reviews (Lemon and Tjian 2000; Zhang and Dent 2005; Berger 2007)). Total H3 and H4 acetylation by histone acetyl transferases (HATs), mainly p300/CBP, at promoter regions

From (Peterlin 2006): The stages of transcription shown as 1) Pre-initiation complex (PIC) formation, 2) promoter clearance by phosphorylation of S5 on the CTD of RNA pol II by TFIH, 3) recruitment of pausing factor NELF and DSIF and human capping enzymes (HCE) to methylate the 5’ cap of the nascent transcript and 4) phosphorylation of DSIF and S2 CTD RNA pol II for productive elongation and recruitment of splicing (SR) and polyadenylation (pA) machinery.
of genes is associated with activation of transcription initiation (see reviews (Santos-Rosa and Caldas 2005; Zhang and Dent 2005)). The initiated phospho-Serine 5 CTD RNA pol II is able to recruit histone methyltransferases (HMTs) that cause H3 K4 and K79 methylation, modifications also associated with transcription initiation (Figure 6) (see reviews (Santos-Rosa and Caldas 2005; Berger 2007; Buratowski 2009). The elongating phospho-Serine 2 CTD RNA pol II recruits SETD2, a HMT that trimethylates H3K36 (H3K36me3), a chromatin modification associated with active transcriptional elongation (Figure 6) (Morris et al. 2005; Sun et al. 2005; Berger 2007; Edmunds et al. 2008; Buratowski 2009). The histone modification, H2B K120 ubiquitination and subsequent deubiquitination, are critical for the transition between initiation and elongation during transcription (see reviews (Henry et al. 2003; Berger 2007; Geng et al. 2012)).

**Figure 6: Chromatin Modifications associated with Transcription**

From (Berger 2007). A) Represents Histone post-translational modifications (PTMs) associated with transcription initiation and early transcription elongation at transcription start sites and in the open reading frame (ORF). H2B K123 represents the yeast version of H2B K120 seen in mammalian cells. B) Represents Histone PTMs associated with later elongation and attenuation of transcription. Abbreviations: ac-acetylated, dac-deacetylated, me-methylated, ub-ubiquitinated, dub-deubiquitinated.
The nucleosomes are critically involved in transcription. In order for RNA pol II to move through a gene that is transcribed, the histones ahead of the elongating complex need to move out of the way, known as nucleosome/histone eviction, otherwise transcription may be blocked (Schwabish and Struhl 2004; Workman 2006). Histones are in a state of flux during transcription. There is continuous partial or total disassembly/reassembly of H2A/H2B dimers and H3/H4 tetramers of the nucleosome during elongation (Thiriet and Hayes 2006; Workman 2006). In fact, some highly transcribed genes experience nucleosome loss and/or replacement with histone variants, such as histone H3.3, that are more easily moved during transcription (see reviews (Hake et al. 2006; Workman 2006; Park and Luger 2008)). Chromatin remodeling complexes including SWI/SNF and FACT, with the help from histone chaperones such as nucleoplasm and nucleolin, assist in removing H2A/H2B dimers and H3/H4 tetramers so the elongation complex can pass through the given gene (Laskey et al. 1993; Chen et al. 1994; Saunders et al. 2003; Angelov et al. 2006; Workman 2006; Schwabish and Struhl 2007; Eitoku et al. 2008; Park and Luger 2008). Now we will focus our attention on the main negative regulators of the p53 protein.

1.3 MDM2

1.3.1) MDM2 Discovery and Regulation

The mdm2 gene was originally discovered in the 3T3DM cell line, a spontaneously transformed line derived from mice, whereby increased copy number of the gene provided a growth advantage to cells (Cahilly-Snyder et al. 1987; Fakharzadeh et al. 1991). MDM2 is mainly characterized as the E3-ubiquitin ligase for p53 (Haupt et al. 1997). In many human cancers, MDM2 protein can be found overexpressed by three different mechanisms: gene amplification, increased transcription and enhanced translation (Oliner et al. 1992; Bueso-Ramos
et al. 1993; Landers et al. 1994). The human *mdm2* gene spans a region of approximately 33kb and has 12 exons under regulation by two different promoters (Oliner et al. 1992; Iwakuma and Lozano 2003). The P1 promoter gives basal levels of *mdm2* transcript and the P2 promoter is inducible increasing levels of *mdm2* transcript when the cells are exposed to stress (Barak et al. 1994; Zauberman et al. 1995). Multiple binding sites exist in the P2 promoter comprising Ets/Ap-1, E-box, RXR, p53, Smad binding sites and GC boxes (see review (Manfredi 2010)). This allows numerous transcription factors to regulate the P2 promoter including p53, estrogen receptor (ER), Smad 2/3, MYCN, RXRγ and Sp1 (Zauberman et al. 1995; Bond et al. 2004; Slack et al. 2005; Bond et al. 2006; Xu et al. 2009; Araki et al. 2010). Interestingly, *mdm2* transcripts derived from the P2 promoter have more translation potential (Barak et al. 1994).

**1.3.2) MDM2 Protein Structure**

The full length MDM2 protein consists of a 491 amino acid polypeptide with a calculated mass of 54kDa (Figure 7) (Fakharzadeh et al. 1991; Chen et al. 1993). However, products of larger sizes exist as seen through the use of human *mdm2* full length cDNA and *in vitro* translation experiments (Chen et al. 1993). The MDM2 protein is divided up into several domains. At the N-terminal region of MDM2, there is a p53-binding domain which binds to the p53 transactivation domain (Chen et al. 1993). In addition, the N-terminal region of MDM2 has sequence homology to the SWIB domain present in the SWI/SNF chromatin remodeling complex (Riccardo Bennet-Lovsey 2002). The central region contains nuclear localization and export signals involved in MDM2 nuclear-cytoplasmic shuttling (see review (Lozano and Montes de Oca Luna 1998; Roth et al. 1998)). There are also acidic and zinc finger domains that can bind many proteins including ribosomal proteins (see review (Chen et al. 2007; Manfredi 2010; Zhang et al. 2010)). The C-terminal region has a RING finger domain that is mainly
responsible for its ubiquitination activity (Boddy et al. 1994; Honda et al. 1997). There is also a cryptic nucleolar localization signal in the C-terminal region (Lohrum et al. 2000). The functions of some of these domains are known to be evolutionarily conserved. (Marechal et al. 1997). The important conserved regions throughout various species include the central acidic domain, known to interact with ribosomal proteins, zinc finger and RING finger domains and the N-terminal p53 binding domain (Marechal et al. 1997).

**Figure 7: Schematic of full-length MDM2 protein**

Adapted from (Okoro et al. 2012). Schematic represents full-length MDM2 protein. At the N-terminal region is the p53 transactivation domain, which has SWIB domain homology. In the central region is the nuclear localization signal (NLS) and nuclear export signal (NES). This is followed by the Acidic and Zinc Finger domains. At the C-terminal region is the RING finger domain and also a nucleolar localization signal (NoLS). The corresponding exons are found above the specified domains.

1.3.3) Alternative/Aberrant Splicing and Isoforms

The *mdm2* gene can produce mRNA in variously spliced forms from either alternative or aberrant splicing (Bartel et al. 2002). Over 40 *mdm2* transcripts have been identified (Bartel et al. 2002). Stressful conditions promote increased alternative splicing of *mdm2* transcripts and alternatively spliced variants are seen in many late stage and aggressive tumors (Sigalas et al. 1996; Lukas et al. 2001; Weng et al. 2005; Chandler et al. 2006; Dias et al. 2006; Sánchez-Aguilera et al. 2006; Lents et al. 2008). Interestingly, the most commonly found and studied *mdm2* variants, *mdm2*-a,-b and –c, are missing exons that encode the p53 binding domain (reviewed in (Okoro et al. 2012)). This implies that these variants have p53-independent functions. Five splice variants, *mdm2* A-E, are able to form protein by *in vitro* translation experiments (Sigalas et al. 1996). However, it is unknown whether all *mdm2* splice variants are
able to form protein \textit{in vivo}. Recently, the MDM2-C isoform was detected as endogenously expressed protein in cancer cells and tissue with a newly developed antibody against the exon 4-10 splice junction amino acid sequence (Okoro et al. 2013). Future work regarding the functions of these endogenous MDM2 isoform proteins may give a more thorough understanding of MDM2 functions in the cell.

1.3.4) The MDM2-p53 Interaction

The high significance of the MDM2-p53 interaction was first discovered using a knockout mouse model. Loss of MDM2 expression leads to embryonic lethality (Jones et al. 1995; Montes de Oca Luna et al. 1995). However, loss of the \textit{p53} gene in mice that have had the \textit{mdm2} gene knocked out is able to rescue the embryonic lethal phenotype demonstrating that MDM2 is required for \textit{p53} regulation (Jones et al. 1995; Montes de Oca Luna et al. 1995). MDM2 is able to regulate \textit{p53} at both the mRNA and protein level (Honda et al. 1997; Naski et al. 2009). Interestingly, the MDM2-\textit{p53} mRNA interaction can enhance or repress translation dependent on the stress applied to the cell via direct binding by the RING finger domain or through the ribosomal protein RPL26 (Candeias et al. 2008; Ofir-Rosenfeld et al. 2008; Naski et al. 2009; Gajjar et al. 2012).

The MDM2 protein is mainly characterized as a negative regulator of the \textit{p53} tumor suppressor protein. The most well-known function of MDM2 is its E3-ubiquitin ligase activity responsible for the ubiquitin-mediated degradation of \textit{p53} via the proteasome (Haupt et al. 1997; Honda et al. 1997). A delicate balance exists between MDM2 and \textit{p53}. The \textit{p53} protein functions as a transcription factor for the \textit{mdm2} gene forming a negative feedback loop (Juven et al. 1993; Wu et al. 1993; Zauberman et al. 1995). The levels of both proteins have been described in a
mathematical model where MDM2 and p53 oscillate allowing a coordinated response to various cellular stresses regulating the feedback loop (Lev Bar-Or et al. 2000).

MDM2 is also characterized to negatively regulate p53 by inhibiting p53 transcriptional activity (Momand et al. 1992; Thut et al. 1997). The well-characterized interaction between the p53 binding domain of MDM2 with the p53 transactivation domain at the N-termini of both proteins is believed to inhibit p53-mediated transactivation (Momand et al. 1992). Additionally, earlier work also suggested a dual mechanism for MDM2 inhibiting p53 transactivation by binding directly to TFIIE and interacting with the p53 transactivation domain to inhibit recruitment of basal transcription machinery (Thut et al. 1997). Pharmacological inhibitors have been made targeting the interaction between the p53 binding domain of MDM2 and the p53 transactivation domain. Nutlin-3, a well-known small-molecule inhibitor, can reactivate p53 signaling in numerous cancer types with MDM2 overexpression (Vassilev et al. 2004; Tovar et al. 2006).

MDM2 co-localizes with p53 on chromatin at p53 target genes (Minsky and Oren 2004). It has been proposed that at p53 REs, cells with basal MDM2 expression have MDM2-p53 complexes on the chromatin that are disrupted with DNA damage treatment to allow transcriptional activation (White et al. 2006). However, transcriptional repression is recovered when the complex is reformed (Figure 8A) (White et al. 2006). Cells that overexpress MDM2 due to a single nucleotide polymorphism (T to G) near the P2 promoter (mdm2 SNP309) have MDM2-p53 complexes on chromatin that are unaffected by DNA damage treatment (Arva et al. 2005). Therefore, it was hypothesized that continued presence of these MDM2-p53 chromatin complexes may block transcription of important downstream apoptotic and cell cycle arrest p53 target genes (Figure 8B) (Arva et al. 2005).
1.3.5) MDM2 Chromatin Functions

As previously mentioned, MDM2 can bind to chromatin when in complex with p53 at p53 REs (Minsky and Oren 2004; Arva et al. 2005; White et al. 2006). However, MDM2 is also reported to interact with chromatin and other chromatin-bound proteins besides p53. The MDM2 N-terminal domain has sequence homology with the SWIB domain, a part of the SWI/SNF chromatin remodeling complex (Riccardo Bennet-Lovsey 2002). This implies MDM2 binds directly to DNA and may be responsible for chromatin remodeling. MDM2 can bind to Nbs-1, a protein that is a part of the DNA repair MRN complex that binds to sites of DNA damage (Alt et al. 2005). Binding of MDM2 to Nbs-1 can delay DNA damage repair facilitated by the MRN complex (Alt et al. 2005). The RING finger domain of MDM2 is reported to ubiquitinate histone
H2B (Minsky and Oren 2004). Interestingly, the RING finger domain of MDM2 can also bind to nucleotides directly (Poyurovsky et al. 2003; Priest et al. 2010). The histone methyltransferases SUV39H1 and EHMT1 are reportedly recruited by MDM2 to facilitate methylation of H3K9 and p53 to regulate p53-mediated transcription (Chen et al. 2010). MDM2 interacts with the histone deacetylase HDAC-1 to mediate deacetylation of p53 (Ito et al. 2002). Interestingly, the MDM2-HDAC-1 interaction on chromatin can also reduce transcription by the Androgen receptor (AR) family of transcription factors (Gaughan et al. 2005). Additionally, MDM2 binds to KAP1 contributing to p53 inactivation (Wang et al. 2005). KAP1 is a protein involved in general transcriptional regulation due to its ability to recruit numerous chromatin-remodeling proteins (Iyengar and Farnham 2011; Iyengar et al. 2011). This provides some evidence that MDM2 may have broader implications in global chromatin functions.

1.4 MDMX

1.4.1) MDMX Discovery and Regulation

MDMX, also known as MDM4, was identified in a mouse cDNA library screen as a novel p53 binding partner (Shvarts et al. 1996). It is encoded by the mdmX/mdm4 gene in humans and mice with a transcript comprised of 11 exons (Shvarts et al. 1996; Shvarts et al. 1997). Several transcription factor binding sites are characterized in the human mdmX promoter region including c-Ets-1, Elk-1 and Aml-1. All are deemed critical for increased MDMX expression in a panel of tumor cell lines (Gilkes et al. 2008). Oncogenic K-Ras and insulin-like growth factor-1 (IGF-1) induce mdmX transcriptional activation in an MAPK/ERK-dependent manner showing MDMX expression can be regulated by mitogenic signaling (Gilkes et al. 2008). In 2006, a p53-binding site was identified within the first intron of the mdmX gene (Wei et al. 2006). Luciferase reporter constructs with this mdmX intron 1 p53-binding site are
responsive to wild-type p53 in a co-transfection assay (Li et al. 2010). Similar to the *mdm2* gene (both human and mouse), the *mdmX* gene has its p53-binding site localized near its P2 promoter (Phillips et al. 2010).

1.4.2) Structural and Functional Comparison of MDMX and MDM2

The MDMX protein was originally identified as a p53-binding protein with some functional properties of MDM2 (Shvarts et al. 1996). Full-length MDMX consists of 490 amino acids and similar to MDM2 has p53-binding, acidic, zinc and RING finger domains (Figure 9) (Shvarts et al. 1996; Shvarts et al. 1997; Blaydes 2010). Human MDMX protein is characterized to have high sequence homology to human MDM2 protein particularly in the N-terminal p53-binding domain and RING finger domain (Shvarts et al. 1997). However, unlike MDM2, MDMX does not have nuclear localization and export signals. This is associated with the mostly cytoplasmic localization of MDMX protein in cells and requirement of MDM2 to translocate MDMX into the nucleus for p53 inhibition (Migliorini et al. 2002). Heterodimerization of MDM2 and MDMX proteins via their RING finger domains stabilizes MDM2 causing more efficient p53 ubiquitination (Sharp et al. 1999; Tanimura et al. 1999). Interestingly, homodimerization of MDM2 has decreased stability compared to heterodimers of MDM2 and MDMX proteins (Sharp et al. 1999; Tanimura et al. 1999). The RING finger domain of MDMX, despite high sequence homology to MDM2, has very little or no E3-ubiquitin ligase activity (Badciong and Haas 2002; Linke et al. 2008). It is suggested that the increase in E3-ligase activity by the MDMX/MDM2 heterodimer, generally when cells are under non-stress conditions, may be due to stabilization of the UbcH5b E2 protein when MDMX is present (Badciong and Haas 2002; Kawai et al. 2007; Poyurovsky et al. 2007; Linke et al. 2008; Lenos...
and Jochemsen 2011). Additionally, MDM2 E3- ligase activity also functions to promote ubiquitination and degradation of MDMX (Pan and Chen 2003).

**Figure 9-Comparison of MDMX and MDM2 protein domains**

![Figure 9](image)

From (Blaydes 2010). Shows the similarity of the domains between MDM2 and MDMX proteins. Both proteins share p53 binding domains, acidic, zinc-finger and RING finger domains. However, MDMX does not have nuclear localization or export signals.

### 1.4.3) The MDMX-p53 Interaction

Multiple regulatory molecules are necessary to control p53 because of its critical function in cells. In addition to MDM2, the MDMX protein regulates p53 activity. Overexpression of MDMX contributes to tumorigenesis via inhibition of p53 tumor suppressor activity (Danovi et al. 2004). Mice that are *mdmX/*4 null are embryonic lethal while still retaining the *mdm2* gene. The lethality is rescued by additional deletion of the *p53* gene showing some non-redundant MDM2 and MDMX regulatory functions (Parant et al. 2001). Specific deletion of either gene in tissue-specific regions in mice reveals that both MDM2 and MDMX proteins have different functions dependent upon cell type (reviewed in (Wade et al. 2010)). Due to the lack of E3-ubiquitin ligase activity, MDMX is more strongly associated with inhibition of p53 transcriptional activity via binding with the p53 transactivation domain (Shvarts et al. 1996). This interaction is also reported to inhibit p53 and p300 binding thereby decreasing p53
acetylation necessary for transactivation (Sabbatini and McCormick 2002). Similar to MDM2, MDMX protein can be found on chromatin at p53 REs (Tang et al. 2008).

The MDMX protein plays a critical role in the p53 response to cellular stress. MDM2 and MDMX work together to regulate p53 activity under cellular stress conditions. Upon DNA damage treatment, there is MDM2-mediated degradation of MDMX resulting in p53 induction (Kawai et al. 2003). Several groups have reported that ATM and Chk2-dependent phosphorylation of MDMX at several C-terminal serine (S) residues contribute to MDM2-mediated degradation of MDMX (Chen et al. 2005; Pereg et al. 2005; Pereg et al. 2006). Phosphorylation of MDMX at tyrosine 99 (Y99) by c-Abl after DNA damage causes dissociation and p53 activation (Zuckerman et al. 2009). Chk1 induced phosphorylation of MDMX at S367 enhances association with 14-3-3γ resulting in increased MDMX cytoplasmic localization and p53 activation (Jin et al. 2006). Interestingly, DNA damage induced phosphorylation of MDMX at S367 was also linked to p53 activation via MDM2-mediated degradation (Okamoto et al. 2005).

1.4.4) Splice Variants and Isoforms of MDMX

The *mdmX* gene can produce transcripts of varying sizes in addition to the full length form. To date, a total of six transcript variants have been identified in tumor tissue and/or tumor cell lines (Mancini et al. 2009). The first transcript variant identified, called *mdmX*-s, results from an internal deletion in exon 6 creating a stop codon and produces a transcript which retains exons only encoding the p53 binding domain (Rallapalli et al. 1999). This variant and isoform, MDMX-S, potently binds to p53 inhibiting p53 transactivation better than MDMX-FL (Rallapalli et al. 1999; Rallapalli et al. 2003). Other characterized variants *mdmX*-a, *mdmX*-g
and \textit{mdmX}-211 reportedly interfere with MDM2 stabilization and degradation functions (de Graaf et al. 2003; Giglio et al. 2005; Mancini et al. 2009). Genotoxic cellular stress conditions also produce alternative splice variants of \textit{mdmX}--ALT-1 (missing exons 5 and 10) and ALT-2 (missing exon 3 and 10), respectively (Chandler et al. 2006). It is unknown if all \textit{mdmX} splice variants are translated into protein isoforms. However, several smaller MDMX protein isoforms were identified in a panel of tumor cell lines (Ramos et al. 2001). This indicates that the variants may be represented in these identified MDMX protein isoforms.

\subsection*{1.5) Chemotherapeutics and MDM2 Overexpressing Cancers}

\subsubsection{1.5.1) Types of Cancer with MDM2 overexpression}

When overexpressed, MDM2 has the potential to cause several malignancies from different primary sites. Indeed, there are over 40 different types of malignancies that can have MDM2 overexpression by increased protein and/or mRNA expression or genomic amplification (see review (Rayburn et al. 2005)). These include, but are not limited to, carcinoma of the brain, esophagus, breast, thyroid, lung, liver, ovaries, bladder, colon, cervix and prostate, melanoma, soft tissue sarcoma, osteosarcoma, and several hematological malignancies including leukemia and lymphomas (Rayburn et al. 2005). Interestingly, MDM2 overexpression can signal for either a better or worse prognosis dependent upon the cancer type making MDM2 a controversial prognostic marker (see reviews (Onel and Cordon-Cardo 2004; Rayburn et al. 2005)).

Most clinicians are unsure of what high MDM2 expression means because MDM2 has both p53-dependent and independent capabilities and functions in diverse cellular processes. This is clearly seen for cases of soft tissue sarcomas with MDM2 overexpression, where there are conflicting reports about how MDM2 correlates with survival and prognosis (Wurl et al. 2004).
in hematological malignancies there is a more definitive link between MDM2 overexpression and poor prognosis, worse survival and resistance to chemotherapy (Gustafsson et al. 1998; Moller et al. 1999; Zhou et al. 2000; Gustafsson et al. 2001). For breast cancers, overexpression of MDM2 is reported to correlate with a worse prognosis (Turbin et al. 2006). However, MDM2 overexpression is generally associated with estrogen receptor (ER) positive cancers which are generally seen to have better prognosis than ER negative breast cancers (Reiner et al. 1990; Berger et al. 1991; Hideshima et al. 1997; Kim et al. 2011). Studies have shown that ER positive breast cancer cells work through MDM2 to increase proliferation (Brekman et al. 2011; Kim et al. 2011). Other studies have shown that MDM2 overexpression is associated with metastases of different tumor types through mechanisms including upregulation of vascular endothelial growth factor (VEGF) and matrix metalloprotease-9 (MMP-9) (Zietz et al. 1998; Patterson et al. 2011; Chen et al. 2013). Finally, there are links between MDM2 overexpression and decreased response to chemotherapy and radiation. In patients and several cell lines with MDM2 overexpression, there are reports of increased resistance to DNA damage (Suzuki et al. 1998; Cocker et al. 2001; Rodriguez-Lopez et al. 2001; Rotterud et al. 2001; Nayak et al. 2007). All the literature points to MDM2 having multiple roles in cancer (Figure 10) (Rayburn et al. 2005).
MDM2 has multiple roles in cancer and cancer therapy.

Figure 10- MDM2 has multiple roles in cancer and cancer therapy.

1.5.2) Chemotherapeutic DNA damage agents used to treat cancers

A plethora of chemotherapeutic anti-cancer agents that target rapidly dividing cells exist for treatment of many tumor types. What these agents mostly have in common is the ability to directly or indirectly cause DNA damage. There are several classes of DNA damage agents which are divided into: alkylating agents, anti-metabolites, and topoisomerase poisons (see review (Cheung-Ong et al. 2013)). Some common drugs that are used in the clinic today include, but are not limited to, cisplatin (alkylating agent), camptothecin, doxorubicin and etoposide (topoisomerase poisons). All have the ability to elicit a DNA damage response (DDR) by activating ATM and ATR kinases which can signal to p53 for activation of cell cycle arrest or cell death (see reviews (Nitiss 2002; Woods and Turchi 2013)).

Taken from (Rayburn et al. 2005). MDM2 can confer metastasis and resistance to chemotherapy and radiation. It has multiple roles in cancer through p53-dependent and independent activities.
In particular, etoposide is classified as a poison for topoisomerase II, an enzyme responsible for binding to DNA at breaks, having the ability to stabilize the DNA-topoisomerase II complex (see reviews (Champoux 2001; Nitiss 2002; Montecucco and Biamonti 2007)). The reported cytotoxic effects are due to this ability causing massive amounts of cellular DNA damage (Montecucco and Biamonti 2007). Due to production of double-strand breaks, etoposide treatment of cells activates the ATM kinase and eventually leads to formation of foci of the Mre11/Rad51/Nbs-1 (MRN) complex involved in DNA repair (Robison et al. 2005). The activation of the ATR-Chk1 pathway is necessary to inhibit DNA replication after etoposide treatment and involves regulation by Nbs-1 (Rossi et al. 2006). The activation of these DNA damage response pathways by etoposide involves cell cycle checkpoint regulation and apoptotic signals, which can work through the p53 pathway (Canman et al. 1994; Kastan et al. 1995; Canman et al. 1998; Nitiss 2002; Montecucco and Biamonti 2007).

1.5.3) Targeted MDM2 inhibitors

Many small-molecule inhibitors in development are targeting the p53-binding domain of MDM2 to prevent an MDM2-p53 interaction (see review (Vu and Vassilev 2011)). One of the first class of potent inhibitors of this interaction called Nutlins, in particular Nutlin-3a, was discovered to reactivate wild-type p53 signaling in some MDM2 overexpressing cancers (Vassilev et al. 2004; Tovar et al. 2006). A modified version of Nutlin-3, RG7112, reported more potent than Nutlin-3 can activate p53 signaling (Tovar et al. 2013; Vu et al. 2013). It is the one of the first MDM2 inhibitors in clinical trials and has already shown some efficacy in patients in a Phase1b trial for acute myeloid leukemia (Martinelli et al. 2013). Another class of spirooxindole-structure compounds called the MI compounds, in particular MI-63, was reported to have similar cell growth inhibition results compared to Nutlin-3 (Vassilev et al. 2004; Ding et
Finally, there are inhibitors that target the RING finger domain of MDM2 to inhibit p53 degradation that need to be developed further (Herman et al. 2011).

1.5.4) Potential of therapeutics that can induce p53-independent cell death

After DNA damage, including chemotherapy and/or radiation treatment, patients can develop resistance and the recurring cancer will be more aggressive than it was before treatment. In many of these cases, there is inactivation of the wild-type p53 pathway either by mutation and/or overexpression of MDM2 or MDMX (Buttitta et al. 1997; Laframboise et al. 2000; Nayak et al. 2007; Jin et al. 2010; Knappskog and Lonning 2012). MDM2 is suggested as a contributor to chemo-and radioresistance (see review (Rayburn et al. 2005)). The targeted chemotherapeutics developed for MDM2 work through activation of p53 signaling although MDM2 has many p53-independent functions (see reviews (Ganguli and Wasylyk 2003; Rayburn et al. 2005; Vu and Vassilev 2011)). There are reported cases of cancers having both MDM2 overexpression and mutant p53 (Cordon-Cardo et al. 1994). Additionally, having wild-type p53 does not always correlate to a better prognosis (Taylor et al. 2000). Therefore, in cases where wild-type p53 cannot be activated through DNA damage or inhibition of MDM2, other treatment options need to be available irrespective of p53 status.

Recently, a nucleoside analogue called 8-amino-adenosine was found to induce p53-independent cell death in metastatic breast cancer cells with mutant p53 (Polotskaia et al. 2012). It was also reported as a potential therapeutic for multiple myeloma because of its cytotoxic effects similar to the RNA-directed actions of 8-chloro-adenosine (Stellrecht et al. 2003; Krett et al. 2004). 8-Amino-adenosine can inhibit general transcription of cells and interfere with RNA metabolism (Frey and Gandhi 2010). Interestingly, the kind of cell death induced by 8-amino-
adenosine varies dependent upon the cell line (Polotskaia et al. 2012). The use of agents that induce p53-independent cell death may function better than standard DNA damaging agents (or MDM2 inhibitors) for cancers with MDM2 overexpression and inactive p53 protein.
Chapter 2: Materials and Methods
2.1 Cell Culture – ML-1 cells were a generous gift from Michael Kastan and the MANCA cells were a generous gift from Andrew Koff. A875 cells were a generous gift from the Arnold Levine laboratory. ML-1, MANCA and A875 cells were grown in RPMI 1640 medium (Mediatech) supplemented with 10% fetal bovine serum (Gemini) and 50U/ml of penicillin and 50μg/ml of streptomycin (Mediatech) in a 5% CO₂ 37°C humidified incubator. Suspension cells (ML-1 and MANCA) for experiments were seeded at 2.5 x 10⁵ cells/mL. Phoenix packaging cells were a generous gift from Scott Lowe. The packaging cells were grown in DMEM (Cellgro) with 10% fetal bovine serum and 50U/ml of penicillin and 50μg/ml of streptomycin in a 5% CO₂ 37°C humidified incubator. MANCA and A875 MLP and MLP/shMDM2 cells were grown under constant selection pressure in complete RPMI media supplemented with 2μg/mL or 3μg/mL of Puromycin.

2.2 Drug Treatments – Cell were treated with: 8μM etoposide (ETOP) (SIGMA) dissolved in sterile DMSO (SIGMA) or sterile DMSO for 6 hours, 5nM Actinomycin D (ActD) (SIGMA) for 24 hours or 15μM 8-aminoadenosine (8AA) for 24 hours dissolved in sterile water.

2.3 Bacterial Culture and Molecular Cloning

2.3.1) Plasmids - The pSM2c plasmids with shRNA, MLP.1224, STGM PGK PURO, pMSCV-rtTA3-PGK-Hygro and Helper expression plasmids were generous gifts from Scott Lowe.

2.3.2) Bacterial Growth - *Echeresia coli* were grown on LB (Luria-Bertani (1% Bacto-tryptone, 0.5% Bacto-yeast extract, 1% NaCL)) - Ampicillin (100μg/ml))-Agar plates overnight at 37°C. Colonies were grown in LB-Broth with Ampicillin (50μg/ml) for 8 hours or overnight at 37°C.

2.3.3) Mini/Maxi preps - Qiagen Mini/Maxi prep kits were used according to manufacturer’s protocol.
2.3.4) Plasmid DNA cloning - Cloning shRNA sequences into MLP.1224 and STGM PGK PURO (MLP and STGM) vectors were done by Jill Bargonetti, Angelika Brekman, Alla Polotskaia and Mandeep Kaur. The shRNA sequences for mdm2, mdmX and p53 were cloned from pSM2c vectors into XhoI and EcoRI restriction sites in miR30 sequence of MLP and STGM vectors.

2.3.5) Digestions - MLP and STGM vectors were digested with EcoRI and XhoI (New England Biolabs, Inc.). Digestions performed with 10X NE EcoRI buffer, BSA, 20U/µL of restriction enzymes and nuclease free water as according to manufacturer’s protocol. Samples were incubated for 3 hours at 37°C followed by heat inactivation at 65°C for 20 min.

2.4 Cellular protein extracts

2.4.1) Whole cell extract - Cells were pelleted at 1100 rpm for 7 min at 4°C, washed three times with ice-cold PBS and resuspended in RIPA buffer (0.1% SDS, 1% IGEPAL NP-40, 0.5% Deoxycholate, 150mM NaCl, 1mM EDTA, 0.5mM EGTA, 50mM Tris-Cl pH 8.0, 1mM PMSF, 8.5µg/ml Aprotinin, and 2µg/ml Leupeptin). The cell suspension was incubated on ice for 30 to 60 minutes to lyse the cells, vortexing occasionally. Additional sonication of lysate for 3 times for 30 seconds/ 30 seconds rest on ice at 98% amplitude was done when deemed necessary. Samples centrifuged at 13,000 rpm for 20 min at 4°C. The supernatants were stored at -80°C.

2.4.2) Cytosolic and nuclear extracts - Cells were pelleted at 1100 rpm for 7 min at 4°C and washed three times with ice-cold phosphate buffered saline (PBS). Cells were then resuspended in 5 packed cell volumes of Buffer A (10 mM Hapes; 1.5 mM MgCl2; 10 mM KCl; 0.5 mM PMSF; 0.5mM DTT; 2µg/ml Leupeptin; and 8.5µg/ml Aprotinin), centrifuged at 1100 rpm for 7 min at 4°C, resuspended in 2 packed cell volumes of Buffer A. The samples were passed through
a 20½ G needle several times and incubated for 10 min on ice. Then the samples were centrifuged at 13,000 rpm for 10 min at 4°C and the supernatant was removed to give the cytoplasmic fraction, which was stored at -80°C. The remaining pellets were resuspended in 1 packed cell volume of Buffer C (20mM Heps; 25% Glycerol; 420mM NaCl; 1.5mM MgCl₂; 0.2mM EDTA; 0.5mM PMSF; 0.5mM DTT; 2µg/ml Leupeptin; 8.5µg/ml Aprotinin) and passed through a 20½ G needle several times. The samples were then rocked for 30 min at 4°C and centrifuged for 30 min at 13,000 rpm at 4°C. The supernatant constituted the nuclear fraction and was stored at -80°C.

2.4.3) Chromatin Fractionations - Cells were pelleted at 1100 rpm for 7 min at 4°C and washed three times with ice-cold phosphate buffered saline (PBS). Cells were suspended in Buffer A (10mM HEPES pH 7.9, 10mM KCl, 1.5mM MgCl₂, 0.34M Sucrose, 10% glycerol, 1mM DTT, 0.5mM PMSF, 2µg/ml Leupeptin, 8.5µg/ml Aprotinin) + 0.1% Triton X-100. Incubated on ice 5 min. Spun down cells 3600 rpm for 5 min at 4°C. Spun down supernatant for an additional 5 min at 13,000 rpm at 4°C to clarify (S1 Fraction). Washed pellet 2 times with Buffer A spinning down at 3600 rpm for 5 min at 4°C. Resuspended nuclei pellet in Buffer B (3mM EDTA, 0.2mM EGTA, 0.5mM PMSF, 2µg/ml Leupeptin, 8.5µg/ml Aprotinin). Samples were incubated on ice 30 minutes with vigorous vortexing every 5 minutes and then spun down 4000 rpm for 5 min at 4°C. The supernatant is nuclear soluble proteins (S2 Fraction) and the pellet is enriched in chromatin. Washed pellet 2 times with Buffer B. Resuspended pellet (P3 Fraction) in RIPA buffer and sonicated 3 times 30 sec/30 sec rest on ice. Froze samples at -80°C.

2.5 Western Blot analysis – Cell lysates were resuspended with lysis buffer and 6X Laemmli Buffer with 12% β-Mercaptoethanol (final concentration 1X) and heated at 95°C for 7 minutes. For resolution of MDM2 and MDMX proteins the lysate was resuspended in 4X NuPAGE LDS
buffer (Invitrogen) and DTT so that final concentrations for components were 1X for NuPAGE buffer and 20mM DTT. The samples were heated at 70°C for 10 minutes and Iodoacetamide was added to a final concentration of 100mM before loading onto gel. Without Iodoacetamide the MDM2 did not run as a sharp band but rather as a large fuzzy band. The samples were then separated by use of 8%, 10%, 12%, 14% or 4-12% (Invitrogen) SDS-PAGE followed by an electro-transfer to nitrocellulose membrane or PVDF membrane for smaller proteins and blocked with 5% milk solution in 0.1% Tween 20–PBS. The membranes were probed with primary antibodies overnight at 4°C. Membranes were washed 3 times with 0.1% Tween 20 - PBS solution. Secondary HRP-conjugated antibodies applied in 1% milk solution of 0.1% Tween 20 – PBS for 1 hour at room temperature followed by an additional three washes of 0.1% Tween 20–PBS solution. The signal was detected by chemiluminescence. Actin-HRP (Santa Cruz) antibody was used in 1% milk solution of 0.1% Tween 20 –PBS for 90-120 minutes at room temperature.

2.6 RNA isolation and quantitative RT-PCR – Cells were centrifuged at 1100 rpm for 7 min at 4°C, washed three times with ice-cold PBS. The RNA was isolated using QIA shredder columns and the RNeasy Mini Kit (Qiagen) following manufacturer’s protocol. RNA was stored at -80°C. 1-5µg of RNA was used for cDNA synthesis using the High Capacity cDNA Archive Kit reagents and protocol (Applied Biosystems), where the RT master mix (containing RT buffer, dNTPs, random primers and MultiScribe reverse transcriptase) along with the RNA was incubated at 25ºC for 10 min and then at 37ºC for 2 hrs. cDNA was stored at -20ºC. Amplification of gene transcripts by quantitative PCR with primer probes for p21(Hs00355782_m1), pig3(Hs00153280_m1), puma (Hs00248075_m1), fas (Hs00538709_m1), gadd45a (Hs00169255_m1), mdm2(3-4) (Hs01069930_m1), mdmx (Hs00910358_s1), and gapdh (4333764) (Applied Biosystems Assays on Demand) combined
with the cDNA and Taqman Universal Master Mix was carried out following the program: one cycle, 2 min, 50°C; one cycle, 10 min, 95°C; and 40 cycles, 15 seconds, 95°C and 1 minute 60°C in a 7500 Sequence Detection System or ViiA7 sequence detection system (Applied Biosystems).

2.7) Immunoprecipitations - 0.5-1 mg of protein was isolated out from protein extracts and adjusted to equal volumes with either Co-IP or RIPA lysis buffer. We used Co-IP lysis buffer when wanted to co-immunoprecipitate proteins. Antibody (2-5µg) was added to samples for overnight rotating incubation at 4 °C. Next day 50µl of 25% bead slurry of Protein A/G+ beads (Santa Cruz) was added to samples and incubated for an additional 2 hours at 4 °C. Spun down 2000 rpm for 3min at 4 °C and then washed beads three times with 1mL of lysis buffer. Added 5X Lammeli buffer (with 10% β-Mercaptoethanol) or 4X NuPAGE buffer (diluted to 2X with components listed in Western blot) to beads and ran SDS-PAGE and western blot analysis. Used either Co-IP lysis buffer (50mM Tris-HCl pH 7.4, 50mM NaCl, 1% Triton X-100, 1mM PMSF, 8.5µg/ml Aprotinin (SIGMA), and 2µg/ml Leupeptin, 1/100 dilution phosphatase inhibitor cocktail 3 (SIGMA) (optional)) or RIPA buffer (0.1% SDS, 1% IGEPAL, 0.5% Deoxycholate, 150 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM Tris-Cl pH 8.0, 1 M PMSF, 8.5µg/ml Aprotinin (SIGMA), and 2µg/ml Leupeptin, 1/100 dilution phosphatase inhibitor cocktail 3 (SIGMA) (optional)).

2.8 Chromatin Immunoprecipitation (ChIP) – Cells were cross-linked for 10 min at 37°C in 1% formaldehyde followed by shaking 5 min at room temperature with 0.125 M Glycine addition to quench the reaction. Cells then washed four times with cold 1X PBS; spun down at 1100 rpm, 7 min at 4°C for suspension cells and 2000 rpm for 15 min at 4 °C for adherent cells; resuspended in 1X RIPA Buffer (0.1% SDS, 1% IGEPAL, 0.5% Deoxycholate, 150mM NaCl,
1 mM EDTA, 0.5 mM EGTA, 50 mM Tris-Cl pH 8.0, 1 M PMSF, 8.5 µg/ml Aprotinin (SIGMA), and 2 µg/ml Leupeptin, 1/100 dilution phosphatase inhibitor cocktail 3 (SIGMA), incubated for 30 minutes on ice; sonicated on ice to shear chromatin into approximately 3500-150 bp fragments; and spun down at 13,000 rpm for 20 min at 4°C. Immunoprecipitations carried out overnight using 400 µg of protein. For p53, Mdm2, RNA pol II and Cdk9 antibodies used 2 µg of antibody per reaction and for H3K36me3 antibody used 5 µg per reaction. The next day, pre-washed in PBS and pre-blocked with 0.3 mg/ml herring sperm DNA (Invitrogen) for 30 min at 4°C, Protein A/G Plus Agarose beads (Santa Cruz) were added for 2 hrs with rocking at 4 °C. For the Covance H5 antibody, used 3 µL (3-5 mg/mL - approximately 9-15 µg) of antibody with 50 µL of anti-mouse IgM Agarose beads (Sigma) diluted to 25% bead slurry (blocked with 1mg/mL BSA and 0.3mg/mL herring sperm DNA). Then the samples were spun down for 2 min at 3,000 rpm at 4°C and were washed with (1) 0.1% SDS, 1% Triton-X, 20 mM Tris pH 8.1, 150 mM NaCl, (2) same as Wash 1 but NaCl is 500 mM, (3) 0.25 M LiCl, 1% IGEPA, 1% Na Deoxycholate, 1 mM EDTA, 10 mM Tris pH 8, (4) TE pH 8, (5) same as Wash 4; every time spinning the beads down between each wash. To obtain DNA from these samples for PCR, added 100 µL of 1X Elution buffer (0.1 M NaHCO3 in 1% SDS) and 1 mg/mL Proteinase K (SIGMA), and incubated overnight at 65°C. DNA fragments were purified using Qiagen PCR Purification kit (Qiagen) according to manufacturer’s protocol and were amplified by quantitative PCR.

Used primers designed to amplify the gene regions p21 5’ p53 binding site and TATA box and puma p53 binding sites/ transcription start site. Sequences for sites are given below for primers and Taqman probes (Applied Biosystems) as described in (Kaeser and Iggo 2002; Kaeser and Iggo 2004).
p21: p53RE (5’) Forward – 5’GTGGCTCTGATTTGGCTTTCTG-3’
  Reverse- 5’CTGAAAAACAGGCGACCCAAG-3’
  Probe- 5’- TGGCATAGAAGAGGGCTGTTGCTATTCTT-3’
  TATA box Forward- 5’CGCGAGGATGCGTGTTC-3’
  Reverse – 5’CATTCACCTGCGCAGAAAA-3’
  Probe - 5’CGGGTGTGTGC-3’
puma p53RE: Forward- 5’-GCAGACTGTGCGCCTTGCT-3’
  Reverse- 5’-CGTCCAGGGTCCACAAAGT-3’
  Probe – 5’- TGTGAGTACATCCTCTGGCTCTTG-3’

Used primers designed to amplify other regions of p21 and puma genes with SYBR Green Dye (Applied Biosystems). Below is a chart of primers used described in (Gomes et al. 2006; Gomes and Espinosa 2010).

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>p21</td>
<td>CCTGGCTGACTTCTGCTGTCT</td>
<td>CCGCGTTGGAGTGGTAGA</td>
</tr>
<tr>
<td>p21</td>
<td>CCTCCCACAATGTGGTAAATACAG</td>
<td>AGTCATGCTGAGCACC</td>
</tr>
<tr>
<td>puma</td>
<td>AGGTGCTGCTCCGCCA</td>
<td>CCCTCTGCCTCTCCAAGGTC</td>
</tr>
<tr>
<td>puma</td>
<td>ACATGATTTTGATATTTCTTTTCAATA</td>
<td>AAAGAGTATCCACTGTTCCAATCTGA</td>
</tr>
</tbody>
</table>

2.9 Antibodies

2.9.1) Western Blot - Mix of mouse monoclonal supernatant antibodies 421, 1801 and 240 for p53; a mix of mouse monoclonal supernatant antibodies 4B2, 2A9 and 4B11 for MDM2; (H-224) rabbit polyclonal RNA Pol II (Santa Cruz); (Ab-1) mouse monoclonal anti-p21 (Calbiochem); Mdm2C 4-10 rabbit polyclonal custom developed by immunizing rabbit with the C4-10 peptide (Biosynthesis, Inc.); rabbit polyclonal MDMX (Bethyl); (C-20) rabbit polyclonal Cdk9 (Santa Cruz); (H14) mouse monoclonal phosphorylated Serine 2 CTD RNA pol II (Covance); mouse monoclonal β-Tubulin (Sigma), rabbit polyclonal Fibrillarin (abcam); goat polyclonal H3 (Santa Cruz); rabbit polyclonal Histone H2B (abcam); rabbit polyclonal anti-actin (Sigma); Secondary anti-mouse, anti-rabbit and anti-goat/sheep HRP antibodies (Sigma);

2.9.2) ChIP - (DO-1) mouse monoclonal anti-p53 (Calbiochem), (N-20) rabbit polyclonal Mdm2 (Santa Cruz); (H-224) rabbit polyclonal Pol II (Santa Cruz); (H5) mouse monoclonal
phosphorylated Serine 5 CTD RNA pol II (Covance); (C-20) rabbit polyclonal Cdk9 (Santa Cruz); goat polyclonal H3 (Santa Cruz); ; rabbit polyclonal anti-RNA polymerase II CTD repeat (phospho S2) ChIP grade (ab5095-abcam); rabbit polyclonal anti-Histone H3K36me3 ChIP grade (ab9050-abcam); Goat IgG (Santa Cruz); Rabbit IgG (Santa Cruz); Mouse IgG (Santa Cruz); Mouse IgM isotype control (Sigma)

2.10 Generation of stable and inducible shRNA cell lines

For stable shRNA knockdown cell lines, infected cells with MLP vector with shRNA using MLP empty vector as control. For doxycycline-inducible shRNA knockdown cell lines, used STGM vector with shRNA co-infected with rtTA3. Doxycycline binds to rtTA protein and together they bind to the doxycycline-regulated promoter (TRE) to induce shRNA expression. Used STGM empty vector co-infected with rtTA3 as control. Both MLP and STGM vectors are Puromycin resistant and rtTA3 is Hygromycin resistant. GFP expression was used to assess shRNA expression.

2.10.1) shRNA sequences

<table>
<thead>
<tr>
<th>Clone ID</th>
<th>Position</th>
<th>shRNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mdm2 151656</td>
<td>1793…1811</td>
<td>CTGTCCTATAAGAGAATTAT</td>
</tr>
<tr>
<td>mdmx 13023</td>
<td>246…264</td>
<td>GGTGAAATGTTCACTGTATA</td>
</tr>
<tr>
<td>p53 2120</td>
<td>2120…2139</td>
<td>GAGGATTTTCATCTCTTGTATA</td>
</tr>
</tbody>
</table>

2.10.2) Transient Transfection of Phoenix Packaging cells to make Retrovirus - Phoenix packaging cells were plated at 5 x 10⁶ cells per 10 cm plate and allowed to incubate (12-24 hours) overnight. Changed media on packaging cells with 9 ml of complete DMEM media supplemented with 25µM chloroquine. Mixed together a DNA solution of 20µg retroviral DNA
(Either MLP or STGM vector with/without shRNA or rtTA3 vector) and 5µg of Helper plasmid DNA with 2M CaCl2 in 0.01M HEPES pH 5.5 and sterile water into 2X BBS (50mM BES, 280mM NaCl, 1.5mM Na2HPO4 at pH 6.95) dropwise with bubbling. Waited 15 minutes for precipitate to form and added 1 mL of this mixture into the packaging cells and incubated plates overnight (no longer than 18 hours). Next day, changed media on packaging cells in the morning and in the evening (7 mL per 10 cm plate) and let incubate overnight. The next day from packaging cells, at five hour intervals, collected media containing virus and replaced with fresh media and filtered through 0.45µm syringe filter to either use or freeze at -80ºC.

2.10.3) Retroviral-mediated Gene transfer

**MANCA cells** – Used viral containing media and added 2µg/mL polybrene (Millipore) to make infection cocktail. Counted MANCA cells using a hemocytometer and resuspended 1.0 x 10^6 cells into 2mL of infection cocktail and added to a six-well plate. The cells in the plate were spun down at 1200 rpm for 120 minutes at 30ºC using a plate rotor. After the spin, the cells were collected in 15mL falcon tubes by spinning down at 1100 rpm for 7 minutes and resuspended in 4mL of complete RPMI media. After transferring to tissue culture flasks, the cells were allowed to grow for 24-72 hours and the drug selection was started.

**A875 cells** - The cells were seeded at approximately 30% confluency the day before infection into a 6 well plate. On the day of infection, combined viral containing media with 4µg/ml polybrene for infection cocktail and added to wells (2mL/well). The plate was spun down at 1800 rpm for 45 min at room temperature and then incubated in a 37ºC incubator for 4 hours. Replaced the infection cocktail in the wells with fresh infection cocktail and proceeded to spin down the cells again at 1800 rpm for 45 minutes. This was followed by an additional 4 hour incubation at 37ºC. The infection cocktail was removed from the cells and replaced with fresh
media. After growing overnight (12-24 hours), the cells were then split into a new plate and allowed to grow for 24 hours. This was followed by the drug selection.

2.10.4) Drug selections of Retroviral-infected cells - After infection both MANCA and A875 cells were selected with either Puromycin or Hygromycin B. Before this was done a kill curve was performed for each of the antibiotics to find the minimum dose that could be tolerated for selection. For MANCA cells, a kill curve was performed with Puromycin comparing the following concentrations 0, 1, 2, 4, 6, and 8µg/mL over a seven day period. The same was done for the MANCA cells with Hygromycin B comparing the following concentrations 0, 400, 500, 600, 700, and 800µg/mL over a seven day period. For A875 cells, a kill curve was performed with Puromycin comparing the following concentrations 0, 1, 2, 3, 4 and 5µg/mL over a seven day period. The same was done for Hygromycin B at the following concentrations 0, 300, 400, 500, 600, 700, 800, and 1000µg/mL over a seven day period. Cell counts using a hemocytometer and/or MTT assay were used for cell viability along with visual microscopic examination.

The following doses and time points were selected for each cell line:

MANCA cells - 2µg/mL of Puromycin for five days

500µg/ml of Hygromycin B for seven days

A875 cells - 3µg/ml of Puromycin for three days

600µg/ml of Hygromycin B for seven days

Double infected MANCA cells with constitutive and inducible mdm2 and mdmx shRNA and rtTA3, selected cells in media with 2µg/ml of Puromycin and 500µg/ml Hygromycin B for seven days.
2.11 MTT Assay - MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) stock solution (5mg/ml in clear HANKs media) was added to treated cells in volume equal to 10% of culture medium. Let cells incubate in 37°C incubator until color developed. Added MTT solubilization solution (90% anhydrous isopropanol, 10% Triton X-100, and .826% 12.1N HCl) to volume equal to original culture medium and pipetted to mix to dissolve formazan crystals. Gently shook for 5 min at room temperature and then analyzed samples with plate spectrophotometer. The absorbance was measured at 550nm and the 620nm absorbance was subtracted for background to allow for quantification.
Chapter 3: G/G \textit{mdm2} SNP309 cancer cells display compromised transcriptional elongation of p53 target genes
3.1- Introduction

MDM2 overexpression is found in over 40 different tumor types (Rayburn et al. 2005). A single nucleotide polymorphism (SNP) at position 309 of the mdm2 gene leads to increased mdm2 transcription and is linked to increased cancer incidence, aggressiveness and accelerated tumor development (Bond et al. 2004; Post et al. 2010). A nucleotide change from thymine to guanine increases the binding affinity of the ubiquituous transcription factor, Sp1, for a site near the P2 promoter of the mdm2 gene (Bond et al. 2004). Patients characterized as homozygous G/G at SNP 309 manifest earlier age of onset of cancer and increased susceptibility to certain cancer types in a gender and hormone specific manner (Bond et al. 2004; Bond et al. 2006). G/G mdm2 SNP 309 cancer cell lines are reported to have an attenuated p53 stress response (Bond et al. 2004). These cells are also described to have resistance to topoisomerase II- targeted chemotherapeutic DNA damaging drugs, compromised p53 transcriptional activity after DNA damage treatment and a disruption of p53-MDM2 oscillations in response to cellular stress (Bond et al. 2004; Arva et al. 2005; Hu et al. 2007; Nayak et al. 2007). This implicates MDM2 overexpression in G/G SNP309 cancers as a driver for increased cancer incidence and chemoresistance to standard DNA damage treatment through inhibition of wild-type p53 signaling.

An integral part of wild-type p53 signaling occurs through its function as a transcription factor. MDM2-p53 chromatin complexes contribute to down-regulation of p53 transcriptional activity (Arva et al. 2005; White et al. 2006). After DNA damage, cells with basal-level MDM2 expression can activate p53 recruitment to chromatin and transactivation of p53 target genes. The MDM2 found in complex with p53 at p53 responsive elements (p53REs) is released upon activation of DNA damage (White et al. 2006). After the initial DNA damage response has
passed, MDM2-p53 complexes reform on chromatin and are associated with repressed p53-mediated transcription (White et al. 2006). Homozygous G/G mdm2 SNP309 cancer cells have excess levels of MDM2 protein and MDM2-p53 chromatin complexes that are not sensitive to DNA damage treatment, and this correlates with the inhibition of p53 transcriptional activity (Arva et al. 2005). Three hours of etoposide treatment in various G/G SNP309 cancer cell lines results in increased MDM2-p53 chromatin complexes compared to wild-type T/T cells (Arva et al. 2005). ML-1 (T/T) cells are suitable for comparison to the G/G SNP309 MANCA and A875 cancer cell lines to examine the molecular signaling involved in compromised p53 transcriptional activity (Arva et al. 2005). Six hours of DNA damage treatment in ML-1 cells, by various agents, represents a critical point in p53 stabilization and peak of transcriptional activity (Abbas et al. 2002; Arva et al. 2005; White et al. 2006). All work done for this thesis was performed at the critical six hour time point to examine compromised p53 transcriptional signaling seen in G/G SNP309 MANCA and A875 cell lines. These cell lines and the ML-1 cell line are described in the table below (Table 1). The purpose of this study was to investigate the mechanism of compromised p53-dependent transcription in G/G SNP309 cancer cells and what role chromatin-bound MDM2 may play in transcriptional regulation.

**Table 1- Cell Lines Examined**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>p53 status</th>
<th>mdm2 SNP309 status</th>
<th>Cell Type</th>
</tr>
</thead>
<tbody>
<tr>
<td>ML-1</td>
<td>wild-type</td>
<td>T/T (wild-type)</td>
<td>chronic myeloid leukemia</td>
</tr>
<tr>
<td>MANCA</td>
<td>wild-type</td>
<td>G/G (homozygous)</td>
<td>Burkitt lymphoma</td>
</tr>
<tr>
<td>A875</td>
<td>wild-type</td>
<td>G/G (homozygous)</td>
<td>Melanoma</td>
</tr>
</tbody>
</table>
3.2 Results

3.2.1) G/G SNP309 cancer cell lines display compromised p53 transcriptional activity and MDM2-p53 complexes on chromatin.

Treatment of G/G SNP309 cancer cell lines with various DNA damaging agents yields compromised transcription of p53 target genes relative to wild-type cells including \textit{p21}, \textit{gadd45} and \textit{fas} (Arva et al. 2005). Additionally, G/G SNP309 cancer cells maintain higher basal levels of wild type p53 that is not degraded efficiently (Arva et al. 2005). We previously published work showing that MANCA and A875 cells have compromised apoptotic signaling as compared to ML-1 cells after 24 hours of DNA damage treatment by etoposide, camptothecin and mitomycin C. FACS analysis shows that, only in ML-1 cells, treatment with DNA damage agents significantly increases sub-G1 populations of cells representing apoptotic cell death (Arva et al. 2005). Therefore, we were interested in comparing apoptotic p53 target genes such as 
\textit{puma}. Using quantitative reverse transcription-polymerase chain reaction (qRT-PCR), we looked at transcript levels of five common p53 target genes in ML-1 cells vs. MANCA and A875 cells after six hours of DNA damage treatment by etoposide (Figure 11). In addition to the p53 target genes \textit{p21}, \textit{gadd45} and \textit{fas}, transcription of the other genes \textit{puma} and \textit{pig 3} was compromised in MANCA and A875 cells in comparison to ML-1 cells. However, the response for the gene \textit{pig 3} was higher in A875 cells as compared to MANCA cells (Figure 11). These data support our previous findings that G/G SNP309 cancer cells display abnormally low p53 transcriptional activity after DNA damage for several types of p53 target genes.
Figure 11 - G/G SNP309 MDM2 overexpressing cells display compromised wild-type p53 transcriptional activity after DNA damage.

ML-1, MANCA and A875 cells were treated with DMSO or 8 μM etoposide for 6 hours. Time point is reflected as the highest point of p53 transcriptional activity in the ML-1 cells. Quantitative RT-PCR for p53 target genes was normalized to gapdh as loading control. Values represent average of six independent experiments with standard error bars for MANCA cells. The number of independent experiments for ML-1 cells varies per gene with n for p21 equal to 5, gadd45 equal to 3, fas equal to 3, puma equal to 4 and pig 3 equal to 5 given with standard error bars. Values for A875 cells represent an average of three independent experiments with standard error bars. The graph is shown on a logarithmic scale.

In G/G SNP309 cancer cells, the p53 protein binds DNA and is phosphorylated at Serine 15, a post translational modification associated with transcriptional activation at p53 response elements (Dumaz and Meek 1999; Arva et al. 2005; Loughery et al. 2014). In contrast to G/G SNP309 cancer cells, ML-1 cells have transcriptionally active p53 protein and less MDM2 protein localized at p53RE’s for p21 and mdm2 target genes after three hours of etoposide treatment (Arva et al. 2005). We assessed the recruitment of p53 and MDM2 to chromatin after a six hour-etoposide treatment, since six hours is the peak for p53 stabilization and transcriptional...
activity in ML-1 cells. All cell lines were examined under these conditions as compared to the three hour time point for \( p21 \) and \( puma \) genes. As expected, ML-1 cells showed increased p53 recruitment to \( p21 \) and \( puma \) p53 response elements after DNA damage by etoposide (Figure 12A). In MANCA cells, there was also increased p53 recruitment after DNA damage at p53 responsive elements for both genes. A875 cells had higher basal p53 recruitment at the \( p21 \) gene than at \( puma \), but both genes had trends of DNA damage induced p53 recruitment (Figure 12A).

MDM2 was also recruited to these sites in all three cell lines. We observed some MDM2 recruitment in ML-1 cells at \( p21 \) and \( puma \) genes associated with the large increase in p53 recruitment after etoposide treatment (Figure 12B). This correlates with the data published for MDM2 recruitment back to chromatin after 6 hours with p53 to re-repress transcription of the \( mdm2 \) gene (White et al. 2006). MANCA cells had a small significant increase in MDM2 recruitment after DNA damage at the \( p21 \) gene, with this trend also seen at the \( puma \) gene (Figure 12B). This corresponds to the increase in p53 recruitment in MANCA cells after DNA damage. A875 cells also displayed a small trend of increased MDM2 recruitment (Figure 12B).

In addition, we directly compared the ratios of MDM2 to p53 on chromatin for ML-1, MANCA and A875 cell lines (Figure 12C). At the \( p21 \) gene, we observed that MANCA cells had the highest ratio of MDM2 to p53 on the chromatin before etoposide treatment, which decreased after treatment. We observed that MANCA cells had the lowest levels of p53 recruitment, while ML-1 cells had higher basal levels of p53 recruitment that increased significantly after etoposide treatment (Figure 12A). Therefore, higher levels of p53 recruitment contributed to the decrease of the MDM2 to p53 ratio. Interestingly, because A875 cells had high basal levels of p53 recruitment to the \( p21 \) gene the ratio of MDM2 to p53 was low and did not change much with etoposide treatment (Figure 12C). At the \( puma \) gene, we observed that before
etoposide treatment MANCA cells also had the average highest ratio of MDM2 to p53 on chromatin (Figure 12C). However, in ML-1 cells we observed that the MDM2 to p53 ratio was higher for puma than the p21 gene before etoposide treatment (Figure 12C). As before, the ratio of MDM2 to p53 in A875 cells did not change with etoposide treatment although the ratios are slightly higher for puma as compared to the p21 gene (Figure 12C). Overall, the data shows that MANCA cells had the highest MDM2 to p53 ratio on chromatin as compared to the ML-1 and A875 cell lines irrespective of etoposide treatment.

In order to address whether G/G SNP 309 cells have more MDM2 protein globally on the chromatin compared to ML-1 cells, we performed a biochemical fractionation assay that separates the cell into chromatin, nuclear soluble and cytosolic fractions and performed Western Blot analysis for p53 and MDM2 protein levels (Figure 12D). We observed higher levels of MDM2 and p53 on the chromatin in MANCA and A875 cells as compared to ML-1 cells. Our data shows that these G/G SNP309 MDM2 overexpressing cancer cells have increased MDM2 and stabilized p53 protein on chromatin globally.
Figure 12- G/G SNP309 cells have increased MDM2 and p53 protein on chromatin and increased p53 recruitment to p53REs after DNA damage.

ML-1, MANCA and A875 cells treated with DMSO or 8 µM etoposide for 6 hours. A and B) Chromatin immunoprecipitations were performed for p53 and MDM2 proteins and analyzed by quantitative PCR using primers for the p21 gene 5’ p53 responsive element and puma gene p53 responsive elements. ML-1 and MANCA results represent four to six independent experiments. A875 results represent two independent experiments. All results given with standard error bars. All chromatin immunoprecipitation samples normalized to IgG and input values. C) The MDM2 to p53 ratio was found by dividing the amount of MDM2 recruited at the p53 RE divided by the amount of p53 recruited at these sites. The graphs represent two to four independent experiments given with standard error bars. D) Cells were subjected to chromatin fractionation. 50 µg of chromatin extract was subjected to SDS-PAGE and Western blot analysis. * represents a p value ≤ 0.05, ** represents p value ≤ 0.01, *** represents a p value ≤ 0.001, **** represents p value ≤ 0.0001. Statistical analysis was done with student’s t test (unequal variance).
3.2.2) Stable knockdown of MDM2 in G/G SNP309 cancer cell lines moderately increases p53 transcriptional activity in a gene-specific manner without affecting p53 degradation.

In order to examine the role of MDM2 overexpression in G/G SNP309 cells in compromised p53 transactivation activity, we engineered MDM2 knockdown cells. Using shRNA targeting exon 12 of mdm2, we created stable MDM2 knockdown MANCA and A875 cell lines by retroviral-mediated gene transfer (Figure 13A). Quantitative Image J analyses showed an approximately 80-90% decrease in MDM2 protein levels (Figure 13B). The cells with MDM2 knockdown remained viable and we were able to use them for all the following experiments. Using MANCA and A875 cells with MDM2 knockdown, we examined p53 transcriptional activity by assessing changes of five common p53 target genes (Figure 13C). Stable knockdown of MDM2 in MANCA cells increased transcription of puma and to a lesser extent pig 3 (Figure 13C). In A875 cells, stable knockdown of MDM2 increased transcription of fas and pig 3 to approximately equal levels (Figure 13C). Induction of DNA damage treatment with six hours of etoposide yielded no additive effect on increased p53 target genes transcription for these cells (data not shown). However, it is of note that the p53 target genes most strongly affected by MDM2 knockdown alone in both cell lines (puma, fas and pig-3) are related to cellular apoptotic functions. These data suggest that in different cell types MDM2 moderately regulates transcription of p53 target genes gene-specific manner.

G/G SNP309 MANCA and A875 cancer cell lines have stabilized p53 protein despite the high MDM2 protein levels (Arva et al. 2005). Therefore, we asked what effect MDM2 knockdown in these cell lines would have on p53 protein levels. The knockdown of MDM2 in MANCA cells caused a small but significant increase in p53 protein levels. In A875 cells, there was variability seen in increased p53 protein levels (Figure 13A and B). In order to directly
address whether this was through the well-characterized ubiquitin-proteasome pathway, we treated MANCA cells with the proteasome inhibitor MG132 for six hours (Figure 13D). Proteasome inhibition by MG132 treatment caused a statistically significant increase in total ubiquitinated protein (Figure 13E). Interestingly, p53 protein levels were not significantly changed with addition of MG132. Additionally, MANCA cells with MDM2 knockdown and MG132 treatment did not show any change in p53 protein levels as compared to MDM2 knockdown alone (Figure 13D and E). There was also a trend for increased MDM2 protein levels with MG132 treatment suggestive of some MDM2 auto-ubiquitination function (Figure 13E). This indicates that MDM2 is not functioning as an E3-ubiquitin ligase for p53 in G/G SNP309 cancer cells, but may serve as an E3-ligase for other proteins besides p53 including itself. This data suggests that cancers with G/G SNP309 do not have MDM2 that functions as a normal E3-ligase for p53 protein targeting it for degradation.
Figure 13- Stable knockdown of MDM2 in MANCA and A875 cells moderately increases p53 transcriptional activity in a gene-specific manner without affecting p53 degradation.

MANCA and A875 cells were infected with retrovirus containing empty vector or vector with mdm2 shRNA and subjected to puromycin selection. Cells were grown under constant selection pressure with puromycin. A) Whole cell extracts of were made and 50 µg of protein was subjected to SDS-PAGE and western blot analysis. Cells were probed with antibodies against MDM2, p53 and Actin proteins and a representative blot is shown above. B) Image J analysis of western blots shown for MDM2 and p53 protein levels normalized to Actin. Results represent an average of three independent experiments with standard error bars. C) cDNA was prepared from the cells and quantitative RT-PCR was performed on the five p53 target genes p21, puma, fas, gadd 45 and pig 3 and normalized to gapdh. All results represent an average of three independent experiments with standard error bars. D) MANCA cells were treated with 10µM MG132 for six hours. Whole cell extracts were made and 50 µg of protein was subjected to SDS-PAGE and western blot analysis with a representative blot shown. E) Image J analysis performed for western blots of samples treated in D. Graphs represent an average of three independent experiments with standard error bars. * represents a p value ≤ 0.05, ** represents p value ≤ 0.01, *** represents a p value ≤ 0.001, **** represents p value ≤ 0.0001. Statistical analysis was done using student’s t test.
3.2.3) Regulation of p53 target genes in G/G SNP309 cancer cells occurs at post-initiation steps.

To discern the mechanisms leading to compromised p53 transcriptional activity in the G/G SNP309 cancer cell lines, we tested the hypothesis that p53 transcriptional activity was compromised at transcription initiation. We analyzed the recruitment of total and initiated (phospho-Serine-5 CTD) forms of RNA pol II at \textit{p21} and \textit{puma} transcription start sites (Figure 14). In ML-1 cells, which have functional p53 transcriptional activity, addition of etoposide increased total and initiated forms of RNA pol II at transcription start sites for both genes (Figure 14A and B). Interestingly, in MANCA cells etoposide treatment increased total and initiated forms of RNA pol II comparable to that of ML-1 cells at transcription start sites (Figure 14A and B). Additionally, A875 cells had approximately equal basal and activated total RNA pol II recruitment to that seen in ML-1 cells after etoposide treatment (Figure 14A). This was also seen for the initiated forms of RNA pol II after etoposide (Figure 14B). The results observed in A875 cells suggest that the studied p53 target genes are poised for transcription irrespective of DNA damage treatment. Altogether, these results suggest that G/G SNP 309 cells exhibit functional transcriptional initiation at p53 target genes. Therefore, the inhibition of these genes may occur at post-initiation steps.
Figure 14-DNA damage induced RNA pol II recruitment at p53 target genes in G/G SNP309 cancer cells indicates functional transcription initiation.

ML-1, MANCA and A875 cells were treated with DMSO or etoposide for 6 hours. A and B) Total RNA pol II and phosphorylated Serine 5 C-terminal domain (S5P) RNA polymerase II chromatin immunoprecipitations were carried out and analyzed using qPCR primers to p21 and puma transcription start sites for both genes. Results for total RNA pol II represent four to six independent experiments. For S5P RNA pol II chromatin immunoprecipitations represent three independent experiments given with standard error bars for ML-1 and MANCA cell and four independent experiments for A875 cells. All chromatin immunoprecipitation samples normalized to IgG and input values. Total and S5P RNA pol II after ETOP for all three cell lines were compared for statistical analysis. * represents a p value \leq 0.05, ** represents p value \leq 0.01, *** represents a p value \leq 0.001, **** represents p value \leq 0.0001. ns signifies not significant. Statistical analysis was done using student’s t-test (unequal variance).

3.2.4) Reduced H3K36 trimethylation near transcription start sites suggests G/G SNP309 cancer cells have compromised transcriptional elongation at p53 target genes.

Since the p53 target genes tested showed functional transcription initiation, we hypothesized that p53 target genes in G/G SNP 309 cancer cells were inhibited during transcription elongation. To address this, we first asked whether p21 and puma genes manifested any defects in CDK9 recruitment. CDK9 functions as the active subunit of P-TEFb, the enzyme
responsible for phosphorylating Serine 2 CTD of RNA pol II and DSIF for productive elongation (see review (Peterlin 2006)). We noticed that in ML-1, MANCA and A875 cells there were no defects in CDK9 recruitment at transcription start sites for both genes (Figure 15A). Second, we examined the elongating form of RNA pol II, phospho-Serine 2 CTD RNA pol II. Since the phosphorylation of Serine 2 on the RNA pol II CTD increases toward the 3’ end of genes (Komarnitsky et al. 2000), we looked at regions of p21 (+7011) and puma (+6014) genes downstream of the transcription start sites near exon 3. As expected, in ML-1 cells there was a very significant increase in phospho-Serine 2 RNA pol II after etoposide treatment (Figure 15B). In MANCA and A875 cells, phospho-Serine 2 RNA pol II was also recruited with trends of increased elongating pol II after etoposide treatment. It was statistically significant for the p21 gene in MANCA cells (Figure 13B). This indicated there was functional transcript being made for p21 and puma genes. However, we still needed to account for the differences in transcript levels between T/T and G/G SNP309 cells. In order to address this, we compared histone H3 Lysine 36 trimethylation (H3K36me3) for both genes. Histone H3K36 trimethylation is a histone post-translational modification mark associated with active transcriptional elongation (Morris et al. 2005). We expected that following DNA damage by etoposide in ML-1 cells we would detect an increase in H3K36 trimethylation. We observed that at transcription start sites for p21 and puma genes there was an increase in H3K36 trimethylation in ML-1 cells after DNA damage treatment (Figure 15C). In MANCA and A875 cells, there was less H3K36 trimethylation at transcription start sites for p21 and puma genes after DNA damage when compared to ML-1 cells (Figure 15C). We observed a large difference in the means when comparing H3K36me3 between ML-1 and MANCA (338 with 95% CI [39-638]) and between ML-1 and A875 (342...
with 95% CI [43-642]) after DNA damage. Together these data indicate that MANCA and A875 cells have less active transcriptional elongation at p53 target genes.

**Figure 15- Reduced H3K36 trimethylation near transcription start sites in G/G SNP309**

cells suggests compromised transcriptional elongation for p53 target genes

ML-1, MANCA and A875 cells treated with DMSO or 8 µM etoposide for 6 hours. A) CDK9 chromatin immunoprecipitations were carried out and analyzed by qPCR at p21 and puma transcription start sites. Results represent four independent experiments with standard error bars. B) Phospho-Serine 2 RNA pol II chromatin immunoprecipitations were carried out and analyzed using qPCR primers to p21 +7011 and puma +6014 regions of the genes. C) H3K36me3 chromat immunoprecipitations were carried out and analyzed by qPCR primers for p21 and puma gene transcription start sites. Results represent at least four independent experiments given with standard error bars. All chromatin immunoprecipitation samples are normalized to input and IgG values. * represents a p value \( \leq 0.05 \), ** represents p value \( \leq 0.01 \), *** represents a p value \( \leq 0.001 \),**** represents p value \( \leq 0.0001 \). Statistical analysis was done using student’s t test.
3.2.5) Stable MDM2 knockdown in MANCA cells alters p53 recruitment and H3K36 trimethylation in a gene- and site-specific manner.

MDM2 knockdown in MANCA and A875 cells moderately increased transcription of p53 target genes and MANCA cells have slightly increased p53 protein levels (Figure 13). Therefore, we asked if knockdown of MDM2 increased p53 levels on chromatin and altered p53 recruitment to p53REs. We also tested if DNA damage with MDM2 knockdown had an additive effect to change p53 recruitment and levels on chromatin. Chromatin was isolated using biochemical fractionation and Western Blot analysis was carried out for MANCA cells with MDM2 knockdown and compared to vector control. Interestingly, p53 protein levels appeared to be approximately equal on chromatin irrespective of etoposide treatment or MDM2 knockdown (Figure 16A). When analyzed for p53 recruitment on chromatin, MANCA cells with MDM2 knockdown had a small but significant (p=0.03) increase (approximately double) in p53 recruitment to the p21 p53 RE. There was also a trend (p=0.06) of this increase in p53 recruitment with etoposide treatment (Figure 16B). For the puma gene, there was a trend (p=0.085) of increased p53 recruitment in MANCA cells after MDM2 knockdown although not as pronounced an effect as that seen for the p21 gene. Also, MDM2 knockdown did not impact DNA-damage induced p53 recruitment to the puma gene (Figure 16B). Interestingly, MDM2 recruitment to p53 response elements was not changed with MDM2 knockdown although overall levels of MDM2 appear to be decreased on the chromatin (Figure 16A and C). For both p21 and puma genes, MDM2 knockdown did not change MDM2 recruitment to chromatin. This is likely due to the concomitant increased p53 recruitment seen in MANCA cells with MDM2 knockdown. This phenomenon can be seen in ML-1 cells, which have increased MDM2
recruitment to chromatin when p53 recruitment is increased by DNA damage treatment (Figure 13).

Figure 16- Stable MDM2 knockdown in MANCA cells increases p53 recruitment to the p21 p53 response element.

MANCA vector and mdm2 shRNA cells were treated with DMSO or 8µM etoposide for 6 hours. A) Cells were subjected to chromatin fractionation. 50 µg of chromatin extract was subjected to SDS-PAGE and Western Blot analysis and probed with MDM2, p53 and Fibrillarin antibodies. B and C) Chromatin immunoprecipitations were performed for p53 and MDM2 proteins and analyzed by quantitative PCR using primers for the p21 gene 5’ p53 responsive element and puma gene p53 responsive elements. Results represent four independent experiments. All chromatin immunoprecipitation samples normalized to IgG and input values. * represents a p value \( \leq 0.05 \), ** represents p value \( \leq 0.01 \), *** represents a p value \( \leq 0.001 \), **** represents p value \( \leq 0.0001 \). Statistical analysis was done with student’s t test (unequal variance).
To investigate the moderate changes in transcription in MANCA cells, we also tested if MDM2 had any effect on H3K36me3 at p53 target genes. We examined whether MDM2 knockdown in MANCA cells changed H3K36me3 at p21 and *puma* genes. MDM2 knockdown had no significant impact on H3K36me3 in MANCA cells at *p21* and *puma* transcription start sites. This was not changed when cells were treated with etoposide (Figure 17A). We also examined *p21* and *puma* polyadenylation sites for changes in H3K36me3. As observed at transcription start sites, there were no significant changes in *p21* or *puma* polyadenylation sites with MDM2 knockdown alone. Interestingly, MDM2 knockdown in MANCA cells displayed a significant, DNA-damage induced increase in H3K36me3 at the *puma* polyadenylation site as compared to vector control (Figure 17B). Our data show that MDM2 knockdown alone was not able to recover H3K36 trimethylation near transcription start sites of p53 target genes. This suggests that the moderate increase in transcription of select p53 target genes resulting from MDM2 knockdown in MANCA and A875 cells does not appear to be through a pathway that results in increased H3K36 trimethylation.
Figure 17- MDM2 knockdown in MANCA cells increases H3K36 trimethylation after DNA damage at the *puma* polyadenylation site.

MANCA vector and *mdm2* shRNA cells were treated with DMSO or 8µM etoposide for six hours. H3K36me3 chromatin immunoprecipitations were carried out and analyzed by qPCR primers for *p21* and *puma* gene transcription start sites (A) and polyadenylation sites (B). Results represent five independent experiments given with standard error bars. All chromatin immunoprecipitation samples are normalized to input and IgG values. * represents a p value ≤ 0.05, ** represents p value ≤ 0.01, *** represents a p value ≤ 0.001, **** represents p value ≤ 0.0001. Statistical analysis was done using student’s t test (unequal variance).

3.2.6) Histone H2B post-translational modifications and cytosolic levels are altered by

**MDM2 knockdown in G/G SNP309 MANCA and A875 cells.**

MDM2 can ubiquitinate histone H2B and inhibit transcription via H2B ubiquitination of the surrounding chromatin near p53 REs (Minsky and Oren 2004). We observed that MDM2 knockdown alone is not sufficient to change histone H3K36 trimethylation in MANCA cells at *p21* and *puma* genes (Figure 17). Therefore, we tested if MDM2 knockdown in MANCA and A875 cells altered H2B post-translational modifications. Using biochemical fractionation and western blot analysis, we looked for changes in total histone H2B in the chromatin-enriched
fractions of MANCA and A875 cells (Figure 18A). We observed a band above the normally described ~17 kDa band for histone H2B, which likely represents a post-translationally modified form of H2B. Given the approximate size of this band, ~25 kDa, it may represent ubiquitinated H2B (Figure 18A). In the chromatin fraction of both MANCA and A875 cell lines, the H2B band at ~25 kDa decreased with MDM2 knockdown. With etoposide treatment, the ~25 kDa was also decreased irrespective of MDM2 knockdown in MANCA cells in the chromatin-enriched fraction (Figure 18A). These data are indicative of MDM2 regulation in H2B post-translational modifications. Cytosolic fractions of MANCA and A875 cells were run alongside chromatin fractions. Interestingly, in both cell lines MDM2 knockdown alone caused significant decreases in cytosolic H2B levels (Figure 18B). In MANCA cells, there was an approximately 60% decrease and in A875 cells a 40% decrease in cytosolic H2B levels. Treatment with etoposide in both cell lines did not change H2B cytosolic levels. Overall, these data imply MDM2 is involved in some aspect of H2B regulation.

In order to address if ubiquitinated H2B could be regulated by MDM2, we asked if H2B K120-Ub, the mammalian form known to be influenced during transcription, could be altered by MDM2 knockdown. We used MANCA cells with MDM2 knockdown and looked for changes in H2B K120-Ub in chromatin-enriched fractions. Interestingly, we did not observe any changes in the H2B K120-Ub protein levels (Figure 18C). The levels of H2B-Ub were also not changed in MANCA cells with etoposide treatment. Cytosolic fractions of these samples were also run alongside for comparison and we observed no H2B K120-Ub in the cytosolic fractions (Figure 18C). Only when the MDM2 knockdown cells were treated with etoposide did we observe an increase in the levels of H2B K120-Ub in the cytosolic fraction. The significance of this is not known at this time.
Figure 18 – Histone H2B is altered by MDM2 knockdown in MANCA and A875 cells.

MANCA and A875 vector and mdm2 shRNA cells were treated with DMSO or 8 µM etoposide for six hours. Cells were subjected to chromatin fractionation. A) 25µg of chromatin protein and 50µg of cytosolic protein were subjected to SDS-PAGE and Western Blot analysis and probed for H2Band Actin and Fibrillarin proteins as loading controls for chromatin and cytosol. A representative image is shown. B) Image J analysis of cytosolic H2B protein levels. Results represent two independent experiments. C) Chromatin and cytosolic proteins were subjected to SDS-PAGE and western blot analysis and probed with H2B K120-Ub and Fibrillarin. A representative image is shown. * represents a p value ≤ 0.05, ** represents p value ≤ 0.01, *** represents a p value ≤ 0.001, **** represents p value ≤ 0.0001. Statistical analysis was done using student’s t test (unequal variance).
3.2.7) MANCA cells with MDM2 knockdown have changes in RNA pol II post-translational modifications

Here, we present data that in MANCA and A875 cells there is functional transcription initiation, but compromised transcriptional elongation because chromatin modifications suggest less active elongation near p53 target gene transcription start sites (Figures 14 and 15). This suggests that the RNA pol II is arrested or stalled at points in transcription elongation and likely accounts for the differences in p53 target gene transcript levels between T/T and G/G SNP309 cells. Published reports describe stalled/arrested RNA pol II complexes can be released through ubiquitination and proteasomal degradation to allow continued transcription for the next functional RNA pol II elongation complexes (Svejstrup 2003). We asked if MDM2 was involved to restore some functional transcription through this pathway.

First, we addressed if there were any differences in RNA pol II ubiquitination in MANCA cells in the presence or absence of MDM2 knockdown. The cells were treated with the proteasome inhibitor MG132 for six hours to inhibit protein degradation. Total RNA pol II was immunoprecipitated from extracts and subsequently checked for differences in ubiquitinated protein (Figure 19A). We observed that MG132 treated MANCA cells with MDM2 knockdown had higher levels of ubiquitinated RNA pol II as compared to vector control. Quantitative Image J analysis showed a difference in the means of 8.89 with 95% CI [5.29-12.49] between MDM2 knockdown and vector control MG132 treated cells after two independent experiments (Figure 19B). Secondly, we observed only slightly lower levels in phospho-Serine 2 CTD RNA pol II with MDM2 knockdown (Figure 19A). Total RNA pol II was immunoprecipitated at approximately equal levels from the whole cell extracts (Figure 19A). Our data suggests that MANCA cells with MDM2 knockdown have higher ubiquitinated RNA pol II. This indicates
that MDM2 may be inhibiting the rescue of stalled/arrested RNA pol II complexes during transcription.

**Figure 19- MANCA cells with MDM2 knockdown appear to have increased RNA pol II ubiquitin modifications.**

MANCA vector or *mdm2* shRNA cells were treated with MG132 for six hours. A) Whole cell extracts were performed and total RNA pol II was immunoprecipitated from 1mg of protein compared to input samples and run for SDS-PAGE and Western Blot analysis. Samples were probed for RNA pol II, phospho-Serine 2 pol II and total ubiquitin protein. Representative images are shown. B) Image J analysis of the ubiquitin from the immunoprecipitated RNA pol II. Graph represents two independent experiments normalized to RNA pol II from the immunoprecipitation.
3.3 Discussion

Among the cancer cells with MDM2 overexpression, those with G/G SNP309 are known to be resistant to DNA damage in part to the cells attenuated p53 stress response (Bond et al. 2004). The compromised p53 transcriptional activity in G/G SNP309 cancer cells contributes to the attenuated stress response (Arva et al. 2005). The experiments described in this chapter explored how p53-mediated transcription in G/G SNP309 cancer cells is compromised and the involvement of MDM2 in p53 transcriptional regulation in these chemoresistant cancers. We recapitulated that G/G SNP309 MANCA and A875 cells had compromised p53-mediated transcription after DNA damage and added the apoptotic target puma and pig-3 to the list of p53 target genes with compromised transcription (Figure 11). The compromised apoptotic signaling seen in MANCA and A875 cells after 24 hours of DNA damage by FACS analysis (Arva et al. 2005) correlates to the compromised transcriptional activity of apoptotic p53 targets after six hours of DNA damage treatment. In addition to the presence of MDM2-p53 chromatin complexes at p53REs, we analyzed the MDM2 to p53 ratio on chromatin and observed that MANCA cells had the highest ratio before etoposide treatment (Figure 12C). We also showed that overall MDM2 protein levels on the chromatin in MANCA and A875 cells were higher than in ML-1 cells (Figure 12D).

MDM2 reportedly inhibits p53-mediated transcription via dual mechanisms with the first through MDM2 binding to the p53 transactivation domain (Momand et al. 1992; Thut et al. 1997). Targeted chemotherapeutics that inhibit MDM2 binding to p53 transactivation domain have been developed and are in clinical trials as methods to reactivate wild-type p53 transcriptional activity (Vu and Vassilev 2011). One of the more common small-molecule inhibitors, Nutlin-3, is reported to have some efficacy at treating MDM2 overexpressing cells.
with *mdm2* genomic amplification when they have wild-type p53 function (Tovar et al. 2006). In A875 cells, Nutlin-3 treatment activates p53-dependent transcription significantly better than etoposide treatment for *mdm2* and *pig-3*, less so for *p21/waf1*, but not for *gadd45* or *puma* genes (Arva et al. 2008). We provide evidence that, similar to MDM2 inhibition by Nutlin-3 treatment, MDM2 knockdown has a selective effect on p53-dependent transcription (Figure 13C).

Knockdown of MDM2 in G/G SNP309 cells moderately increased select p53 target genes in a cell-type specific manner with no additive effect after DNA damage treatment (Figure 13C and data not shown). In p53-null H1299 lung carcinoma cells with inducible wild-type p53, excess full-length MDM2 has a selective inhibition on p53 target genes *pig-3* and *14-3-3σ* (Ohkubo et al. 2006). The second reported mechanism for MDM2 inhibition of p53 transcription is through binding TFIIE and thus inhibiting pre-initiation complex formation (Thut et al. 1997). However, our data suggest that G/G SNP309 MDM2 overexpressing cells have functional transcription initiation (Figure 14). Both MANCA and A875 cells, in comparison to ML-1 cells, had similar total and initiated forms of RNA pol II recruited to p53 target gene start sites. In fact, for A875 cells, there was significantly higher initiated RNA pol II recruited than in ML-1 cells for *p21* and *puma* genes. A875 cells treated with Nutlin-3 also had similar RNA pol II recruitment to cells treated with DNA damage (data not shown). The work implicating MDM2 repressing p53-dependent transcription by binding TFIIE and p53 was carried out in an *in vitro* system without chromatin (Thut et al. 1997). In this system, it would be difficult to account for the chromatin environment of p53 target genes which obviously has a profound effect on its transcription.

When examining all this information it indicates a complex relationship between MDM2 and p53-dependent transcriptional regulation.
In cancer cells with MDM2 overexpression, there is a concomitant increase in \textit{mdm2} splice variants and protein isoforms and in G/G SNP309 cancer cell lines several \textit{mdm2} splice variants are found (Bartel et al. 2002; Arva et al. 2008; Okoro et al. 2012). In fact, MANCA cells are characterized with higher levels of the \textit{mdm2-c} splice variant compared to full-length \textit{mdm2} transcript (Okoro et al. 2013). When excess exogenous \textit{mdm2-c} is transfected into H1299 lung carcinoma cells, there is no significant effect on co-transfected wild-type p53 transcriptional activity (Okoro et al. 2013). The knockdown of MDM2 protein in G/G SNP309 cancer cells, due to the shRNA targeting exon 12 of \textit{mdm2}, likely alters all forms of MDM2 protein isoforms present. The moderate effects on p53 transcriptional activity are consistent with higher MDM2 protein isoforms than full-length MDM2 protein in our G/G SNP309 cancer cells.

Promoter pausing and elongation are increasingly seen as the main regulatory events during transcription (see review (Smith and Shilatifard 2013)). Our data show that MANCA and A875 cells have CDK9 and phospho-Serine 2 CTD RNA pol II recruited during transcription of p53 target genes (Figure 15A and B). However, the data also suggest that p53 target genes in G/G SNP309 cancer cells, due to lower H3K36 trimethylation, have less active transcriptional elongation (Figure 15C). Nearly 75\% of all genes in embryonic stem cells are poised for transcription initiation and, interestingly, only a fraction of the studied genes are able to efficiently elongate (Guenther et al. 2007). Environmental responsive genes are poised for transcriptional activation with paused RNA pol II near transcription start sites (Hendrix et al. 2008; Sawarkar et al. 2012). Combined the data suggest that, similar to stem cells, G/G SNP309 cancer cells have p53 target gene regulation at post-initiation steps. The exact mechanism to account for compromised transcriptional elongation is unknown and warrants further study.
Due to compromised elongation we were interested in how MDM2 may account for the moderate changes in transcription with MDM2 knockdown alone. We asked if there was an associated increase in H3K36 trimethylation in MANCA cells with MDM2 knockdown at p53 target genes. We observed that MDM2 knockdown did not significantly change H3K36 trimethylation at p21 and puma transcription start sites (Figure 17B). These data indicate that in G/G SNP309 cancer cells MDM2 knockdown alone is likely not sufficient to reactivate p53-dependent transcriptional elongation. Interestingly, we noticed that MDM2 knockdown increased ubiquitinated RNA pol II after MG132 treatment (Figure 19). Since RNA pol II ubiquitination is reported to clear stalled/arrested elongation complexes (Somesh et al. 2005; Somesh et al. 2007), it is likely there is some MDM2 involvement in compromised elongation through inhibited ubiquitination of RNA pol II. MDM2 knockdown in MANCA cells also altered DNA damaged induced H3K36me3 at the puma polyA site. This may not necessarily be related to transcription, but have more to do with MDM2 involvement in DNA damage response pathways. H3K36 trimethylation, in addition to active transcriptional elongation, is also associated with DNA damage repair by mismatch and homologous recombination repair leading to genomic stability (Li et al. 2013; Pfister et al. 2014). MDM2 interacts with NBS-1 of the MRN complex to delay DNA repair (Alt et al. 2005), therefore MDM2 knockdown in MANCA cells may facilitate quicker DNA repair after etoposide treatment. Additionally, extrachromosomal histone H2B serves as a sensor for cellular DNA damage (Kobiyama et al. 2013). We observed that after MDM2 knockdown, MANCA and A875 cells had a significant decrease in cytosolic H2B protein levels (Figure 18B). This is indicative of MDM2 involvement G/G SNP 309 cancers cells in the DNA damage response pathway likely changing DNA damage sensing and/or signaling.
We also tested the E3 ligase functions for MDM2 in G/G SNP309 cancer cells. Interestingly, MG132 treatment in MANCA cells did not increase p53 protein levels or have an additive effect with MDM2 knockdown (Figure 13D and E). These data demonstrate that p53 in G/G SNP309 cells is not affected by proteasome-dependent degradation. This implicates MDM2 is not functioning as an E3-Ubiquitin ligase for p53 in these cells. In H1299 cells, exogenously transfected *mdm2*-C has no effect on co-transfected wild-type p53 protein levels (Okoro et al. 2013). In addition to ligase function, MDM2 can also regulate p53 mRNA (Candeias et al. 2008; Naski et al. 2009). The RING finger domain of MDM2 is reported to enhance p53 mRNA translation with the phosphorylation of Serine 395 on MDM2 by ATM seemingly necessary for this function (Candeias et al. 2008; Gajjar et al. 2012). Interestingly, the ligase function of MDM2 toward the ribosomal protein RPL26 can also attenuate p53 mRNA translation (Ofir-Rosenfeld et al. 2008). The small but significant increase in p53 protein levels in MANCA cells with MDM2 knockdown (Figure 13B) may be due to slight increases in p53 mRNA levels. The most commonly found *mdm2* splice variants are missing exons that code for the p53 binding domain, but retain exon 12 that encodes the RING finger domain (Okoro et al. 2012). The RING finger domain contains P loop motifs associated with nucleotide binding proteins and activity implying DNA and RNA functions (Poyurovsky et al. 2003; Priest et al. 2010). In G/G SNP309 cancer cells, MDM2 isoform proteins likely outnumber MDM2-FL protein and therefore may not contribute much to the normal p53 regulatory functions.
Chapter 4- MDMX Regulation in G/G SNP309 MDM2 Overexpressing Cancer Cells
4.1-Introduction

MDM2 and MDMX are inextricably linked in p53 regulation and regulate each other. MDM2 protein is a well-known E3-ubiquitin ligase and promotes the degradation of MDMX through this pathway (Pan and Chen 2003). We were interested to determine if the reason we did not see robust activation of p53 after knockdown of MDM2 was due to an increase in MDMX. The MDMX protein is involved in mdm2 gene regulation promoting p53-mediated transactivation of mdm2 under cellular stress conditions (Biderman et al. 2012). Cellular stress conditions, such as DNA damage, decrease MDMX protein levels through pathways associated with MDM2-mediated degradation of MDMX protein (Kawai et al. 2003). Phosphorylation of several residues on MDMX by DNA damage induced kinases, Chk1 and Chk2, can induce MDM2-mediated degradation of MDMX and subsequently p53 activation (Chen et al. 2005).

We wanted to address how MDMX is regulated under conditions with MDM2 overexpression. This was a critical question because the cells did not die when we decreased MDM2 protein levels and because MDMX is a target of MDM2. The G/G SNP309 MDM2 overexpressing cell lines used in this study were a vehicle to address this question. Both cell lines used in this study are characterized to be among the cancer types (Burkitts’ lymphoma and melanoma) by several groups to have MDMX overexpression (Gembarska et al. 2012; Leventaki et al. 2012). We characterized the basal MDMX expression levels in the MANCA and A875 cells and proceeded to address if MDMX contributed toward compromised p53 activity in these G/G SNP309 cancer cell lines by assessing the influence of MDM2 knockdown on MDMX levels.
4.2- Results

4.2.1) MANCA cells with MDM2 knockdown have increased MDMX protein stability

Both MANCA and A875 cell lines overexpress MDM2 protein. We asked what the basal levels of MDMX protein were in both cell lines. MANCA cells had higher basal levels of MDMX protein compared to A875 cells (data not shown). Next, we asked if there would be changes in MDMX levels with decreased MDM2. When addressed in the context of MDM2 knockdown, there were no changes to MDMX protein levels in A875 cells (data not shown). Interestingly, in MANCA cells the knockdown of MDM2 caused an approximate 11 fold increase in MDMX protein levels (p=0.03) (Figure 20A and B). We wanted to see if the dramatic increase in MDMX protein levels correlated to an increase of mdmX transcript levels in MANCA cells. With MDM2 knockdown, we observed no statistically significant increase in mdmX transcript levels (Figure 20C). This implies that MDM2 in MANCA cells is involved in regulating MDMX protein levels, but not mRNA.

Due to the importance of MDMX and MDM2 in the p53 stress response, we also treated MANCA cells in the presence and absence of MDM2 knockdown with DNA damage using etoposide. MDMX transcript and protein is reported to decrease with DNA damage agents (Kawai et al. 2003; Markey and Berberich 2008). DNA damage significantly decreased MDMX protein levels by approximately 80% (p < 0.0001). With MDM2 knockdown, the high levels of MDMX protein decreased slightly after DNA damage, although it was not statistically significant (Figure 20A and B). Interestingly, in MANCA cells treated with etoposide we did not observe a decrease in mdmX transcript levels (Figure 20C). The data indicates DNA damage regulation of MDMX in MANCA cells is through protein degradation.
**Figure 20- MANCA cells have increased MDMX protein stability with MDM2 knockdown**

A) MANCA vector and mdm2 shRNA cells were treated with 8µM etoposide for six hours. Whole cell extracts were performed and subjected to SDS-PAGE and Western Blot analysis. The samples were probed for MDM2, MDM2, p53 and Actin. A representative image is shown. B) Quantitative analysis by Image J was performed for analyzing MDMX protein levels normalized to Actin. Graph represents four independent experiments given with standard error bars. C) Transcript levels of mdmX were analyzed by quantitative RT-PCR and normalized to gapdh. Student’s t-test done for statistical analysis. * represents a p value \( \leq 0.05 \), ** represents p value \( \leq 0.01 \), *** represents a p value \( \leq 0.001 \), **** represents p value \( \leq 0.0001 \)

**4.2.2) MDM2 knockdown regulates MDMX protein levels in different subcellular compartments.**

Since MDMX protein levels were dramatically increased in MANCA cells by MDM2 knockdown, we wanted to locate the increased MDMX levels. We addressed this question by performing a subcellular fractionation on the MANCA cells with constitutive mdm2 shRNA compared to vector control in the presence and absence of etoposide-induced DNA damage. Using Western Blot analysis, chromatin and cytosolic protein fractions were compared for differences in MDMX protein levels. With MDM2 knockdown, we observed a statistically
significant increase in MDMX protein levels in the cytosolic fraction (p < 0.0001). Additionally, there was a trend (p=0.07) of decreased MDMX protein levels after etoposide treatment (Figure 21A and C). In the chromatin fraction, there was a trend (p=0.12) of increased MDMX after MDM2 knockdown. However, with etoposide treatment there was a large variability of MDMX protein on chromatin (Figure 21A and B). This data indicates MDM2 knockdown is associated with increased MDMX protein in different subcellular compartments in MANCA cells.

**Figure 21- MDM2 knockdown in MANCA cells alters MDMX protein levels in different subcellular compartments.**

MANCA vector and mdm2 shRNA cells were treated with 8µM etoposide for six hours. A) Cells were subjected to biochemical subcellular fractionation. Chromatin and cytosolic fractions were run for SDS-PAGE and Western Blot analysis and probed for MDMX, Fibrillarin and Actin. A representative image is shown. B) Image J analysis was done for the chromatin fractions of the samples normalized to Fibrillarin. Results represent four independent experiments with standard error bars. C) Image J analysis was done for the cytosolic fractions of the samples normalized to Actin. Results represent four independent experiments with standard error bars. Student’s t-test done for statistical analysis. * represents a p value ≤ 0.05, ** represents p value ≤ 0.01, *** represents a p value ≤ 0.001, **** represents p value ≤ 0.0001
4.2.3) MDM2 and MDMX protein regulation is linked in MANCA cells

The above result led us to further investigate in MANCA cells if MDMX might have a role in p53 regulation. We were interested in the roles of MDMX and MDM2 proteins in relation to each other and p53 regulation. We infected MANCA cells with constitutive and inducible \( mdm2 \) and \( mdmX \) shRNA and compared this to vector controls. First, we addressed if we could knockdown basal MDMX protein levels using constitutive \( mdmX \) shRNA and observed a decrease in MDMX protein (Figure 22, lanes 1 and 3). As before, constitutive \( mdm2 \) shRNA knockdown increased MDMX protein levels (Figure 22, lanes 1 and 2). We were also interested in decreasing MDM2 and MDMX protein levels simultaneously. The approach was to use the constitutive \( mdmX \) shRNA cell line and infect those cells with inducible \( mdm2 \) shRNA. In that way, there should be a manner to control the levels of MDM2 protein in an already low baseline background of MDMX. We tested the inducible \( mdm2 \) shRNA cell line compared to vector control in the presence and absence of the low \( mdmX \) background. Several unexpected issues were encountered in the MANCA cells during this process. The inducible \( mdm2 \) shRNA alone decreased MDM2 protein levels although it appeared there was decrease before induction (Figure 22, lanes 7-10). At doses above 2µg/mL, the drug doxycycline used to induce \( mdm2 \) shRNA proved to be quite cytotoxic to MANCA cells. Interestingly, in the background of low basal MDMX levels, MDM2 protein levels were comparable to vector controls before and after \( mdm2 \) shRNA induction (Figure 22, lanes 7, 8 to 11, 12). Therefore, the \( mdm2 \) shRNA did not have an effect on MDM2 protein levels in cells with constitutive \( mdmX \) shRNA. Similar to MDMX protein levels with MDM2 knockdown, the stability of MDM2 protein in MANCA cells increased slightly with MDMX knockdown. Our data indicate that MDM2 and MDMX are involved in the others protein regulation.
We also asked how the p53 stress response may differ in the MANCA cell lines made using etoposide to induce DNA damage. As expected, MDMX protein levels decreased with DNA damage (Figure 20 lanes 1,4 and 7,13). The p53 protein levels were not affected by constitutive \textit{mdmX} shRNA alone (Figure 22, lanes 1 and 3). The basal levels of p53 protein appeared to increase in the MANCA cells with low basal MDMX levels and inducible \textit{mdm2} shRNA (Figure 22 lanes 7,8 to 11,12). The levels of p53 protein were also increased with DNA damage treatment in all cell lines treated (Figure 22). The knockdown of either MDM2 or MDMX did not change DNA damage induced p53 protein levels. However, because simultaneous knockdown on MDM2 and MDMX proteins could not be achieved in MANCA cells it is hard to infer any conclusions about p53 regulation or the stress response in these cells.

\textbf{Figure 22- MDM2 and MDMX protein regulation are linked in MANCA cells}

MANCA cells were infected with vector or constitutive \textit{mdm2} or \textit{mdmX} shRNA. The MANCA cells were also infected with inducible vector or \textit{mdm2} shRNA. Constitutive \textit{mdmX} shRNA MANCA cells were also infected with inducible \textit{mdm2} shRNA. Cells with inducible shRNA and vector control were treated with 2 \(\mu\)g/mL of doxycycline for 10 days. The above cell lines were treated with 8 \(\mu\)M etoposide for six hours. Whole cell extracts were performed on the cells and subjected to SDS-PAGE and Western Blot analysis. Samples were probed for MDM2, MDMX, p53 and Actin proteins. Image J analysis was done and shown below the blot and the samples were normalized to Actin.
4.3) Discussion

MDMX protein is a major negative regulator of p53, homologous to the MDM2 protein (Shvarts et al. 1996). We wanted to explore if MDMX may play a role in p53 regulation in cancer cells with MDM2 overexpression. First, we observed basal MDMX protein expression was higher in MANCA cells than A875 cells (data not shown). In A875 cells, MDM2 knockdown did not affect the levels of MDMX protein (data not shown). However, in MANCA cells MDMX protein levels were dramatically increased with MDM2 knockdown (Figure 20). Interestingly, mdmX transcript levels did not correlate with the increase in MDMX protein levels as mdmX transcript levels were not affected by MDM2 knockdown (Figure 20). MDM2 reportedly functions as an E3-Ubiquitin ligase toward MDMX protein (Pan and Chen 2003). It is likely that MDM2 in MANCA cells is responsible for degrading MDMX protein.

After subcellular fractionation of MANCA cells, we observed MDMX protein in the cytosolic fraction and consistent increases after MDM2 knockdown (Figure 21). MDMX protein is characterized as a cytosolic protein with MDM2 necessary to traffic MDMX to the nucleus (Migliorini et al. 2002). The data presented here is consistent with MDMX being a cytosolic protein. However, MDMX protein is more associated with inhibition of p53 transcriptional activity because the RING finger domain of MDMX is not capable E3 ligase activity (Shvarts et al. 1996; Ghosh et al. 2003). MDMX protein can be found on chromatin at p53 response elements (Tang et al. 2008). We observed variability in the protein levels in the chromatin-enriched fraction especially with etoposide treatment (Figure 21). This is suggestive of MDMX protein being in flux between being chromatin-bound and in the nuclear soluble fraction. This also implies that other proteins besides MDM2 are likely involved in bringing MDMX protein into the nucleus.
Due to the structural protein homology of MDM2 and MDMX proteins, we hypothesized that in MANCA cells the increase in MDMX levels by MDM2 knockdown was substituting for the lack of MDM2 protein to regulate p53 activity. Using shRNA, we attempted to create MANCA cells with knockdown of both MDM2 and MDMX proteins (Figure 22). We observed that knockdown of MDMX protein levels in MANCA cells caused a slight increase in MDM2 protein levels. As in Figure 20, we saw the levels of MDMX increase with knockdown of MDM2. However, successful knockdown of both MDM2 and MDMX proteins simultaneously was not achieved in MANCA cells. MDM2 and MDMX protein can bind via the RING finger domains (Tanimura et al. 1999), therefore implying that MDMX and MDM2 may be involved in each other’s protein regulation. Our data also suggests that it may be lethal to get rid of both MDM protein family members at the same time. Both MDM2 and MDMX depleted may allow for activation of p53 and cell death.

MDM2 and MDMX work together after DNA damage to regulate p53 activation (see reviews (Chen 2012; Shadfan et al. 2012)). DNA–damage induced phosphorylation of MDMX leads to MDM2-mediated degradation resulting in p53 activation (Kawai et al. 2003; Chen et al. 2005; Okamoto et al. 2005). Our results in MANCA cells were consistent to published results. We observed, under etoposide-induced DNA damage conditions, a significant decrease in MDMX protein levels (Figure 20). Also, suggestive of MDM2-mediated MDMX protein degradation is the lack of change seen in \textit{mdmX} transcript levels after DNA damage treatment (Figure 20). We observed that knockdown of MDMX protein in MANCA cells did not change p53 protein levels (Figure 20). We also observed no significant changes in DNA-damage induced p53 protein levels with MDM2 or MDMX knockdown (Figures 20 and 22). This is consistent with MDMX not affecting p53 protein levels and with the compromised p53 protein levels.
transcriptional activity still seen in MANCA cells after DNA damage. However, when we checked if knockdown of MDMX with DNA damage could induce p53 transcriptional activity we observed no significant changes in transcript levels of p21, puma or pig 3 (data not shown). Although the p53 characterized in G/G SNP309 MANCA and A875 cells is genetically wild-type, its function in these cells is not. Knockdown of either of the major negative regulators of p53 in MANCA cells was not able to restore a functional p53 stress response. Overall, the data presented here suggests a complex relationship between MDM2 and MDMX protein in cancer cells with MDM2 overexpression and wild-type p53.
Chapter 5- G/G SNP309 cancer cells are sensitive to p53-independent cell death
5.1 Introduction

The most common chemotherapeutic agents utilize DNA damage pathways that can activate wild-type p53 to promote cell death (see reviews (Levine 1997; Woods and Turchi 2013)). However, resistance to DNA damage in some cancer types can develop if there are p53 mutations or overexpression of MDM2 and/or MDMX to functionally inactivate the wild-type p53 protein (Buttitta et al. 1997; Laframboise et al. 2000; Nayak et al. 2007; Jin et al. 2010; Knappskog and Lonning 2012). Specific pharmacological agents can switch p53 from a mutant to a wild-type conformation for restoring wild-type p53 function (Foster et al. 1999). Small-molecule inhibitors of the MDM2-p53 interaction at the N-termini of both proteins are also in development to reactivate wild-type p53 function in cancers that overexpress MDM2 (see review (Vu and Vassilev 2011)). Additionally, there are inhibitors that target the RING fingers of both MDM2 and MDMX proteins to inhibit p53 degradation (Herman et al. 2011). This suggests that reactivation of wild-type p53 in cancer cells is a targetable pathway to induce cell death in aggressive and/or chemoresistant cancers. However, in cases where wild-type p53 function cannot be restored, alternative treatments need to be sought to induce cell death in these cancer types. Here we explored treatment options for chemoresistant G/G SNP309 cancer cells.
5.2 Results

5.2.1) G/G SNP309 cancer cells are sensitive to p53-independent cell death induction

G/G SNP309 cancer cells are known to be resistant to various types of common chemotherapeutic DNA damage agents in part due to compromised p53 transcriptional activity (Arva et al. 2005). The knockdown of MDM2 by siRNA in G/G SNP309 MANCA and A875 cancer cells was reported to sensitize the cells to etoposide as shown by lower half maximal inhibitory (IC50) concentrations after treatment (Nayak et al. 2007). Using the MDM2 knockdown MANCA cell line, we tested the sensitivity of the cells to 24 hours of etoposide treatment at varying concentrations. First, we observed that MDM2 knockdown in MANCA cells caused a slight trend of decreased cell proliferation over a 96 hour period (Figure 23A). Next, we treated the cells with varying doses of etoposide and observed that only at 1 µM concentration was there a moderately smaller amount of cell growth with MDM2 knockdown as compared to vector control (Figure 23B). Using an MTT assay to assess cell viability, we observed no difference in sensitivity to different doses of etoposide with MDM2 knockdown (Figure 23C).

Contrary to mdm2 siRNA in G/G SNP309 MANCA and A875 cancer cells (Nayak et al. 2007), our data show that MDM2 knockdown by constitutive shRNA did not sensitize G/G SNP309 MANCA cells to etoposide treatment. This may be due to the increase in MDMX protein levels after MDM2 knockdown in MANCA cells (Figure 20). MDMX may contribute to the DNA damage resistance in a redundant manner to MDM2 protein.
Figure 23 - Knockdown of MDM2 in MANCA cells does not change sensitivity to DNA damage by etoposide.

A) MANCA vector and mdm2 shRNA cells were grown over a 96 hour period and counted using a hemocytometer at 24, 48, 72 and 96 hour time points seeded at 1.0 x 10^5 cells/mL. The p values at each time point are as follows: 24 p = 0.093, 48 p = 0.17, 72 p = 0.196, 96 p = 0.067. Results represent an average of four independent experiments given with standard error bars. B) Cells were seeded at 2.5x10^5 cells/mL at treated with varying concentrations of etoposide. After 24 hours cells were counted using a hemacytometer. Results represent two independent experiments with standard error bars. C) Cells used in B were also subjected to an MTT assay and assessed for changes in cell viability. Results represent two independent experiments with standard error bars.

Since MDM2 knockdown by shRNA did not sensitize cells to etoposide, we tested if other treatment options known to induce other cell death pathways would be more effective than standard DNA damaging agents. Inhibitors of general transcription and RNA synthesis have been suggested as efficacious treatment options for cancers (see review (Stellrecht and Chen 2011)). For example, actinomycin D at low doses is reported to activate the p53 pathway.
(Choong et al. 2009). Using 5nM actinomycin D, we treated G/G SNP309 MANCA and A875 cells for 24 hours and also addressed if MDM2 knockdown would further sensitize the cells to treatment. Microscopic examination revealed that actinomycin D treatment caused MANCA cells to stop aggregating, while in A875 cells there was no visible change compared to controls (Figure 24A). Using an MTT assay, we tested for cell viability. In MANCA cells, low dose actinomycin D caused an approximate 50% decrease in cell viability, while in A875 cells it caused no change in cell viability (Figure 24B). MDM2 knockdown had no effect on cell sensitivity to actinomycin D treatment. In MANCA and A875 cells, we observed that actinomycin D treatment increased p53 protein levels (Figure 24C, lanes 1, 2 and 7, 8). Interestingly, with MDM2 knockdown actinomycyn D treated MANCA cells had p53 protein levels decrease while in A875 cells p53 levels did not change (Figure 24C, lanes 2, 5 and 8, 11).

To address if there was any apoptotic cell death in both cell lines, we asked if there was PARP cleavage after actinomycin D treatment. In both MANCA and A875 cell lines, there was no PARP cleavage in the presence or absence of MDM2 knockdown after treatment (Figure 24C, lanes 1,2 and 4,5; lanes 7,8 and 10,11). This indicates that methods used to reactivate the p53 pathway in G/G SNP309 cancers cells may not be the best option for treatment of these chemoresistant cancers.

Another inhibitor of general transcription and RNA synthesis is the nucleoside analog 8-amino-adenosine (Frey and Gandhi 2010; Stellrecht and Chen 2011). It is cytotoxic to cancer cells in a p53-independent manner (Polotskaia et al. 2012). We used 15µM 8-amino-adenosine, previously reported to be effective for killing metastatic breast cancer cells (Polotskaia et al. 2012). MANCA and A875 cells were treated with 8-amino-adenosine in the presence or absence of MDM2 knockdown for 24 hours. Microscopic examination revealed that both MANCA and
A875 responded to treatment. However, A875 cells appeared more responsive to 8-amino-adenosine than MANCA cells (Figure 24A). When testing for cell viability using MTT assay, we found that both cell lines had a significant reduction (~70%) in viability (Figure 24B). MDM2 knockdown in MANCA or A875 cell lines did not further sensitize the cells to treatment (Figure 24B). We also observed that 8-amino-adenosine treatment did not increase p53 protein levels in either MANCA or A875 cells and therefore did not activate the p53 pathway (Figure 24C, lanes 1,3 and 7,9). In order to address if the decrease in cell viability was associated with apoptotic cell death we looked for PARP cleavage in both cell lines. In MANCA cells, there was no PARP cleavage with 8-amino-adenosine with or without MDM2 knockdown (Figure 24C lanes 1,3,4,6). When A875 cells were treated with 8-amino-adenosine there was a band below the main PARP band indicating cleavage (Figure 24C lanes 7, 9). Interestingly, treated A875 cells with MDM2 knockdown had the band that indicated PARP cleavage decreased (Figure 24C lanes 9, 12). Our data imply that targeting G/G SNP309 cancer cells with an inducer of p53-independent cell death, like 8-amino-adenosine, may be a more effective treatment option that trying to reactivate the wild-type p53 pathway.
Figure 24- G/G SNP309 cancer cells are sensitive to 8-aminoadenosine.

MANCA and A875 with constitutively expressed *mdm2* shRNA or vector control were treated with 5nM Actinomycin D (ActD) and 15 µM 8-aminoadenosine (8AA) for 24 hours. A) After treatment, pictures of cells under 10X objective were taken. B) MTT assay was performed on the cells after treatment. Results represent three independent experiments with standard error bars. C) Whole cell extracts performed and subjected to SDS-PAGE and Western Blot analysis. Shown are representative images. Statistical analysis performed using student’s t-test. * represents a p value ≤ 0.05, *** represents p value < 0.001, **** represents p value < 0.0001.
5.3 Discussion

Chemotherapeutic DNA damaging agents are in common use in the clinic to treat a multitude of cancers. Many of these drugs respond via activation of the DNA damage pathway signaling to p53 (see reviews (Nitiss 2002; Woods and Turchi 2013)). Reactivation of p53 signaling through DNA damage can signal transcriptional cell death targets in cancer cells (see reviews (Vousden 2002; Woods and Turchi 2013)). When cancer cells develop a resistance to these drugs, there is no response via the normal signaling pathways. Knockdown of MDM2 by siRNA in G/G SNP309 MANCA and A875 cancer cells can decrease IC50 concentrations after etoposide treatment and was therefore reported to sensitize cells to treatment (Nayak et al. 2007). We were interested in whether MDM2 knockdown by shRNA showed a similar sensitivity to overcome resistance to etoposide. Our results showed that in MANCA cells the knockdown of MDM2 by shRNA did not sensitize the cells to etoposide treatment (Figure 23). However, constitutive MDM2 knockdown increases MDMX protein levels (Figure 20). This may account for the contrary results because MDMX protein may have redundant roles for MDM2 protein and contribute to DNA damage resistance. This suggests that other methods of treating chemoresistant G/G SNP309 cancer cells besides DNA damage agents need to be considered.

Inhibitors of general transcription and RNA synthesis are being used as a new therapeutic targets for cancer because they display differential sensitivity between non-transformed and transformed cells (see review (Stellrecht and Chen 2011)). The drug actinomycin D can bind DNA and interfere with elongating RNA of actively transcribing genes (Sobell 1985). At low doses actinomycin D can reactivate the p53 pathway (Choong et al. 2009). This reportedly occurs through MDM2 binding to ribosomal proteins released from the nucleolus after ribosomal stress thus preventing p53 degradation (Choong et al. 2009; van Leeuwen et al. 2011). We
observed that actinomycin D treatment in MANCA and A875 cells increased p53 protein levels (Figure 24C); however, cell viability was not affected in A875 cells while decreasing viability approximately 50% in MANCA cells (Figure 24B). When we treated MANCA and A875 cells with the nucleoside analog 8-amino-adenosine, we observed an approximate 70% decrease in cell viability and no change with MDM2 knockdown (Figure 22B). 8-Amino-adenosine inhibits multiple mechanisms of transcription and RNA synthesis associated with a decrease in cellular ATP levels (Frey and Gandhi 2010). The nucleoside analog 8-amino-adenosine is cytotoxic to cancer cells in a p53-independent manner (Polotskaia et al. 2012). In metastatic breast cancer cells, 8-amino-adenosine treatment activates differing types of cytotoxic reactions with markers for both autophagy and apoptosis dependent upon the cell line (Polotskaia et al. 2012). We observed a marker for apoptosis by PARP cleavage in A875 cells after treatment with 8-amino-adenosine, but in MANCA cells no evidence was presented for apoptotic cell death (Figure 24C). This suggests 8-amino-adenosine targeted different cell death pathways in the different cell types. Therefore, targeting chemoresistant G/G SNP309 cancer cells with p53-independent transcriptional/RNA synthesis inhibitors is suggested as a better option for therapy compared to other chemotherapeutics that reactivate the wild-type p53 pathway.
Chapter 6- Future Directions
6.1) Future Directions

In closing, the evidence collected by the experiments described in this thesis indicates several interesting phenomenon. We describe in chapter 3 that G/G SNP309 cancer cells have functional transcriptional initiation as evidenced by efficient recruitment of total and initiated forms of RNA pol II at transcription start sites for p53 target genes (Figure 14). After comparing H3K36 trimethylation (H3K36me3), there was evidence of compromised transcriptional elongation. In comparison to MANCA and A875 cells, we showed that ML-1 cells had higher H3K36me3 after etoposide treatment at p21 and puma transcription start sites (Figure 15). The reduced H3K36me3 in MANCA and A875 cells, as compared to ML-1 cells, suggested less active elongation. This should be followed up by looking at additional histone modification marks associated with transcriptional elongation such as H3K79 dimethylation (H3K79me2). Less active elongation likely suggests a decreased rate of transcription elongation for p53 target genes. This could be tested by employing a newer technique developed to measure rates of transcriptional elongation in cells, 4sUDRB-seq, which involves tagging newly transcribed RNA with 4-thiouridine followed by high throughput sequencing (Fuchs et al. 2014). However, the mechanism in G/G SNP309 cancer cells that may be associated with less transcriptional elongation at these p53 target genes needs to be explored.

The enzyme responsible for H3K36me3 is SETD2, a histone methyltransferase preferentially recruited to chromatin by phosphorylated S5/S2 CTD RNA pol II (Li et al. 2005; Sun et al. 2005). The p53 protein interacts with SETD2 to selectively regulate transcription of p53 target genes (Xie et al. 2008). The overexpression of SETD2 cooperates with p53 to upregulate transcription of select p53 target genes including p21 and puma, while down-regulating mdm2. However, the knockdown of SETD2 by siRNA has the opposite effect (Xie et
SETD2, and the associated H3K6me3 mark, recruits the FACT chromatin complex (Carvalho et al. 2013). FACT (Facilitates Chromatin Transcription) is a chromatin-specific elongation complex that facilitates nucleosome disassembly and reassembly during transcription (Orphanides et al. 1998; Orphanides et al. 1999; Belotserkovskaya and Reinberg 2004). The FACT complex is proposed to displace H2A-H2B dimers from nucleosomes destabilizing its structure and reassembling nucleosomes after elongating RNA pol II has passed over the gene during transcription (Belotserkovskaya and Reinberg 2004; Formosa 2008; Xin et al. 2009). Therefore, SETD2 is a likely target in attempting to explain the discrepancy in H3K36me3 and lower transcript levels in G/G SNP309 cancer cells for p53 target genes as compared to wild-type cells.

In G/G SNP309 cancer cells, we hypothesize less SETD2 recruitment at p53 target gene transcription sites as compared to wild-type cells. SETD2 recruitment can be tested by performing chromatin immunoprecipitation analysis. As compared to ML-1 cells, MANCA and A875 cells may have defects in SETD2 recruitment that would account for differences in H3K36me3 at p21 and puma genes. Less SETD2 recruitment likely contributes to the compromised transcriptional elongation at p53 target genes due to decreased FACT recruitment leading to less displacement of H2A-H2B dimers from nucleosomes and less nucleosome disassembly. This can be tested by performing chromatin immunoprecipitation analysis of a FACT subunit, Spt16, one of the components found to be affected by SETD2 at several genes during transcription (Carvalho et al. 2013). Nucleosome organization can also be observed for both p21 and puma genes by observing changes in H2B with chromatin immunoprecipitation analysis along the length of the p53 target genes. Previously, it was observed that H2B levels are affected in cells in a SETD2-dependent manner during transcription (Carvalho et al. 2013).
order to determine if SETD2 is indeed the dependent factor for transcription elongation, overexpression of SETD2 in G/G SNP309 MANCA and A875 cancer cells should be able to overcome transcription of p53 target genes. This can be achieved by transfecting or infecting exogenous SETD2 into G/G SNP309 cancer cells followed by etoposide treatment to observe if this causes an increase in transcription of p53 target genes. We can then determine if in G/G SNP309 cancer cells this is due to differences in H3K36me3 and SETD2 recruitment using chromatin immunoprecipitation analysis. Additionally, we can also determine if treating G/G SNP309 cancer cells with different therapeutic agents known to active p53 transcriptional activity including Nutlin-3 and actinomycin D also have similar differences in H3K36me3 and SETD2 recruitment in comparison to wild-type cells.

A smaller, but interesting, finding in chapter 3 was observed involving ubiquitinated RNA pol II. After total RNA pol II immunoprecipitation, we observed that constitutive knockdown of MDM2 in MANCA cells treated with proteasome inhibitor had increased ubiquitinated RNA pol II (Figure 19). Proteasome inhibitor treatment was added to increase the overall total pool of ubiquitinated protein in the cells. Why ubiquitinated RNA pol II increased with MDM2 knockdown is not known, however, we can speculate the reason this may occur. Ubiquitination of RNA pol II is a mechanism used by cells to target arrested transcriptional RNA pol II complexes during transcription elongation to clear the complexes for the next elongating RNA pol II complex (see reviews (Svejstrup 2003; Wilson et al. 2013)). Distinct E3-ubiquitin ligases are responsible for ubiquitination of RNA pol II in a two-step mechanism (Harreman et al. 2009). NEDD4 cooperates with the ElonginA/B/C-Cullin 5 complex for RNA pol II poly-ubiquitination (Harreman et al. 2009). Recently, NEDD4-1 was found to interact with MDM2 targeting it for ubiquitination and degradation (Xu et al. 2015). Interestingly, since MANCA
cells have an overabundance of mdm2 mRNA and protein (including splice variants and protein isoforms) there could be binding of MDM2 to NEDD-4 and targeted degradation. In Figure 13, we did indeed observe that MDM2 was a target of the ubiquitin-proteasome pathway because proteasome inhibitor treatment increased MDM2 protein levels (Figure 13). It is likely that in MANCA cells, the knockdown of MDM2 could shift the equilibrium of NEDD4 E3 ligase activity toward the binding and ubiquitination of RNA pol II. This could be tested by first performing co-immunoprecipitation analysis for NEDD4 and RNA pol II and NEDD4 and MDM2 in MANCA cells. Hypothetically, the knockdown of MDM2 would increase the amount of NEDD4 protein immunoprecipitated with RNA pol II and hence increased ubiquitinated RNA pol II.

In chapter 4, we observed that G/G SNP309 MANCA cells had a substantial increase in MDMX protein levels after MDM2 knockdown with no significant increases in mdmX transcript levels (Figure 20). MDM2 functions as an E3-ligase for MDMX targeting it for degradation (Pan and Chen 2003). The data indicates MDM2-mediated degradation of MDMX protein. It would be interesting to follow-up by treating the MANCA cells with and without MDM2 knockdown with a proteasome inhibitor to observe if it causes an increase in MDMX protein levels. This will determine if MDMX protein is indeed a target of the ubiquitin-proteasome pathway. Since MDMX increased only in MANCA cells, we should also ask if this can occur in other hematological malignancies. We can screen a panel of MDM2 overexpressing hematological cancer cell lines and infect them with mdm2 shRNA to determine if MDM2 knockdown will increase MDMX protein levels. It will help determine if this is cell-type specific and also indicate if MDMX has redundant roles for MDM2 in hematological malignancies.
Finally, in chapters 3 and 5 we observed that in G/G SNP309 cancer cells the normal p53-dependent associated functions of MDM2 were not functioning properly. The knockdown of MDM2 did not increase p53 protein stability and only moderately increased transactivation of p53 target genes (Figure 13). Additionally, MDM2 knockdown combined with etoposide treatment did not sensitize the cells further to treatment or cause an additive effect on p53 transcriptional activity (data not shown). Low dose actinomycin D, used as an alternative to DNA damage for p53 activation, was not able to cause a robust cell death response in both MANCA and A875 cell lines (Figure 24). G/G SNP309 cancers have an attenuated p53 stress response (Bond et al. 2004), and the evidence presented here shows that these cancers cannot have the p53 pathway reactivated. To pursue this further we need to compare T/T and G/G SNP309 cells for differences in response to treatment after activation of both p53-dependent and p53-independent therapeutics including etoposide, Nutlin, actinomycin D and 8-amino-adenosine. We can assess for cell viability, transcription levels of p53 target genes, p53 protein levels and different markers of cell death. T/T SNP309 cells will likely be responsive to both p53-dependent and p53-independent chemotherapeutic treatments. Evidence presented here suggests that G/G SNP309 cancer cells will be more responsive to p53-independent therapeutic treatments. Below is a model describing the predictions for response to treatment in both T/T and G/G SNP309 cells (Figure 25).
MDM2 has both p53-dependent and independent functions in cancer. MDM2 in G/G SNP309 cancer cells likely contributes to chemoresistance in a p53-independent manner. This may be due to delays in DNA repair associated with MDM2, the regulation of general transcription by inhibiting RNA pol II ubiquitination or the many functions of the MDM2 isoform proteins that are still unknown. These are just several avenues that can be explored to correlate MDM2 to chemoresistance in a p53-independent manner.
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