

# **MdmX Suppresses Chromosome Loss and Multipolar Mitosis in p53-Independent Manner**

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## Abstract

Mdm2 and MdmX are homologous proteins that function as oncogenes by negatively regulating the tumor suppressor p53. The overexpression of each of the MDM proteins has been linked to the tumorigenesis and metastasis of a significant portion of human cancers. Recent *in vitro* and *in vivo* studies have shown that MdmX can also function as a tumor suppressor by promoting genome stability in a p53-independent manner. Data has now further identified that the Zinc Finger (ZnF) region of MdmX is the key determinant to this function. This project aimed to generate, validate, and functionally analyze mouse epithelial tumor cell lines suitable for the future identification of MdmX interactive protein partners. Also of interest was to explore whether the role of MdmX in supporting genome stability requires intact Mdm2 function. For both aims, triple knockout (TKO) mouse tumor cells (p53<sup>-/-</sup>, Mdm2<sup>-/-</sup>, and MdmX<sup>-/-</sup>) were transfected with expression vectors containing FLAG-tagged MdmX in full-length form or with the ZnF region deleted. The results indicate that a transduction of full length MdmX leads to the reduction of multipolar mitosis and chromosome loss in TKO cells. It also supports that this function is independent of the presence of Mdm2. Moving forward, these stably transfected cell lines will be used for the identification of protein partners interacting with MdmX. This will advance the understanding of the mechanisms by which MdmX supports genome stability, something that is important to the development of effective drugs for cancer treatment.

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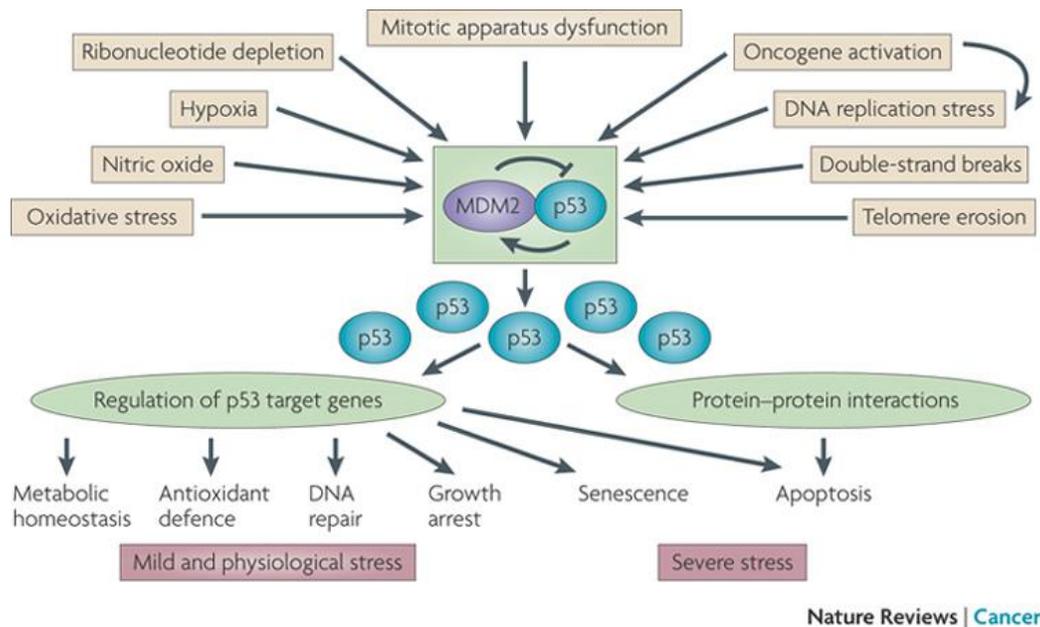
Finally, I would also like to thank Mike Carr who was always a plethora of knowledge and expertise. He was invaluable with the validation experiments and always lent an ear when I was working through new experiments. Thanks also to Mike Buckholt, my WPI advisor, for meeting with me whenever I needed as well as for reading and helping me to finalize my paper.

# 1.0 Background

## 1.1 The p53 Tumor Suppressor

The p53 protein is a 53kDa tumor suppressor, encoded by the TP53 gene, found in both human and mouse cells. Under normal conditions it functions as a sequence-specific transcriptional activator that responds to both the mild and severe stress encountered by a cell. Depending on the severity of the stress, p53 will become upregulated and activate cascade channels, often to either suspend the cell cycle until any damage has been repaired, or induce cellular suicide if the damage is too severe (Figure 1) (Vogelstein, 2010). Given the crucial role p53 plays in ensuring cells do not grow or proliferate when they are damaged, mutation or upregulation by proteins that restrict its ability to function can have serious repercussions. According to the International Cancer Genome Consortium, the TP53 gene is the most frequently mutated cancer-related gene, altered in over 50% of human cancers (Surgent, 2013). Due to this, p53 has become a significant target for research and development of cancer therapeutics.

Endogenous levels of p53 are usually very low within the cell until internal or external stimuli indicates stress, upregulating p53 to prevent and repair damages (Figure 1). p53 has been shown to respond to numerous stress inducers including DNA damage, hypoxia, telomere erosion, and nutrient deprivation (Vousden et al., 2007). In its mutated form, p53 loses its function, generally becoming oncogenic in nature (Muller et al., 2013). For example, breast tumors and neuroblastomas are often caused by mutant p53 proteins being incorrectly positioned outside the nucleus, losing its ability to function as a tumor suppressor (Vogelstein, 2010). However, in many cancers the TP53 gene is unaltered but the p53 protein becomes inactivated due to the interacting proteins, Mdm2 and MdmX. Because of this, many cancer therapeutics are now focusing on targeting these MDM family members to prevent or alter their interaction with p53 (Muller et al., 2013).



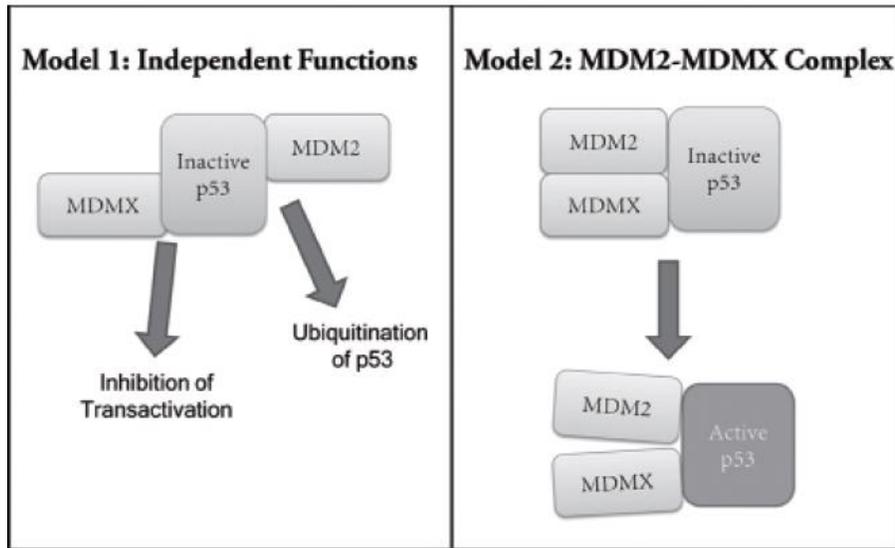
**Figure 1: Activation Pathway of p53.** Both mild and severe stress can activate p53 upregulation and in turn result in a variety of different outcomes to protect and repair the cell (Surgent, 2013).

## 1.2 Mdm2 and MdmX Regulation of p53

MdmX and Mdm2 are homologous RING domain proteins that act to inhibit p53 activity. Under normal conditions, both proteins function to prevent p53 from permanently arresting the cell cycle or inducing apoptosis when the cell has repaired damages and can continue safely (Shadfan et al., 2012). Both proteins are vital to normal cellular function because they prevent p53 from over suppression of cellular functions once the stress-inducing conditions have been resolved (Wade et al., 2013).

Mdm2 functions by either binding to p53's transactivation domain, thereby inhibiting it from downstream transcription of normal activation targets, or by acting as an E3 ligase targeting p53 for ubiquitination and proteosomal degradation (Shadfan et al., 2012). Mdm2 is frequently found overexpressed in many cancers, like soft-tissue sarcomas and osteosarcomas, which often retain wild-type p53 (Shadfan et al., 2012). MdmX can also function to inhibit the transactivation of p53, but it also

plays a crucial role in stabilizing Mdm2 (Figure 2). Like Mdm2, MdmX is often overexpressed in tumors retaining wild-type p53, including lymphoblastic leukemia, carcinoma, retinoblastoma, and some breast tumors (Shadfan et al., 2012).



**Figure 2: Proposed Models of MdmX and Mdm2.** The MDM proteins have different proposed mechanisms for the regulation of p53 (Shadfan & Lopez-Pajares, 2012).

Maintaining the balance and proper regulation of p53 and MDM protein levels is vital to healthy cellular functioning. Without sufficient Mdm2 or MdmX to restrict p53 functions, the cell is unable to grow or differentiate and often ends up dying (Shadfan et al., 2012). Absence of proper MDM protein levels has been proven embryonic lethal in mice; this is then rescued when coupled with the removal of p53 (Migliorini et al., 2002) (Jones et al., 1995). In contrast, overexpression of MDM proteins causes a decrease in the ability of p53 to serve as a tumor suppressor, resulting in oncogenesis and tumor formation (Jones et al., 1995).

### 1.3 Role of MdmX in Tumorigenesis

Recently studies have indicated that MdmX can also play a role in tumor suppression in mice lacking p53 (Matijasevic et al., 2008). Mice without both p53 and MdmX expression developed tumors

significantly faster than mice only deficient for p53. A two-fold increase in multipolar spindle formation, as well as reduced ploidy from chromosome loss, were also found in mouse embryonic fibroblast (MEF) cells lacking both MdmX and p53 as compared to those only lacking p53. With the reintroduction of MdmX into double knockout (p53<sup>-/-</sup>, MdmX<sup>-/-</sup>) MEF cells, occurrence of multipolar spindles and chromosome number stabilizes to that of p53-null state (Matijasevic et al., 2008).

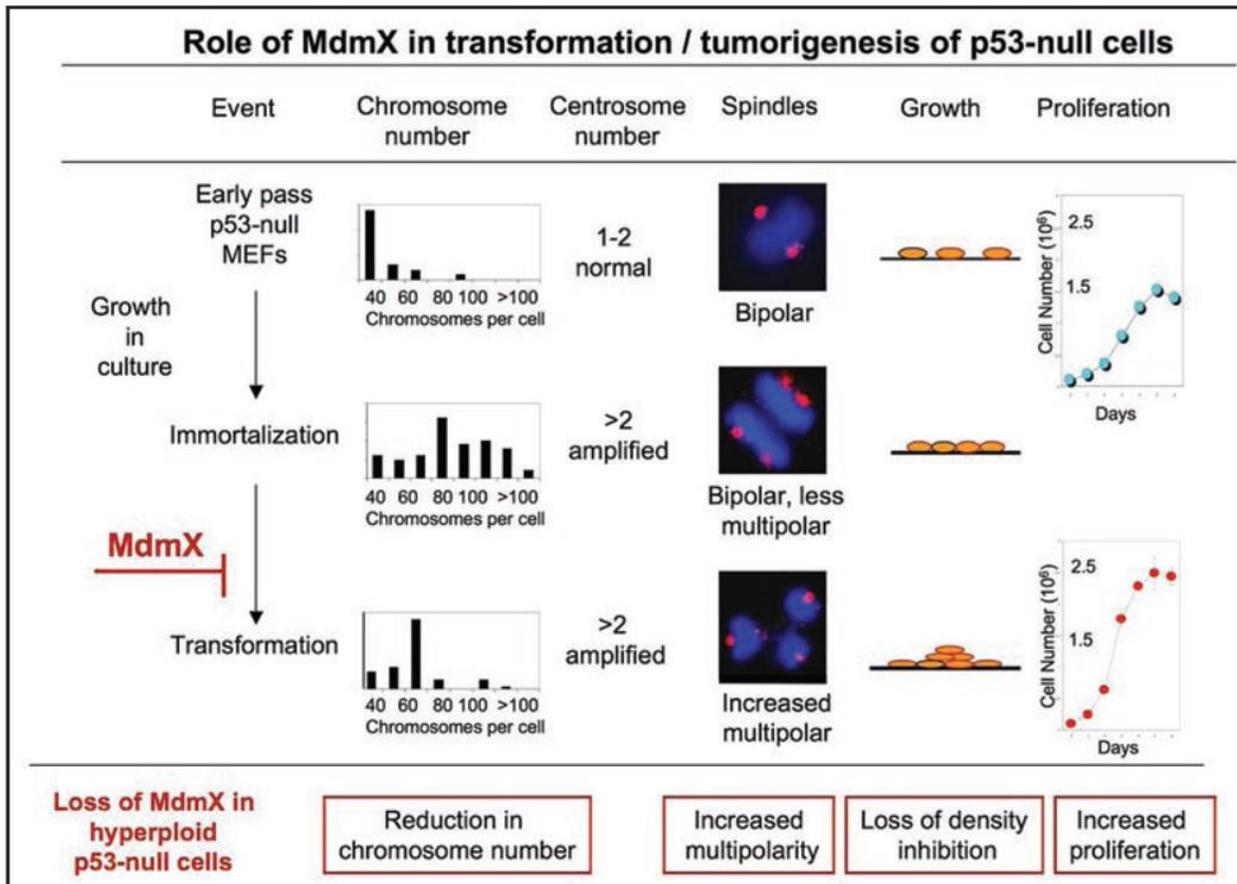


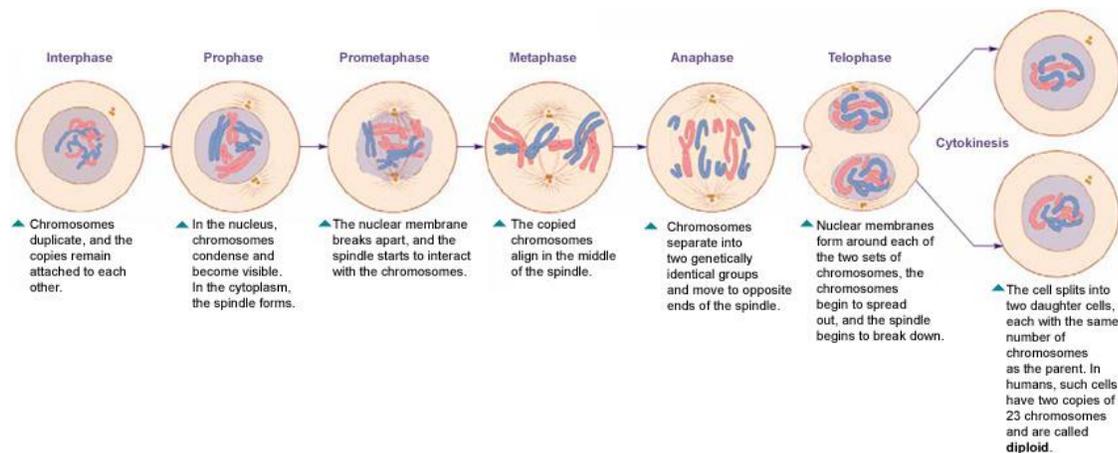
Figure 3: Role of MdmX in Transformation/Tumorigenesis of p53-null Cells. Summary of events observed in p53-null mouse embryonic fibroblast cells as triggered by the deletion of MdmX (Matijasevic et al., 2008).

In contrast to MdmX, Mdm2 has not been shown to have a p53-independent role in tumor suppression. No difference was found between p53-deficient and both p53 and Mdm2-deficient fibroblasts for cell proliferation, cell cycle control, or tumorigenesis (Jones (2) et al., 1996). However, there are no publications yet indicating whether or not Mdm2 is required to support MdmX in its role in genome stability.

Due to the unique nature of MdmX, studies have now focused on understanding the tumor-suppressive role of MdmX in p53-null conditions. Further research has indicated that the Zinc Finger domain (ZnF) plays a quintessential role in suppression of chromosome loss and multipolar mitosis within p53-deficient mouse and human cells (Matijasevic et al., unpublished). This ZnF has also been found to be required in the ability for MdmX to suppress tumor progression and decrease metastatic potential for tumor cells *in vivo* (Matijasevic et al., unpublished). This research is important because it introduces new information about the tumor suppressive role MdmX can play when p53 is mutated.

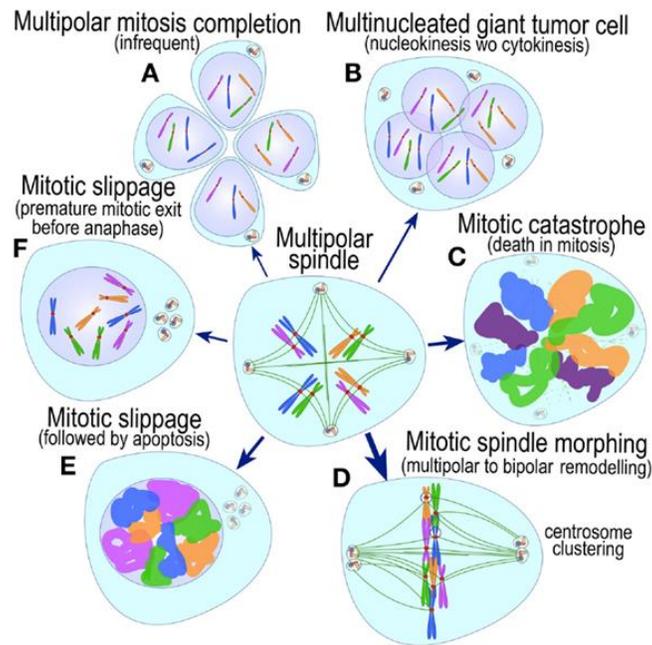
### 1.4 Cancer and Genomic Instability

The role of MdmX in supporting genome stability is thought to manifest during mitosis (Matijasevic et al., 2008). Before a cell undergoes mitosis, the genome and centrosomes are duplicated in Interphase. The genetic material then condenses and aligns along the metaphase plate. Microtubules attach the chromosomes to the centrosomes located on opposite poles of the mitotic spindle, one centrosome on each pole, and begin separating the chromosomes equally. This results in bipolar cell division and the formation of two daughter cells (Figure 3).



**Figure 4: Stages of Normal Mitosis Resulting in Bipolar Chromosome Segregation.** Illustrative model with descriptions of the process by which somatic cells divide into diploid daughter cells (Machlek, 2005).

However, cells in a cancerous states often have more than two centrosomes which can cause asymmetrical segregation during mitosis (Ganem et al., 2009). This leads to abnormal chromosome numbers and/or cellular death of the resulting daughter cells (Figure 4). Cells that survive this aberrant division have irregular chromosome numbers (ploidy) which will result in subsequent daughter cells likely presenting with either hyperploidy or hypoploidy (more or less than normal numbers of chromosomes). This escalation in frequency of centrosomes has been shown to increase chromosomal instability and promote tumor development *in vivo* and *in vitro* (Ganem et al., 2009) (Fujiwara et al., 2005). In some instances, cells are able to cluster overexpressed chromosomes to opposite poles leading to pseudo-bipolar mitosis where the chromosomes are more likely to separate equally into daughter cells (Figure 4, Panel d).



**Figure 5: Potential Results of Multipolar Mitosis.** Cells undergoing mitosis with too many centrosomes often result in inviable daughter cells and/or apoptosis unless centrosome clustering can occur (Pihan, 2013).

## 2.0 Statement of Purpose

Previous research has established not only that MdmX plays an important role in promoting genome stability in a p53-independent manner, but also that this function can be specifically associated to its Zinc Finger domain (ZnF). This project aimed to generate, validate, and functionally analyze mouse epithelial tumor cell lines suitable for the future identification of MdmX interactive protein partners. Also of interest was to explore whether the ZnF mediated role of MdmX in supporting genome stability requires intact Mdm2 function. For both aims triple knockout (TKO) mouse cells (p53<sup>-/-</sup>, Mdm2<sup>-/-</sup>, and MdmX<sup>-/-</sup>) were transfected with expression vectors containing FLAG-tagged MdmX in full-length form and a deletion mutant lacking the ZnF region.

## 3.0 Methods

### 3.1 Plasmid Isolation and Digestion

Frozen *E.coli* stocks were grown in LB Broth with Carbenicillin overnight at 30°C, plasmid DNA was isolated using Quigen Plasmid Purification Midiprep Kit and analyzed with NanoDrop for DNA concentration. Isolated plasmids were digested under NEB protocol for respective enzyme(s) then run on 1% Agarose Gel. Table 1 includes all plasmids used and Figure 6 illustrates the respective differences in size between FL (full-length) and  $\Delta$ ZnF $\Delta$ RING (double-deleted Zinc Finger and RING domains) MdmX constructs.

Table 1: Plasmid Files for MdmX Constructs

NAME	DESCRIPTION	ABBREVIATION
SJ156	Puro-Babe containing Puromycin resistance gene	<b>Puro-R</b>
SJ385	pcDNA3.1 Full Length MdmX	<b>MdmX-FL</b>
SJ416	pOZ expression vector with FLAG-tagged Full Length MdmX t	<b>pOZ-FL</b>
SJ424	pOZ expression vector with FLAG-tagged double deleted ZnF and RING domain MdmX	<b>pOZ-dd</b>

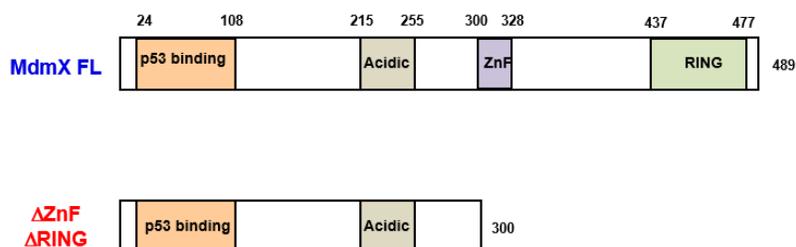


Figure 6: MdmX FL & MdmX dd Proteins. Illustrative representations of both MdmX constructs, full length (FL) and double deleted Zinc Finger and RING domains (dd), with respective amino acid lengths.

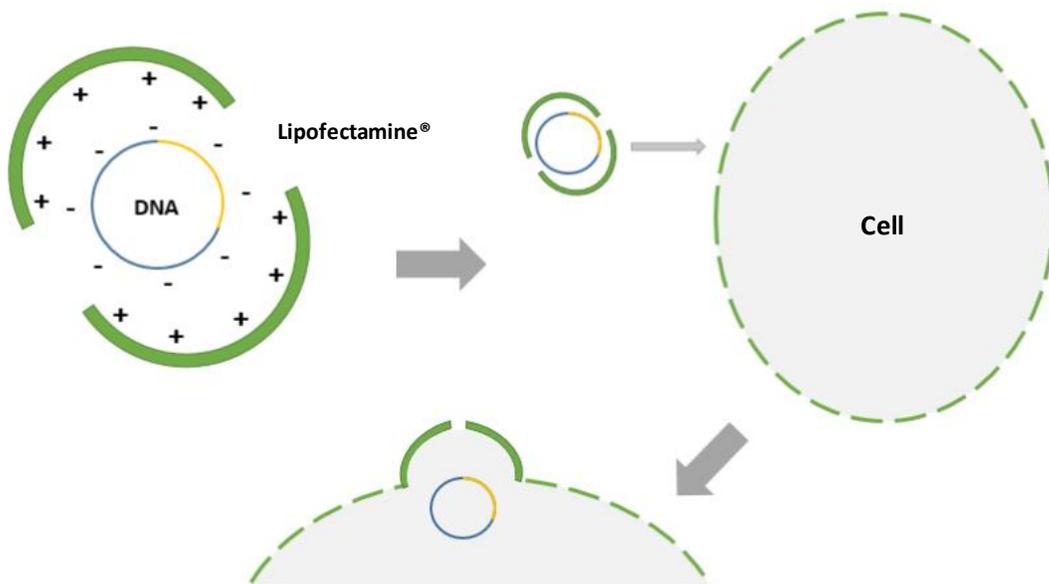
### 3.2 Cell Culture

Human embryonic kidney-derived cells (HEK293) and mouse TKO (triple knockout for p53, Mdm2, and MdmX) adherent cell lines were used for this project. Cells were cultured in Dulbecco's

Modified Eagles Media (DMEM) containing 10% Fetal Bovine Serum (FBS) and ampicillin /streptomycin antibiotic and grown at 37°C in a 5% CO<sub>2</sub> incubator.

### 3.3 Transfection

Cells in 6-well plates were transfected at 70% confluency in antibiotic-free media in accordance with Invitrogen Lipofectamine<sup>®</sup> protocol. 4µg of total DNA was used (in a 1:5 ratio for Puro/MdmX plasmids) with 10µL Lipofectamine<sup>®</sup> 2000 per well. Cells were incubated at 37°C in a CO<sub>2</sub> incubator and media was changed after 8 hours. For transient transfection cells were grown for additional 30 to 48 hours and tested for transgene expression. For stable transfection, cells were re-plated to 10cm plates 24h after transfection in preparation for Puromycin selection (3µg/mL) 24 hours later. Puro-resistant clones were then pooled, propagated, and used for further analyses.



**Figure 7: Lipofectamine<sup>®</sup> Transfection Reagent Mechanism.** The transfection reagent surrounds negatively charged DNA in order to allow passage into the positively charged cell membrane. These positively transfected cells, now with Puromycin Resistance (Puro-R), are then selected for using Puromycin and propagated to create a stably transfected cell line.

### 3.4 Immunofluorescent Staining

Transfected cells were grown on coverslips to 60% confluency and washed in cold PBS. Cells were then fixed in pre-cooled methanol and stored at -20°C until staining. Cells were removed from methanol, rehydrated in PBS for 5 minutes at room temperature and blocked (3% PBS, 0.5% BSA, 0.5% Tween 20) for one hour at room temperature. Coverslips were then incubated in a wet chamber with a 1:500 dilution of primary antibodies (mouse anti-alpha-tubulin and rabbit anti-gamma-tubulin from Sigma Aldrich) in blocking buffer overnight at 4°C. Cells were washed in 1% Buffer (1% PBS, 1% BSA, and 0.5% Tween) 3x10 minutes in, incubated with secondary antibodies (goat anti-mouse ALEXA 488 and goat anti-rabbit ALEXA 594 from Molecular Probes) at 1:2000 dilution for 2-3 hours at room temperature. After washing, cells were incubated with DAPI for 10 minutes and mounted on slides with a drop of Prolong® Gold Antifade Reagent (Life Technologies) for imaging.

### 3.5 Mitotic Spreads

Cells grown on 10cm dishes to 80% confluency were incubated with 0.02µg/mL Colcemid solution (Gibco) for 90 minutes and then trypsinized and spun down. Most supernatant was aspirated and cells were resuspended in remaining liquid. 10mL of 37°C hypotonic solution (0.075M KCl) was added and incubated for 15 minutes at 37°C. Three drops of fixative (3:1 methanol to acetic acid) was then added, centrifuged (120RCF for 8 minutes), and supernatant was aspirated. 1mL of fixative was added (dropwise with agitation) then incubated on ice for 60 minutes. Cells were then spun 45RCF for 5 minutes, aspirated, and given another 1mL of fixative dropwise- this was repeated 4 times. Mitotic spreads were prepared by dropping cell solution at a distance of about 90cm onto glass slides, which were then flamed and heat-dried. After drying, slides were rinsed with PBS and stained for 15 minutes

with Giemsa (Sigma GS-500) to physically color the condensed chromosomes, permitting visualization and counting with light microscope.

### 3.6 RT-PCR

Cells were harvested using RLT Plus Buffer (Quiagen) containing Beta-Mercaptoethanol ( $\beta$ ME) and RNA was isolated using RNeasy® Plus Micro Kit with RNase-free DNase (Quiagen). IScript™ Reverse Transcription Supermix (BioRad) for RT-PCR was used with 2 $\mu$ g RNA for first strand cDNA synthesis of each sample. A “RNA Control” sample was generated using IScript™ No Reverse Transcriptase Supermix. Reaction protocol: incubation of 5 minutes at 25°C, 30 minutes at 42°C, 5 minutes at 85°C, and hold at 4°C was applied using PCR machine.

Table 2: Primer Sequences

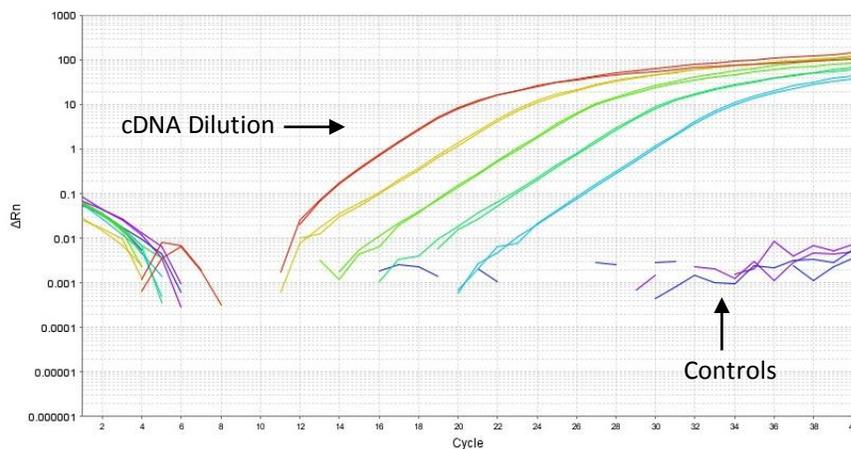
NAME	SEQUENCE
ACTIN	<b>F</b> 5' – TCC TGT GGC ATC CAT GAA ACT – 3'
	<b>R</b> 5' – GAA GCA CTT GCG GTG CAC GAT – 3'
MDMX	<b>F</b> 5' – TCT CGC ACA GGA TCA CAC TAT GGA – 3'
	<b>R</b> 5' – TCA TCT GCT CTG GAG TCT CTG CAT – 3'
MDMX BZ	<b>F</b> 5' – GAC CGA CTG AAG CAC GGT GCA A – 3'
	<b>R</b> 5' – AAC AAG GCA GGC CAG CAA CG – 3'

12.1 $\mu$ L Syber Green® Master Mix was then added to 2 $\mu$ L synthesized cDNA and 0.2 $\mu$ L of each forward and reverse primers with total volume reaching 25 $\mu$ L with nuclease-free water. Two different MdmX primers were used against endogenous control actin primers. All samples were loaded in triplicates into a 96-well plate, run on RT-PCR, and analyzed using Applied Biosystems StepOne® Plus RT-PCR software. MdmX expression was normalized to actin control and then paired t-test was performed against Mock samples to determine statistical significance of transfected TKO cells.

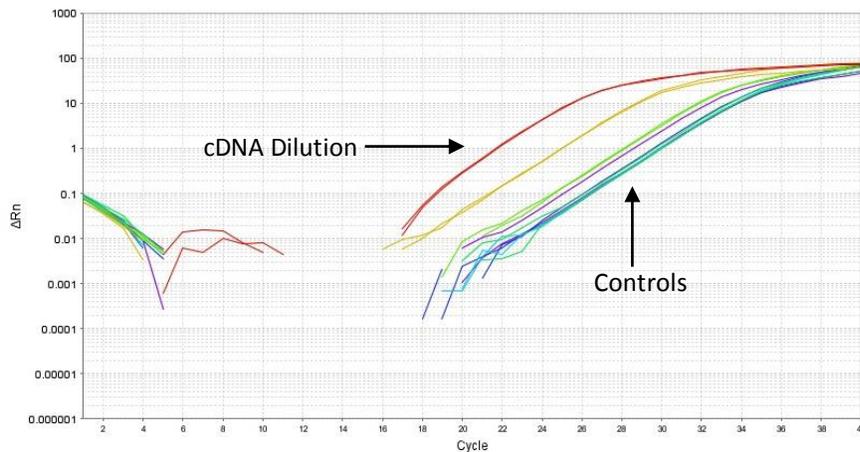
### 3.6.1 Troubleshooting

Although the RT-PCR results showed a small difference between the MdmX levels between transfected (pOZ-FL and pOZ-dd) and Mock control cells, there was still more expression in Mock than expected as well as only a 50% increase of MdmX expression in transfected samples relative to Mock (Figure 13). From this, troubleshooting experiments were performed in order to validate primers used and to trace possible sample contamination.

A series of 10 fold dilution of cDNA (100 $\mu$ g to 0.01 $\mu$ g) was run with both MdmX primers and with actin primer in parallel with Water Control (containing dH<sub>2</sub>O, SyberGreen<sup>®</sup> Master Mix, and primer) and RNA Control (IScript<sup>®</sup> No Reverse Transcriptase-generated “cDNA”, SyberGreen<sup>®</sup>, and primer), and cDNA Control (IScript<sup>®</sup> Reverse Transcriptase-generated “cDNA” using water instead of RNA, SyberGreen<sup>®</sup>, and primer).



**Figure 8: Amplification Curve of Troubleshooting MdmX Primers- Endogenous Control (Actin).** Dilution series (100 $\mu$ g to 0.01 $\mu$ g) of unrelated cDNA (red, orange, green, teal, light blue) and water, RNA, and cDNA controls (purple, dark blue, and dark purple).



**Figure 9: Amplification Curve of Troubleshooting MdmX Primers- MdmX BZ.** Dilution series (100 $\mu$ g to 0.01 $\mu$ g) of unrelated cDNA (red, orange, green, teal, light blue) and water, RNA, and cDNA controls (purple, dark blue, and dark purple).

Figures 8 and 9 show the respective amplification curves of the endogenous actin control and MdmX BZ primers on a dilution of cDNA unrelated to the experiment. While the cDNA dilutions with actin primers gave satisfactory amplification results relative to Water and RNA controls (Figure 8), the cDNA and controls for the MdmX primers showed amplification, which indicates possible sample contamination (Figure 9). This validation experiment was repeated three times with varying alterations and similar results.

### 3.7 Western Blot

Cells were harvested and lysed with NP-40 Lysis buffer containing protease and phosphatase inhibitors. A Bradford Protein assay was then performed to determine protein concentration. 60 $\mu$ g of each protein extract was then heated to 95 $^{\circ}$ C for 5 minutes in 4x Laemmli buffer then cooled on ice. Protein extracts were then loaded on a 4-15% GPROTEAN<sup>®</sup> TGX<sup>™</sup> gel (BioRad) with 1X Running Buffer (1L 10X Running Buffer: 30.3g Tris, 144g glycine, 10g SDS in dH<sub>2</sub>O) and run at 80V for about 2 hours. Gel was then transferred onto PVDF membrane overnight at 20V in a gel transfer chamber with 1X Transfer Buffer with 20% Methanol (1L 10X Transfer Buffer: 30.3g Tris, 144g glycine). After transfer phase, the

membrane was washed 3x5min in TBS-T (1L 10X TBS-T: 100mL 2M Tris pH 7.5, 87.66g NaCl, 10mL Tween20 in dH<sub>2</sub>O), and blocked for 2 hours with Blocking Buffer (15mL 10X TBS, 135mL dH<sub>2</sub>O, 7.5g nonfat dry milk, 150μL Tween-20) at room temperature. Membrane was then washed 3x5min in TBS-T then incubated with a 1:750 dilution of primary anti-MdmX antibody (Mouse, MX-82, Sigma Aldrich) for 3 hours with rocking at room temperature. Next, membrane was thoroughly washed 3x10min with TBS-T and treated with secondary antibody anti-mouse (sheep), at a 1:4000 dilution, for 1 hour with rocking at room temperature, then finally washed 3x5min in TBS-T. Blot was then treated for 5 minutes with Clarity™ Western ECL Substrate (BioRad) then imaged.

## 4.0 Results

Recent studies have indicated a previously unknown function of the oncogene MdmX in suppressing tumorigenesis in a p53-independent manner (Matijasevic et al., 2008). This has been further investigated in mouse cells to reveal the role of the Zinc Finger (ZnF) region of MdmX in supporting genome stability (Matijasevic et al., unpublished). Specifically, these results have indicated that the ZnF region of MdmX aids in the reduction of multipolar mitosis and prevention of chromosome loss that occurs in mouse and human tumor cells lacking p53. The next phase for this study is to explore the protein partners interacting with MdmX in order to understand the mechanisms by which it provides genome stability in a p53 independent manner.

The purpose of this experiment was to prepare stably transfected cell lines in mouse TKO cells (Triple Knockout MdmX<sup>-/-</sup>, Mdm2<sup>-/-</sup>, and p53<sup>-/-</sup>) with pOZ expression vectors containing FLAG-tagged MdmX in full-length form and with the ZnF region deleted. These cell lines were then validated for MdmX expression and a functional analysis was performed in respects to multipolarity and chromosome retention. Following this, the next phase for this study will be to utilize affinity purification to isolate the tagged fusion proteins interacting with the MdmX.

### 4.1 Plasmid Purification and Digestion

All plasmids used contained ampicillin resistance and were grown in *E.coli* in media containing carbenicillin, an ampicillin derivative. Table 1 (see Methods) details information on all plasmids used for the purpose of this experiment. Plasmids intended for application throughout the experiment were purified and evaluated through enzyme digestion in order to ensure identity. DNA of puromycin resistance (Puro-R), MdmX Full Length (MdmX-FL), and dZnFdRING (MdmX-dd) plasmids were extracted,

dual-digested with restriction enzymes HindIII and XhoI, and run on 1% agarose gel (Figure 10). Resulting bands were evaluated based on plasmid maps and identity was supported for each plasmid.

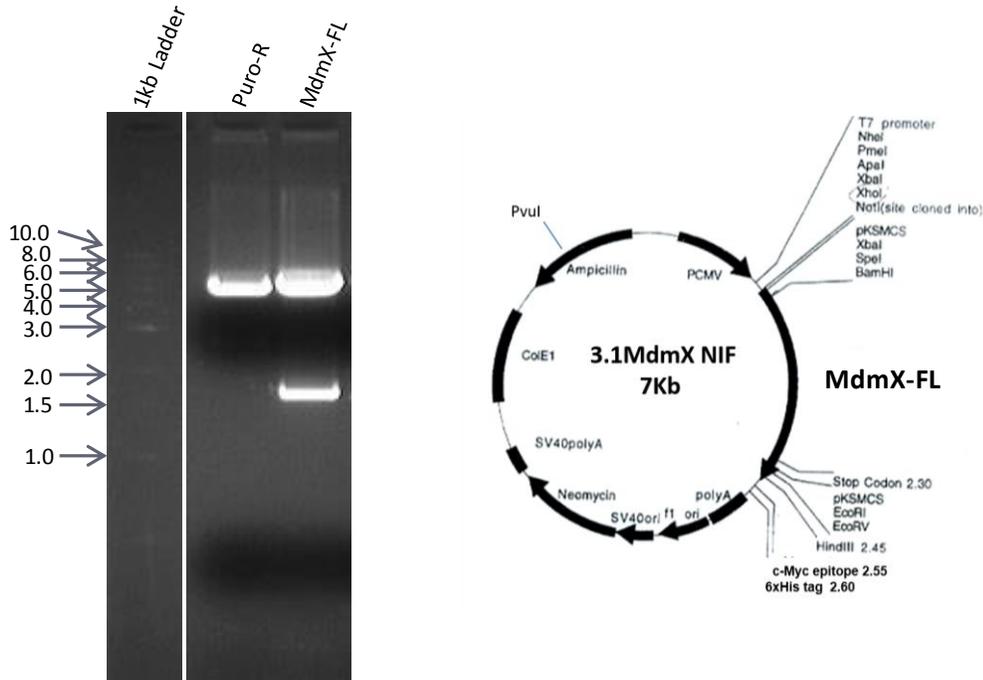
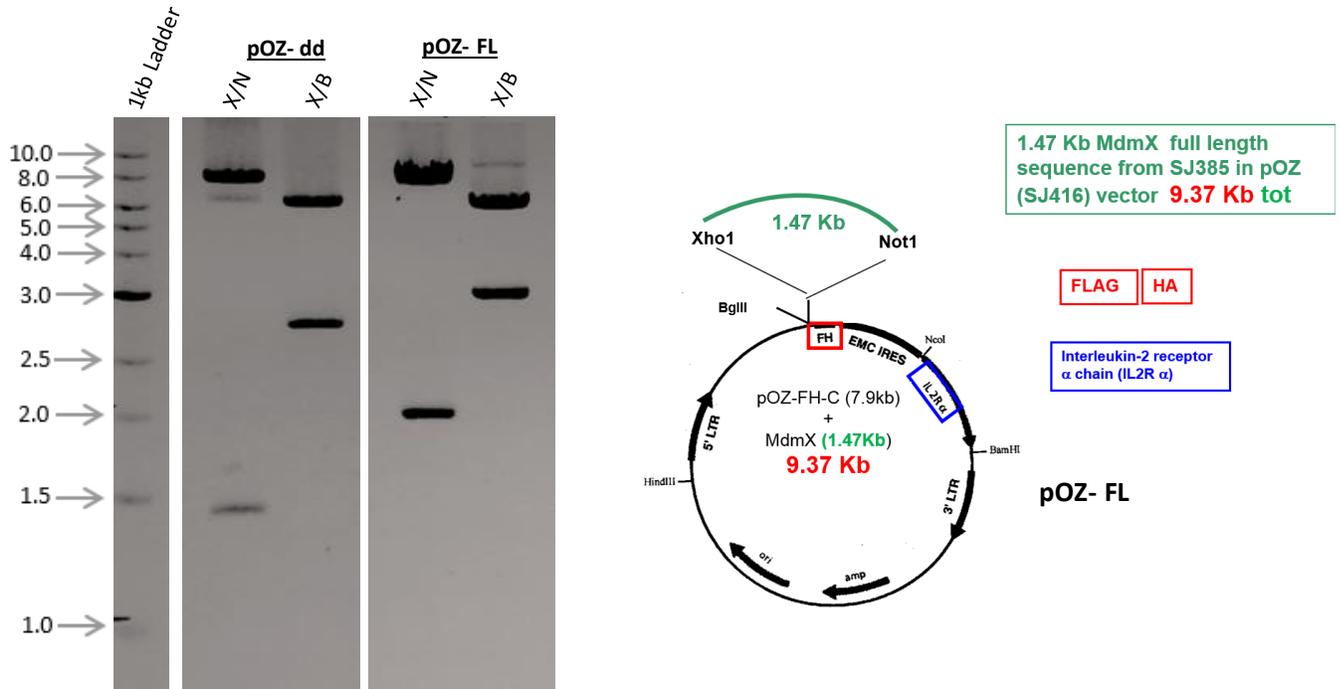


Figure 10: Digestion of Puro-R and MdmX-FL Plasmid (Left); MdmX-FL Plasmid Map (Right). Plasmid DNA was double-digested with HindIII/XhoI restriction enzymes to confirm identity and size.

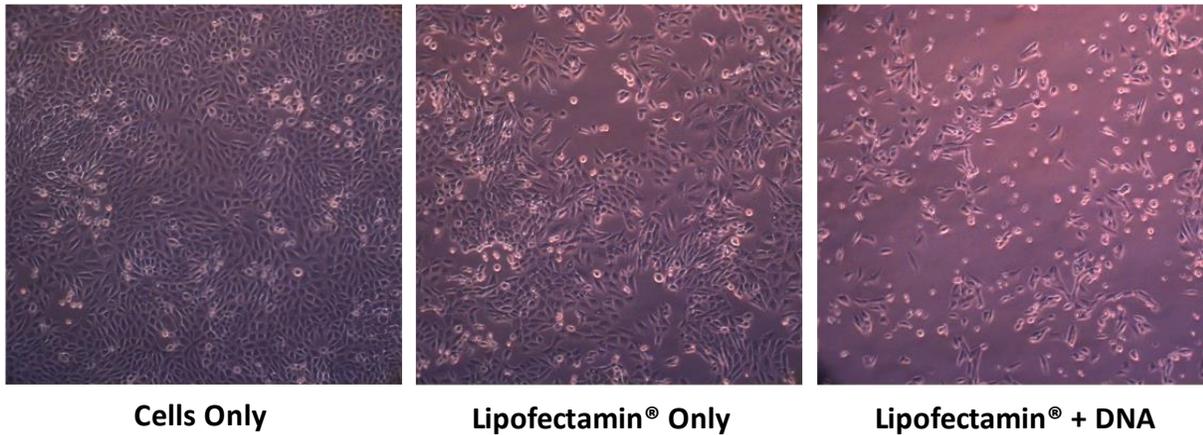
For transfection experiments with TKO mouse cells, the DNA of pOZ expression vectors containing FLAG-tagged full length (pOZ-FL) and double-deleted Zinc Finger and RING domains (pOZ-dd) was extracted, dual-digested with XhoI/NotI and XhoI/BamHI, and run on 1% agarose gel. Resulting bands were evaluated based on plasmid maps and identity was supported for each plasmid (Figure 10).



**Figure 11: Digestion of pOZ-dd and pOZ-FL with Plasmid Map.** MdmX expression vector DNA was double-digested with XhoI/NotI (X/N) and XhoI/BamHI (X/B) restriction enzymes to confirm identity and size.

## 4.2 Stable Transfections

After plasmids were purified and identity was confirmed through digestion analysis, pOZ- based MdmX constructs with full length (pOZ-FL) and deletion mutant (pOZ-dd) were selected for use in transfection experiments because they already possessed the FLAG-tagging required for further experiments. These plasmids were then linearized with BamHI and co-transfected with Puro-R plasmid into triple knockout (TKO) cells using Lipofectamin® transfection reagent. Mock was transfected with only Puro-R plasmid as a control for validation and functional analysis.



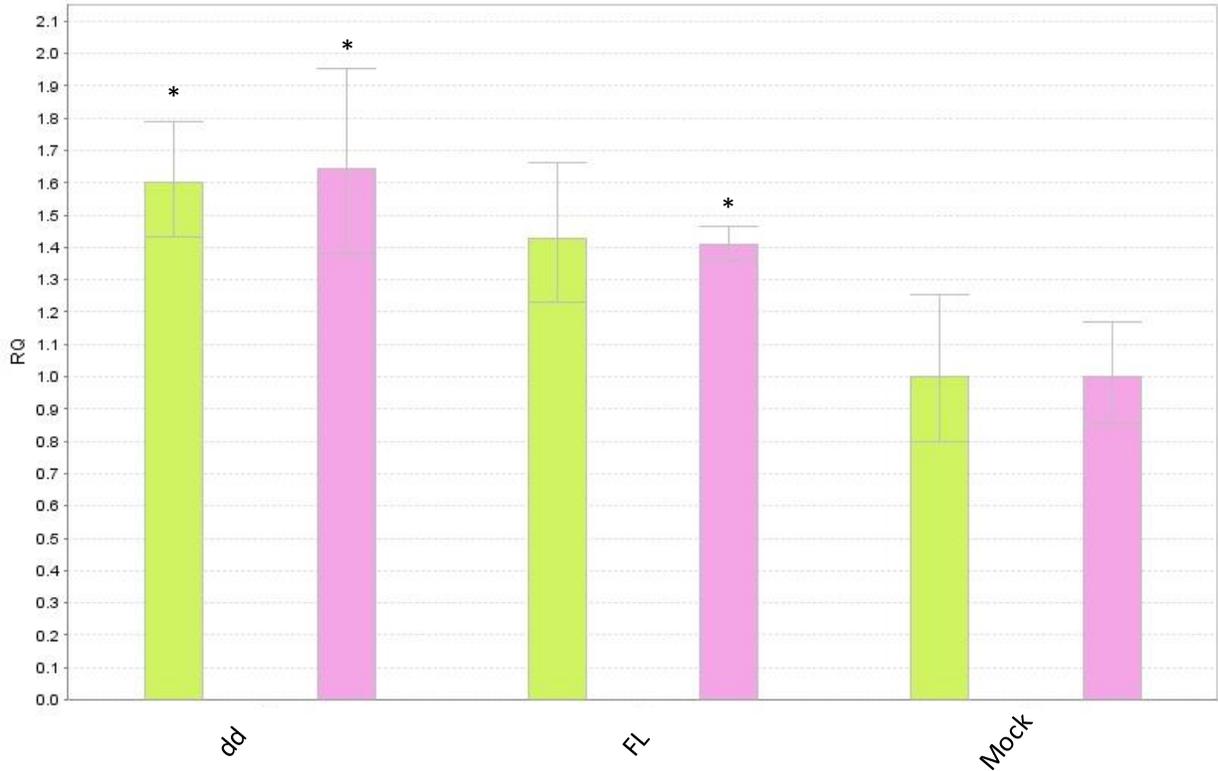
**Figure 12: Mouse TKO Cells 24hr Post-Transfection with pOZ/MdmX constructs and Puro-R.** TKO cells were imaged under light microscope after transfection, those treated with transfection reagent and DNA showed slightly reduced proliferation.

24 hours after transfection, cells were visualized to examine the growth of untransfected and transfected cells before Puromycin selection. The cells treated with Lipofectamine® transfection reagent appeared to have slightly reduced proliferation, with more significant reduction observed in plates treated with both transfection reagent and DNA. At 48 hours, Puromycin was added to initiate selection and surviving clones were pooled and propagated in DMEM medium for freezing and further analysis.

## 4.3 Validation

### 4.3.1 RT-PCR

In order to validate transfection of the MdmX constructs into mouse TKO cells, quantitative RT-PCR was performed. Results were analyzed using Applied Biosystems StepOne® Plus RT-PCR software and RQ (normalized to actin control) for both MdmX primers was evaluated (Figure 13). Paired t-tests were then performed against Mock samples to evaluate statistical significance.

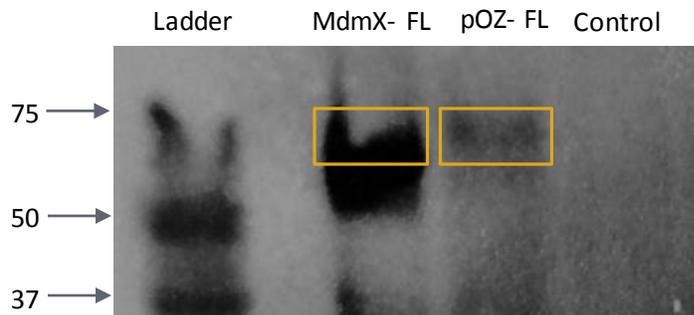


**Figure 13: MdmX Levels in Mouse TKO cells Transfected with pOZ/MdmX Expression Plasmids.** Results from RT-PCR (normalized to endogenous actin control) of RNA extracted from TKO cells transfected with MdmX constructs analyzed with both MdmX (green) and MdmX BZ (purple) primer sets. Statistical significance (\*) of MdmX expression comparative to Mock control was indicated for dd (p value = 0.027) and FL (p value = 0.013) with MdmX BZ primer and dd (p value = 0.044) with MdmX primer. Bars indicate standard error between triplicate samples.

A significant difference was indicated between the MdmX levels, comparative to Mock, for both full length (FL) and deletion mutant (dd) RNA samples with MdmX BZ primer, and with deletion mutant (dd) with MdmX primer. No statistical significance was found for MdmX expression of full length with MdmX primer (p value = 0.10). However, the expression difference between Mock samples and those transfected was much lower than anticipated. Also of concern was the relatively high levels of MdmX expression within Mock. Troubleshooting experiments of primers validation were then performed to further evaluate these unclear results (see Methods).

### 4.3.2 Western Blot

To further explore MdmX expression, pOZ- based vector containing FLAG-tagged full length MdmX (pOZ- FL) and pcDNA3.1-based vector containing full length MdmX (MdmX- FL) were transiently transfected into HEK293 cells and Western Blot analysis was performed on protein extracts. 60µg of protein extracted from the transient transfections was run on 4-15% gel, transferred, blocked, and treated with a 1:750 dilution of primary antibody (anti-MdmX mouse) and a 1:4000 dilution secondary antibody (anti-mouse). The control used was protein from HEK293 cells treated only with transfection reagent, and therefore with no expected MdmX expression.



**Figure 14: Western Blot of Protein Extracts from Transiently Transfected HEK Cells.** Both MdmX- FL and pOZ- FL showed expression of MdmX (yellow boxes) comparative to the control.

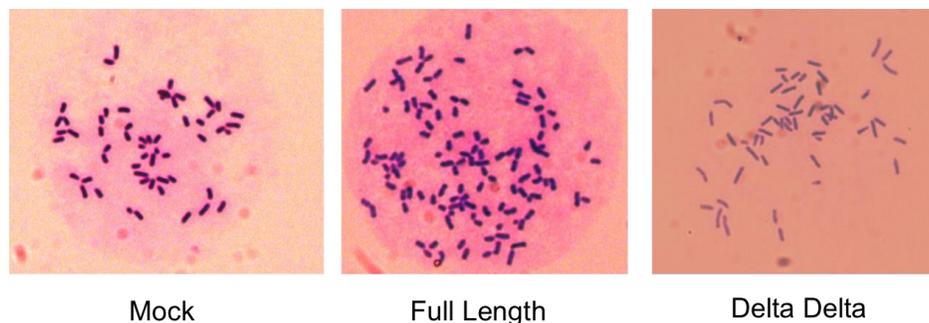
The western blot was visualized and yellow boxes indicate the MdmX band. Although the dual-color protein ladder from this Western Blot broke, the expected bands at around 60kDa can be seen for both pOZ-FL and MdmX-FL with no expression observed for the control. Potential error in loading equal amounts of protein for all samples could account for the lower intensity shown with the pOZ- FL sample. However, this supports a technically successful transfection and subsequent expression of MdmX from both pOZ-FL and MdmX-FL vectors.

## 4.4 Functional Analysis

To examine the effects of exogenous FLAG-tagged MdmX constructs on genome stability of transfected TKO (triple knockout for MdmX, Mdm2, and p53) cells, a functional analysis was performed. This included quantification of chromosomes by metaphase analysis and spindle organization by immunofluorescent staining. Cells only transfected with Puro-R (Puromycin resistance marker) were used as a control (Mock).

### 4.4.1 Chromosome Analysis by Metaphase Spreads

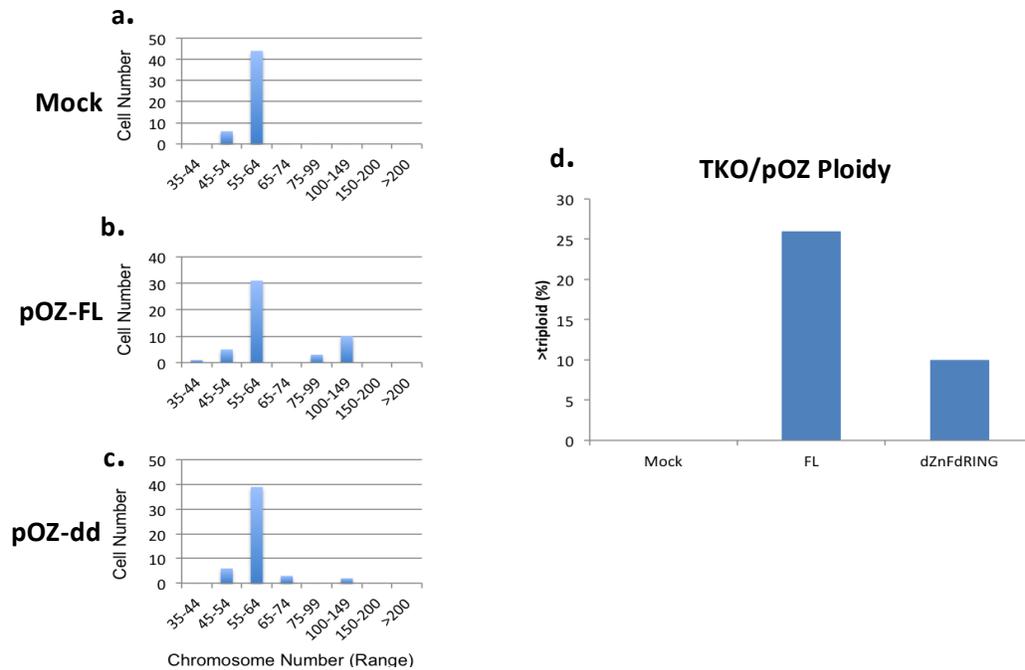
Stably transfected TKO cells were grown to 80% confluency and treated with the microtubule-depolymerizing drug Colcemid, arresting cells in metaphase. Samples were then incubated in a hypotonic solution to fracture the cells, permitting the condensed chromosomes to be spread on a glass slide. Mitotic spreads were stained with Giemsa stain, observed under the light microscope and photographed. Figure 15 is representative of the differences in ploidy found between the Mock control and both MdmX constructs.



**Figure 15: Metaphase Spread Images of Mouse TKO Cells Transfected with pOZ/MdmX Expression Plasmids.** Sample images of metaphase spreads for the quantification of chromosomes.

For quantification of chromosome number, at least 50 mitotic cells were randomly chosen from each cell line and chromosomes were counted. Figure 16, panel a, b, and c, depicts the histograms

created based on cell counts within indicated ranges. In figure 16 d the results of chromosome counts are distilled and presented as a percent of cells with higher than triploid chromosome content.

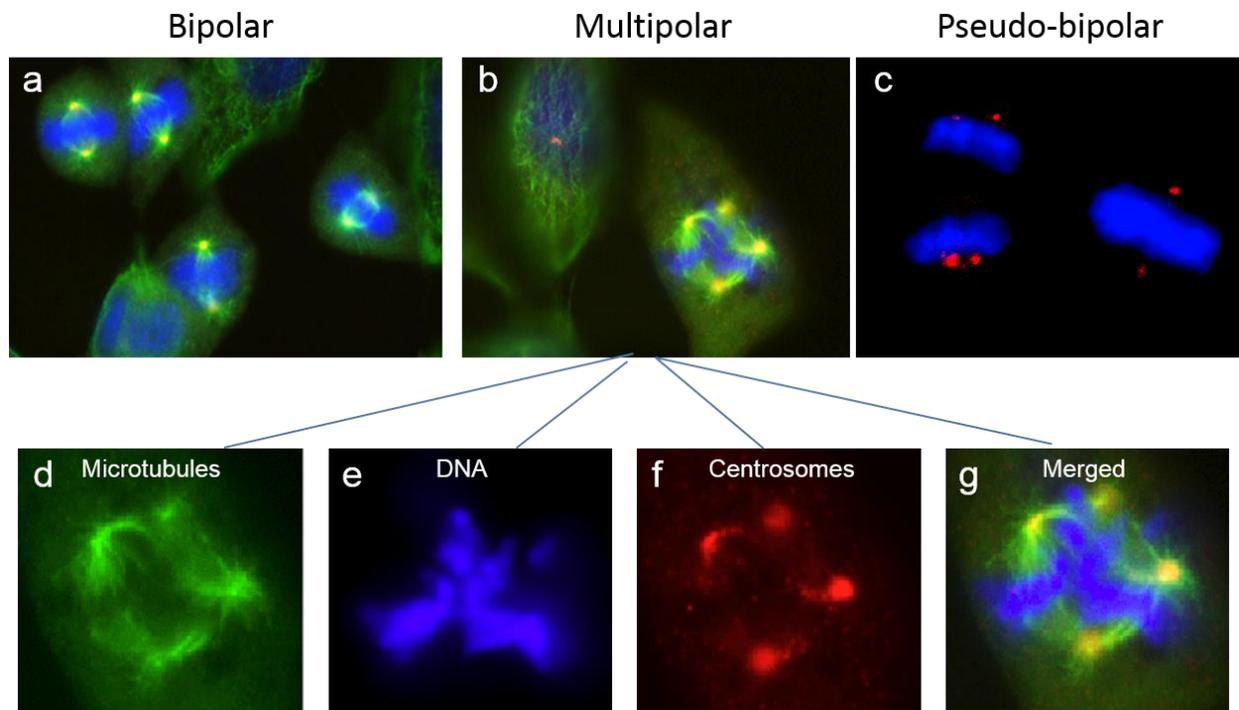


**Figure 16: Chromosome Count Data of Mouse TKO cells Transfected with pOZ/MdmX Expression Plasmids.** Panels a, b, c: Histogram of chromosome counts for TKO cells transfected with Mock, pOZ-FL, and pOZ-dd respectively. Panel d: Summary of chromosome counts presented as percent of cells with greater than triploid genome.

Through chromosome analysis, 26% of cells transfected with full length MdmX (pOZ-FL) showed greater than triploid counts. Transfection with MdmX deletion mutant (pOZ-dd) showed only 10% of cells greater than triploid (Figure 16, Panel d.). These data indicate that the FLAG-tagging of MdmX, as expressed from the pOZ vector, increases ploidy and reduces chromosome loss. The deletion of the ZnF/RING region of MdmX shows to (at least partially) abrogate this effect. This parallels previous results with endogenous MdmX in MEFs and tumor cells (Matijasevic et al., 2008) and exogenous MdmX expressed from pcDNA3.1-based constructs (Matijasevic, unpublished).

#### 4.4.2 Spindle Analysis by Immunofluorescent (IMF) Staining

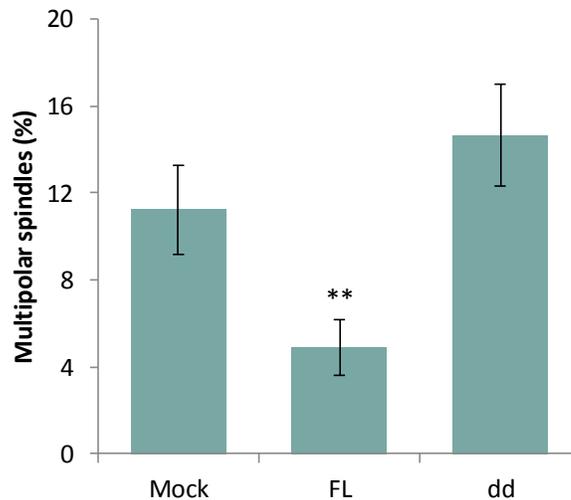
In order to examine the effect of FLAG-tagged MdmX on mitotic spindle organization, immunofluorescent analyses were performed on stably transfected TKO cells grown on glass coverslips.. Cells were stained with anti-alpha tubulin antibodies for microtubules (green), anti-gamma tubulin antibodies for centrosomes (red), and with diamino-phenylindole (DAPI) for DNA (blue) and observe under fluorescent microscope.



**Figure 17: Immunofluorescent Staining of Transfected TKO Cells.** Cells undergoing bipolar and multipolar mitosis are shown stained for microtubules (green), DNA (blue), and centrosomes (red). Individual stains are shown for multipolar mitosis to illustrate the process of overlaying. Pseudo-bipolar mitosis, stained for DNA (blue) and centrosomes (red) is also depicted.

Representative images of mitotic spindles presenting bipolar, multipolar, and pseudo-bipolar mitosis are shown in Figure 17. The images of bipolar and multipolar mitosis, in panels a and b, are merged images of cells stained for microtubules (green), DNA (blue), and centrosomes (red). Images in panels d, e, and f are showing the individual staining of multipolar mitosis from panel b. Figure 17, panel c shows merged images for centrosomes and DNA staining of pseudo-bipolar mitosis in late anaphase

stage (left) and bipolar mitosis in metaphase stage (right). Pseudo-bipolar mitosis is demonstrated in the picture by the clustering of amplified centrosomes (2+) around opposing poles allowing for chromosome segregation in a bipolar manner.



**Figure 18: Spindle Polarity in MdmX-transfected TKO Cells.** Percent of multipolar spindles were quantified from a sample of 300 mitotic events of immunofluorescent stained slide for each transfected cell line. Error bars indicate standard deviation within samples.

Spindle quantification was done by calculating the percent of multipolar spindles found in a sample of 300 mitotic events for each Mock, pOZ-FL, and pOZ-dd transfected TKO cells. Expression of full length MdmX (pOZ-FL) significantly reduced the percentage of multipolar spindles relative to the Mock control (p value = 0.0109). In comparison, there was no statistically significant difference between MdmX double deletion (pOZ-dd) and the Mock control (p value = 0.182).

#### 4.5 Conclusion

The results of functional analysis of TKO cells transfected with pOZ expression vectors for FLAG-tagged MdmX constructs indicate that neither the FLAG-tagging to the C-terminus or the absence of Mdm2 impedes MdmX in supporting genome stability. Data also confirmed that the ZnF region of MdmX plays an important role in its p53-independent function in reducing multipolar mitosis and chromosome

loss. RT-PCR and Western blot experiments provided some support to the validation of MdmX expression, however more tests should be done to confirm the relative levels of tagged-MdmX protein in the transduced cells.

## 5.0 Discussion

MdmX is an oncoprotein that negatively regulates the tumor suppressor p53. However, it has recently been shown that MdmX also plays a role in tumor suppression and supports genome stability by reducing chromosome loss and multipolar mitosis in p53-deficient cells *in vivo* and *in vitro* (Matijasevic et al., 2008). This has been further explored to find that the Zinc Finger (ZnF) region of MdmX protein plays a critical role in this function (Matijasevic et al., unpublished). This project focused on developing and evaluating cells that would be suitable for identification of MdmX binding proteins in order to learn more about the mechanism of MdmX-mediated tumor suppression.

Experiments were done with MdmX  $-/-$ , Mdm2  $-/-$ , p53  $-/-$  triple knockout (TKO) mouse cells stably transfected with FLAG-tagged full length or deletion mutant MdmX expression vectors. The TKO cells were chosen as they are null for both known MdmX binding proteins, p53 and Mdm2, whose presence would impede identification of other interacting proteins. The FLAG-tagging of MdmX will allow for future affinity purification with anti-FLAG magnetic beads in order to isolate and later identify the MdmX binding proteins.

If the functional analysis results parallel that of the previous experiments (Matijasevic et al., unpublished) it will also indicate that the FLAG-tagging of MdmX does not impede its function in genome stabilization. In addition, this would provide insight as to whether Mdm2 is required for MdmX to suppress multipolar mitosis and/or preclusion of chromosome loss. The results of functional analyses indicate that: 1) C-terminal FLAG-MdmX fusion does not obviate MdmX function in genome stability; 2) MdmX role in genome stability is Mdm2-independent; and 3) the results confirm that the Zn Finger domain of MdmX is important for maintenance of genome stability.

Based on the evidence from spindle analyses (Figure 18), it can be postulated that the role of MdmX in genome stability, most likely regulated by its ZnF domain, is based on its ability to cluster

amplified centrosomes around opposite poles in hyperploid cells. This clustering of centrosomes and formation of pseudo-bipolar spindles allows cells to go through mitosis, decreasing the likelihood of losing chromosomes, which often happens with multipolar mitosis.

## 5.1 Experimental Setbacks

Although the RT-PCR results indicated a difference between MdmX expression in the transfected cells versus the control cells, there was still a nominal amount of MdmX expression in the control cells that was unexpected (Figure 13). Troubleshooting experiments were performed in order to validate each primer set used and to identify possible reasons for such an outcome. While the RT-PCR primer validation done with a dilution series of unrelated cDNA gave satisfactory amplification curves for the actin primer, the results with both MdmX primers suggested possible sample cross-contamination (Figures 8 & 9). Further troubleshooting should be done to explore this so that the TKO cells with MdmX expression vectors can be accurately validated. This should involve repeating cDNA synthesis and RT-PCR steps, or if necessary, growing TKO cell lines again and extracting new RNA.

Given the time restrictions for this project, an alternate method was used to validate the expression of MdmX from the FLAG-tagged pOZ vector. Western blot analysis of transiently transfected human HEK293 cells was done with the pOZ-FL plasmid in parallel to a pcDNA3.1-based MdmX-FL construct. The results confirmed expression from both constructs, although there was less band intensity for the pOZ-FL sample, most likely as a result of an error in protein concentration estimates leading to less protein being loaded. This could be tested by re-probing for loading control (e.g. alpha-tubulin).

## 5.2 Future Experiments

Due to the experimental setbacks, it was not possible to proceed to the next step, the immunoprecipitation and identification of MdmX-FLAG-tagged interaction proteins, as initially planned. Therefore immediate future experiments should focus on confirming the MdmX expression in the transfected TKO cell lines. The transfected TKO cells should then be used for affinity purification experiments based on FLAG-protein fusion. This will allow for the isolation and identification of the proteins interacting with the FLAG-tagged MdmX full length and deletion mutant. This data would aid in the understanding of proteins interacting with MdmX under p53 and Mdm2-null conditions as well as potentially indicate the differences in protein partners between full length and double deletion MdmX in transfected TKO cell lines. This could provide a basis for study of the specific mechanisms by which MdmX and its ZnF maintain genome stability. A mechanistic understanding of the role of MdmX in these processes would further support the hypothesis that MdmX regulates centrosome clustering in hyperploid cells to promote pseudo-bipolar mitosis in situations where the cell would otherwise undergo multipolar mitosis.

## 5.3 Significance for Cancer Therapeutics

Since p53 is such a prevalent subject of interest for research as a target for cancer therapeutics, having extensive understanding of its functions, and the proteins that interact with it, is vital. Since both MDM proteins negatively regulate p53, they have become prime targets for cancer-fighting drugs. Already there are proposals and trials being conducted to explore methods of reducing cellular levels of MdmX and/or Mdm2 as well as blocking their abilities to interact with p53 (Wade et al. (2), 2010). Since MdmX and Mdm2 are homologous proteins, assumptions are often made about their functional similarity. However, the role of MdmX in genome stability has proved to be not only completely independent of p53, and Mdm2, but also that this function is unique to MdmX (Jones (2) et al., 1996).

Future design of targeted therapies for those cancers that display normal p53 alleles but contain overexpressed MdmX must consider both its p53-dependent and p53-independent effects.

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