MQP-BC-DSA-9096

MDM2 IS NOT REQUIRED FOR THE P53-INDEPENDENT ROLE OF MDMX IN GENOME STABILITY AND CELL TRANSFORMATION *IN VITRO*

A Major Qualifying Project Report

Submitted to the Faculty of the

WORCESTER POLYTECHNIC INSTITUTE

in partial fulfillment of the requirements for the

Degree of Bachelor of Science

in

Biochemistry

by

Anika Blodgett

April 26, 2012

APPROVED:

Stephen Jones, Ph.D.
Department of Cell Biology
UMASS Medical School
MAJOR ADVISOR

David Adams, Ph.D.
Biology and Biotechnology
WPI Project Advisor

ABSTRACT

It was previously established that MdmX, negative regulator or tumor suppressor p53, promotes genome stability and suppresses proliferation and tumorigenesis in a p53-independent manner. The purpose of this project was to determine whether protein Mdm2, another p53 regulator previously shown to interact with MdmX, is required for the p53-independent role of MdmX in genome stabilization and suppression of cell transformation *in vitro*. Triple knock-out (TKO) cells derived from the tumors of mice lacking p53, Mdm2, and MdmX were transfected with an MdmX expression plasmid. Compared to control cells, TKO cells ectopically expressing MdmX show decreased cell proliferation, a longer cell cycle, increased chromosome numbers and bipolar mitotic spindles, and decreased foci formation. Thus, MdmX, even in the absence of Mdm2, plays a role in genome stability and proliferation. This is crucial to consider in regards to potential cancer treatments aimed to suppress Mdm2 and/or MdmX in order to reactivate p53.

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ACKNOWLEDGEMENTS

Primarily, I would like to express my genuine gratitude to Dr. Stephen Jones, who has graciously allowed me to work in his laboratory for many months. This has allowed me to complete the most crucial part of my undergraduate degree in a comfortable and educationally valuable environment. Thank you for your accommodation of my project, as well as your advice on the world of science and life in general.

I would like to thank the members of the Jones' lab – Kathy, Hugh, Michael, and Marilyn – who have always been more than willing to lend me constant help; whether it be as simple as finding a reagent, analyzing a piece of data, or offering expert advice. Thank you for allowing me to work (and sometimes struggle) alongside you as you perform your important and sometimes stressful work.

I absolutely cannot fully express my appreciation for Zdenka Matijasevic, who worked tirelessly to make sure that I experienced everything I could from this project. She has in essence allowed me to experience fully what it is like to complete her own work, and to truly understand what scientific research entails, all while remembering that I am a stressed college student. Thank you very much for your time and effort, your understanding of my insane schedule, and for the amount of responsibility you've entrusted to me when it came to your work. Thank you for being a strong guide, a mentor, and a friend. I have learned an enormous amount under your guidance.

Finally, a very sincere thank you to Dave Adams for guiding me to Dr. Jones, it has been a great experience. Thank you for your help in shaping and editing this report, which must be a tedious and time-consuming task, especially when you have about thirty of them! And most of

all, thank you for being a very knowledgeable, friendly, and helpful professor and advisor, not only for this MQP, but for the four-year duration of the completion of my degree.

I am of course forever grateful to WPI, an institution that has shaped me as a prospective scientist, performer, and person.

1.0 BACKGROUND

1.1 Protein p53

p53 is a well-known and intensely studied protein in cancer biology, as it functions as a tumor suppressor. The p53 gene is the most commonly mutated gene in human cancers. In fact, 50 percent of all human cancers contain an alteration in the p53 gene. Alternatively, cancers may also result from a mutation or deregulation of proteins that directly or indirectly interact with p53. In addition to the importance of p53 as a tumor suppressor, an increasing number of new roles for p53 have recently been reported, including the ability to regulate metabolism, fecundity, and various aspects of differentiation and development (Vousden and Prives, 2009).

Activity of p53 in normal cells is low. However, when the cell experiences DNA damage, p53 becomes active, initiating the expression of genes involved in DNA damage, cell cycle arrest or apoptosis (programmed cell death) (reviewed in Okorokov et al., 2006). p53 has a role in many non-stress situations as well, including the regulation of glycolysis and autophagy, cell survival, regulation of oxidative stress, cellular senescence and angiogenesis (Vousden and Lane, 2007).

p53 primarily functions as a transcription factor. It is activated by various stress signals including DNA damage, oncogene activation, telomere erosion, nutrient deprivation and hypoxia. p53 regulates the expression of an array of different genes that then mediate the p53 response (**Figure 1)**.

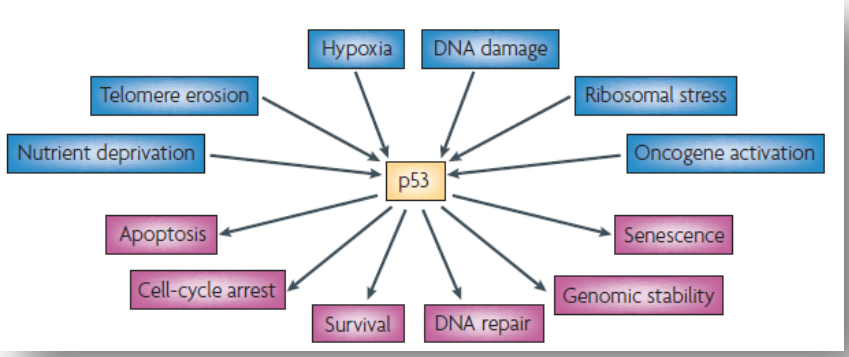


Figure 1: Overview of p53 Activation and Response. p53 is activated by many different situations, both stress and non-stress, and triggers pathways which result in various cellular responses (Vousden and Lane, 2007).

Upon DNA damage, protein kinases ATM and Chk2 phosphorylate p53, preventing its binding to Mdm2 (a negative regulator that promotes p53 degradation), thus increasing the cellular levels of p53 protein. ATM and Chk2 participate in a phosphorylation cascade which induces cell cycle arrest at the G_2 checkpoint. p53 prolongs the cell cycle arrest process by activating transcription of the gene coding for $p21^{Cip1}$, a cyclin-dependent kinase inhibitor. This CKI binds to the Cdk-cyclin complex and inhibits the G_1/S and G_2/S transitions. Irreparably damaged cells have a high p53 activity, which activates proteins responsible for apoptosis (Voet et al., 2008).

p53 may be activated by aberrant growth signals which cause the inappropriate activation of transcription factors. For example, Myc activates the transcription of the gene coding for $p14^{ARF}$. This protein binds and inhibits a negative regulator of p53, Mdm2. Therefore, p53 is stabilized, which triggers p53-dependent cell cycle arrest programs and apoptosis (Voet et al., 2008).

Similarly, DNA-damaging chemotherapeutics, protein kinase inhibitors, and UV-radiation can activate the protein kinase ATR, which phosphorylates p53 and reduces its affinity for Mdm2, resulting in cell cycle arrest and apoptosis (Voet et al., 2008).

p53 also possesses transcriptionally-independent activities through direct interaction with other proteins. For example, p53 interacts with members of the BCL2 family of proteins (BAX) to elicit apoptosis (Vousden and Lane, 2007).

The outcome of p53 activation, among other factors, depends on the extent of damage, and the duration of stress **(Figure 2)** (Vousden and Prives, 2009).

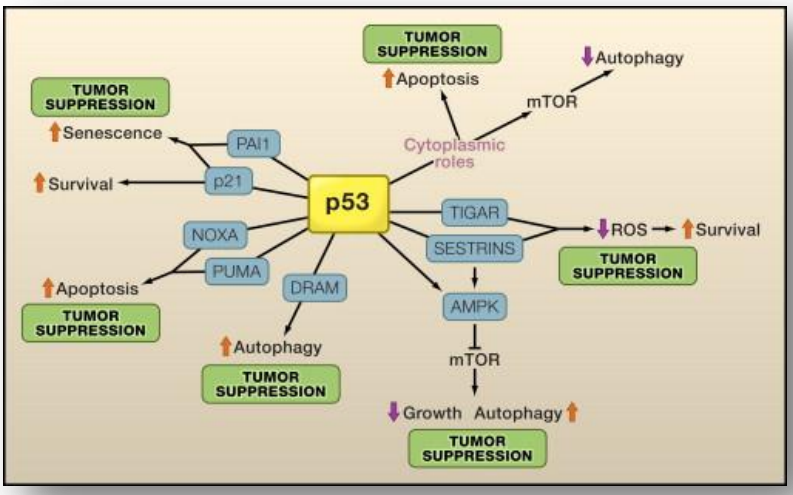


Figure 2: Tumor Suppressing Response of p53. The p53 response involves the regulation of several target genes that mediate cell survival, proliferation, and death (Vousden and Prives, 2009).

1.2 Mdm2 and MdmX – Negative Regulators of p53

During normal development, the inhibitory effect of p53 on cell growth must be held in check. Therefore, several molecules exist that act as negative regulators or "controllers" of p53. These include ubiquitin ligases that control p53 stability, kinases and acetylases that affect post-

translational modification of the molecule, and transcriptional co-activators that modulate the transcriptional activity of p53 (Vousden and Lane, 2007). Most prominent negative regulators of p53 are the Mdm2 and MdmX proteins. Deletion of either Mdm2 or MdmX results in embryonic lethality, a result that is rescued by co-deletion of p53 (reviewed in Wade et al., 2010).

Mdm2 functions as an E3 ligase, and specifically ubiquinates p53, marking it for proteolytic degradation by the proteosome. In normal cells, p53 is regulated by a feedback loop where increased levels of p53 result in higher levels of Mdm2, which negatively regulates p53. Both Mdm2 and MdmX bind to the N-terminal activation domain of p53, promoting modifications that inhibit p53 transactivation function. Although structurally similar to Mdm2 **(Figure 3)**, the MdmX protein does not have a pronounced E3 ligase ability or effect on p53 stability (Wade et al., 2010).

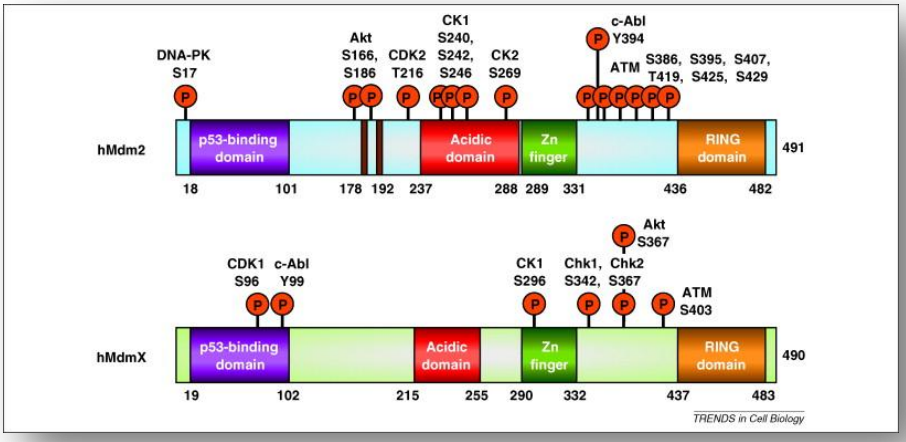


Figure 3: The Structure of Human Mdm2 and MdmX. (Wade et al., 2010).

Mdm2 can form homo-oligomers, or it can hetero-oligomerize with MdmX, but MdmX alone is monomeric (Tanimura et al., 1999). Mdm2/MdmX heterodimer formation **(Figure 4)**, is mediated by the C-terminal RING domain of both (Linke et al., 2008).

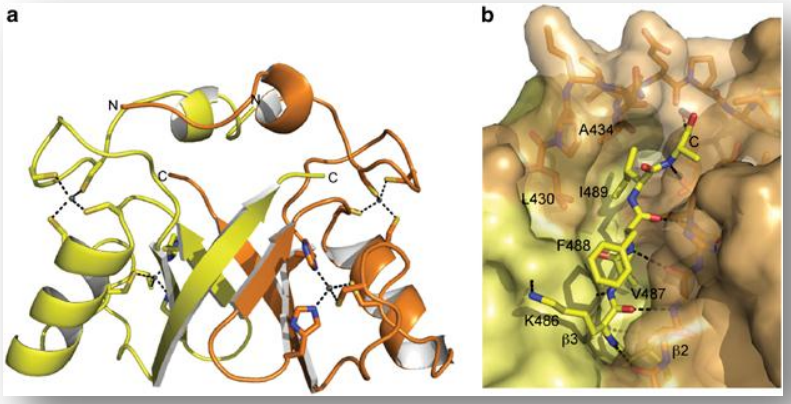


Figure 4: Diagram of Mdm2/MdmX Heterodimer. (Linke et al., 2008)

Studies suggest that the Mdm2/MdmX hetero-oligomer is a more effective ligase for p53 *in vitro* than Mdm2 alone (Linares et al., 2003), leading to the conclusion that MdmX contributes to p53 control by stabilizing the levels of Mdm2 (Linke et al., 2008). Following DNA damage, Mdm2 oligomers are phosphorylated and destabilized, leading to increased p53 levels. Mdm2 then degrades itself and MdmX, removing Mdm2-Mdm2 and Mdm2-MdmX oligomers, resulting in maximum p53 accumulation. When DNA damage signaling ceases, kinase inhibition and phosphate activation remove the phosphorylated Mdm2 and MdmX which leads to their stabilization. The oligomers then continue to reduce p53 levels (Wade et al., 2010).

1.3 p53-Independent Roles of MdmX

As a negative regulator of p53, over-expression of MdmX is potentially oncogenic, but surprisingly, MdmX was also found to have p53-independent roles in *suppressing* tumorigenesis and transformation (Matijasevic et al., 2008a). Mouse tumor cells double-null for p53 and MdmX show faster proliferation, increased genome stability, and an increased rate of

spontaneous tumorigenesis compared to p53-null cells/animals with intact MdmX (Matijasevic et al., 2008a).

While early passage p53-null and MdmX/p53-null cells show similar growth rates, the serial passaging of primary cells reveals an increase in growth rate of the MdmX/p53-null cells. Double-null cells are able to grow when plated at low densities and form much larger colonies than p53-null cells. A transformation assay shows that double-null cells form foci when allowed to grow to saturation density while p53-null cells do not (Matijasevic et al., 2008a).

MdmX not only suppresses cell proliferation, but helps maintain genome stability. Fluorescence Activated Cell Sorting (FACS) analysis comparing p53-null and double-null cells shows that the latter undergo a large reduction in ploidy. Double-null cells also undergo a reduction in chromosome number. Spindle formation in p53-null cells is mostly bipolar, with equal segregation of chromosomes, while double-null cell population shows an increased rate of multipolar spindles. Reintroduction of MdmX into double-null cells increases chromosome clustering and reduces multipolar spindle formation (Matijasevic et al., 2008b).

Mapping of the MdmX protein domain(s) responsible for the observed p53-independent functions using MdmX deletion mutants, is currently in progress in the Jones lab.

Inhibition of the interaction of p53 with its negative regulators has been proposed as a potential cancer treatment. Yet the fact that MdmX also plays a role in the suppression of tumorigenesis and proliferation, and maintaining chromosome stability, shows that further knowledge is needed regarding the p53-independent MdmX roles to be sure the MdmX-inhibiting treatments will not hinder the important characteristics of MdmX function (Matijasevic et al., unpublished).

1.4 Project Purpose

Proteins MdmX and Mdm2 have been shown to interact within the cell, and most previous studies have focused on the role of this interaction in the negative regulation of p53 function and stability to increase tumorigenesis. Several p53-independent functions have also been described for MdmX, including MdmX-mediated *decrease* in tumorigenesis (Matijasevic et al., 2008a). It remains to be determined whether Mdm2 has a role in some of the p53-independent MdmX functions described. This project seeks to begin an investigation of whether MdmX retains its tumor-suppressing characteristics in an Mdm2/p53-independent manner. The goal of this project is to investigate *in vitro* the Mdm2 requirement for the p53-independent MdmX role in suppression of transformation and in genome stability.

2.0 EXPERIMENTAL DESIGN

Tumor cells cultured from chest epithelium of Mdm2/MdmX/p53 triple knockout (TKO) mice will be stably transfected with an MdmX expression plasmid and co-transfected with a plasmid containing a puromycin-resistance gene. “Mock” control cells will be transfected with a plasmid containing the puromycin-resistance gene. Transfectants will be selected on puromycin, then examined for MdmX expression by qPCR, and subjected to functional analysis, including cell proliferation, foci formation, cell cycle length, chromosome number, and spindle formation of each cell line. The phenotype of MdmX-transfected TKO cells will be compared to the previously obtained results with MdmX-transfected MdmX/p53-double knockouts (DKO) cells in order to determine the Mdm2 requirement for MdmX-mediated effects.

3.0 MATERIALS AND METHODS

3.1 Cells

All experiments were performed with epithelial tumor cells isolated from mice deleted for p53, Mdm2, and MdmX (TKO, triple knockout). Cells were grown on DMEM media supplemented with 10% FBS and 10% antibiotic as attached culture.

3.2 Plasmid Purification, Digestion, and Linearization

Plasmid DNA was isolated and purified using a Qiagen Maxiprep kit from 300 μL of bacterial culture. Plasmid identity was confirmed by digestion with restriction enzymes PVU, Xho and HindIII (**Figure 5**). According to the plasmid map, the double digest for the MdmX expression plasmids would be expected to produce bands at approximately 1.6 kb and 5 kb, which was confirmed by the digest gel.

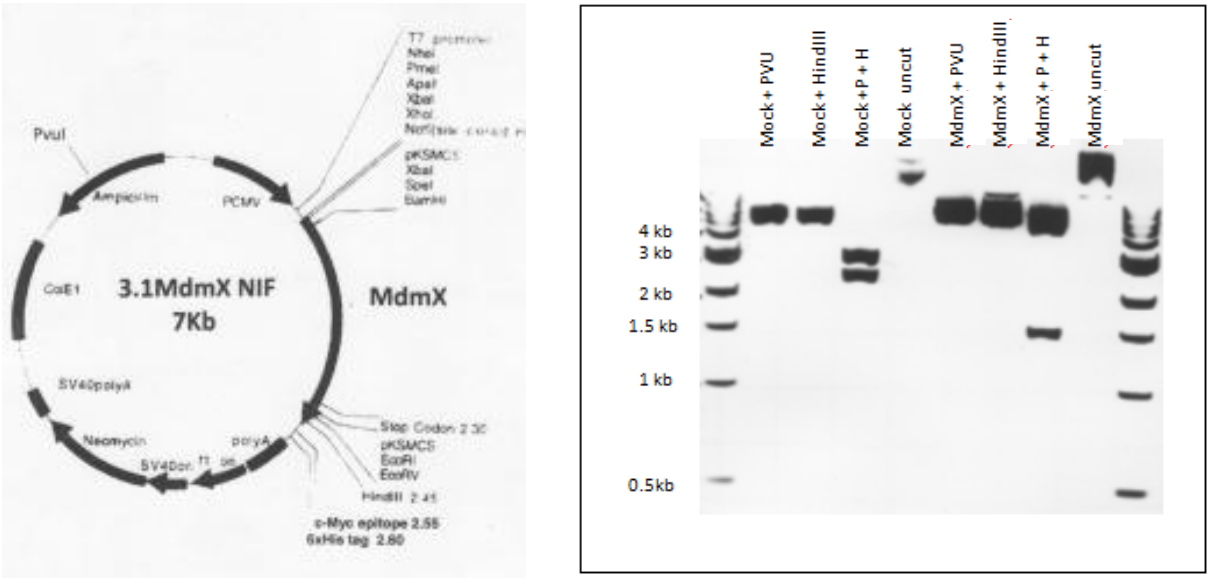


Figure 5: Digestion of MdmX Expression Plasmids. Plasmid DNA was digested with PVU and HindIII, then electrophoresed on an agarose gel. Positive clones containing the MdmX cDNA show bands at 1.6 kb and 5 kb.

3.3 TKO Transfections

Cells were plated in a 6-well format and grown to 70% confluency in media with no antibiotics. The DNA to be transfected (MDMX expression construct and/or puromycin expression construct pBABE-Puro at a 6:1 molar ratio; or GFP) was diluted in 50 uL of DMEM without serum, and mixed. An appropriate amount of Lipofectamine-2000 (Invitrogen) was diluted in DMEM and incubated for 5 minutes at room temperature. The DNA and Lipofectamine were mixed and allowed to incubate at room temperature for 20 minutes. 100 uL of the mixture was added to each well and rocked gently. Cells were incubated at 37ºC. The media was changed 6 hours later. The cells were re-plated 24 hours after transfection, and selected for puromycin resistance.

3.4 qRT-PCR

RNA Extraction

Cells were grown in a 6-well plate format and collected with 0.5 mL of Trizol. Then 0.2 mL of $CHCl_3$ was added, and the cells were incubated for 3 minutes at room temperature, then shook vigorously for 15 seconds. The tubes were centrifuged for 15 minutes at 12,000 X g at 4ºC and the clear supernatant was transferred to a fresh tube. The tubes were combined with 0.5 mL of isopropanol, incubated for 10 minutes at room temperature, then centrifuged for 10 minutes at 12,000 X g at 4ºC. The supernatant was removed, and 1.0 mL of 75% ethanol in diethylpyrocarbonate (DEPC) water was added to the pellet and inverted several times. The tubes were centrifuged for 5 minutes at 7.5 X g at 4ºC, the ethanol was removed, and the pellet was dried for 5 minutes. 50 uL of DEPC water was added to the pellets which were then left on ice for 30 minutes. The RNA was then quantified by spectrophotometry.

cDNA synthesis

The following mixture was prepared for each RNA sample: 5 ug RNA, 5 uL random hexamers (50 ng/uL), 1 uL 10 mM dNTP mix, and DEPC water to bring the volume to 10 uL. The samples were placed in a Themocycler for incubation for 5 minutes at 65ºC, then cooled for one minute. The following mixture was prepared (enough for each sample): 2 uL 10X RT buffer, 4 uL 25 mM $MgCl_2$, 2 uL 0.1 M DTT, and 1 uL RNaseOUT Recombinant Ribonuclease Inhibitor. 9 uL of this mixture was added to each sample, mixed, and incubated for 2 minutes at 25ºC. 1 uL (50 units) of SuperScript II RT was added to each tube. The tubes were placed in a Thermocycler and incubated at 25ºC for 10 minutes, 42ºC for 50 minutes, 70ºC for 15 minutes,

then chilled. 1 uL of RNase H was added to each tube and then incubated for 20 minutes at 37ºC. All reagents were from Invitrogen.

qRT-PCR

The master mix for PCR tubes was prepared for each well: 12.1 uL GoTag gPCR Master Mix (Promega), 10.5 uL H_2O, 0.2 uL forward primer, and 0.2 uL reverse primer. 23 uL of the master mix was added to each well in the PCR plate. 2 uL of each cDNA sample was added to each well containing the master mix. The PCR tubes were capped and placed into the PCR machine.

3.5 Functional Analysis

Transfected cells were assayed for the effects of MdmX expression on cell proliferation, foci formation, cell cycle length, chromosome number, and spindle formation as follows:

Proliferation Assay

Cells were plated at $.01X10^6$ cells per well in a six-well plate format. Each well was fed every three days with fresh media. Each day, two wells per sample were harvested with recorded volumes of trypsin and media, then were counted in triplicate. The number of cells per plate was calculated for each well per day.

Chromosome Spreads

Cells were grown to 1 X 10^6 in 150cm2 dishes. Pre-warmed media was added with colcemid solution (10 ug Gibco 15210-040) at a concentration of 0.02 ug/mL. The cells were

incubated at 37ºC for 90 minutes. The cells were harvested with trypsin, counted and centrifuged. The media and PBS used to harvest the cells were centrifuged to keep dislodged cells and added to the harvested cells. 10 mL of 37ºC hypotonic solution (0.075 M KCl) was added to the cells, which were then incubated at 37ºC for 18 minutes. A few drops of fixative (3:1 methanol : acetic acid) was added. Cells were collected by centrifugation, mixed with 1 mL of fixative added drop by drop while agitating and incubated on ice for 30 minutes. The cells were centrifuged at 500 RPM for 5 minutes and 1 mL of fixative was added. To prepare mitotic spreads, about 20 uL of the fixed swollen cells was dropped onto a glass microscope slide run quickly through a flame and placed on a 60ºC warmer. The cell density of the slides was checked and the last step repeated to determine the appropriate final volume of fixative. The slides were stained with DAPI (1 ug/mL) in PBS (200 uL DAPI/100 mL PBS) for 30-60 seconds, and then rinsed with PBS. Several drops of Permount solution were added to the slides along with a cover slip. After one hour the slides were examined with a 40x oil immersion microscope.

Mitotic Spreads

Cells were grown in 6-well plates on top of coverslips. Coverslips were removed with cells from media, dipped briefly in pre-warmed 37°C PBS, and fixed in -20°C pre-cooled methanol for 5 minutes. Cells were removed and placed in room temperature PBS for 5 minutes. The PBS was removed, and PBS with 3% BSA and 0.5% Tween 20 was added at room temperature for one hour. The primary antibody (Sigma mouse monoclonal anti-alpha tubulin and Sigma rabbit polyclonal anti-gamma tubulin) were diluted 1:4000 in PBS, 3% BSA, 0.5% Tween 20. Coverslips were removed and placed cell-side down on 100 uL drop of primary antibody, and then incubated overnight at 4°C. The coverslips were washed 3 times with PBS,

1% BSA, 0.5% Tween 20. The secondary antibodies (molecular probes goat anti-mouse ALEXA 488 and molecular probes goat anti-rabbit ALEXA594) were diluted 1:1000 in PBS, 3% BSA, 0.5% Tween 20. Coverslips were placed cell-side down on 100 uL drop of secondary antibody and incubated for 1 hour at 37 °C. The coverslips were washed 3 times with PBS, 1% BSA, 0.5% Tween 20 and incubated on a 100 uL drop of Hoescht 33258 solution (made 10 ug/mL in PBS) for 5-15 minutes, and then washed for 10 minutes in PBS. The coverslips were placed cell-side down on 7 uL mounting media (50:50 PBS:Glycerol) on a slide and sealed with clear nail polish. The slides were examined with a 100x oil immersion microscope.

Soft Agar Assay (Foci Formation)

A bottom layer of 1% agar in 1xDMEM/FBS was solidified in 60mm plates. Cells were grown, harvested and counted. Then 13,000 cells were added to the 45dC soft agar (0.5% final) in DMEM/FBS and poured on the top of the bottom layer. The plates were placed into the 37°C incubator. Once the upper layer was solid, 0.75 ml of 1xDMEM, 10% FBS were added to all plates. The cells were fed with 1mL of 1xDMEM, 10% FBS twice per week. Cells were fixed with methanol and stained with crystal violet to evaluate foci formation.

4.0 RESULTS

The purpose of this project is to determine whether protein Mdm2, previously shown to interact with MdmX, is required for the p53-independent promotion of genome stability and suppression of cell transformation by MdmX. Triple knockout cells were derived from tumors of mice lacking three proteins: p53, Mdm2, and MdmX. MdmX was then ectopically expressed in these cells by transformation with an MdmX expression plasmid. Transfectants were screened by RT-PCR for the expression of MdmX. The cells were then assayed for various transformation phenotypes, including cell proliferation, cell cycle length, chromosome number, spindle formation, and foci formation. The results of these functional assays were compared to those of cells transfected with a mock plasmid.

4.1 Transfection Efficiency and Cell Viability

To test the transfection efficiency and possible cytotoxicity of the transfection reagent, cells were transiently transfected (without selection) with an expression plasmid encoding Green Fluorescent Protein (GFP) and a plasmid encoding MdmX, using two different transfection reagents (X-treme GENE Roche and Lipofectamine 2000, Invitrogen). Human Embryonic Kidney (HEK) cells were used as a positive control. Transfection efficiency was determined as a ratio of GFP-positive cells to total cells from the fractions of 6-well plate images (**Figure 6** and **Table 1**). Cell survival was determined by counting total cell number of harvested cells in the control and transfected wells. The data showed that the cells with the lower transfection rate had a higher survival, while cells with higher transfection rate showed a lower survival, suggesting

that the MdmX over-expression can affect cell survival and that cells cannot tolerate high levels of MdmX.

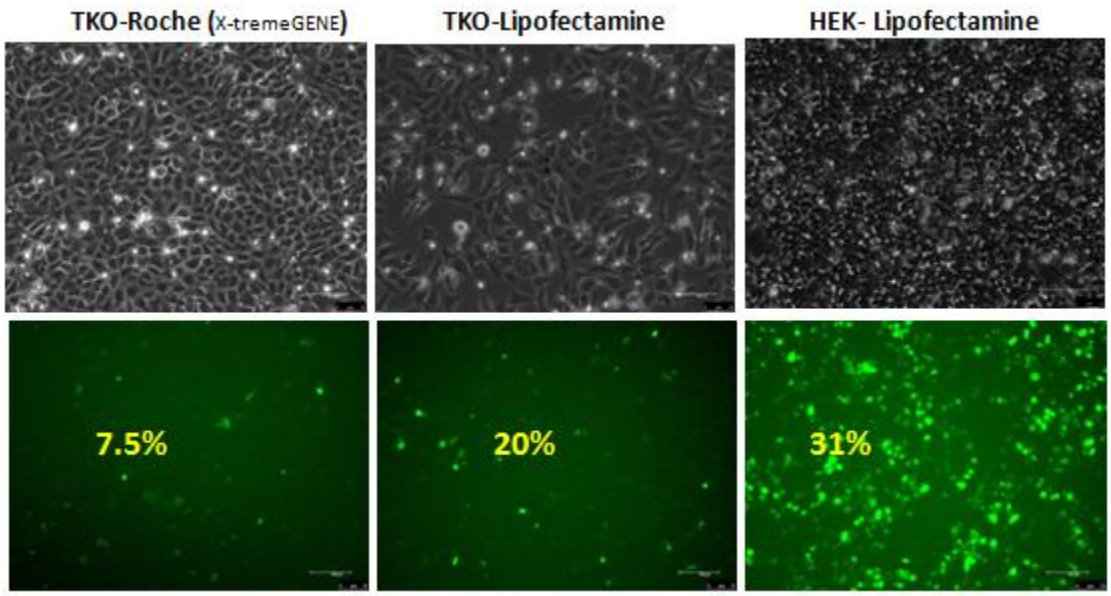


Figure 6: Transfection Efficiency and Cell Survival. Two different transfection reagents (R or L) were used to co-transfect cells with MdmX and GFP plasmids. The percent of GPF-positive cells and the percent surviving cells are shown.

Table 1: Transfection Reagent Toxicity

Reagent	*Conc. (µL/well)*	*Transfection Reagent + MdmX*		*Transfection Reagent Only*	
		30 hours		*25 hours*	*44 hours*
		GFP-pos.	*survival*	*survival %*	*survival %*
No TR				100	100
Roche	10	7.5%	72%	87	83
Roche	15			83	58
Lipo	10	20%	32%	100	83
Lipo	15			93	83

The results show that mouse tumor cells transfected at lower efficiency than HEK cells and that Lipofectamine is more efficient and a less toxic tranfection reagent under the experimental conditions.

4.2 RT-PCR

An expression plasmid containing the MdmX cDNA and/or puromycin resistance was stably transfected into TKO tumor cells using Lipofectamine. The transfected cells were selected for puromycin resistance. Positive transfectants were screened for levels of MdmX mRNA using qRT-PCR (**Figure 7**). Cells transfected with the plasmid encoding MdmX showed much higher levels of MdmX than parental TKO cells or cells transfected with the control plasmid.

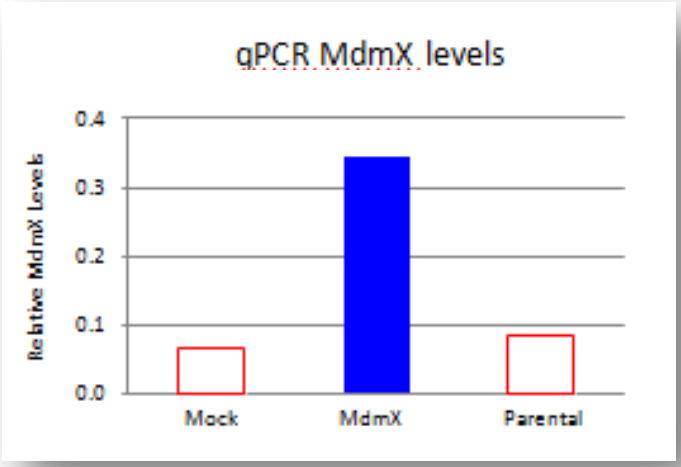


Figure 7: qRT-PCR of MdmX mRNA Levels in TKO Transfectants. Cells transfected with the plasmid encoding MdmX showed much higher levels of MdmX than parental TKO cells or cells transfected with the control plasmid.

4.3 Effect of MdmX on Cell Proliferation

A cell proliferation assay was performed on TKO cells transfected with mock plasmid or with full length MdmX (**Figure 8**). When plated at an initial low density, both cell lines seemed to behave similarly, but at higher densities over a 7-day period, the proliferation rate of cells

transfected with MdmX was significantly slower than the mock control, suggesting that MdmX plays a role in suppressing cell proliferation in an Mdm2-independent manner, and that the plating density affects the cell growth.

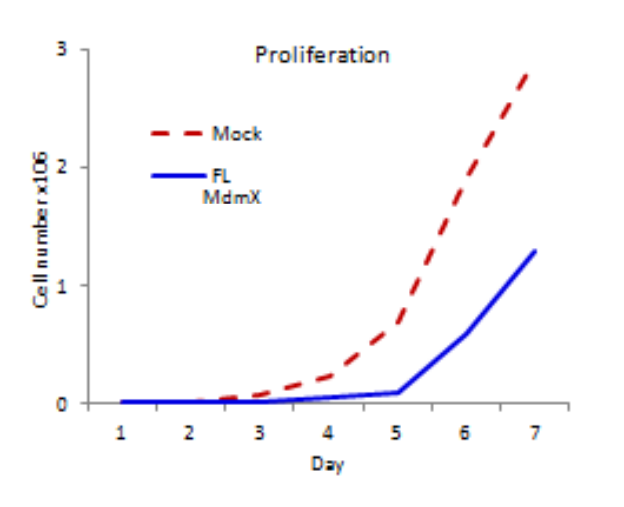


Figure 8: Effects of MdmX Expression on Cell Proliferation. Cells derived from tumors of mice deficient for p53, Mdm2, and MdmX (triple knockouts, TKO) were transfected with plasmid not encoding MdmX (mock) or encoding MdmX (FL).

4.4 Effect of MdmX on Cell Cycle Length

In collaboration with Sluder's lab at UMMS, TKO cells transfected with MdmX and control cells were subjected to time lapse videomicroscopy to determine the cell cycle length at the single cell level. The cell cycle length was calculated as the time from anaphase of one cell to the anaphase of the daughter cell (**Figure 9**). The data showed that cells ectopically expressing MdmX have a significantly longer cell cycle length compared to cells without MdmX. The

cycle lengths of 12.94 and 14.2 might not seem like a large difference, but an unpaired *t* test proves that the difference in time is extremely statistically significant. This increase in cell cycle length likely explains the slower proliferation of the cells ectopically expressing MdmX, and again confirms that MdmX plays a role in suppressing cell proliferation in an Mdm2-independent manner.

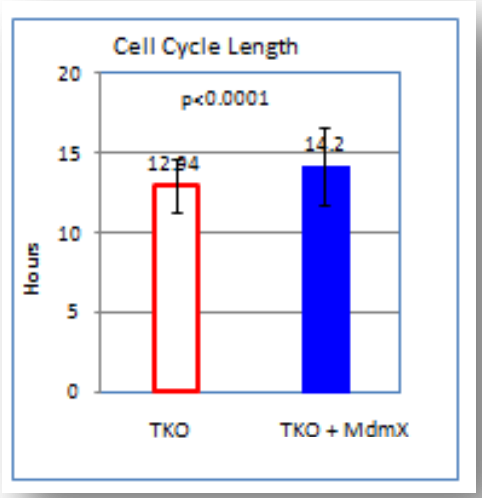
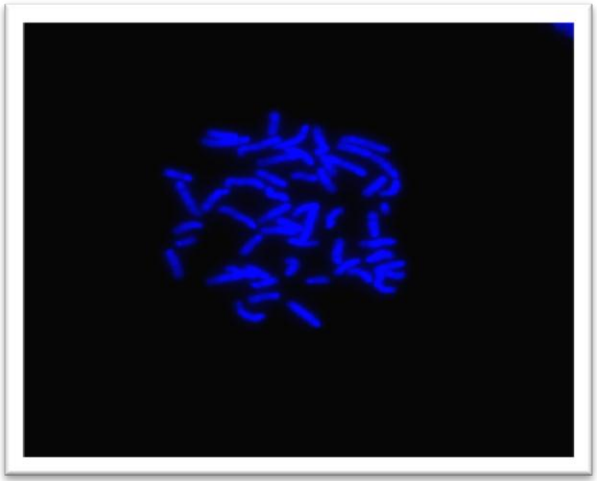


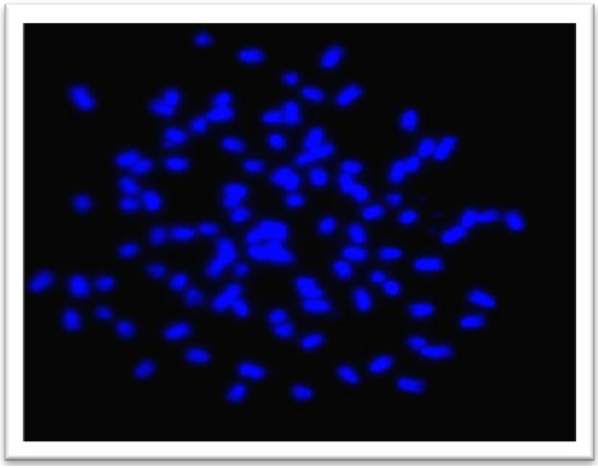
Figure 9: Effects of MdmX Expression on Cell Cycle Length.
Tumor cells derived from mice deficient for p53, Mdm2, and MdmX were transfected with plasmid not encoding MdmX (mock) or encoding MdmX (FL), and their cell cycle length measured by time lapse video microscopy.

4.5 Effect of MdmX on Chromosome Number

Chromosome spreads were prepared for mock and MdmX transfected cell clones, stained with DAPI, and observed under a fluorescent microscope (**Figure 10**). Chromosome number in at least sixty cells per cell line were determined. The results are expressed as the percent of cells with a larger than triploid genome (more than 60 chromosomes). Cells transfected with an

MdmX plasmid have almost twice as many cells with a larger than triploid genome, suggesting that the MdmX suppression of chromosome loss is not Mdm2-dependent.





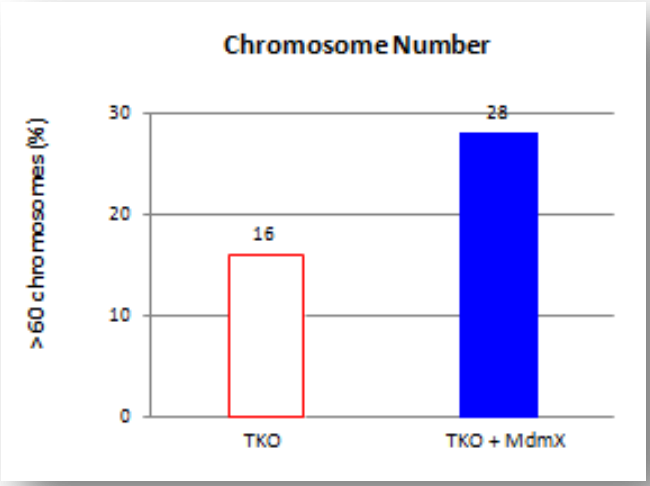


Figure 10: Effects of MdmX Expression on Chromosome Number. Chromosome spreads were prepared from TKO cells ectopically expressing MdmX and for control cells. Cells that lack MdmX show a decreased chromosome number (top picture: chromosome <60; bottom picture: chromosomes >60).

4.6 Effect of MdmX on Mitotic Spindles

MdmX was previously found to have a p53-independent role in maintaining genome stability, partly in terms of promoting bipolar spindle formation during mitosis in p53-deficient cells. To determine the requirement for Mdm2 in spindle polarity in MdmX-transfected TKO cells, mock and clones were stained with alpha-tubulin for microtubules, gamma-tubulin for centrosomes, and DAPI for DNA, and were observed under a fluorescent microscope (**Figure 11**). The upper panel shows examples of the various shapes of spindles that were observed. Normal spindle shape is bipolar with two centrosomes (left column), however normal cells can also contain some spindles with amplified centrosomes that were still bipolar (third column). Some had amplified centrosomes and were multipolar (second column). Another broad category included asymmetrical spindles that were elongated and were often seen in pairs or tandems.

The spindles observed were separated into one of three categories: bipolar, multipolar, or asymmetric. When comparing cells that will presumably divide correctly to those that will not, both biopolar spindles and spindles with amplified centrosomes that are still bipolar were placed together. This category was then compared to both obviously multipolar spindles and spindles that were considered multipolar and/or asymmetric (grouped as "irregular"). This is because it is unknown for sure whether the asymmetric spindles will divide incorrectly. The middle panels show the number of multipolar or "irregular" spindles for mock and MdmX clones calculated as a percent of all spindles observed. Cells ectopically expressing MdmX showed a decreased number of abnormal spindles. This suggests that MdmX plays a role in promoting bipolar spindle formation even in the absence of Mdm2.

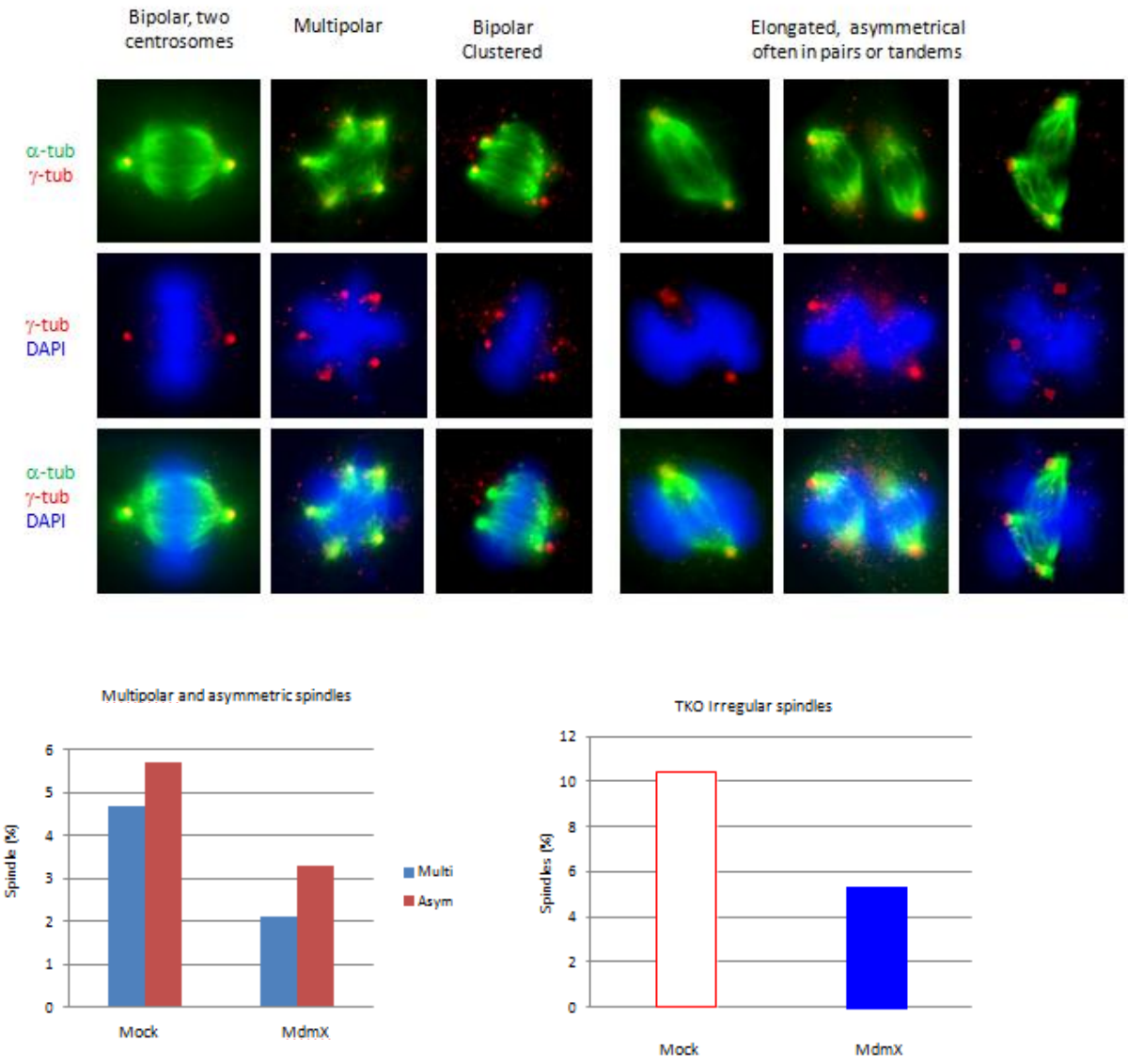
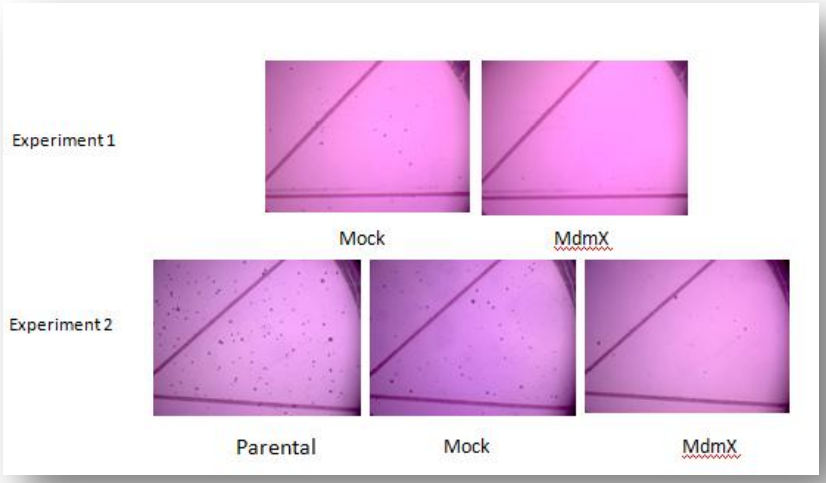


Figure 11: Effects of MdmX Expression on Irregular Spindle Formation. Cells were stained with alpha-tubulin for microtubules, gamma-tubulin for centrosomes, and DAPI for DNA, and were observed under a fluorescent microscope. Cells ectopically expressing MdmX show a reduction in irregular spindles.

4.8 Effect of MdmX on Cell Transformation *in vitro* (Soft Agar Analysis)

A soft agar assay determines the ability of cells for anchorage-independent growth, manifested as foci formation in soft agar. A soft agar assay was conducted using the mock and MdmX transfected TKO cell clones. MdmX expressing clones showed a great reduction in both

size and number of foci compared to the parental or mock transfected cells in two separate experiments (**Figure 12**). This shows that similar to DKO cells, MdmX suppresses foci formation in TKO cells, suggesting that Mdm2 is not required for MdmX to suppress cell transformation *in vitro*.



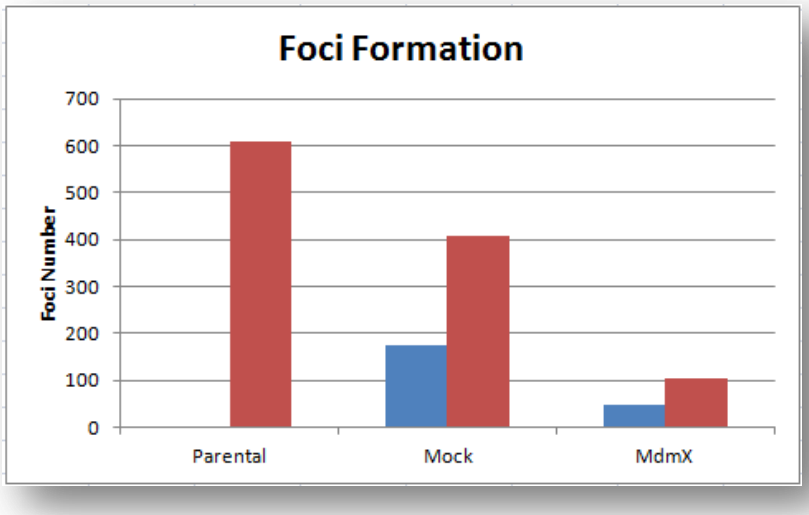


Figure 12: Effects of MdmX Expression on Foci Formation (Soft Agar Assay). Cells derived from triple knockout p53, Mdm2, and MdmX-deficient mice were transfected with a control plasmid or encoding MdmX and their foci formation was assayed in soft agar. Cells ectopically expressing MdmX show a lower rate of foci formation (red and blue indicate two different experiments).

5.0 DISCUSSION

5.1 Conclusions

The purpose of this project was to determine whether protein Mdm2, previously shown to interact with MdmX, is required for the p53-independent promotion of genome stability and suppression of cell transformation by MdmX. TKO cells were derived from tumors of mice lacking p53, Mdm2, and MdmX. MdmX was then ectopically expressed in these cells by transformation with an MdmX expression plasmid. MdmX expression was confirmed by qRT-PCR.

The cells were assayed for various transformation phenotypes. Cells ectopically expressing MdmX that were plated at a known low number over seven days showed slower growth when compared to control cells. This difference in growth rates is confirmed by the analysis of cell cycle length by video microscopy, showing that cell expressing MdmX have a significantly longer cell cycle when compared to control cells. Similar to p53-null cells, p53/Mdm2-null cells maintained an increased chromosome number. Cells expressing MdmX had a higher occurrence of bipolar spindle formation, while mock cells frequently produced multipolar or asymmetric spindles. Finally, cells ectopically expressing MdmX showed lower foci formation in soft agar than did control cells.

These data strongly parallel data obtained through similar studies. These were done with double-knockout cells that were p53/MdmX-null. Serial passage of primary cells revealed an increase in growth rate of the MdmX/p53-null cells when compared to p53-null cells. Double-null were able to form foci when allowed to grow to saturation density while p53-null cells do not (Matijasevic et al., 2008a). Double-null cells also underwent a reduction in chromosome number. Spindle formation in p53-null cells was mostly bipolar, with equal segregation of

chromosomes, while the double-null cell population shows an increased rate of multipolar spindles. Reintroduction of MdmX into double-null cells increased chromosome clustering and reduced multipolar spindle formation (Matijasevic et al., 2008b).

The conclusions of the DKO and TKO experiments regarding the role of MdmX strongly parallel each other. Despite the fact that MdmX and Mdm2 often form heterodimers, the role of MmdX in genome stability and cell transformation seems to remain unaltered whether in the presence or absence of Mdm2. This provides strong evidence that MdmX retains its important role in promoting genome stability and suppressing cell transformation in the absence of both p53 and Mdm2 *in vitro*.

5.2 Experimental Setbacks

The Jones' lab had also begun mapping of the MdmX protein domain(s) responsible for the observed p53-independent functions using MdmX deletion mutants. So far, the results suggest that the role of MdmX in suppressing proliferation is separated from its role in genome stability, with the RING domain regulating proliferation, and the RanBP Zn-finger domain regulating genome stability. The original goal of this MQP project was not only to examine the effect of MdmX on TKO cells, but also to examine whether the RING and RAN domain retain their respective roles in an Mdm2-null background. Deletion constructs were transfected into TKO cells and tested alongside the full length MdmX transfectants. However, no solid conclusions could be drawn from any of the assays using the deletion constructs. Whether this was due to an unsuccessful transfection or inconsistency of assay preparation is unknown.

FACS analysis was also performed on the full length MdmX transfectants as well as deletion construct transfections. Despite multiple attempts with different transfections, all cell lines appeared to have identical ploidy. The reason for this is again unknown.

It was attempted in earnest to achieve the detection of MdmX through Western Blots using an antibody to probe for MdmX, yet none were successful. HEK cells, as a positive control, showed high transfection efficiency and high levels of MdmX expression through qPCR, and was also able to be detected through Western Blot. Yet with MdmX-transfected TKO cells, despite having confirmed levels of MdmX through qPCR, even endogenous levels of MdmX could not be detected. Despite different antibodies and conditions, the detection of MdmX was on a whole unsuccessful.

5.3 Future Experiments

These experiments have only begun the long process of fully understanding the relationship between p53, Mdm2 and MdmX in tumor suppression. In the continuation of this reasearch, it would be beneficial to attempt further transfections with the deletion mutants in an attempt to achieve the expected results from functional analysis. Here one can discover whether the roles of the MdmX domains behave similarly in the absence of Mdm2, or whether Mdm2 might actually be required for their respective effects on the cell.

Most importantly, these conclusions are strong when it comes to *in vitro* conditions, but there are many factors that can affect the interactions studied here when they are introduced into a living organism. These *in vivo* studies should be done using mice as test subjects as a continuation of the previous DKO experiments (as described in Matijasevic et al., 2008b). Only

in vivo studies will confirm the relationship of MdmX and Mmd2 that was concluded by this project.

5.4 Medical Importance

Due to the fact that it is a negative regulator of p53, the suppression of MdmX and Mdm2 has been proposed as a potential cancer therapy in several ways; first, by reducing the cellular levels of MdmX; second, by developing small molecules that relieve the MdmX-dependent inhibition of p53, which would restore p53 function; and third, by using existing Mdm2 antagonists with agents that sensitize cells to p53-dependent apoptosis (Wade et al., 2009).

Yet it has also been previously shown that MdmX plays a role in the *suppression* of tumorigenesis and genome stability, suggesting that further knowledge is needed regarding the p53-independent MdmX roles to be sure the MdmX-inhibiting treatments will not hinder the important characteristics of MdmX function (Matijasevic et al., unpublished). This project provides even more insight into the relationship between p53, Mdm2, and MdmX, so that the effects of the removal of either Mdm2 or MdmX can be taken into consideration when contemplating these potential cancer therapies.

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