

IDENTIFICATION OF NOVEL REGULATORY AND TARGET PROTEINS IN THE P53
PATHWAY: APC2 AND PFK2

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ABSTRACT

Yizhou Joseph He: IDENTIFICATION OF NOVEL REGULATORY AND TARGET PROTEINS IN THE P53 PATHWAY: APC2 AND PFK2
(Under the direction of Yanping Zhang)

The Mdm2 proto-oncoprotein is the primary negative regulator for the tumor suppressor p53. While it is believed that Mdm2 degradation is regulated via its own E3 ubiquitin ligase activity, recent development of knock-in mouse models demonstrate that *in vivo* Mdm2 E3 ligase function is dispensable for the degradation of Mdm2 itself. Here, we show that the anaphase promoting complex/cyclosome (APC/C) is an E3 ubiquitin ligase for Mdm2 degradation. We demonstrate that APC2, a scaffold subunit of APC/C, binds to Mdm2 and is required for Mdm2 polyubiquitination and proteasomal degradation. Downregulation of APC2 by RNAi results in transcription-independent accumulation of Mdm2 and attenuation of stress-induced p53 stabilization, leading to decreased senescence and increased cell survival. Furthermore, APC2 expression is frequently downregulated in human cancers and in tumor cell lines, and often correlates with Mdm2 overexpression. Our study shows the regulation of Mdm2 by APC/C E3 ubiquitin ligase, modifying our understanding of Mdm2 degradation *in vivo*, and providing important therapeutic implications for tumors with Mdm2 overexpression.

Although nucleotide shortage can result in genomic instability and cancer development, relatively little is known regarding the mechanisms responsible for coordinating nucleotide shortage and cell metabolism to maintain a nucleotide pool amenable to DNA replication and DNA damage repair. Here, we provide evidence supporting a model whereby p53-dependent regulation of phosphofructokinase-2 (PFK2) is essential for the redirection of glucose from glycolysis to the pentose phosphate pathway (PPP) under nucleotide shortage stress. Our data

show that the suppression of PFK2 is specific to nucleotide shortage. Decreased expression of PFK2 resulted in a decrease in the rate of glycolysis and an increase in PPP activity, leading to an increased nucleotide pool and improved DNA damage repair efficiency. Importantly, exogenously supplied nucleosides effectively rescued the DNA damage repair defect caused by p53 inactivation, further suggesting that the maintenance of the nucleotide pool is an important function of p53. These findings underscore an essential role for p53 in modulating glucose metabolism in response to nucleotide shortage stress, and suggest that the tumor suppressive function of p53 is linked to its role in responding to nucleotide shortage and coordinating metabolic adaptation.

PREFACE

Chapter 2 is adapted from a research article accepted for publication in *Cell Cycle*. The concept of the project was developed by me and Yanping Zhang. Tae-Hyung performed the half-life assay, Laura Tollini performed the MG-132 analysis, and Yoko Itahana performed the in vitro ubiquitin ligase assay. The rest of the experiment was performed by me. The manuscript was written by myself and Laura Tollini and finalized by Yanping Zhang.

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Chapter 3 is adapted from a research article currently in preparation. The concept of the project was developed by me and Yanping Zhang. All experiment was performed by me. The manuscript was written by myself and Patrick Leslie and finalized by Yanping Zhang.

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TABLE OF CONTENTS

LIST OF FIGURES	xi
LIST OF ABBREVIATIONS AND SYMBOLS	xiii
1. CHAPTER 1. INTRODUCTION	1
The discovery of the tumor suppressor gene <i>p53</i>	1
The discovery of Mdm2 as the major negative regulator of p53.....	3
p53 is a stress activated transcription factor	4
Regulating Mdm2 to modulate p53 function.....	6
Regulation of Mdm2 degradation	8
The Mdm2-p53 pathway is altered in human cancer	10
p53 plays a role in regulation of cell cycle arrest and apoptosis	12
p53 and Mdm2 play a role in mitotic regulation.....	13
Anaphase Promoting Complex and Spindle Assembly Checkpoint.....	15
p53 plays a role in glucose metabolism	18
Glycolysis, Nucleotide Shortage and Cancer	20
2. CHAPTER 2. IDENTIFICATION APC2 AS E3 UBIQUITIN LIGASE FOR MDM2	25
INTRODUCTION	25
RESULTS.....	27
Mdm2 E3 ubiquitin ligase function is dispensable for Mdm2 self-degradation under physiological conditions	27
Mdm2 interacts with APC2	29
APC2 is important for mdm2 degradation.....	30
APC2 is important for stress induced p53 stabilization and activation	31
APC2 downregulation correlates with cancer development.....	32
DISCUSSION	34
EXPERIMENTAL PROCEDURE	36
Cell culture, transfection.....	36
Double thymidine block synchronization	36

DNA plasmids, siRNA and lenti-viral based shRNA	36
SDS-Page and western blotting.....	36
IP-Western blotting.....	37
Half-life assay	37
Beta-Gal staining	37
Luciferase assay	38
Antibodies	38
<i>In vitro</i> ubiquitination assay.....	38
<i>In vivo</i> ubiquitination assay	38
FIGURES	40
Figure 2-1. Mdm2 E3 ubiquitin ligase function is dispensable for Mdm2 autodegradation under physiological conditions.	40
Figure 2-2. Mdm2 E3 dead mutant still degraded in proteasome dependent manner.....	41
Figure 2-3. Cell lysate contains E3 ubiquitin ligase activity for Mdm2 polyubiquitination in vitro.	42
Figure 2-4. Mdm2 oscillates in a p53 independent manner in Serum Starvation synchronized cells.	43
Figure 2-5. Mdm2 oscillates in a p53 independent manner in Double Thymidine Block synchronized cells.	44
Figure 2-6. Mdm2 interacts with APC2 and Cullin 1.	45
Figure 2-7. Mdm2 interacts with APC2.	46
Figure 2-8. Mdm2 interacts with Fzr1.....	47
Figure 2-9. APC2 Knocking Down led to extended Mdm2 half-life.	48
Figure 2-10. APC2 Knocking Down leads to Mdm2 accumulation.....	49
Figure 2-11. APC2 is important for Mdm2 degradation.	50
Figure 2-12. APC2 knocking down leads to reduced cell death and senescence.	51
Figure 2-13. APC2 knocking down leads to attenuated p53 accumulation.....	52
Figure 2-14. APC2 is down regulated in Cancer cell lines expressing high level of Mdm2.....	53
Figure 2-15. APC2 gene copy is lost in multiple cancers.....	54
Figure 2-16. APC2 is under-expressed in multiple cancers.....	55

3. CHAPTER 3. IDENTIFICATION PFK2 AS P53 SUPPRESSION TARGET	
GENES	56
INTRODUCTION	56
RESULTS	59
Identification of <i>PFK2</i> as a p53 transcriptional suppression target	59
Nucleotide shortage leads to p53-dependent suppression of PFK2	62
PFK2 suppression leads to increased DNA damage repair and cell survival	63
<i>PFK2</i> suppression leads to decreased glycolytic flux and increased PPP activity	64
<i>PFK2</i> suppression leads to increased PPP activity and nucleotide production	64
PFK2 depletion-mediated increase in DNA damage repair rate and cell survival requires nucleotide production from the PPP	65
DISCUSSION	67
MATERIALS AND METHODS	70
Chromatin immunoprecipitation assay	70
Measurement of fructose-2, 6-bisphosphate levels	70
Measurement of cellular nucleotide levels	70
Cell culture and reagents	70
Plasmids and adenovirus	71
Microarray analysis	71
Immunofluorescence assays	71
Antibodies	72
DNA damage repair efficiency assay	72
Lentivirus-based shRNA and siRNA treatment	72
FIGURES	74
Figure 3-1. Cellular PFK2 mRNA and protein expression is suppressed upon p53 activation.	74
Figure 3-2. UV and Nutlin-3 induced p53 activation lead to PFK2 suppression in p53 dependent manner.	75
Figure 3-3. p53 binds to the first PFK2 intron and suppresses PFK2 transcription.	76
Figure 3-4. Not All stress activates p53 lead to PFK2 suppression.	77
Figure 3-5. Nucleotide shortage activates p53 and suppresses PFK2 expression.	78
Figure 3-6. PFK2 suppression leads to increased DNA damage repair.	79

Figure 3-7. p53 promote DNA damage repair depend on its ability to suppress PFK2 suppression.	80
Figure 3-8. PFK2 suppression leads to increased cell survival.	81
Figure 3-9. PFK2 suppression leads to decreased glycolytic flux.	82
Figure 3-10. PFK2 suppression leads to increased PPP activity and nucleotide production.	83
Figure 3-11. PFK2 suppression leads to increased nucleotide production.	84
Figure 3-12. PFK2 suppression promotes DNA damage repair and cell survival in a PPP-dependent manner.	85
Figure 3-13. Nucleotide supply is a rate limiting factor for UV induced DNA damage repair and cell survival.	86
Figure 3-14. PFK2 suppression promotes DNA damage repair and cell survival in a nucleotide-dependent manner.	87
4. CHAPTER 4. SUMMARY AND FUTURE DIRECTIONS	88
Mdm2 protein level is cell cycle regulated by APC/C	88
APC/C may mediate the tetraploidy checkpoint response to Mdm2-p53	90
Mdm2 may promote Metaphase/Anaphase transition.....	92
Implications of APC/C mediated Mdm2 degradation in cancer diagnosis and treatment.....	95
In response to nucleotide shortage, p53 suppresses glycolysis to promote nucleotide synthesis	97
Potential use of nucleoside and glucose supplements in sunburn treatment.....	98
RB-E2F pathway contributes to tumor initiation by inducing nucleotide shortage	99
From nucleotide shortage to nucleotide overproduction in cancer development	101
APC/C and Cul1-betaTRCP may regulate the nucleotide shortage response through the Mdm2-p53-PFK2 pathway	103
FIGURES	106
Figure 4-1. Prometaphase arrest leads to decrease of Mdm2 protein level in proteasome dependent manner.....	106
Figure 4-2. Mdm2 overexpression leads to irregular nuclear shape.	107
Figure 4-3. Mdm2 knock down lead to increased G2/M and round up cell independent of p53.	108
Figure 4-4. Mdm2 knock down lead to increased mitotic metaphase cell independent of p53.	109

Figure 4-5. p53 knock out lead to tetraploidy and p53 Mdm2 double knock out
lead to aneuploidy. 110

Figure 4-6. p53's function in tetraploidy control, cell cycle arrest, apoptosis,
glucose metabolism and nucleotide shortage response. 111

LIST OF FIGURES

Figure 2-1. Mdm2 E3 ubiquitin ligase function is dispensable for Mdm2 autodegradation under physiological conditions.	40
Figure 2-2. Mdm2 E3 dead mutant still degraded in proteasome dependent manner.	41
Figure 2-3. Cell lysate contains E3 ubiquitin ligase activity for Mdm2 polyubiquitination in vitro.	42
Figure 2-4. Mdm2 oscillates in a p53 independent manner in serum starvation synchronized cells.	43
Figure 2-5. Mdm2 oscillates in a p53 independent manner in double thymidine block synchronized cells.	44
Figure 2-6. Mdm2 interacts with APC2 and Cullin 1.	45
Figure 2-7. Mdm2 interacts with APC2.	46
Figure 2-8. Mdm2 interacts with Fzr1.	47
Figure 2-9. APC2 Knock down lead to extended Mdm2 half-life.	48
Figure 2-10. APC2 Knock down leads to Mdm2 accumulation.	49
Figure 2-11. APC2 is important for Mdm2 degradation.	50
Figure 2-12. APC2 knock down leads to reduced cell death and senescence.	51
Figure 2-13. APC2 knock down leads to attenuated p53 accumulation.	52
Figure 2-14. APC2 is down regulated in cancer cell lines expressing high level of Mdm2.	53
Figure 2-15. APC2 gene copy is lost in multiple cancers.	54
Figure 2-16. APC2 is under-expressed in multiple cancers.	55
Figure 3-1. Cellular PFK2 mRNA and protein expression is suppressed upon p53 activation.	74
Figure 3-2. UV and Nutlin-3 induced p53 activation lead to PFK2 suppression in p53 dependent manner.	75
Figure 3-3. p53 binds to the first PFK2 intron and suppresses PFK2 transcription.	76
Figure 3-4. Not all stress activates p53 to lead to PFK2 suppression.	77
Figure 3-5. Nucleotide shortage activates p53 and suppresses PFK2 expression.	78

Figure 3-6. PFK2 suppression leads to increased DNA damage repair.....	79
Figure 3-7. p53 promotion of DNA damage repair depends on its ability to suppress PFK2 expression.....	80
Figure 3-8. PFK2 suppression leads to increased cell survival.....	81
Figure 3-9. PFK2 suppression leads to decreased glycolytic flux.	82
Figure 3-10. PFK2 suppression leads to decreased glycolytic flux and increased PPP activity.....	83
Figure 3-11. PFK2 suppression leads to increased nucleotide production.....	84
Figure 3-12. PFK2 suppression promotes DNA damage repair and cell survival in a PPP-dependent manner.....	85
Figure 3-13. Nucleotide supply is a rate limiting factor for UV induced DNA damage repair and cell survival.	86
Figure 3-14. PFK2 suppression promotes DNA damage repair and cell survival in a nucleotide-dependent manner.....	87
Figure 4-1. Prometaphase arrest leads to the decrease of Mdm2 protein levels in a proteasome dependent manner.	106
Figure 4-2. Mdm2 overexpression leads to irregular nuclear shape.	107
Figure 4-3. Mdm2 knock down leads to increased G2/M and rounded up cell morphology independent of p53.....	108
Figure 4-4. Mdm2 knock down lead to increased mitotic metaphase independent of p53.....	109
Figure 4-5. p53 knock out leads to tetraploidy and p53, Mdm2 double knock out leads to aneuploidy.	110
Figure 4-6. Function of p53 in tetraploidy control, cell cycle arrest, apoptosis, glucose metabolism, and nucleotide shortage response.	111

LIST OF ABBREVIATIONS AND SYMBOLS

4-OHT	4-Hydroxytamoxifen
5-FU	5-Fluorouracil
Act D	actinomycin D
APC/C	anaphase promoting complex/cyclosome
ATP	adenosine triphosphate
ARF	alternative reading frame
cDNA	complementary deoxyribonucleic acid
ChIP	chromatin immunoprecipitation
CMV	cytomegalovirus
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNDP	nucleoside-diphosphate
dNTP	deoxynucleotide triphosphates
DTT	dithiothreitol
FBS	fetal bovine serum
Fzr1	fizzy related 1
G6P	Glucose 6-phosphate
G6PD	Glucose-6-phosphate dehydrogenase
GFP	green fluorescent protein
IgG	immunoglobulin G
IP	immunoprecipitation
kDa	kiloDalton
mRNA	messenger ribonucleic acid
Mdm2	Mouse double minute 2

MEFs	mouse embryo fibroblasts
NAD	nicotinamide adenine dinucleotide
NADP	nicotinamide adenine dinucleotide phosphate
NDP	Nucleoside-diphosphate
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFK2	Phosphofructokinase 2 (PFKFB3)
PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PMSF	phenylmethanesulfonylflouride
PPP	Pentose Phosphate Pathway
preRCs	Pre-replication complex
qRT-PCR	quantitative real time polymerase chain reaction
RING	really interesting new gene
RNA	ribonucleic acid
ROS	reactive oxygen species
rRNA	ribosomal ribonucleic acid
SAM	significance analysis of microarrays
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
shRNA	short hairpin ribonucleic acid
siRNA	small interfering ribonucleic acid
SV-40	simian vacuolating virus 40
TCGA	The Cancer Genome Atlas
UV	Ultraviolet
WT	Wild type

CHAPTER 1. INTRODUCTION

The discovery of the tumor suppressor gene *p53*

The tumor suppressor gene *TP53* (*p53* hereafter) was initially identified as an oncogene for three very good reasons: 1. *p53* was first discovered via co-immunoprecipitation with the oncogenic viral SV-40 large T-antigen (Chang, Simmons et al. 1979; Kress, May et al. 1979; Lane and Crawford 1979; Linzer and Levine 1979). 2. *p53* cDNA cloned from cancer lines was found to be capable of transforming both primary cells (Jenkins, Rudge et al. 1984) and normal embryonic fibroblasts in cooperation with the activated form of the known oncogene Ha-RAS (Eliyahu, Raz et al. 1984; Parada, Land et al. 1984). 3. *p53* inhibition by anti-sense RNAi or antibody micro-injection was found to inhibit cell cycle progression and proliferation (Mercer, Nelson et al. 1982; Mercer, Avignolo et al. 1984; Reich and Levine 1984; Shohat, Greenberg et al. 1987). At the time, this evidence collectively pointed towards a role for *p53* as an oncogene capable of promoting malignant transformation.

It was later revealed that oncogenic viral SV-40 large T-antigen interaction with *p53* inhibits *p53*'s tumor suppressor function (Radna, Caton et al. 1989; Hara, Tsurui et al. 1991; Lin and Simmons 1991; Olson and Levine 1994). The transformation activity of *p53* cDNA cloned from cancer lines were the result of mutant *p53*, rather than wild-type *p53* (Eliyahu, Goldfinger et al. 1988; Finlay, Hinds et al. 1988; Hinds, Finlay et al. 1989). Finally, cell lines demonstrating slower proliferation after *p53* knockdown were observed to contain a dominant negative mutant *p53*, and micro-injection of *p53* antibody led to activation of *p53* transcriptional activity instead of inhibition of *p53* (Hupp and Lane 1994; Hupp and Lane 1994; Hupp, Sparks et al. 1995).

The expression of wild-type *p53*, in conjunction with activated RAS and MYC or adenovirus E1A oncogenes, in rat embryonic fibroblasts caused a marked reduction in

transformed foci, suggesting that p53 is actually a tumor suppressor gene (Eliyahu, Michalovitz et al. 1989; Finlay, Hinds et al. 1989). Genetic analysis of colorectal cancer reveals a very high rate of heterozygous loss of the short arm of chromosome 17, which carries the p53 gene (Vogelstein, Fearon et al. 1988). Loss of 17p is a very frequent feature in many of the major forms of human cancers—including breast cancer (Mackay, Steel et al. 1988), astrocytoma (James, Carlbom et al. 1989), small cell lung carcinoma (Yokota, Wada et al. 1987) and chronic myeloid leukemia (Borgstrom, Vuopio et al. 1982). PCR analysis and sequencing of the remaining p53 allele shows that it often contains a point mutation (Hollstein, Hergenhahn et al. 1999). Similar observations have been made in the case of lung cancer and many other cancer types (Takahashi, Nau et al. 1989). As of today, we understand that the p53 gene itself is mutated in approximately 50% of all human tumors (Vousden and Lu 2002; Vazquez, Bond et al. 2008).

Considering the high mutation rate of p53 in human cancers and the fact that cells with wild type p53 were originally considered to be p53 negative cells, it is not surprising that the initial cloning and experimentation made use of mutant p53. Because p53 is a short lived protein, wild-type p53 protein is almost undetectable in conventional immunochemical and immunohistochemical assays. In contrast, mutant p53 is often more stable and high expression frequently correlates with tumor development (Lane and Benchimol 1990). Cells with wild type p53 were therefore believed to be negative for p53 in early studies, further contributing to the identification of p53 as an oncogene.

Despite early studies mistakenly identifying p53 as oncogene, many of these early discoveries point to a link between p53 and cancer. This link directly led to increased effort and funding spent in p53 study, eventually leading to the discovery of the fact that p53 is widely mutated in human cancer. Due to current limitations in our understanding of the involved genes and pathways, the research presented here may not all be correct, but I will be grateful if one

day they are proved to point out a good direction of future study and bring us one step closer to a comprehensive understanding of these pathways and systems.

The discovery of Mdm2 as the major negative regulator of p53

Murine Double Minute 2 (Mdm2) was originally identified from purified acentric chromosomes, also known as double minutes, in a spontaneously transformed mouse 3T3-DM cell line together with Mdm1 and Mdm3 (Cahilly-Snyder, Yang-Feng et al. 1987). These amplified double minutes often contain genes conferring a selective growth advantage to cells. Mdm2, but not Mdm1 and Mdm3, was identified to have tumorigenic potential when ectopically overexpressed (Fakharzadeh, Trusko et al. 1991). Later, Mdm2 was demonstrated to bind to p53 and block p53 mediated transactivation (Cahilly-Snyder, Yang-Feng et al. 1987; Finlay, Hinds et al. 1989; Fakharzadeh, Trusko et al. 1991; Momand, Zambetti et al. 1992; Oliner, Kinzler et al. 1992; Buolamwini, Addo et al. 2005). Human Mdm2 gene was mapped to chromosome 12q13-14, and was shown to be amplified in a subset of soft tissue sarcomas and osteosarcomas (Oliner, Kinzler et al. 1992). As of today, Mdm2 is found to be mutated or overexpressed in up to 50% of tumors (Vousden and Lu 2002; Vazquez, Bond et al. 2008).

In addition to binding to p53 to block p53 mediated transactivation through its N-terminus p53 binding domain, the C-terminus of Mdm2 was found to contain intrinsic E3 ubiquitin ligase activity, which promotes the ubiquitination and degradation of p53 (Haupt, Maya et al. 1997; Honda, Tanaka et al. 1997; Deshaies and Joazeiro 2009). On the other hand, *Mdm2* is a transcriptional target of p53 and its transcription is directly promoted by p53, forming a regulatory feedback loop between Mdm2 and p53 to maintain cellular homeostasis (Barak, Juven et al. 1993; Wu, Bayle et al. 1993).

Despite existing controversy about whether Mdm2 alone is sufficient to poly-ubiquitinate p53 or if an E4 ubiquitin ligase is required for this function (Shi, Pop et al. 2009; Wu and Leng 2011), the role of Mdm2 in the promotion of p53 mono-ubiquitination is generally accepted.

Moreover, this mono-ubiquitination is generally agreed to be required to at least initiate p53 polyubiquitination and subsequent degradation. Mice generated by homologous recombination to carry a null allele for Mdm2 died during development, just prior to embryo implantation (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995). This embryonic lethal phenotype could be rescued by concomitant deletion of p53, suggesting a role for Mdm2-directed p53 inhibition during murine development (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995). Furthermore, induction of p53 using a conditional hypomorphic allele of Mdm2 in mice increased radio-sensitivity in a fraction of tissues where increased apoptosis was observed suggest reduced expression of Mdm2 result in insufficient suppression of p53 and lead to p53 activation (Mendrysa, McElwee et al. 2003). Collectively this work helped to establish Mdm2 as the major negative regulator of p53 *in vivo*.

p53 is a stress activated transcription factor

The tumor suppressor p53 is a transcription factor that is activated in response to a wide variety of stressors, including ribosomal stress, oncogene activation, DNA damage, nutrient stress, oxidative stress, ribonucleotide depletion, as well as many others (Levine, Hu et al. 2006). Once activated, p53 protein levels increase enhancing its ability to bind to p53-responsive DNA sequence elements in the genome. The p53 binding consensus DNA sequence half-site is RRRCWWGYYY, where R is a purine, W is adenine or tyrosine, and Y is a pyrimidine (Levine, Hu et al. 2006). A p53-responsive element is composed of two of these consensus half-site, separated by a spacer of 0–21 base pairs (Levine, Hu et al. 2006). A mismatch in the WW motif in the middle of the p53 binding consensus DNA sequence half-site has been reported to convert a p53-inducible sequence to a p53 suppression sequence *in vitro* (Wang, Xiao et al. 2009). p53 binding consensus sequences are often located 5' to the p53-inducible target gene or in the first or second intron of p53-repressed target gene (el-Deiry, Kern et al. 1992).

The functions of the p53-response genes fall into several categories: cell cycle arrest and senescence (p21 (el-Deiry, Tokino et al. 1993), 14-3-3 sigma (Hermeking, Lengauer et al. 1997), GADD-45 (Carrier, Smith et al. 1994; Zhan, Bae et al. 1994), PIG-3 (Flatt, Polyak et al. 2000), CDC25C(Krause, Haugwitz et al. 2001)), apoptosis (Fas (Owen-Schaub, Zhang et al. 1995), killer/DR5 (Wu, Burns et al. 1997), APAF-1 (Moroni, Hickman et al. 2001), bax (Miyashita, Krajewski et al. 1994; Selvakumaran, Lin et al. 1994), noxa (Oda, Ohki et al. 2000) and PUMA (Nakano and Vousden 2001), PERP (Attardi, Reczek et al. 2000), scotin (Bourdon, Renzing et al. 2002), P53AIP1 (Oda, Arakawa et al. 2000), BID (Sax, Fei et al. 2002), Caspase6 (MacLachlan and El-Deiry 2002), BIRC5 (Hoffman, Biade et al. 2002)), DNA damage repair (P53R2 (Nakano, Balint et al. 2000), DDB2 (Hwang, Ford et al. 1999), XPC (Adimoolam and Ford 2002),REDD1 (Ellisen, Ramsayer et al. 2002)), and glucose metabolism (TIGAR (Bensaad, Tsuruta et al. 2006), PGAM (Ruiz-Lozano, Hixon et al. 1999), HexoKinase (Mathupala, Heese et al. 1997), AIF (Ohiro, Garkavtsev et al. 2002), GLS2 (Suzuki, Tanaka et al. 2010), SCO2 (Matoba, Kang et al. 2006)). A number of p53-responsive or p53-regulated genes produce secreted proteins such as IGF-BP3 (Buckbinder, Talbott et al. 1995), PAI (Kunz, Pebler et al. 1995) maspin (Zou, Gao et al. 2000), thrombospondin (Dameron, Volpert et al. 1994), and TSAP-6(Amzallag, Passer et al. 2004). Finally, a large number of p53-responsive genes (p21, WIP-1, SIAH-1, PTEN, TSC-2, IGF-BP-3, cyclin G, p73delta N, Mdm2, COP-1 and Pirh-2) initiate positive or negative feedback loops with the p53 stress response pathway (Harris and Levine 2005).

It has become clear that in response to different types of stress signals p53 promotes the transcription or suppression of different subsets of its target genes(Zhao, Gish et al. 2000). In addition, p53 regulates the transcription of target gene expression in cell type specific or tissue type specific manner. We do not currently understand what mediates these different regulatory responses, but previous study suggests that post translational modifications and

recruitment of transcription co-factors, as well as differences in the DNA sequences of response elements could play a role (Levine, Hu et al. 2006).

Regulating Mdm2 to modulate p53 function

Most stress signals lead to post translational modification and subsequently inhibit the interaction of Mdm2 with p53, thereby resulting in p53 stabilization, transactivation, and the induction of downstream genetic programs to resolve the crisis cause by the stress (Meek 2009). For instance, DNA damage activates the ataxia-telangiectasia mutated (ATM) and ataxia-telangiectasia and Rad-3 related (ATR) protein kinases. Activation of ATM and ATR promotes CHK1 or CHK2 phosphorylation and activation, which in turn results in phosphorylation of p53 and Mdm2 (Maya, Balass et al. 2001; Shiloh 2003; Gannon, Woda et al. 2012). p53 activation as a result of the ATM and ATR pathways promotes induction of genes involved in cell cycle arrest and DNA repair. Cells from ataxia-telangiectasia patients that harbor a mutation in *ATM* often demonstrate defective double-strand break repair, defective cell cycle control, and enhanced sensitivity to ionizing radiation (Shiloh 2003).

There are a number of amino acid residues in p53 that are post-translationally modified and have been postulated to promote p53 stability (Xu 2003). These modifications may act as a signal for directing the response of p53 to different upstream signals (Murray-Zmijewski, Slee et al. 2008). Serine 15 (Banin, Moyal et al. 1998; Canman, Lim et al. 1998; Nakagawa, Taya et al. 1999) and Serine 20 (Shieh, Taya et al. 1999; Unger, Juven-Gershon et al. 1999) of p53 have been reported to be primary targets of ATM/ATR kinases upon DNA damage. Phosphorylation of p53 at these sites was hypothesized to disrupt the ability of Mdm2 to bind p53, thereby promoting p53 transactivation of target genes. Mutation of Ser18 (Serine 15 in human) to alanine in a knock-in mouse model, suggested that this phosphorylation site was necessary for an optimal response to DNA damage (Chao, Saito et al. 2000). However, unlike p53 knockout mice, *p53^{S18A}* mice did not demonstrate early-onset tumors, which suggests that

phosphorylation of p53 Ser18 is not required for p53-dependent tumor suppression (Armata, Garlick et al. 2007). Other mouse knock-in studies have also shown that phosphorylation at Ser23 (Ser20 in human) may not be essential for p53 response to DNA damage (Wu, Earle et al. 2002). Together, these studies call into question the necessity of p53 post-translational modification for its tumor suppressor and DNA damage function, and indicate the existence of other mechanisms and pathways for modulating the p53 response.

In addition to DNA damage, nucleolar stress (also known as ribosomal stress) and oncogenic stress also lead to p53 activation through Mdm2. RPL5 was first reported to bind to Mdm2 in a 5S rRNA-RPL5-Mdm2-p53 ribonucleoprotein complex, but at the time, the significance of such an interaction was not understood (Marechal, Elenbaas et al. 1994). Nearly a decade later, ribosomal proteins RPL5, RPL11, and RPL23 were all reported to bind to Mdm2, blocking the E3 ubiquitin ligase function of Mdm2, and promoting p53 accumulation (Lohrum, Ludwig et al. 2003; Zhang, Wolf et al. 2003; Bhat, Itahana et al. 2004; Dai and Lu 2004; Dai, Zeng et al. 2004; Jin, Itahana et al. 2004). Later studies provided evidence supporting the roles of additional ribosomal proteins, including RPS7 (Chen, Zhang et al. 2007; Zhu, Poyurovsky et al. 2009), RPL26 (Ofir-Rosenfeld, Boggs et al. 2008), and RPS3 (Yadavilli, Mayo et al. 2009), as Mdm2 binding partners. A number of reports investigating RP-Mdm2 binding have used “nucleolar stress” as the upstream signal responsible for induction of the RP-Mdm2-p53 response. “Nucleolar stress” may refer to any stress that leads to perturbations of ribosome biogenesis and the subsequent breakdown of nucleolar structure, resulting in activation of p53. These observations have therefore led to the hypothesis that the nucleolus functions as a central stress response regulator for p53 activation (Rubbi and Milner 2003).

ARF, a tumor suppressor transcribed from an alternate reading frame of *CDKN2A*, has been demonstrated to bind to Mdm2 and inhibit Mdm2-mediated p53 ubiquitination and degradation in response to oncogenic stress such as RAS activation, c-myc overexpression, or RB deficiency (Sherr 2001; Sharpless 2005). ARF null mice are highly prone to spontaneous

tumor development (Kamijo, Zindy et al. 1997). Mutation or epigenetic silencing of the *CDKN2A* locus encoding ARF is a common occurrence in mouse-derived tumors (Eischen, Weber et al. 1999; Schmitt, McCurrach et al. 1999). However, the mechanisms through which oncogenic signals act to trigger ARF activation remain unclear.

Mdm2 is also reported to be modified through many different types of posttranslational modifications such as ubiquitination, sumoylation, and phosphorylation (Meek and Knippschild 2003). The amino terminus of Mdm2 contains two clusters of phosphorylation sites that may be modified by AKT (Ser166, Ser186), cyclinA-CDK1/ 2 (Thr219), c-Abl (Tyr294), and CK2 (Ser269) (Hay and Meek 2000). DNA damage is known to activate ATM kinase-induced phosphorylation of Mdm2 at Ser395 (Maya, Balass et al. 2001). Mdm2 phosphorylation inhibits Mdm2 directed destabilization of p53. An Mdm2 Y394F mutant that prevented c-Abl-dependent phosphorylation of Mdm2 at Tyr394 was shown to increase Mdm2 activity and subsequently down-regulate p53 transactivation, supporting the hypothesis that Mdm2 phosphorylation can also block Mdm2 from binding to p53, which results in increased p53 stabilization and activity (Goldberg, Vogt Sionov et al. 2002).

Altogether, it becomes clear that Mdm2 is the major negative regulator of p53 stability and activity and is itself subject to complex regulatory mechanisms. Differences in post-translational modifications or interaction with various inhibitory proteins in response to different sources of stress signal lead to difference in p53 activation and the appropriate downstream genetic programs.

Regulation of Mdm2 degradation

The importance of Mdm2 in proper p53 regulation makes the understanding of its own regulation of critical concern. In order for the Mdm2-p53 feedback loop to function properly, tight regulation of Mdm2 degradation is essential. Previous *in vitro* and overexpression studies have demonstrated that Mdm2 regulates its own degradation by autoubiquitination, targeting itself for

proteasome-mediated degradation (Fang, Jensen et al. 2000; Honda and Yasuda 2000). However, recent studies characterizing *Mdm2*^{C462A/C462A} knock-in mice have challenged the Mdm2 autoubiquitination dogma (Itahana, Mao et al. 2007; Clegg, Itahana et al. 2008). In *Mdm2*^{C462A/C462A} mouse embryonic fibroblasts (MEFs) the mutant Mdm2^{C462A} protein is degraded as rapidly as wild type Mdm2, while p53 degradation is blocked, indicating that Mdm2 E3 ligase activity is not required for its own degradation when endogenously expressed (Itahana, Mao et al. 2007; Clegg, Itahana et al. 2008). This suggests that other E3 ubiquitin ligases likely exist to regulate Mdm2 stability.

In light of the *in vivo* data, the potential for an outside E3 ubiquitin ligase to function in Mdm2 regulation has been more expressly studied. The Cullin1/β-TRCP E3 ubiquitin ligase complex was identified to interact with Mdm2 and this interaction was demonstrated to lead to poly-ubiquitination and degradation of Mdm2 (Inuzuka, Tseng et al. 2010). However, the regulation of Mdm2:Cullin1/β-TRCP interaction suggests that this interaction only occurs following DNA damage (Inuzuka, Tseng et al. 2010). Furthermore, knock down of Cullin1-βTRCP does not block p53 activation; instead, it affects the regulation of Mdm2 and p53 during the recovery of cells to following exposure to stress (Inuzuka, Tseng et al. 2010). More recently, NEDD4-1 was biochemically identified to function as an E3 ligase and contribute to the regulation of Mdm2 protein stability in cells (Xu, Fan et al. 2014), however, NEDD4-1 catalyzes the formation of K63-type polyubiquitin chains on Mdm2 that are distinct from the K48-type polyubiquitin chains typically required for proteasomal degradation (Xu, Fan et al. 2014). Notably, K63-type polyubiquitination by NEDD4-1 competes with K48-type polyubiquitination on Mdm2 in cells, and as a result, NEDD4-1-mediated ubiquitination stabilizes Mdm2. However, the E3 ubiquitin ligases responsible for the regulation of Mdm2 stability under physiological conditions remain unknown.

The Mdm2-p53 pathway is altered in human cancer

Inactivation of the p53 tumor suppression pathway is frequently observed in human cancer. Generally, p53 is estimated to be mutated in more than 50% of cancer cases (Vousden and Lu 2002; Vazquez, Bond et al. 2008), but the specific rate of direct p53 mutation varies between tumor types with the highest frequency observed in cancer of the colon and lung (60-65%) and the lowest in leukemias (10%) (Soussi 1996). While inactivation of p53 alone is generally insufficient to cause tumorigenesis, the combination of p53 loss with oncogene activation or loss of a second tumor suppressor will promote cellular transformation (Hahn and Weinberg 2002). p53 loss or mutation is thought to lead to accumulation of genetic lesions and often observed in benign tumors that become metastatic. When left unchecked through inefficient DNA repair, lack of apoptosis and senescence, selection and maintenance of growth promoting mutations can promote genetic instability and ultimately malignant potential (Soussi 1996).

Approximately 90% of point mutations in p53 occur in the DNA binding domain, with about 20% of these point mutation occurring on “hotspot” codons (175, 245, 248, 249, and 273) (Hainaut, Soussi et al. 1997). Missense mutations, or insertions/deletions, lead to expression of mutant p53 protein 90% of the time, and nonsense mutations leading to the absence of protein in the remaining 10%. DNA binding activity of these point mutants often correlates with suppression of cell growth, indicating that p53 transactivation of its downstream target genes is an essential component to tumor suppression (Ory, Legros et al. 1994; Rolley, Butcher et al. 1995).

In cancers where wild-type p53 is retained, modifications to upstream components of the pathway are often observed; specifically, gene amplification of Mdm2 is estimated at 7% overall for all spectra of cancer types, with the highest rates of gene amplification observed in soft tissue sarcomas (20%) and osteosarcomas (16%). Furthermore, the prevalence of Mdm2 amplification and p53 mutation were found to be, for the most part, mutually exclusive events,

implying that Mdm2 overexpression is sufficient to inactivate the p53 pathway (Momand, Jung et al. 1998).

In multiple studies, Mdm2 protein overexpression, independent of gene amplification, has been observed, which suggests that assessment of gene amplification alone greatly underestimates the occurrence of Mdm2 protein overexpression in human cancer. For example, despite the observation that only about 16% of glioblastomas are reported to contain Mdm2 gene amplification, around 60% of glioblastomas demonstrate Mdm2 protein over-expression (Ghimentì, Fiano et al. 2003). Another study found no Mdm2 gene amplification in Childhood Acute Lymphoblastic Leukemia but detected Mdm2 protein overexpression in tumors with wild type p53 (Zhou, Yeager et al. 1995). A similar study in melanoma found that 27% of *in situ*, and 56% of invasive primary and metastatic melanomas contain Mdm2 overexpression, however, only 1% of *in situ* and none of the metastatic cases contained Mdm2 gene amplification (Polsky, Bastian et al. 2001). These studies suggest that the majority of Mdm2 protein overexpression is due to misregulation of Mdm2 at the post-transcriptional level.

As a small protein inhibitor of Mdm2, ARF blocks Mdm2-mediated inhibition of p53, thereby promoting p53 dependent growth inhibition (Sherr 2001). ARF is often lost or mutated in many cancers, including melanoma where p53 mutation is rare. The Ink4a-ARF locus often undergoes genetic modification to disable one or both components, p16^{INK4A} and p14^{ARF}; Germline mutations that alter both p14^{ARF} and p16^{INK4A} occur in proximately 40% of familial melanomas (Rizos, Darmanian et al. 2001). Furthermore, both p14^{ARF} and p16^{INK4A} are altered in approximately 40% of sporadic melanomas, and p14^{ARF} is exclusively targeted in an additional 11% (Rizos, Puig et al. 2001); one point mutation observed in melanoma, located in exon 2 of the Ink4a-ARF locus, is shared by both p16^{INK4A} and p14^{ARF}. Strikingly, some of these mutants alter the cellular localization of p14^{ARF}, which can affect Mdm2:ARF interaction, and reduce p53 activation (Rizos, Darmanian et al. 2001).

p53 plays a role in regulation of cell cycle arrest and apoptosis

Soon after the discovery of p53 as an SV40 large T antigen binding protein, it was found that T-antigen binding inhibits both p53 and the tumor suppressor RB simultaneously (Hara, Tsurui et al. 1991; Nevins 2001; Sherr and McCormick 2002). The RB-E2F and MDM2-p53 pathways were soon discovered to be disabled in most, if not all, human tumors (Nevins 2001). Tumorigenic viral strains including SV40 (Bryan and Reddel 1994), adenovirus, and human papillomavirus (HPV) use large T antigen, E1A/E1B (Jones 1990; Debbas and White 1993), and E6/E7 (Vousden 1990; Moody and Laimins 2010; Jiang and Yue 2014), respectively, to inhibit the RB-E2F pathway and the Mdm2-p53 pathway simultaneously, suggesting that either of these two pathways may be sufficient for suppressing tumor development. Although both the RB pathway and p53 pathway play important roles in regulating cell cycle arrest and apoptosis (Polager and Ginsberg 2009; Udayakumar, Shareef et al. 2010), the role of p53 in cell cycle arrest and apoptosis has been more extensively studied.

Following upstream activation and protein stabilization, p53 directs downstream events to regulate the cell cycle. One of the most well characterized target genes of p53 is *p21* (WAF1, Cip-1) (el-Deiry, Tokino et al. 1993). Multiple p53 binding sites were identified near the p21 transcription start site, making p21 a high affinity target for p53. After induction, p21 binds to multiple cyclin-CDK complexes to block their kinase activity on RB. The result is inhibition of the G1/S transition of the cell cycle, preventing DNA replication from initiation (Xiong, Hannon et al. 1993).

p53 is reported to directly induce transcription of Bax, Noxa, and Puma. Bax was the first pro-apoptotic gene shown to be directly transcriptionally induced by p53 and through heterodimerization with Bcl-2, functions to accelerate apoptosis (Miyashita and Reed 1995). Noxa encodes a BH3-only member of the Bcl-2 family and inhibits pro-survival Bcl-2 members to promote mitochondrial outer membrane permeabilization (MOMP) (Oda, Ohki et al. 2000). Puma, another BH3 domain containing protein, can bind to Bcl-2 and promote MOMP-mediated

cytochrome c release (Nakano and Vousden 2001). In addition, p53 induces transcription of APAF-1, a critical component of the apoptosome, and caspase-6, an executioner caspase that acts downstream of the activated apoptosome (Kannan, Kaminski et al. 2001; MacLachlan and El-Deiry 2002) to facilitate the formation and function of the apoptosome.

Although the role of p53 in cell cycle arrest and apoptosis is evident, and connection of cell cycle arrest and cancer seems to be obvious, recent studies using mouse models have suggested that the p53-mediated upregulation of genes that promote cell cycle arrest, senescence, and apoptosis is dispensable for its tumor suppression activity (Li, Kon et al. 2012; Valente, Gray et al. 2013), implicating other functions of p53 as the key to its tumor suppressor function.

p53 and Mdm2 play a role in mitotic regulation

Tetraploidy, characterized as cells carrying precisely twice the normal number of chromosomes, is the consequence of cell fusion, endoreduplication, cytokinesis failure or mitotic slippage (Aylon and Oren 2011). Tetraploidy can lead to cellular transformation and tumor formation, and is observed in early stage cancers, preceding chromosome instability and aneuploidy (Galipeau, Cowan et al. 1996; Olaharski, Sotelo et al. 2006). Tetraploid mouse cells generated by inhibition of cytokinesis can initiate tumor formation when transplanted into immunocompromised mice, whereas isogenic diploid cells do not (Fujiwara, Bandi et al. 2005). Importantly, this can occur only in the absence of wild type p53, since tetraploids expressing wild type p53 fail to propagate (Fujiwara, Bandi et al. 2005). Although multiple centrosomes in tetraploid cells tend to cluster into two functional poles (Quintyne, Reing et al. 2005; Kwon, Godinho et al. 2008), clustered bipolar spindles in tetraploid cells demonstrate an increased occurrence of lagging chromosomes and segregation errors (Ganem, Godinho et al. 2009; Silkworth, Nardi et al. 2009), likely to promote aneuploid formation. Despite the distinct correlation between tetraploidy and aneuploidy, fusion of two normal diploid cells into one

tetraploid cell does not routinely end up in aneuploidy (Lengauer, Kinzler et al. 1997; Stukenberg 2004). An increase in tetraploid and polyploid tumor cells is specifically correlated with loss of p53 function in multiple mouse and human cancer models (Ramel, Sanchez et al. 1995; Galipeau, Cowan et al. 1996). p53^{-/-} mice rapidly develop thymic lymphoma, which are usually composed of aneuploid cells (Donehower, Harvey et al. 1992; Jacks, Remington et al. 1994). Furthermore, lymphomas and sarcomas from p53^{+/-} mice with loss of heterozygosity of wild type p53 allele, exhibit more chromosomal instability than tumors that retain the wild type p53 allele (Venkatachalam, Shi et al. 1998). Additionally, cells from p53^{-/-} animals (Fukasawa, Choi et al. 1996), as well as cells deficient in p53 downstream transcriptional targets such as p21 (Mantel, Braun et al. 1999) or Gadd45 (Hollander, Sheikh et al. 1999), accumulate aberrant chromosomal numbers, even before demonstration of a malignant phenotype (Fukasawa, Wiener et al. 1997).

It is clear that p53 plays a critical role in preventing the proliferation of tetraploid cells. However, the mechanism through which p53 is able to sense tetraploid or mitotic slippage remains unclear. Recent studies suggest that one possible way that p53 may sense tetraploid or mitotic slippage is through its major negative regulator Mdm2. Two different p53 wild-type cancer cell lines (U2OS and HCT116) treated with Nutlin-3, a Mdm2 inhibitor that disrupt Mdm2-p53 interaction, for 24 hours accumulated 2N and 4N DNA content, suggestive of G1 and G2 phase cell cycle arrest (Shen, Moran et al. 2008). However, upon removal of Nutlin-3, 4N cells entered S phase and re-replicated their DNA, indicating that the previously observed G2 arrest is the result of mitotic slippage induced tetraploid G1 arrest. Despite the wild type p53 status of these cells, inhibition of Mdm2-p53 interaction is sufficient to induce mitotic slippage, and restoration of Mdm2-p53 interaction afterwards clearly bypasses the tetraploid checkpoint function of p53. A separate study also shows elevated Mdm2 expression leads to increase polyploid and aneuploidy, correlating with age, in mice (Lushnikova, Bouska et al. 2011). Overexpression of MDM2 in mammary epithelial cell was also demonstrated to cause polyploidy

(Lundgren, Montes de Oca Luna et al. 1997). Together these results indicate that upstream signals from Mdm2 may regulate the p53 response in the tetraploidy and aneuploidy checkpoint, yet how Mdm2 is regulated during mitosis remains unknown.

Anaphase Promoting Complex and Spindle Assembly Checkpoint

The anaphase-promoting complex or cyclosome (APC/C) is a multi-subunit E3 ubiquitin ligase complex that controls the degradation of many proteins. The protein levels of most of these proteins oscillate through the cell cycle, controlling multiple cell cycle transitions, including the metaphase-anaphase transition and mitotic exit (Peters 2006; Thornton and Toczyski 2006; Yu 2007). Binding of APC/C to one of its two co-activators, Cdc20 or Fzr1, activates its ubiquitin ligase activity, and contributes to its substrate recognition and specificity. Cdc20 is the mitotic activator of APC/C while Fzr1 mainly interacts with APC/C in telophase and G1 (Yu 2007). Securin and mitotic cyclins are the major substrates of APC/C-Cdc20 during metaphase-anaphase transition; degradation of securin and cyclin B will lead to the activation of separase, which cleaves the cohesin complex and triggers the separation of two sister-chromatids. Cyclin B degradation also leads to inactivation of Cdk1 and promotes mitotic exit (Kim and Yu 2011). Inactivation of CDK1 activity reduces inhibitory phosphorylation of Cdh1 (Lukas, Sorensen et al. 1999; Keck, Summers et al. 2007; Lau, Inuzuka et al. 2013), and leads to activation of APC/C-Fzr1 activity (Harper, Burton et al. 2002; Peters 2006).

The major role of APC/C-Cdc20 is to promote the metaphase to anaphase transition, while APC/C-Cdh1 plays an important role in promoting mitotic exit by mediating the degradation of Cdc20 (Huang, Park et al. 2001; Hyun, Sarantuya et al. 2013), Plk1 (Lindon and Pines 2004), Aurora A (Littlepage and Ruderman 2002), Aurora B (Nguyen, Chinnappan et al. 2005; Stewart and Fang 2005) and Tpx2 (Stewart and Fang 2005). APC/C-Fzr1 also plays an important role in maintaining cell cycle progression through the G1 phase by sustaining low CDK activity by mediating the degradation of the mitotic cyclins (Irniger and Nasmyth 1997),

Cdc25A (Donzelli, Squatrito et al. 2002), Skp2 (Bashir, Dorrello et al. 2004; Wei, Ayad et al. 2004) and Cks1 (Bashir, Dorrello et al. 2004). In addition, APC/C-Fzr1 may control the G1/S transition by controlling the destruction of the replication regulators Geminin (McGarry and Kirschner 1998) and Cdc6 (Petersen, Wagener et al. 2000). APC/C also promotes degradation of its own E2, UbcH10 (Rape and Kirschner 2004), which lead to the stabilization of Cyclin A and inactivation of APC/C-Fzr1. Therefore, APC/C is not only a mitotic E3 ubiquitin ligase, but also a key regulator in governing the length of the G1 phase, in part by complex with its co-activator Fzr1 to direct the timely loading of Pre-replication complexes (preRCs) at the origins of DNA replication in S phase (Zhang, Wan et al. 2014).

Maintenance of genome integrity is critical for cell division, the precise process through which two sets of chromosome separate into two daughter cells. Cells replicate their chromosomes in S phase; cohesion serves to tether the sister chromosomes together. In pro-metaphase, the spindle checkpoint monitors the microtubule attachment to two opposing kinetochores of all pairs of sister chromatids (Bharadwaj and Yu 2004; Musacchio and Salmon 2007). During this process, a single unattached kinetochore is sufficient to activate the spindle checkpoint, block APC/C-Cdc20 activation, and therefore block anaphase transition. Only after all pairs of sister kinetochores are properly captured by spindle microtubules and are under tension, is APC/C-Cdc20 activated, leading to the degradation of securin and cyclin B, activation of separase, removal of sister-chromatid cohesion, and sister-chromatid separation (Zhang, Wan et al. 2014).

The core components of the mitotic spindle checkpoint include the Mitotic Arrest Deficiency (Mad) 1–3 and Budding Uninhibited by Benomyl (Bub) 1–3 proteins (Bharadwaj and Yu 2004; Musacchio and Salmon 2007). Upon checkpoint activation, Mad2 and BubR1 inhibit APC/C-Cdc20, through binding and sequestration of Cdc20, leading to inhibition of APC/C-CDC20 (Fang, Yu et al. 1998; Tang, Bharadwaj et al. 2001) (Meraldi, Draviam et al. 2004). In addition, Bub1 phosphorylates Cdc20, which also leads to inactivation of APC/C-CDC20 (Yu

2007). The Mitotic Checkpoint Complex (MCC) contains Mad2, BubR1, Bub3, and Cdc20, and inhibits APC/C-Cdc20 synergistically *in vivo* (Sudakin, Chan et al. 2001; Yu 2002). Depletion of either Mad2 or BubR1 from human cells shortens the mitotic duration between nuclear envelope breakdown and anaphase onset.

The mitotic checkpoint often referred to as the spindle assembly checkpoint, delays completion of mitosis until all chromosomes have been properly aligned and separated. Altered expression of mitotic checkpoint components has been documented in many human cancers including leukemia, breast, colorectal, ovarian and lung (Kops, Weaver et al. 2005), suggesting the importance of their function in tumor suppression. Despite this protective mechanism, cells that are exposed to a spindle damaging agent such as nocodazole, eventually exit from mitosis (“mitotic slippage” or “mitotic catastrophe”) without undergoing cytokinesis (Castedo, Perfettini et al. 2004). Normal cells stably arrest in the subsequent G1 phase with 4N DNA content, in contrast, cells that lack functional p53 enter the S phase and initiate DNA replication regardless of an abnormal number of chromosomes, resulting in tetraploidy and aneuploidy (Hirano and Kurimura 1974; Cross, Sanchez et al. 1995; Minn, Boise et al. 1996; Khan and Wahl 1998; Lanni and Jacks 1998; Casenghi, Mangiacasale et al. 1999; Stewart, Leach et al. 1999; Borel, Lohez et al. 2002).

The mitotic checkpoint obtains upstream signals from spindle assembly progression, and controls its downstream target APC/C to regulate mitotic progression accordingly. Mitotic slippage terminates mitotic progression in the middle of mitosis leaving duplicated chromosomes, duplicated centrosomes, and inappropriately degraded and un-degraded APC/C substrates. Cell fusion and cytokinesis block experiments suggest that duplicated chromosomes and duplicated centrosomes are not sufficient to activate p53 and lead to tetraploidy G1 arrest (Stukenberg 2004). Therefore, I hypothesize that the inappropriately degraded and un-degraded APC/C substrates may provide upstream signal for p53 activation, likely involving Mdm2.

p53 plays a role in glucose metabolism

Recent studies indicate that p53 plays an important role in suppressing glucose consumption and glycolysis at multiple levels. For example, p53 reduces the expression of the glucose transporter gene GLUT3 through the inhibition of the IKK/nuclear factor- κ B pathway, which stimulates GLUT3 expression (Kawauchi, Araki et al. 2008). p53 also transcriptionally suppresses glucose transporter GLUT1 and GLUT4 (Schwartzberg-Bar-Yoseph, Armoni et al. 2004), by direct binding to its promoter region.

The committing step of glycolysis is catalyzed by phosphofructokinase 1 (PFK1), which converts fructose 6-phosphate to fructose 1,6-bisphosphate. PFK1 is inhibited by ATP and citrate and activated by AMP (Sola-Penna, Da Silva et al. 2010). The feedback inhibition of PFK1 can be overridden by fructose 2,6-bisphosphate, a metabolite whose levels are controlled by the bi-functional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK2/FBPase). p53 induces the expression of the TP53-induced glycolysis and apoptosis regulator (TIGAR), which exhibits bisphosphatase activity and is able to reduce levels of fructose 2,6-bisphosphate (Bensaad, Tsuruta et al. 2006). Through up-regulation of TIGAR p53 can slow down the glycolytic rate. TIGAR can also be expressed independently of p53 in a number of tumor cells and plays a role in tumorigenesis (Cheung, Athineos et al. 2013).

Phosphoglycerate mutase (PGM) acts at the middle stage of glycolysis, converting 3-phosphoglycerate to 2-phosphoglycerate. p53 promotes the degradation of PGM proteins through an undefined mechanism (Kondoh, Leonart et al. 2005) in a cell type-specific manner. For example, while p53 reduces PGM protein in embryonic fibroblast cells, it transcriptionally activates PGM expression in muscle cells (Ruiz-Lozano, Hixon et al. 1999).

In addition to generating pyruvate through the glycolysis pathway, glucose is shunted to the pentose phosphate pathway (PPP). The PPP consists of two stages: (1) An irreversible, oxidative stage generating ribose-5-phosphate (R5P) and two NADPH molecules per glucose and (2) a reversible, non-oxidative stage where R5P is converted to fructose-6-phosphate and

glyceraldehyde 3-phosphate and fed into an intermediate step of glycolysis. R5P is required for *de novo* synthesis of nucleotides and many other metabolites including NAD(P)⁺ and ATP (Wamelink, Struys et al. 2008). NADPH provides reducing power for multiple anti-oxidant systems, and supplies electrons for reductive biosynthesis of lipids, deoxynucleotides and cholesterol (Riganti, Gazzano et al. 2012; Stanton 2012). Glucose-6-phosphate dehydrogenase (G6PD) catalyzes the rate limiting step of PPP; p53 is observed to inhibit G6PD activity in various cell lines and mouse tissues (Jiang, Du et al. 2011). p53 deficient cells show increased PPP flux, NADPH production and glucose consumption in the absence of stress, which suggests that p53 inactivation contributes to increased glucose consumption and nucleotide production through a hyperactive G6PD. Interestingly, p53 binds to the G6PD protein and converts it from an active dimer to an inactive monomer (Jiang, Du et al. 2011). Moreover, p53 can inactivate G6PD at a sub-stoichiometric ratio through transient interaction, suggesting a “catalytic” function of p53 in promoting protein conformational changes (Jiang, Du et al. 2011). More importantly, only the cytoplasmic transcriptional inactivate form of the p53 monomer can inactivate G6PD (Jiang, Du et al. 2011). p53 activation leads to the formation of p53 tetramers, nuclear localization, and loss of its ability to inactivate G6PD.

p53-mediated induction of TIGAR, and perhaps also inactivation of PGM, lead to accumulation of glucose-6-phosphate, which upon p53 activation indirectly raises the PPP flux. Although p53 seems to be able to both promote and inhibit PPP (Jiang, Du et al. 2011), p53-mediated inhibition of G6PD and the induction of TIGAR differ temporally. Upon activation, p53 promotes PPP through induction of TIGAR and inactivation of PGM, resulting in increased glucose consumption and nucleotide production; the mechanism for this action remains unknown. When p53 is inactivated, it inhibits PPP through inhibition of G6PD to reduce glucose consumption and nucleotide production. Upon activation, p53 promote PPP through induction of TIGAR and inactivation of PGM to promote glucose consumption and nucleotide production for reason we don't currently understand.

Glycolysis, Nucleotide Shortage and Cancer

Otto Warburg was the first to demonstrate that glucose consumption in tumor cells is greatly elevated compared with normal cells, and thus this phenomenon is referred to as the Warburg effect. Interestingly, tumor cells demonstrate elevated aerobic glycolysis even in the presence of adequate oxygen and generate lactate rapidly (Warburg 1956). It was believed that altered glucose metabolism is the major cause of the cancer development, however, the link between glucose metabolism and genome instability or cancer initiation has not been expressly demonstrated. The Warburg effect is also observed in rapidly dividing normal cells, indicating that it likely represents a metabolic adaptation to support cell proliferation, rather than a defect of tumor cells. Since glycolysis produces less ATP from glucose, but provides more of the building blocks required for cell proliferation, cancer cells (and normal proliferating cells) have been proposed to be in need of an activated glycolysis pathway, despite the presence of oxygen, to proliferate (Lopez-Lazaro 2008). Today, mutations in oncogenes and tumor suppressor genes are known to be responsible for malignant transformation, and the Warburg effect is now considered to be the consequence of rapid growth characteristic of cancer cells rather than a cause of cancer (Bertram 2000).

Despite the fact that during development normal cells can sustain the metabolic needs of fast growth, for these cells to achieve a high glycolytic metabolism and become tumorigenic they must acquire additional mutations (IDH1, IDH2, SDH, FH, PKM2, PFK2 etc.) in the metabolic pathway. The diversity of mechanisms affected by these mutations suggests that high glycolytic metabolism is not due to the natural adaptation of fast growth in cancer cells, but instead, contributes to cancer development in a way not yet understood.

As a consequence of high glycolysis, the glucose flux to PPP is reduced, resulting in reduced NADPH production and nucleotide production PPP (Boada, Roig et al. 2000; Perez, Roig et al. 2000; Bensaad, Tsuruta et al. 2006). This change may compromise the ability of the

cell to respond to oxidative stress and nucleotide shortage. Although research studies suggest that oxidative stress can induce DNA damage, direct evidence supporting a link between oxidative stresses induced DNA damage and cancer development is still missing. On the other hand, H₂O₂ is known to cause oxidative stress but has been demonstrated to be safe for teeth whitening and wound treatment, rather than acting as a carcinogen that could cause skin cancer. Furthermore, antioxidant treatments have not been demonstrated to have an effect in cancer prevention or treatment (Pais and Dumitrascu 2013). Oxidative stress is elevated in neurodegenerative diseases including Lou Gehrig's disease (Motor Neuron Disease or Amyotrophic Lateral Sclerosis), Parkinson's disease, Alzheimer's disease, and Huntington's disease.(Nunomura, Castellani et al. 2006; Boskovic, Vovk et al. 2011; Patel and Chu 2011). Furthermore, cumulative oxidative stress with disrupted mitochondrial respiration and mitochondrial damage is correlated to these neurodegenerative diseases (Ramalingam and Kim 2012). Surprisingly, all three diseases demonstrate decreased risk of multiple cancers; while Parkinson's disease correlates to a lower rate of other cancers, and increased risk of melanoma is observed (Kerr 2002; Zanetti, Rosso et al. 2007; Bajaj, Driver et al. 2010; Garber 2010; Devine, Plun-Favreau et al. 2011; Lai, Liao et al. 2013). These clinical data argue against the role of oxidative stress in promoting cancer development.

On the other hand, recent studies have suggested that nucleotide shortage during DNA replication leads to replication stress and DNA damage (Bester, Roniger et al. 2011; Beck, Nahse-Kumpf et al. 2012). Replication stress and the resulting genomic instability are prominent driving forces of early-stage cancer development since low concentrations of dNTP lead to increased strand breakage and mismatch repair, resulting in consequent spontaneous random mutations. Exogenously supplied nucleosides can relieve nucleotide shortage and rescue premature replication-induced DNA damage, effectively preventing cell transformation. These results illustrate the importance of the nucleotide supply for the suppression of spontaneous DNA damage and for the maintenance of genomic integrity. Interestingly, p53 is activated in

response to nucleotide shortage (Linke, Clarkin et al. 1996), but how p53 alters the transcriptional landscape to generate more nucleotides and relieve nucleotide shortage-induced DNA damage remains poorly understood.

Unlike chemical induced DNA damage, nucleotide shortage induced replication stress and consequent DNA damage is native spontaneous cellular stress. This may explain why human and mice live in climate controlled environment still get cancer without exposure to carcinogen. Nucleotide shortage during DNA replication will lead to increased mismatch and strand breakage, together resulting in infidelity in DNA replication and genome instability. Nucleotide shortage can happen following exposure to natural stressors such as UV induced DNA damage, premature activation of DNA replication, interrupted glucose supply, and others.

UV irradiation from the sun is the major source of DNA damage for most people in their daily lives. Ultraviolet radiation causes sunburns and increases the risk of three types of skin cancer: melanoma, basal-cell carcinoma, and squamous cell carcinoma (Section on and Balk 2011). UV-induced direct DNA damage leads to increased dNTP consumption in DNA damage repair and nucleotide shortage (also referred to as “deoxynucleoside triphosphate pools imbalance”). Nucleotide shortage can lead to replication stress and indirect DNA damage, which can lead to tumorigenesis.

Premature activation of DNA replication can also lead to nucleotide shortage. Abnormal activation of E2F leads to premature initiation of DNA replication even in the presence of an insufficient pool of nucleotides. Inhibition of the RB pathway results in a nucleotide shortage. The RB pathways are disabled in most, if not all, human tumors (Nevins 2001), suggesting that nucleotide shortage is a common feature in cancer development.

Although, tetraploidy and aneuploidy are common features of cancers, the detailed mechanism connecting tetraploidy and aneuploidy to tumorigenesis is incompletely understood. The extra chromosome copy in tetraploid cells leads to a great increase in the need for nucleotides; the increased demand for nucleotides challenges the nucleotide synthesis pathway

and can lead to increased risk of nucleotide shortage during DNA replication, and as a consequence result in genome instability.

De novo synthesis of cellular nucleotides is dependent on glucose supply, making prolonged interruption of glucose supply another cause of nucleotide shortage. A causal relationship between malignant cancers and thrombosis has been known since the 19th century (Sutherland, Weitz et al. 2003), and the association between cancer and excessive blood coagulation has since attracted much attention. Because blood coagulation and cancer development are both hard to detect until very late, it is difficult to determine whether cancer causes blood coagulation or if blood coagulation contributes to cancer development. It is easy to understand how malignant cancers can block blood vessels and cause blood coagulation, therefore it is widely accepted that metastatic cancer cells cause blood coagulation, and consequently ischemia or stroke (Sutherland, Weitz et al. 2003). However, blood coagulation can effectively reduce the regional glucose supply and lead to nucleotide shortage, thereby potentially increasing the risk of cancer initiation.

People with diabetes are at significantly higher risk for multiple type cancer. In fact, type 2 diabetes and cancer share many common risk factors, however, the potential biologic links between diabetes and cancer is poorly understood (Giovannucci, Harlan et al. 2010). Although diabetic patients have high blood glucose, glucose cannot enter the cell due to the lack of insulin or insulin resistance. Lack of cellular glucose can block nucleotide synthesis and lead to nucleotide shortage induced cancer initiation. Consistent with this idea, increased DNA double-strand breaks and p53 activity is observed in Type 2 diabetes and congenital hyperinsulinism (Tornovsky-Babeay, Dadon et al. 2014), indicative of nucleotide shortage induced DNA damage response.

Here, we demonstrate that the mitotic Cullin APC2 regulates Mdm2 stability, and consequently p53 activation potential. This finding links the Mdm2-p53 pathway to mitotic regulation, illuminating a possible explanation for the polyploidy phenotype of Mdm2

overexpression and p53 loss. We also demonstrate p53 suppression of PFK2 expression in response to nucleotide shortage results in the shutdown of glycolysis. Decreased glycolysis reroutes the glucose flux to PPP and promotes nucleotide synthesis to resolve nucleotide shortage and promote DNA damage repair. Our study serves to link metabolic regulation with maintenance of genome stability, and provides a potential explanation for the Walberg effect.

CHAPTER 2. IDENTIFICATION APC2 AS E3 UBIQUITIN LIGASE FOR MDM2

INTRODUCTION

Mdm2 is well characterized as the major negative regulator of the tumor suppressor p53 (Momand, Zambetti et al. 1992; Oliner, Pietenpol et al. 1993; Manfredi 2010). The C-terminus of Mdm2 has an intrinsic E3 ubiquitin ligase activity, which promotes the ubiquitination and degradation of p53 (Haupt, Maya et al. 1997; Honda, Tanaka et al. 1997; Kubbutat, Jones et al. 1997; Brooks and Gu 2006). Mdm2 is a transcriptional target of p53, forming a regulatory feedback loop between Mdm2 and p53 to maintain cellular homeostasis (Barak, Juven et al. 1993; Juven, Barak et al. 1993; Perry, Piette et al. 1993; Honda, Tanaka et al. 1997; Lahav 2008). The importance of Mdm2 in proper p53 regulation makes the understanding of its own regulation of critical concern. In order for the Mdm2-p53 feedback loop to function properly, tight regulation of Mdm2 degradation is essential. Previous *in vitro* and overexpression studies have demonstrated that Mdm2 regulates its own degradation by autoubiquitination, targeting itself for proteasome-mediated degradation (Fang, Jensen et al. 2000; Honda and Yasuda 2000). However, recent studies of *Mdm2*^{C462A/C462A} knock-in mice have challenged the Mdm2 autoubiquitination dogma. In *Mdm2*^{C462A/C462A} mouse embryonic fibroblasts (MEFs) the mutant Mdm2^{C462A} protein is degraded as rapidly as the wild type Mdm2 while p53 degradation is blocked, indicating that Mdm2 E3 ligase activity is not required for its own degradation when endogenously expressed (Itahana, Mao et al. 2007; Clegg, Itahana et al. 2008), suggesting that other E3 ubiquitin ligases regulate Mdm2 stability.

In light of the *in vivo* data, the potential for an outside E3 ubiquitin ligase to function in Mdm2 regulation has been more expressly studied. The Cullin1/β-TRCP E3 ubiquitin ligase

complex was identified to interact with Mdm2 and this interaction was demonstrated to lead to poly-ubiquitination and degradation of Mdm2 (Inuzuka, Tseng et al. 2010). However, the regulation of Mdm2:Cullin1/ β -TRCP interaction suggests that this interaction only occurs following DNA damage. Furthermore, knocking down Cullin1- β TRCP did not block p53 activation; instead, it affected the regulation of Mdm2 and p53 during the recovery of cells to basal conditions following exposure to stress (Inuzuka, Tseng et al. 2010). More recently, NEDD4-1 was biochemically identified to contribute to the regulation of Mdm2 protein stability in cells by functioning as an E3 ligase (Xu, Fan et al. 2014). However, NEDD4-1 catalyzes the formation of K63-type polyubiquitin chains on Mdm2 that are distinct from the K48-type polyubiquitin chains typically required for proteasomal degradation. Notably, K63-type polyubiquitination by NEDD4-1 competes with K48-type polyubiquitination on Mdm2 in cells. As a result, NEDD4-1-mediated ubiquitination stabilizes Mdm2. Our study was designed to identify E3 ubiquitin ligases responsible for the regulation of Mdm2 stability under physiological conditions.

RESULTS

Mdm2 E3 ubiquitin ligase function is dispensable for Mdm2 self-degradation under physiological conditions

To examine the role of Mdm2 E3 ligase function in Mdm2 self-degradation *in vivo*, we compared degradation dynamics of Mdm2 in MEFs expressing wild type (WT) Mdm2 or the E3 ligase inactive mutant Mdm2. We generated two Mdm2 knock-in mouse models with inactivated Mdm2 E3 ubiquitin ligase activity towards p53: the *Mdm2*^{Y487A} (corresponding to human Mdm2^{Y489A}) and *Mdm2*^{C462A} (corresponding to human Mdm2^{C464A}) (Itahana, Mao et al. 2007). Both of the mutated *Mdm2* genes are under control of native promoter and hence results in physiological levels of *Mdm2* expression. While both the Mdm2^{Y487A} and Mdm2^{C462A} proteins display disrupted E3 ubiquitin ligase activity for p53, the mechanism of disruption in each of these mutants is unique. The Mdm2Y487A mutation located to the C-terminal of the RING domain, in a region previously demonstrated to be critical for Mdm2 E3 ubiquitin ligase activity towards p53, however, this mutation retains an intact RING domain (Uldrijan, Pannekoek et al. 2007). In contrast, the Mdm2C462A mutation affects one of the four critical cysteine residues responsible for maintaining the RING domain structure; disruption of the Mdm2 RING domain structure results in the loss of E3 ubiquitin ligase activity (Uldrijan, Pannekoek et al. 2007). Because the *Mdm2*^{C462A} mutation results in early embryonic lethality in homozygous mice, we crossed *Mdm2*^{C462A/+} mice with mice expressing inducible p53ER^{TAM} (p53ER hereafter); the inducible nature of the p53ER fusion protein allows for generation of mice and MEFs with inactive p53 (Martins, Brown-Swigart et al. 2006). The addition of 4-Hydroxytamoxifen (4-OHT) activates p53ER to allow for transactivation of Mdm2 and the study of Mdm2 dynamics in the otherwise lethal *Mdm2*^{C462A} background. In the presence of 4-OHT, the half-life of p53ER was approximately 30 minutes, and the half-life of Mdm2 approximately 15 minutes in *Mdm2*^{+/+};p53^{ER/-} MEFs (Figure 2-1B). The disruption of Mdm2 E3 ligase activity, greatly extended the half-life of p53ER, as observed in *Mdm2*^{Y487A/Y487A};p53^{ER/-} and

Mdm2^{C462A/C462A};*p53*^{ER/-} MEFs (Figures 2-1C, 2-1D). These results support previous findings and indicate that *in vivo* the *Mdm2*^{Y487A} and *Mdm2*^{C462A} mutant proteins have indeed lost E3 ubiquitin ligase activity for p53 degradation.

To evaluate the importance of Mdm2 E3 ligase function in the degradation of Mdm2 itself, we assessed the half-life of Mdm2 in *Mdm2*^{+/+};*p53*^{ER/-}, *Mdm2*^{Y487A/Y487A};*p53*^{ER/-} and *Mdm2*^{C462A/C462A};*p53*^{ER/-} MEFs. Despite disrupting p53 degradation, *Mdm2*^{Y487A} and *Mdm2*^{C462A} protein itself was degraded normally when compared with WT Mdm2 (Figures 2-1B, 2-1C, 2-1D). Furthermore, treatment with the proteasome inhibitor MG132 led to similar stabilization of WT Mdm2, *Mdm2*^{Y487A}, and *Mdm2*^{C462A} (Figure 2-2A), suggesting that each of these proteins is degraded, at a similar rate, in a proteasome dependent manner. To eliminate the possible aberrant effect of p53ER on transcriptional regulation of Mdm2, *Mdm2*^{+/+};*p53*^{-/-}, *Mdm2*^{Y487A/Y487A};*p53*^{-/-}, and *Mdm2*^{C462A/C462A};*p53*^{-/-} mice were generated, and MEFs were isolated and similarly subjected to MG132 treatment. As observed in the *p53*^{ER/-} genetic background, in the absence of p53, a similar level of Mdm2 stabilization was observed regardless of the E3 ligase status (Figure 2-2B). These results suggest that while Mdm2 E3 ligase function is necessary for the degradation of p53, under physiological conditions, it is dispensable for its own degradation through the proteasome-dependent pathway.

In light of the normal, proteasome-dependent Mdm2 degradation dynamics observed in *Mdm2* E3 inactive MEFs, we postulated that alternative E3 ubiquitin ligase(s) is responsible for Mdm2 degradation under physiological conditions. To test this hypothesis, we used an *in vitro* ubiquitin ligase assay to assess the E3 ubiquitin ligase activity of HeLa cell lysate towards bacterial purified Mdm2 protein. Consistent with previous studies (Fang, Jensen et al. 2000), and similar to most other RING finger domain-containing proteins that confer E3 ligase activity (Deshaies and Joazeiro 2009), purified WT Mdm2 protein demonstrated intrinsic autoubiquitination activity in the *in vitro* ubiquitin ligase assay; in contrast, *Mdm2*^{C464A} mutant protein does not demonstrate intrinsic autoubiquitination activity (Figure 2-3A). Interestingly,

following addition of HeLa cell lysate to the Mdm2^{C464A} protein reaction samples, strong polyubiquitin chain formation was observed (Figure 2-3A). The ability for Mdm2^{C464A} to be polyubiquitinated in the presence of HeLa cell lysate suggests that an E3 ubiquitin ligase(s) capable of polyubiquitinating Mdm2 is present in the lysate. To rule out the possibility that WT Mdm2 present in the HeLa lysate is responsible for the observed polyubiquitination of Mdm2^{C464A}, we assessed polyubiquitin chain formation in cell lysate isolated from *WT*, *Mdm2*^{+/+};*p53*^{-/-} and *Mdm2*^{-/-};*p53*^{-/-} MEFs. Polyubiquitin chain formation in *WT*, *Mdm2*^{+/+};*p53*^{-/-} and *Mdm2*^{-/-};*p53*^{-/-} MEF cell lysate occurs at similar levels (Figure 2-3B), indicating that a component in the MEF cell lysates, independent of both Mdm2 and p53, is capable of promoting Mdm2 polyubiquitination.

We then tested if this polyubiquitin chain formation is dependent on E1 and E2 supplied *in vitro* by individually removing each of these components. The polyubiquitin chain formation is dependent on E2 supplied *in vitro*, but it seems the HeLa cell lysate contained sufficient E1 ubiquitin activating enzyme to support the *in vitro* ubiquitination reaction (Figure 2-3C). Furthermore, we found that the E3 ubiquitin ligase activity from HeLa cell lysate is diminished in the presence of SDS, providing further evidence that an enzymatic activity in the HeLa lysate is essential for the observed polyubiquitin chain formation (Figure 2-3D).

Mdm2 interacts with APC2

Previous studies have indicated that Mdm2 protein levels oscillate with the cell cycle (Gu, Ying et al. 2003; Inuzuka, Tseng et al. 2010). Accordingly, we also found that in *WT* and *p53*^{-/-} MEFs synchronized by serum starvation Mdm2 protein levels oscillated in a manner correlated to cyclin B1 oscillations (Figures 2-4A, 2-4B). Similar results were also observed in U2OS cells synchronized with serum starvation (Figure 2-4C), and HeLa and HCT116/*p53*^{-/-} cells synchronized by double thymidine block (Figures 2-5A, 2-5B). Together, these results suggest that Mdm2 levels might be regulated during mitosis, presumably by a member of Cullin family

E3 ubiquitin ligases, in a manner similar to cyclin B1. Furthermore, the observed oscillation of Mdm2 in p53 null cells suggests that this manner of Mdm2 regulation is p53-independent.

To test the possibility that a Cullin family E3 ubiquitin ligase is responsible for the ubiquitination and degradation of Mdm2, we first determined the binding affinity of Cullin family E3 ubiquitin ligases with Mdm2 via immunoprecipitation. As shown in Figure 2-6A, Mdm2 interacted with Cullin1 when co-overexpressed, providing additional support for previous studies characterizing Cullin1:Mdm2 binding (Inuzuka, Tseng et al. 2010). Additionally, we observed Mdm2 interaction with APC2, a scaffold subunit of APC/C, which is a multi-subunit E3 ubiquitin ligase complex that controls the degradation of many proteins involved in cell cycle regulation (Figure 2C). Most APC/C substrates oscillate their protein levels through the cell cycle, including the metaphase-anaphase transition and mitotic exit (Peters 2006; Thornton, Ng et al. 2006; Yu 2007). We confirmed APC2:Mdm2 interaction with reciprocal immunoprecipitation of ectopically expressed proteins (Figures 2-7A, 2-7B), as well as with immunoprecipitation of endogenous Mdm2 in 293T and H1299 cells. 293T and H1299 cells express different levels of Mdm2, which allowed for correlation of the levels of Mdm2 with the levels of APC2 being co-immunoprecipitated (Figure 2-7C).

APC2 is important for mdm2 degradation

To test if APC/C is a physiological E3 ubiquitin ligase for Mdm2, we knocked down APC2 in human tumor cell lines and examined the levels of Mdm2. Knockdown of APC2 by siRNA in both U2OS (p53 positive) and HCT116/p53^{-/-} (p53 negative) cells led to accumulation of Mdm2 (Figures 2-10A, 2-10B), as well as extension of Mdm2 protein half-life (Figures 2-9A, 2-9B). The limited increase of Mdm2 level upon APC2 knockdown suggested that there are other E3 ligases, in addition to APC2, degrade Mdm2. To further rule out the possibility that the increase in Mdm2 is due to increased transcription of *Mdm2* rather than decreased degradation, we generated stable cell lines expressing GFP-tagged WT Mdm2 (GFP-Mdm2) or GFP-tagged

Mdm2^{C464A} mutant under the control of the CMV promoter. As shown in Figure 2-10C, knockdown of APC2 by siRNA led to a strong accumulation of ectopic GFP-tagged Mdm2, indicating that the accumulation of Mdm2 is independent of transcriptional regulation. More importantly, knockdown of APC2 by siRNA also led to a strong accumulation of ectopic GFP-tagged Mdm2^{C464A}, which suggests that the Mdm2 accumulation observed following downregulation of APC2 is independent of Mdm2 E3 ligase activity (Figure 2-10D). Disruption of proteasome-mediated degradation by MG132 treatment resulted in an accumulation of Mdm2 in U2OS cells transfected with a control knockdown siRNA (siCtrl), but did not further stabilize Mdm2 in cells with knocked down APC2 (siAPC2), which had higher levels of Mdm2 prior to MG132 treatment (Figure 2-11A). Together, these results indicate that APC2 is important for Mdm2 degradation in a manner independent of p53 status and *Mdm2* transcriptional regulation.

APC2 is important for stress induced p53 stabilization and activation

Because Mdm2 is the primary regulator for p53, mechanisms functioning to regulate Mdm2 will inevitably affect p53 function as well. To investigate the functional connections of APC2 with p53 activity, we examined the effect of downregulation of APC2 on p53 induced growth inhibition. We generated MEF cells with stable knockdown of APC2 in both *WT* and *p53*^{-/-} genetic backgrounds. Stable knockdown of APC2 in the *WT*, but not *p53*^{-/-} background, corresponded to a higher number of cells displaying flattened and enlarged morphology, indicative of senescence. Assessment of β -galactosidase activity was utilized to determine whether the flattened and enlarged MEF cells were indeed senescent. MEFs with APC2 knocked down exhibited significantly weaker activity of senescence-associated β -galactosidase than did control cells (Figure 2-12A). Additionally, quantitative analysis indicated that APC2 knockdown decreased the percentage of cells in senescence by more than half (Figure 2-12A). We then analyzed the effect of APC2 knockdown on p53 activity by examining p53-dependent

transcription using a p21-luciferase assay. As shown in Figure 2-12B, p53-dependent activation of the p21 promoter was greatly reduced by knockdown of APC2.

To investigate the role of APC2 in the p53 stress response, we knocked down APC2 by shRNA in *WT* MEFs and examined cell survival following exposure to ultraviolet (*UV*) radiation. While APC2 knockdown did not affect cell survival under unstressed conditions, it increased cell survival upon UV induced DNA damage (Figure 2-12C). This increase in cell survival was attenuated when Mdm2-p53 interaction was disrupted by Nutlin-3 (Figure 2-12D), suggesting that the effect of APC2 on cell survival following UV radiation is p53-dependent.

To determine whether APC2 might regulate stress-induced p53 expression, siRNA was used to knock down APC2 in U2OS cells, followed by treatment with genotoxic agents to challenge the cells. Indeed, downregulation of APC2 substantially reduced the level of p53 induced by the DNA damaging agents, as observed following treatment with UV (Figure 2-13A) and doxorubicin (Figure 2-13B), as well as in response to ribosomal stress, as induced by actinomycin D (Figure 2-13C). These results indicate that APC2 plays an important role in p53 stabilization in response to genotoxic stress.

APC2 downregulation correlates with cancer development

To further investigate the physiological relevance of APC2 regulation of Mdm2, we assessed the expression levels of APC2 and Mdm2 in various cancer cells. Interestingly, JAR and SJSA cells expressed very low levels of APC2 and conversely very high levels of Mdm2 compared to HepG2, MCF7, HeLa, A375, U2OS and H1299 cells, which expressed higher levels of APC2 and corresponding lower levels of Mdm2 (Figure 2-14A). To investigate the clinical relevance of variation in APC2 expression, we interrogated available tumor data sets for APC2. mRNA levels of APC2 were significantly decreased in breast, liver, and lymphocyte cancer tissues compared to that observed in corresponding normal tissue based on an analysis of the Oncomine database (<http://www.oncomine.com>) (Figure 2-15A). Investigation of breast,

ovarian, lung, thyroid, liver, and renal cancer tissues in The Cancer Genome Atlas (TCGA) also revealed that *APC2* gene copy number was decreased compared to the gene copy number observed in normal tissues (Figure 2-16A). The decreased level of *APC2* mRNA and gene copy number in a variety of cancer types suggests that *APC2* may be clinically relevant in the development or progression of cancer.

DISCUSSION

Here, we report that Mdm2 protein stability is regulated by the APC/C E3 ubiquitin ligase complex through APC2:Mdm2 interaction. Various biochemical analyses, including protein binding and degradation assays, as well as functional assays, clearly identify Mdm2 as a substrate of APC2. Mdm2 protein levels increases by depletion of APC2 resulting in increased levels of Mdm2, which in turn leads to attenuation of stress-induced p53 stabilization, and increased cell survival.

Although the Mdm2-p53 feedback loop is well established, our data suggests that Mdm2 accumulation induced by APC2 knockdown does not necessarily lead to decreased p53 protein levels under unstressed conditions. Instead, we see p53 accumulation is decreased after stress. Through Mdm2, knockdown of APC2 results in inhibition of p53 activation which leads to reduced stress induced cell death and senescence. In light of our research, we hypothesize that the ability of Mdm2 to inhibit p53 transcriptional activity via direct binding is the primary mechanism through which Mdm2 functions to regulate p53 in the absence of stress. Mdm2 E3 ubiquitin ligase activity only regulates p53 stability after stress. This hypothesis is consistent with the observation that Mdm2^{Y487A/Y487A} mice survive normally but unable to recover from low dose IR induced DNA damage as wild type mice do. APC2 knockdown also recapitulate human cancers with Mdm2 overexpression that high level of Mdm2 binds and inhibit p53 activity instead of lead to decreased p53 protein level.

Mitotic inhibitors that activate the spindle assembly checkpoint by perturbing microtubule function are commonly used in the treatment of breast, ovarian, and lung cancers (Montero, Fossella et al. 2005); however, as activation of the spindle assembly checkpoint does not suppress APC/C activity completely, it is believed that inhibitors directly targeting APC/C could provide a better therapeutic method for inducing mitotic arrest (Zeng, Sigoillot et al. 2010). In contrast, our study suggests that inhibition of APC/C activity by downregulation of APC2 can result in oncogenic consequences due to accumulation of Mdm2, and the resulting inactivation

of p53. In light of this finding, the prospect of pharmacologic inhibition of APC/C as an anti-cancer therapeutic needs to be carefully reevaluated. The observation that APC2 depletion effectively stabilizes Mdm2, and leads to inhibition of p53 activation indicates that cautious consideration of the p53 status should be taken prior to use of cancer treatment targeting APC/C related pathways.

Microarray and gene sequencing are relatively easy and sensitive methods to screen for alterations in Mdm2 that may affect p53 activity. However, these methods are only capable of detecting Mdm2 overexpression due to gene amplification. Our study has identified the APC/C complex as an E3 ubiquitin ligase capable of regulating Mdm2 protein levels post-translationally, making the components of the APC/C complex and APC/C regulators potentially useful new targets for microarray screening and gene sequencing to predict Mdm2 overexpression and p53 misregulation.

EXPERIMENTAL PROCEDURE

Cell culture, transfection

All cells were cultured at 37°C, and 5% CO₂ in DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 ug/mL streptomycin. DNA transfections were carried out using the Calcium phosphate method (Sambrook and Russell 2006) and Fugene-6 (Roche).

Double thymidine block synchronization

Cells were cultured with 2 mM thymidine for 19 hours, washed once with 1 X PBS, incubated with fresh media without addition of thymidine, and then 2mM thymidine was added back 8 hours later. After another 16 hours, cells were washed once with 1 X PBS, fresh media without thymidine addition was added back. Cells were collected every 2 hours and analyzed by western blotting.

DNA plasmids, siRNA and lenti-viral based shRNA

All cloned constructs were confirmed by DNA sequencing. siRNA duplexes targeting APC2 (5'-AACGATCTGCAGGCCAACATC-3'), and nonspecific control siRNA (5'-CAGUCGCGUU UGCGACUGG-3') were manufactured by Dharmacon. Stealth siRNAs targeting APC2 (HSS121004, HSS179082, HSS179083) were manufactured by Invitrogen. Lenti-viral based shRNA clones were purchased commercially from Open Biosystems (pLKO.1 shRNA sets RMM4534-NM_175300). Transfections of siRNA were performed with RNAiMax (Invitrogen # 13778) according to the manufacturer's instructions 48 hours before additional treatments

SDS-Page and western blotting

Cells were lysed in 0.5% NP-40 lysis buffer (50 mM Tris-HCl pH7.5, 150 mM NaCl, 0.5%

NP-40, 50 mM NaF, 1 mM NaVO₃, 1 mM DTT, 1 mM PMSF, 1x Protease inhibitor cocktail), lysates were resolved on a 12.5% polyacrylamide gel and transferred onto a 0.45 μM nitrocellulose membrane. Membranes were blocked for at least 30 minutes in blocking buffer (PBS with 0.1% Tween-20 and 5% nonfat dried milk). Membranes were incubated for overnight in primary antibody diluted in blocking buffer, incubated for 1-2 hours in secondary HRP-conjugated antibody diluted in block buffer, and exposed after incubate in Supersignal West Pico or Dura (Pierce) for 5 minutes.

IP-Western blotting

MEF cells were lysed in 0.5% NP-40 lysis buffer. The lysates were pre-cleared with Sepharose CL4B beads (Sigma) for 30 min and then immunoprecipitated with the specified antibody overnight at 4°C, followed by incubation with protein A/G beads (Pierce) for 2 hours at 4°C. The beads were washed four times with cold 0.5% NP-40 lysis buffer, and analyzed by SDS-PAGE and Western Blotting.

Half-life assay

Cells were treated with cycloheximide (100-150 μg/mL) for the indicated length of time and then harvested using 1X SDS lysis buffer (50mM Tris-HCl, pH 7.5, 0.5mM EDTA, 1% SDS, and 1 mM DTT). The Mdm2 level was analyzed by western blotting, and the intensity of the bands in the linear range of exposure was quantified by densitometry and normalized to Actin using ImageJ.

Beta-Gal staining

Beta-Gal staining was done with Senescence β-Galactosidase Staining Kit (Cell Signaling #9860) according to the manufacturer's instructions.

Luciferase assay

Beta-Gal staining was done with Dual-Light® Luciferase & β -Galactosidase Reporter Gene Assay System (Life technologies #T1004) according to the manufacturer's instructions.

Antibodies

Rabbit anti-APC2 antibody was kindly provided by Dr. Yue Xiong (UNC at Chapel Hill). HA (12CA5), myc (9E10), Mdm2 (2A10), and Mdm2 (4B11) antibodies were made from the culture medium of the hybridoma cell line. The following antibodies were purchased commercially: Actin (Chemicon #MAB1501R), hp53 (DO-1 Labvision AB-6 #MS-187 - P), mp53 (Leica # NCL-p53-505), FLAG (M2 Sigma #F3165). Anti-UB (Covance #PRB-268C), Cyclin B1 (GNS1 Santa Cruz #sc0245)

***In vitro* ubiquitination assay**

Bacterial purified GST (Glutathione S-transferase) or GST tagged Mdm2^{C464A} bound to glutathione were incubated with 50 ng E1 (UBE1, Millipore, #23-021), 50 ng E2 (bacterial purified UBCH5C), E3 (cell lysate or APC2 immuno-complex), 5 ug Ubiquitin, and reaction buffer (50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 2 mM DTT, 5 mM MgCl₂, 2 mM ATP). Reaction mixes were incubated at 37°C for 1 hour, the beads were washed with three times with washing buffer (50 mM Tris-HCl pH 8.0, 0.1 M NaCl, 1 mM DTT, 5 mM MgCl₂, 1% TritonX-100, 1 mM PMSF), and analyzed by western blotting.

***In vivo* ubiquitination assay**

293T cells were transfected with the indicated plasmids and incubated for 24 hours at 37°C, 5% CO₂. Cells were then treated with 25 uM MG132 for 4 hours and then lysed in 1%

SDS lysis buffer (50mM Tris-HCl, pH 7.5, 0.5mM EDTA, 1% SDS, 1mM DTT) and boiled at 95°C for 10 minutes. Cell lysates were centrifuged and supernatant was diluted ten times with 0.5% NP-40 lysis buffer. The diluted lysates were pre-cleared with Sepharose CL4B beads (Sigma) for 30 min and then immunoprecipitated with anti-FLAG antibody overnight at 4°C, followed by incubation with protein A beads (Pierce) for 2 hours at 4°C. The beads were washed four times with cold 0.5% NP-40 lysis buffer. Protein bound to beads was eluted by incubation in 1× SDS sample buffer at 95° C, followed by western blot analysis.

FIGURES

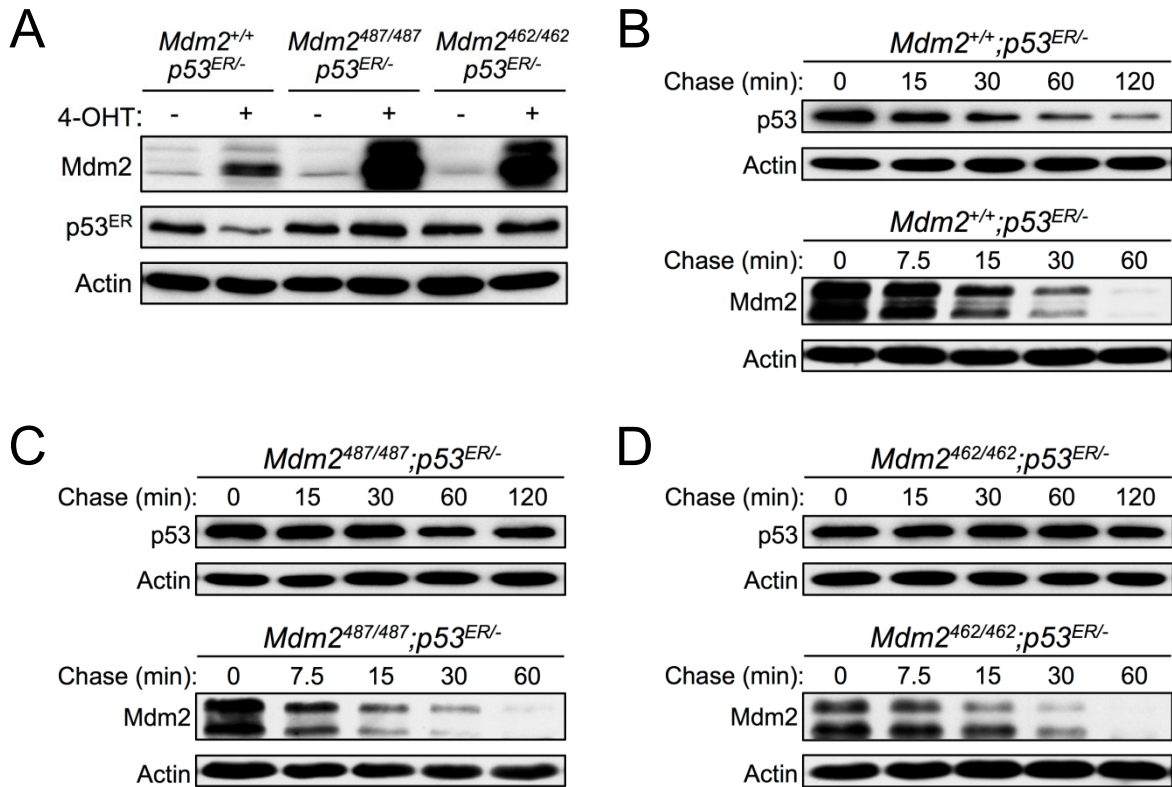


Figure 2-1. Mdm2 E3 ubiquitin ligase function is dispensable for Mdm2 autodegradation under physiological conditions.

A. *Mdm2*^{+/+};*p53*^{ER/-}; *Mdm2*^{Y487A/Y487A};*p53*^{ER/-}, and *Mdm2*^{C462A/C462A};*p53*^{ER/-} MEFs were treated with 100 nM 4OHT for 4 hours, and analyzed by western blot for the indicated proteins. Actin was used as a loading control.

B. *Mdm2*^{+/+};*p53*^{ER/-} MEFs were treated with 100 nM 4OHT for 24 hours before treatment with 150 μ g/ml cycloheximide. Cell lysates were collected at the indicated times following cycloheximide treatment and p53ER and Mdm2 levels were analyzed by western blot. Actin was used as a loading control.

C. *Mdm2*^{Y487A/Y487A};*p53*^{ER/-} MEFs were treated and analyzed as described in (B).

D. *Mdm2*^{C462A/C462A};*p53*^{ER/-} MEFs were treated and analyzed as in (B).

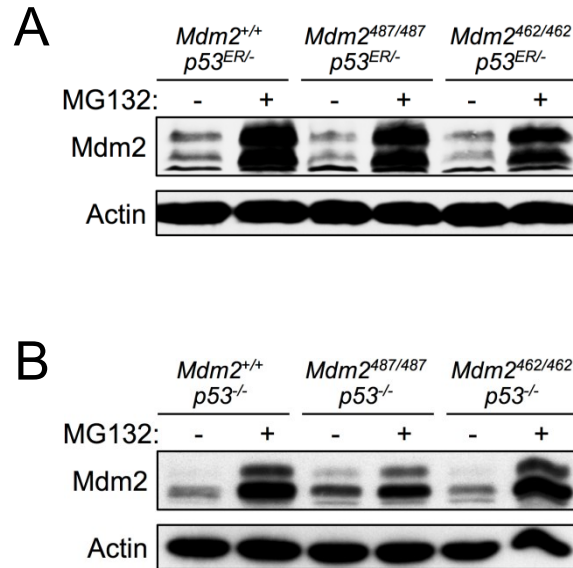


Figure 2-2. Mdm2 E3 dead mutant still degraded in proteasome dependent manner.

A. *Mdm2*^{+/+}; *p53*^{ER/-}; *Mdm2*^{Y487A/Y487A}; *p53*^{ER/-}, and *Mdm2*^{C462A/C462A}; *p53*^{ER/-} MEFs were treated with 100nM 4OHT for 24 hours, and then treated with 25 uM MG132 for 4 hours as indicated. Mdm2 levels were analyzed by western blot. Actin was used as a loading control.

B. *Mdm2*^{+/+}; *p53*^{-/-}; *Mdm2*^{Y487A/Y487A}; *p53*^{-/-}, and *Mdm2*^{C462A/C462A}; *p53*^{-/-} MEFs were treated with 25 uM MG132 for 8 hours as indicated. Mdm2 levels were analyzed by western blot. Actin was used as a loading control.

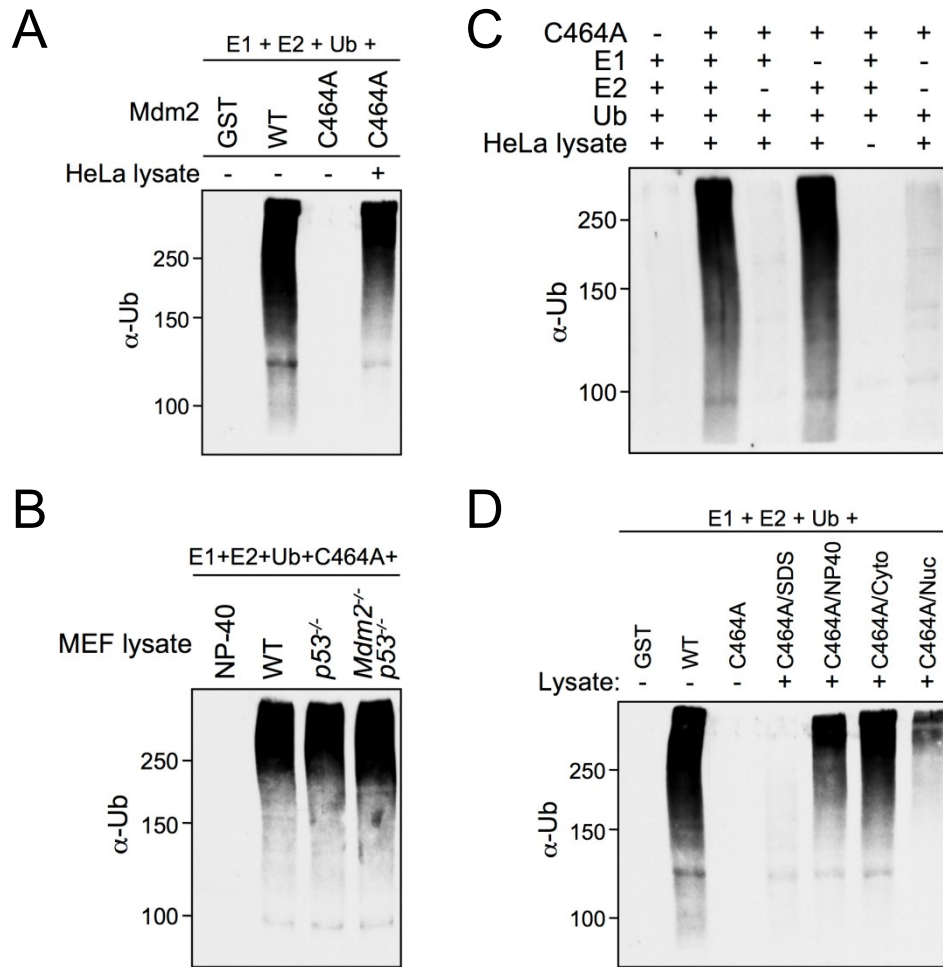


Figure 2-3. Cell lysate contains E3 ubiquitin ligase activity for Mdm2 polyubiquitination in vitro.

A. Bacterial purified GST, GST tagged wild type Mdm2, and GST tagged Mdm2C464A protein bound to glutathione beads was incubated with E1, E2 and ubiquitin (Ub) with or without addition of HeLa cell lysate for 1 hour at 37°C as indicated. Ubiquitin was analyzed by western blot.

B. Experiment is done as in (A) except cell lysate from wild type (WT), p53^{-/-}, or Mdm2^{-/-};p53^{-/-} MEFs is used as indicated.

C. Experiment is done as in (A) except Mdm2C464A, E1, E2, UB, and HeLa cell lysate is added as indicated.

D. Experiment is done as in (A) except HeLa cell lysate is prepared as indicated and analyzed as in (A)

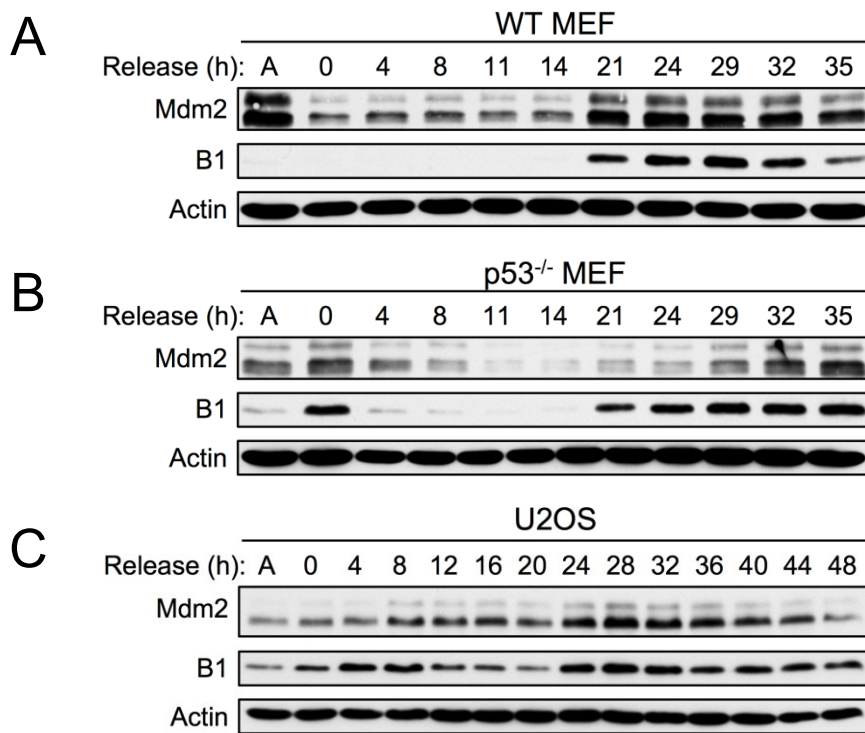


Figure 2-4. Mdm2 oscillates in a p53 independent manner in serum starvation synchronized cells.

- A. *Mdm2*^{+/+}; *p53*^{+/+} (WT) MEFs were incubated with DMEM with 0.1% FBS for 48 hours for serum starvation, media was then replaced with fresh DMEM with 10% FBS. Cells were harvested at the indicated time points and analyzed by western blot. Actin was used as a loading control.
- B. *Mdm2*^{+/+}; *p53*^{-/-} (*p53*^{-/-}) MEFs were treated, harvested, and analyzed as in (A).
- C. U2OS cells were treated, harvested, and analyzed as in (A).

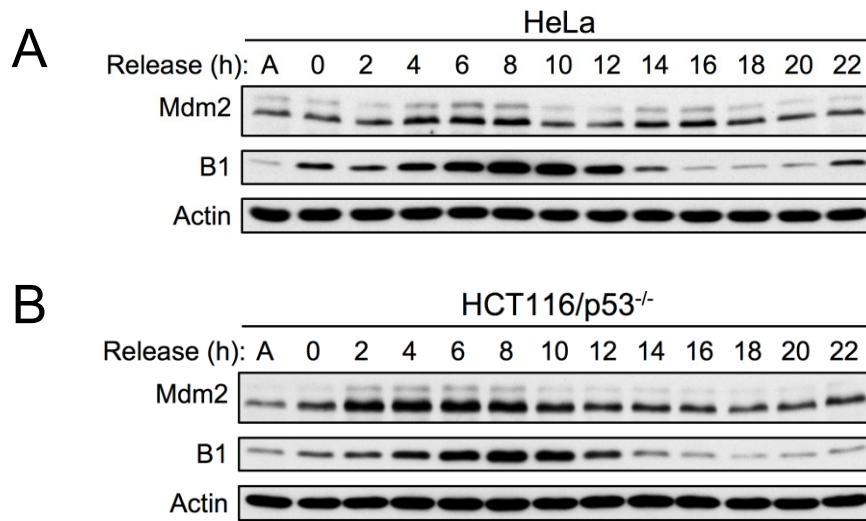


Figure 2-5. Mdm2 oscillates in a p53 independent manner in double thymidine block synchronized cells.

- A. HeLa cells were synchronized by double thymidine block. Cells were harvested after release at the indicated time points and analyzed by western blot. Actin was used as a loading control.
- B. HCT116/p53^{-/-} cells were synchronized with a double thymidine block, harvested, and analyzed as in (A).

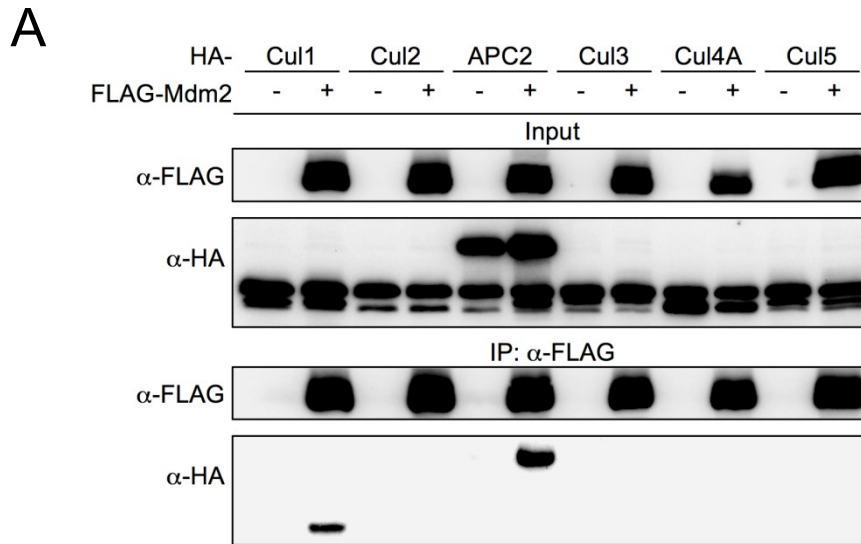


Figure 2-6. Mdm2 interacts with APC2 and Cullin 1.

A. 293T cells were transfected with FLAG-tagged Mdm2 together with HA-tagged Cullin1 (Cul1), Cullin2 (Cul2), APC2, Cullin3 (Cul3), Cullin4A (Cul4A), or Cullin5 (Cul5) as indicated. Cell lysate was immunoprecipitated and immunoblotted with the indicated antibodies. Input represents 4% of total cell lysate utilized for IP.

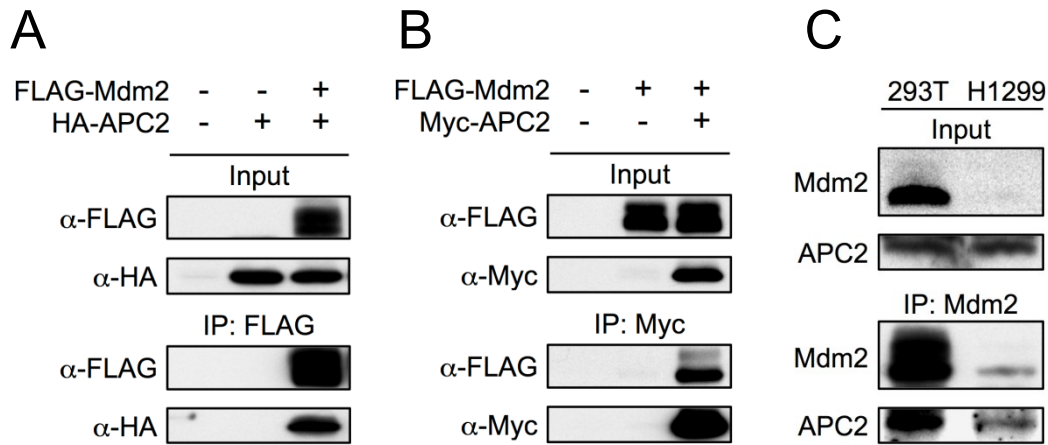
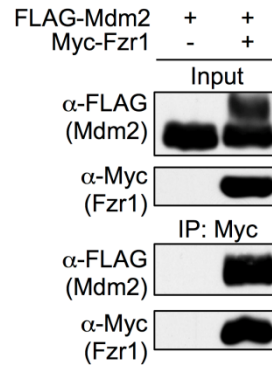


Figure 2-7. Mdm2 interacts with APC2.

- A. 293T cells were transfected with FLAG-tagged Mdm2 and HA-tagged APC2 as indicated. Cell lysate was immunoprecipitated with anti-FLAG and immunoblotted as indicated. Input represents 4% of total cell lysate utilized for IP.
- B. 293T cells were transfected with FLAG-tagged Mdm2 and Myc-tagged APC2 as indicated. Cell lysate was immunoprecipitated with anti-Myc antibody and immunoblotted as indicated. Input represents 4% of total cell lysate utilized for IP.
- C. 293T cell and H1299 cell lysates were immunoprecipitated with anti-Mdm2 antibody and immunoblotted as indicated. Input represents 2% of total cell lysate utilized for IP. H1299 cells, with a low level of Mdm2, were used as a negative control.

A**B****Figure 2-8. Mdm2 interacts with Fzr1.**

A. 293T cells were transfected with FLAG-tagged Mdm2 and Myc-tagged Fzr1. Cell lysate was immunoprecipitated with anti-FLAG antibody and immunoblotted as indicated. Input represents 4% of total cell lysate utilized for IP.

B. 293T cells were transfected with FLAG-tagged Mdm2 and Myc-tagged Fzr1. Cell lysate was immunoprecipitated with anti-Myc antibody and immunoblotted as indicated. Input represents 4% of total cell lysate utilized for IP.

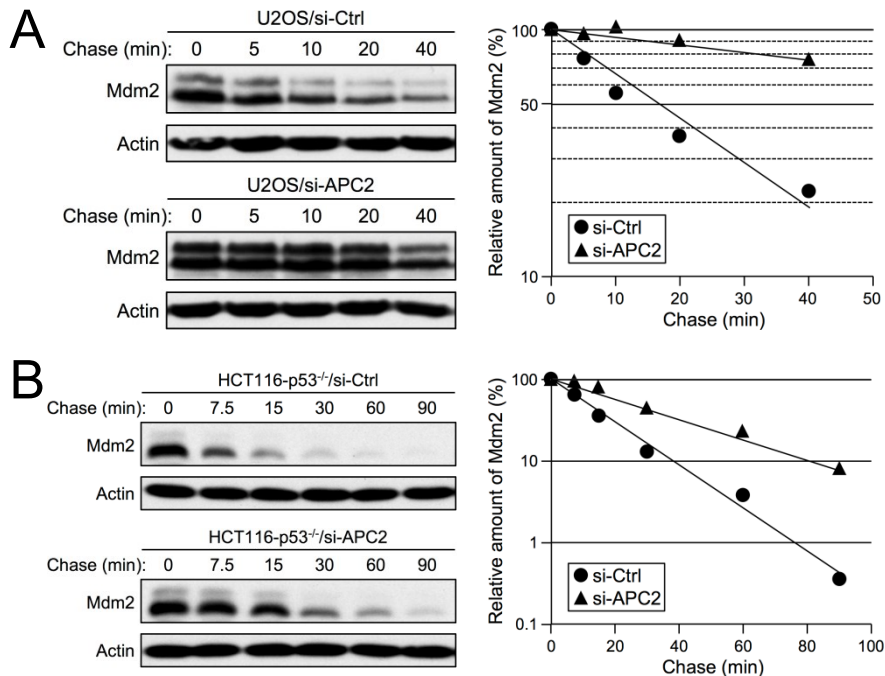


Figure 2-9. APC2 Knock down lead to extended Mdm2 half-life.

A. U2OS cells were transfected with control siRNA (si-Ctrl) or APC2 siRNA (si-APC2) for 24 hours, cells were split and 150 ug/ml cycloheximide was added 48 hours later. Cell lysates were collected at the indicated times following cycloheximide treatment and Mdm2 levels were analyzed by western blot. Actin was used as a loading control. Mdm2 levels were measured by densitometry, normalized to Actin, and plotted.

B. HCT116/p53^{-/-} cells were transfected with control siRNA (si-Ctrl) or APC2 siRNA (si-APC2) for 24 hours, cells were split and 150 ug/ml cycloheximide was added 48 hours later. Cells were collected and analyzed as in (A).

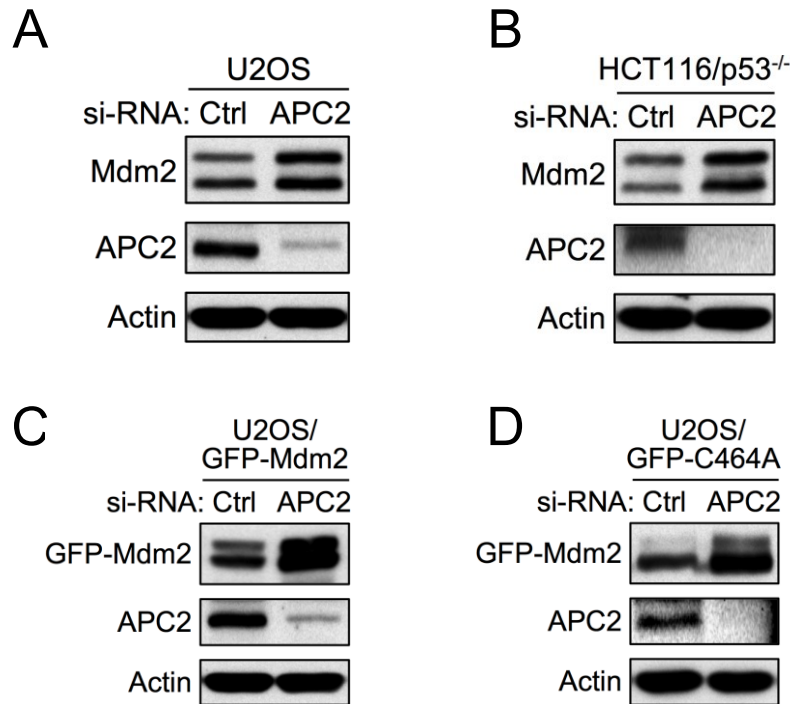


Figure 2-10. APC2 Knock down leads to Mdm2 accumulation.

A. U2OS cells were transfected with control siRNA (si-Ctrl) or APC2 siRNA (si-APC2) for 72 hours. Cell lysates were analyzed by western blotting for Mdm2 and APC2. Actin was used as a loading control.

B. HCT116/p53^{-/-} cells were transfected, harvested, and analyzed as in (A).

C. Clonal stable cell lines stably expressing a GFP-Mdm2 fusion protein under the control of CMV promoter in U2OS cells were transfected, harvested, and analyzed as in (A).

D. Clonal stable cell lines stably expressing a GFP-Mdm2^{C464A} fusion protein under the control of CMV promoter in U2OS cells were transfected, harvested, and analyzed as in (A).

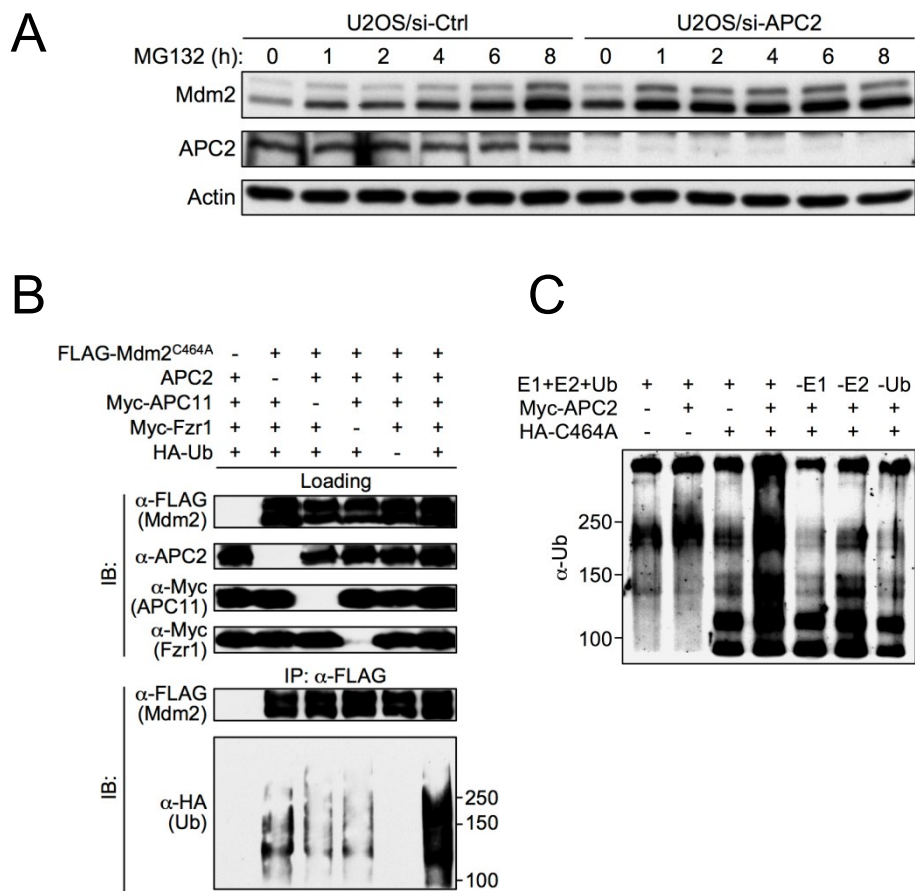


Figure 2-11. APC2 is important for Mdm2 degradation.

- A. U2OS cells were transfected with control siRNA (si-Ctrl) or APC2 siRNA (si-APC2) for 24 hours, cells were then split and 25 μ M MG132 was added 48 hours later. Cells were collected at indicated time after MG132 treatment and analyzed by western blot. Actin was used as a loading control.
- B. *In vivo* ubiquitin ligase assay. 293T cells were transiently transfected with FLAG-tagged Mdm2^{C464A}, HA-ubiquitin, and APC/C core components (APC2, APC11, and Fzr1) for 24 h. Cells were treated with 25 μ M MG132 for 4 hours and lysates were immunoprecipitated and immunoblotted as indicated.
- C. *In vitro* ubiquitin assay. 293T cells were transiently transfected with empty vector or Myc-tagged APC2 for 24 hours and E1, E2, ubiquitin (Ub), and bacterial purified GST tagged Mdm2^{C464A} were incubated with lysate as indicated. Cell lysate was immunoprecipitated with anti-Myc antibody and analyzed by western blot for ubiquitin.

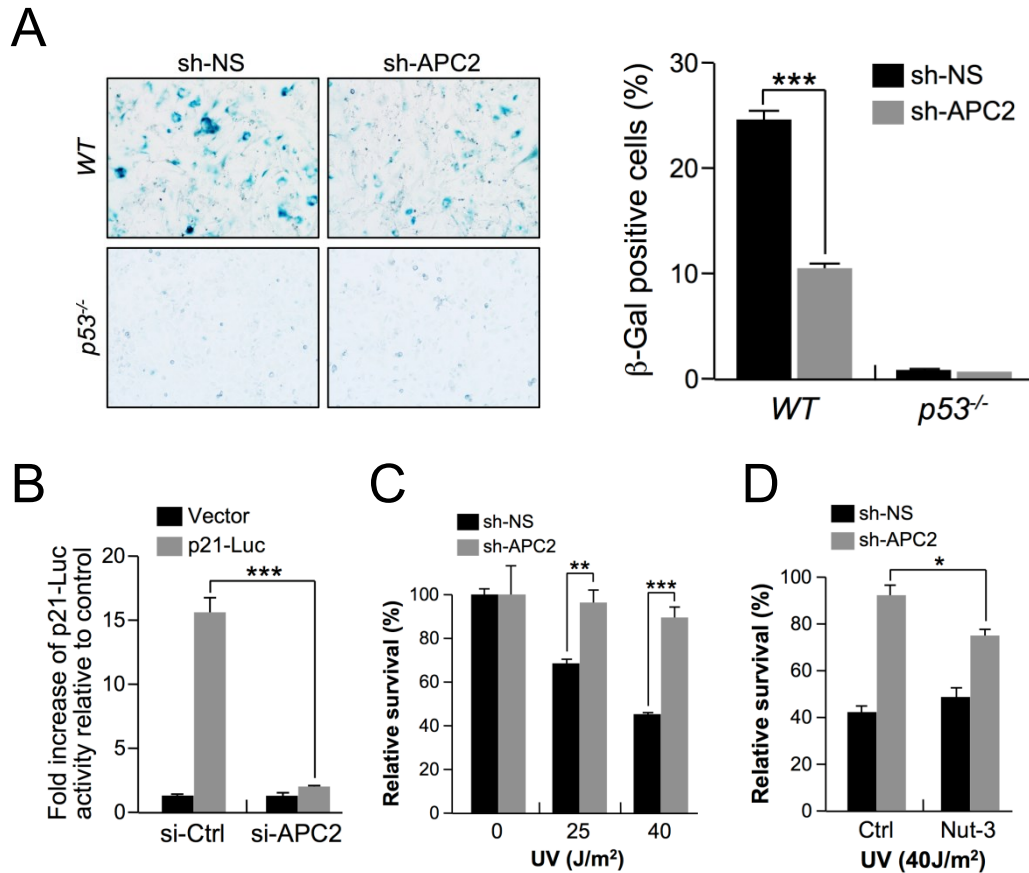


Figure 2-12. APC2 knock down leads to reduced cell death and senescence.

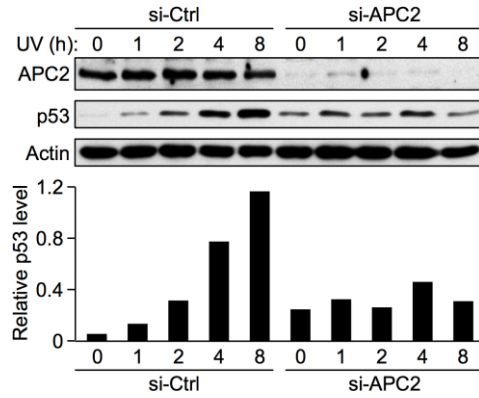
A. WT MEFs infected with non-specific (sh-NS) or APC2 (sh-APC2) Lentiviral based shRNA were cultured for 12 passages. Cells were stained with beta-gal and the percentage of beta-gal positive cells was quantified and plotted.

B. U2OS cells were transiently transfected with control siRNA (si-Ctrl) or APC2 siRNA (si-APC2) for 24 hours. Cells were split, followed by transfection with a pGL3-Basic luciferase reporter plasmid containing the wild-type p21 promoter (p21-Luc) or an empty vector (Vector). Cells were collected 24 hours later and assayed for luciferase activity.

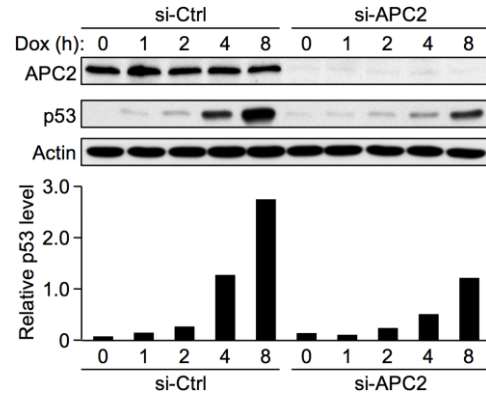
C. WT MEFs infected with non-specific (sh-NS) or APC2 (sh-APC2) Lentiviral based shRNA were treated with different dosages of UV as indicated. Cells were collected 24 hours after UV treatment, stained with trypan blue, and counted to determine cell survival.

D. WT MEFs infected with non-specific (sh-NS) or APC2 (sh-APC2) Lentiviral based shRNA were treated with 40J/m² UV and 8uM Nutlin-3. Cells were collected 24 hours after treatment, stained with trypan blue, and counted to determine cell survival.

A



B



C

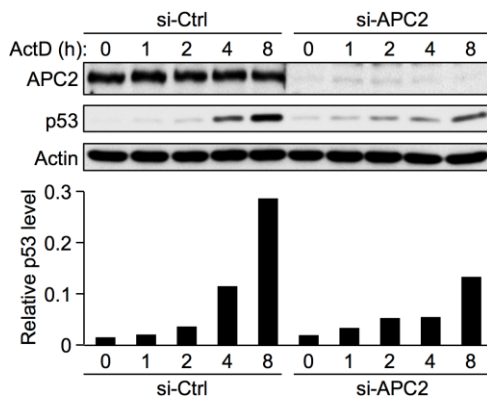


Figure 2-13. APC2 knock down leads to attenuated p53 accumulation.

A. U2OS cells were transfected with control siRNA (si-Ctrl) or APC2 siRNA (si-APC2) for 24 hours, cells were split, and 15J/m² UV was added 48 hours later. Cells were collected at the indicated time points after UV treatment, and analyzed for Mdm2 and p53 by western blot. Actin was used as a loading control. Bottom: p53 levels were measured by densitometry, normalized to actin, and plotted.

B. U2OS cells were treated and analyzed as described in (E) except treatment is 0.5ug/mL doxorubicin.

C. U2OS cells were treated and analyzed as described in (E) except treatment is 5nM Act D.

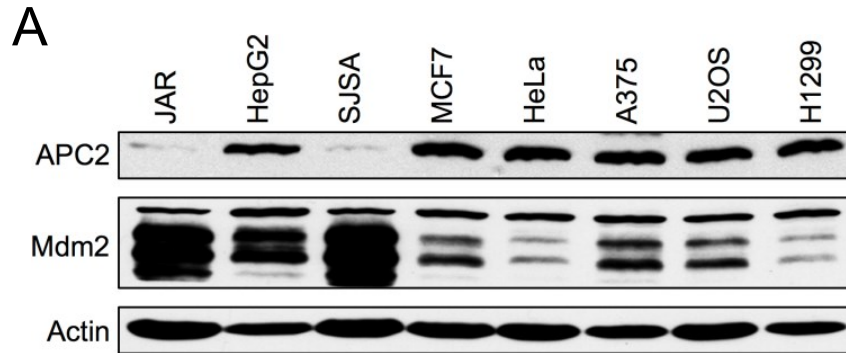


Figure 2-14. APC2 is down regulated in cancer cell lines expressing high level of Mdm2.

A. Western blot was used to assess APC2 and Mdm2 in JAR, HepG2, SJSA, MCF7, HeLa, A375, U2OS and H1299 cancer cell lines. Actin was used as a loading control.

A

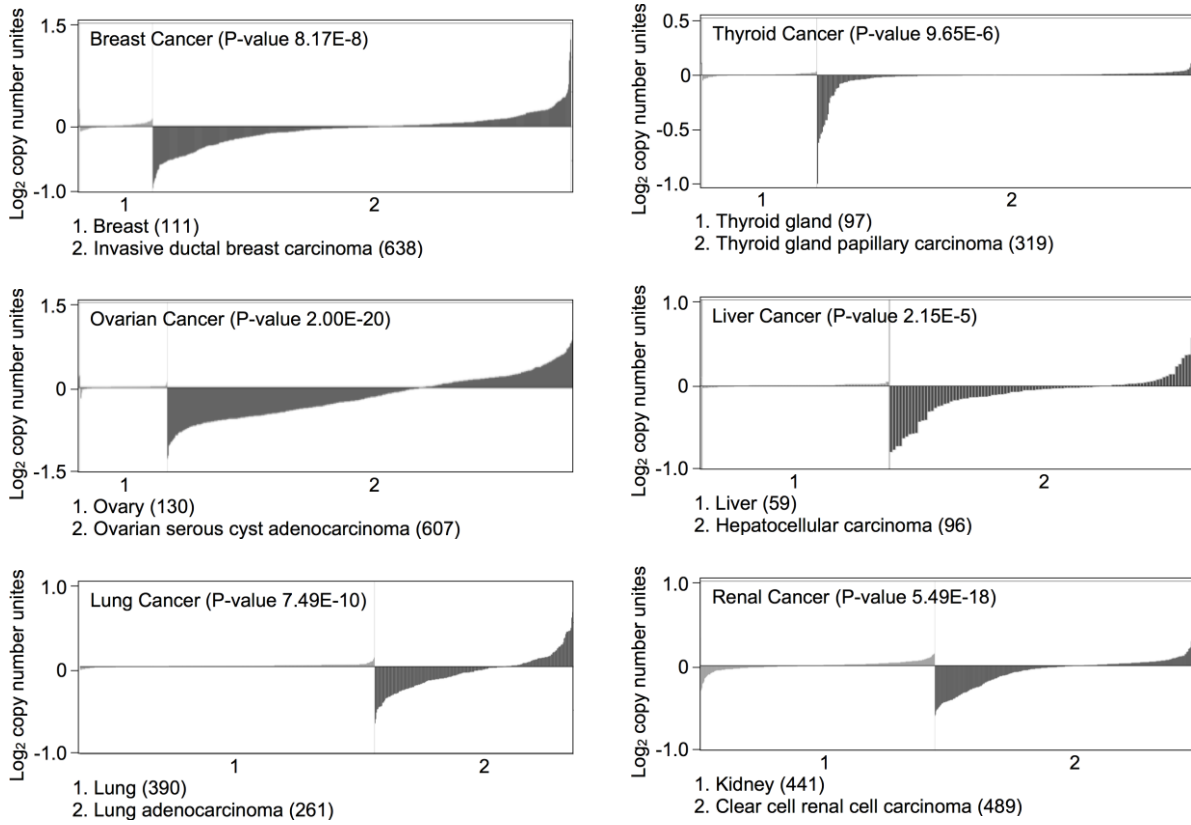


Figure 2-15. APC2 gene copy is lost in multiple cancers.

A. TCGA Gene copy number data for APC2 in breast, ovarian, lung, thyroid, liver, and renal cancer. Data was obtained from Oncomine.

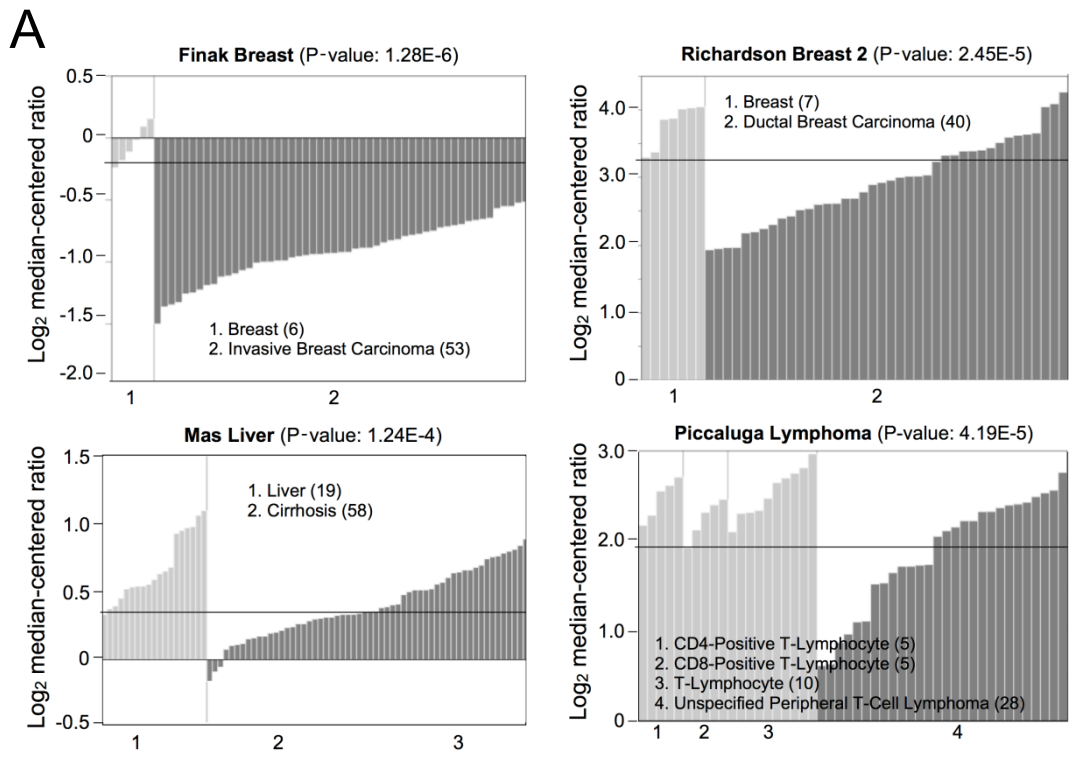


Figure 2-16. APC2 is under-expressed in multiple cancers.

A. mRNA microarray data for APC2 in Invasive breast carcinoma (Finak Breast), liver cancer with cirrhosis (Mas Liver), ductal breast carcinoma (Richardson Breast 2), and unspecified peripheral T-cell lymphoma (Piccaluga Lymphoma). Data was obtained from Oncomine.

CHAPTER 3. IDENTIFICATION PFK2 AS P53 SUPPRESSION TARGET GENES

INTRODUCTION

The tumor suppressor p53 is a transcription factor that is activated in response to a wide variety of stresses including ribosomal stress, oncogene activation, DNA damage, nutrient stress, ribonucleotide depletion, and many others (Levine, Hu et al. 2006). In response to these stresses, p53 transcriptionally promotes and/or represses the transcription of specific subsets of genes to facilitate DNA damage repair, cell cycle arrest, senescence, apoptosis, and/or metabolic regulation. Recent studies using mouse models have suggested that the p53-mediated upregulation of genes that promote cell cycle arrest, senescence, and apoptosis is dispensable for its tumor suppression activity (Li, Kon et al. 2012; Valente, Gray et al. 2013), which implies that other functions of p53 such as its role in metabolic regulation and DNA damage repair could be key factors in p53-mediated tumor suppression. p53-deficient mice raised in a controlled environment without obvious source of DNA damage develop tumors as early as three months of age (Donehower, Harvey et al. 1992). Furthermore, although the Warburg effect suggests a correlation between cancer and glucose metabolism deregulation, a comprehensive understanding of metabolic alterations that contribute to cancer development remains unclear.

Recent studies have suggested that nucleotide shortage during DNA replication leads to replication stress and DNA damage (Bester, Roniger et al. 2011; Beck, Nahse-Kumpf et al. 2012). Replication stress and the resulting genomic instability are prominent driving forces of early-stage cancer development, and importantly, an exogenous supply of nucleosides can relieve nucleotide shortage and rescue premature replication-induced DNA damage effectively

preventing cell transformation. These results illustrate the importance of the nucleotide supply for the suppression of spontaneous DNA damage and for the maintenance of genomic integrity. Interestingly, p53 is activated in response to nucleotide shortage (Linke, Clarkin et al. 1996), but how p53 alters the transcriptional landscape to generate more nucleotides and relieve nucleotide shortage-induced DNA damage remains poorly understood.

De novo synthesis of nucleotides *in vivo* from glucose occurs through the pentose phosphate pathway (PPP) (Tozzi, Camici et al. 2006). The PPP generates ribose-5-phosphate and NADPH, both of which are required for the *de novo* synthesis of nucleotides. Interestingly, the PPP is required for DNA damage repair (Cosentino, Grieco et al. 2011), although how PPP activity contributes to DNA damage repair remains unclear. It is clear, however, that DNA damage indeed elicits a cellular decrease in the dNTP pool (Das, Benditt et al. 1983; Newman and Miller 1983; Newman and Miller 1983; Suzuki, Miyaki et al. 1983; Chabes, Georgieva et al. 2003; Niida, Shimada et al. 2010). Previous studies have also shown that p53 induces p53R2 to promote synthesis of dNTPs from ribonucleotides (Niida, Shimada et al. 2010). Because the cellular ribonucleotide concentration is significantly larger than the dNTP pool, whether the cell requires an increase in the generation of ribonucleotides through the PPP upon DNA damage remains unclear.

Although the role for p53 in the activation of the PPP is not known, several studies have shown that p53 can regulate glucose metabolism by inhibiting glycolysis (Schwartzberg-Bar-Yoseph, Armoni et al. 2004; Kondoh, Leonart et al. 2005; Bensaad, Tsuruta et al. 2006; Matoba, Kang et al. 2006). The glycolysis pathway competes with the PPP for glucose consumption, and inhibition of glycolysis leads to the accumulation of intracellular G6P and a concomitant increase in flux through the PPP (Boada, Roig et al. 2000; Perez, Roig et al. 2000; Bensaad, Tsuruta et al. 2006). Therefore, p53-mediated suppression of glycolysis presumably ensures that a larger portion of the glucose supply is funneled through the PPP. Although multiple enzymes involved

in metabolic regulation are p53 transcription target genes, the upstream stress signals that activate p53 to trigger these metabolic regulations have not been thoroughly characterized.

To better understand how the role of p53 in metabolic regulation relates to its tumor suppression activity, we mined a previously described microarray dataset (Deisenroth, Itahana et al. 2011) and identified PFK2 as a potential p53 suppression target gene. PFK2 has been described as a master regulator of glycolysis that is commonly overexpressed in cancer cells (Hamilton, Callaghan et al. 1997) (Chesney, Mitchell et al. 1999) (Atsumi, Chesney et al. 2002), suggesting that PFK2 may play an important role in the p53-mediated coordination of DNA damage repair and nucleotide production.

RESULTS

Identification of *PFK2* as a p53 transcriptional suppression target

To identify novel p53 transcriptional targets, we analyzed a microarray dataset from a unique knockout/knockin mouse system developed in our lab, which has been previously described (Deisenroth, Itahana et al. 2011). In contrast with other systems that activate p53 by treating cells with genotoxic stress, which causes DNA damage, our system induces p53 activation by knocking out *Mdm2*, the primary negative regulator of p53. Many if not all stress signals that activate p53 pass through MDM2 or are accompanied by MDM2 inhibition. By knocking out *Mdm2*, p53 can be relatively specifically activated without generally stressing the cells. Although *Mdm2* knock out leads to embryonic lethality, *Mdm2* knockout mice can be rescued by concomitant inactivation of p53. Therefore, our *Mdm2* knockout mice were engineered to harbor a single *p53ER* allele, which has been previously described (Martins, Brown-Swigart et al. 2006), and no wild-type p53. The *p53ER* allele encodes WT p53, to which the membrane target domain of estrogen receptor is fused at the c-terminus. p53ER protein is inactive under basal conditions, which permits the normal development of *Mdm2* knockout mice. However, p53ER protein can be activated upon addition of 4-hydroxytamoxifen (4-OHT), which binds to the ER moiety and permits the transcription of p53 target genes. Using the p53ER system, microarrays probing differences in the expression of p53 target genes were assessed for WT MDM2, MDM2 null, and the MDM2 E3-dead point mutant C464A (Christophorou, Martin-Zanca et al. 2005).

After analyzing the microarray data by SAM (significance analysis of microarrays), we identified 919 genes whose expression were significantly altered with a 1.45% false discovery rate. One gene that was significantly downregulated was *PFKFB3* (*PFK2*), which we found particularly interesting based on the strong association with PFK2 overexpression in cancer cells and the fact that PFK2 controls a rate limit step of glycolysis.

To verify that *Pfk2* mRNA is suppressed upon p53 activation, qRT-PCR was conducted using the *p53^{ER/-}* MEF system. To rule out the possibility of transcriptional suppression induced by the estrogen receptor and/or 4-OHT, we included *p53^{-/-}* MEFs as a negative control. Induction of p53 activity by 4-OHT treatment resulted in the reduction of *Pfk2* mRNA expression in *p53^{ER/-}* MEFs but induced only a slight change in *p53^{-/-}* MEFs (Figure 3-1A). Concomitant *Mdm2* knock out in *p53^{ER/-}* MEFs caused a further decrease in *Pfk2* mRNA expression upon p53 activation by 4-OHT. These results suggest that p53 activation leads to the suppression of *Pfk2* transcription.

To further confirm that PFK2 suppression is observed at the protein level, we analyzed PFK2 protein expression by western blot using *p53^{ER/-}* MEFs expressing WT or mutant *Mdm2*. WT MEFs express significantly less PFK2 protein than *p53^{-/-}* MEFs under normal culture conditions (Figure 3-1B). PFK2 protein expression is not significantly affected by 4-OHT treatment in WT or *p53^{-/-}* MEFs. Restoration of p53ER activity by 4-OHT resulted in a reduction in PFK2 protein expression yet caused no detectable change in PFK2 expression in WT and *p53^{-/-}* MEFs. Further activation of p53 by *Mdm2* knockout leads to stronger suppression of PFK2 at the protein level. Activation of p53 by the *Mdm2* inhibitor Nutlin-3 in MEFs also resulted in decreased PFK2 protein expression in a p53-dependent manner (Figure 3-2A). Activation of p53 by UV in HCT116 cells (human colon cancer cell line) also led to decreased expression of PFK2 protein in a p53-dependent manner (Figure 3-2B). Our data also showed a decrease in PFK2 mRNA and protein expression in response to UV irradiation in WT MEFs but not in *p53^{-/-}* MEFs, suggesting that p53-mediated PFK2 suppression occurs in normal cell lines as well as cancer cell lines (Figure 3-2C and 3-2D).

To determine whether p53 directly suppresses *PFK2* transcription, a chromatin immunoprecipitation (ChIP) assay was performed in U2OS cells to assess the ability of p53 to bind directly to *PFK2* promoter regions. We observed no p53 binding to potential p53 consensus response elements upstream of the *PFK2* translation start site. However, we observed that p53

bound to a predicted p53 response element located between the first and the second exons of the *PFK2* locus (Figure 3-3A). p53 consensus sequences are generally strong candidates for p53 binding and regulation if they consist of two p53 response elements (RRRCWWGYYY) separated by 0-2 bps. Based on computer analysis (p53mh and p53scan), we were unable to identify consecutive p53 response elements within the first intron that satisfied these criteria (Hoh, Jin et al. 2002; Smeenk, van Heeringen et al. 2008). However, we identified a cluster of four p53 response elements half sites separated by 6-13-bp spacers within the first intron of *PFK2*. This observation is consistent with previous findings that have suggested that p53 suppression target genes tend to have longer spacers between the response elements than p53-induced target genes (Riley, Sontag et al. 2008). Closer analysis revealed that the p53 response elements in question harbor an insertion or mismatch in the WW motif in the center of each response element (Figure 3-3B). A mismatch in the WW motif has been reported to convert a p53-inducible sequence to a p53 suppression sequence *in vitro* (Wang, Xiao et al. 2009). Altogether, these results suggest that p53 binds directly to the *PFK2* locus within the first intron to suppress its transcription.

To determine whether p53 binding to the first intron of *PFK2* leads to suppression of *PFK2* transcription, we cloned the p53 binding regions pulled down from the ChIP assay into a luciferase reporter vector (pGL3-promoter) expressing luciferase under the control of a CMV promoter without CMV enhancer. The luciferase assay was performed in H1299 cells (p53-null human non-small cell lung carcinoma cell line) and revealed that WT p53 coexpression leads to strong inhibition of luciferase transcription that is not observed in the absence of p53. Consistent with a direct p53-mediated transcriptional control mechanism, cotransfection with a p53 DNA binding mutant R273H resulted in no change in luciferase expression (Figure 3-3C). These results suggest that direct p53 binding to the first intron of *PFK2* suppresses *PFK2* transcription.

Nucleotide shortage leads to p53-dependent suppression of PFK2

To our surprise, metabolic stresses such as H₂O₂, actinomycin D, and glucose starvation, which are known to activate p53, did not suppress *PFK2* protein expression in WT MEFs. Furthermore, DNA damaging agents UV and 5-fluorouracil (5-FU) but not ionizing radiation, doxorubicin, or mitomycin C resulted in the suppression of *PFK2* transcription and protein expression in WT MEFs (Figure 3-4A). Because UV-induced DNA damage decreases the cellular pool of deoxynucleotide triphosphate (Newman and Miller 1983) and because 5-FU inhibits thymidine synthesis (Longley, Harkin et al. 2003), we hypothesized that p53 responds to nucleotide shortage by suppressing *Pfk2* transcription. Nucleotide shortage activates p53 (Linke, Clarkin et al. 1996), but the transcriptional changes elicited by p53 in response to nucleotide shortage remain unclear. Ionizing radiation-mediated activation of p53 results in the upregulation of p53R2, a ribonucleotide reductase responsible for the conversion of Nucleoside-diphosphate (NDP) to deoxynucleotide diphosphate (dNDP) to generate the deoxynucleotides necessary for DNA damage repair (Nakano, Balint et al. 2000; Tanaka, Arakawa et al. 2000). However, when the cellular NDP supply becomes depleted, the mechanisms through which the cell maintains the nucleotide pool remain unknown.

To test the hypothesis that p53 responds to nucleotide shortage by suppressing PFK2 expression, we first confirmed that 5-FU induces PFK2 suppression in WT MEFs but not in *p53*^{-/-} MEFs (Figure 3-5A). Next, we treated WT and *p53*^{-/-} MEFs with the pyrimidine synthesis inhibitor leflunomide. Consistent with our hypothesis, leflunomide treatment resulted in the suppression of PFK2 expression in WT MEFs but not in *p53*^{-/-} MEFs (Figure 3-5B). To further confirm that nucleotide shortage suppresses *Pfk2* expression, we treated WT MEFs and U2OS cells with UV and followed the UV treatment with or without supplementation of nucleosides. As shown in Figure 3-5C and Figure 3-5D, nucleoside supplementation reverses PFK2 suppression after UV treatment in both cell lines. These results suggest that p53 drives the cellular response to nucleotide shortage through the suppression of PFK2 expression.

PFK2 suppression leads to increased DNA damage repair and cell survival

Based on our observation that UV irradiation induces p53-dependent suppression of PFK2, we hypothesized that PFK2 suppression could result in faster resolution of DNA damage lesions and improved cell survival. To test this hypothesis, U2OS cells were transfected with PFK2 siRNA and were assayed for the expression of the DNA damage marker phospho-histone H2AX (gamma-H2AX, γ -H2AX, or g-H2AX) by western blot (Mah, El-Osta et al. 2010). Our results showed that PFK2 knockdown resulted in less overall γ -H2AX signal, which disappeared more rapidly, suggesting that PFK2 suppression promotes the resolution of DNA damage lesions (Figure 3-6A). U2OS cells stably infected with lentiviral particles harboring PFK2-specific shRNA constructs were also subjected to the DNA damage repair assay. Relative to cells infected with non-specific shRNA constructs, the percentage of γ -H2AX-positive cells (non-repaired cells) decreased faster when PFK2 was knocked down (Figure 3-6B). These results suggest that PFK2 suppression in response to UV-induced DNA damage facilitates DNA damage repair.

To evaluate the importance of PFK2 suppression in the p53-induced DNA damage repair response, we performed a DNA damage repair assay using p53^{ER/-} MEFs infected with lentiviral particles harboring *Pfk2*-specific or non-specific shRNA constructs. As expected, p53 inactivation results in an increase in the proportion of cells that fail to repair DNA lesions. Consistent with the importance of PFK2 in the nucleotide shortage response, PFK2 knockdown completely rescued the DNA damage repair deficiency caused by p53 inactivation (Figure 3-7A).

Because UV-induced DNA damage leads to PFK2 suppression in a p53-dependent manner, consequences of the DNA damage response when PFK2 expression cannot be suppressed by p53 was explored. p53^{ER/-} MEFs infected with lentiviral particles harboring a GFP construct or a PFK2-GFP fusion construct was subjected to a DNA damage repair assay. Because PFK2 cDNA expression in these cells is under control of the LTR promoter and lacks the p53 response element found in the first intron, the expression of PFK2 in these cells is not

affected by p53 activation. Restoration of p53 activity by 4-OHT treatment resulted in a decrease in the proportion of cells that failed to repair DNA damage lesions; however, this decrease is reversed when PFK2 expression is enforced (Figure 3-7B).

As a consequence of improved DNA damage repair, PFK2 knockdown led to increased cell survival after UV-induced DNA damage in U2OS cells (Figure 3-8A) and p53^{-/-} MEFs (Figure 3-8B). Conversely, overexpression of PFK2 by adenovirus led to a decrease in cell survival after UV treatment (Figure 3-8C). Altogether, these data indicate that p53-mediated suppression of PFK2 in response to DNA damage promotes DNA damage repair in order to maintain genomic integrity.

PFK2 suppression leads to decreased glycolytic flux and increased PPP activity

To better understand why UV in particular induces *Pfk2* suppression, the consequences of *Pfk2* regulation were explored. As expected, p53 activation by 4-OHT in p53^{ER/-} MEFs resulted in decreased intracellular fructose-2, 6-bisphosphate (F-2, 6-BP) levels (Figure 3-9A). PFK2 knockdown resulted in decreased extracellular lactate levels (Figure 3-9B). Upon UV-induced DNA damage, intracellular F-2,6-BP and extracellular lactate levels decreased in a p53-dependent manner (Figure 3-9C, 3-9D). These results suggest that p53-mediated suppression of PFK2 leads to decreased glycolytic flux.

PFK2 suppression leads to increased PPP activity and nucleotide production

Current evidence directly linking glycolysis regulation to the DNA damage response is scarce; however, the PPP is well known to be required for DNA damage repair (Cosentino, Grieco et al. 2011). Inhibition of glycolysis can lead to accumulation of intracellular glucose-6-phosphate, which can lead to the activation of the PPP (Boada, Roig et al. 2000; Perez, Roig et al. 2000; Bensaad, Tsuruta et al. 2006). Therefore, we determined whether PFK2 suppression could lead to increased PPP activity.

NADPH is a major product of the PPP, as elevated PPP activity results in an increase in cellular NADPH levels and a concomitant decrease in cellular NADP⁺. Therefore, PPP activity can be assayed by measuring the cellular NADPH/NADP⁺ ratio. Consistent with our suspicions, suppression of PFK2 by lentivirus-mediated shRNA knockdown resulted in an increase in the NADPH/NADP⁺ ratio (Figure 3-10A), suggesting that PFK2 suppression correlates with an increase in PPP activity.

A precursor of nucleotides, ribose-5-phosphate is another major product of the PPP. Consistent with the increase in NADPH production, suppression of *Pfk2* by shRNA knockdown results in an increase (~20%) in cellular nucleotides in p53^{-/-} MEFs (Figure 3-10B). Furthermore, overexpression of PFK2 results in a decrease (~20%) in cellular nucleotides in p53^{-/-} MEFs (Figure 3-10C). PFK2 knockdown or p53 activation by 4-OHT also leads to increased nucleotide production in p53^{ER/-} MEFs (Figure 3-11A). In cancer cell lines, *PFK2* knockdown by siRNA results in a 40% increase in cellular nucleotide levels, and UV-induced DNA damage results in an 80% increase in cellular nucleotide levels (Figure 3-11B). These results suggest that suppression of PFK2 in response to nucleotide shortage can promote nucleotide production.

PFK2 depletion-mediated increase in DNA damage repair rate and cell survival requires nucleotide production from the PPP

To test our hypothesis that PFK2-mediated acceleration of DNA damage repair and improvement in cell survival is due to PPP activity, we blocked the PPP by using the glucose-6-phosphate dehydrogenase inhibitor dehydroepiandrosterone (DHEA) (Dworkin, Gorman et al. 1986; Frolova, O'Neill et al. 2011). In the absence of DHEA, PFK2 knockdown resulted in more rapid DNA damage repair and an increase in cell survival (Figure 3-12A and 3-12B). However, upon inhibition of the PPP by DHEA treatment, PFK2 knockdown did not significantly affect DNA damage repair and cell survival. These results indicate that PFK2 suppression promotes DNA damage repair and cell survival in a manner that depends on PPP activity.

Because the PPP generates NADPH and ribose-5-phosphate, both of which have the potential to promote DNA damage repair and cell survival, we sought to determine whether either PPP product is dispensable for our observations. Ribose-5-phosphate is not cell permeable and thus cannot be used directly in rescue experiments. Therefore, instead of using ribose-5-phosphate, a nucleoside mixture containing adenosine, guanosine, cytidine, and uridine is often used to increase the nucleotide precursor pool, as exogenous nucleosides can be used to rescue phenotypes caused by PPP inhibition (Dworkin, Gorman et al. 1986; Yoshida, Honda et al. 2003; Frolova, O'Neill et al. 2011). We found that upon the addition of nucleosides, the DNA damage repair rate increased in a dose-dependent manner, whereas the addition of NADPH had little effect (Figure 3-13A) in U2OS cells following UV treatment.

Furthermore, nucleoside treatment increased the cell survival rate following UV-induced DNA damage in a dose-dependent manner (Figure 3-13B). These results suggest that the nucleotide supply (ribose more specifically) is a rate-limiting factor in the DNA damage response.

To determine whether the observed PFK2 suppression-mediated increase in the DNA damage repair rate is dependent on nucleotides generated by the PPP, U2OS cells infected with lentivirus expressing non-specific or PFK2-specific shRNA were assayed for DNA damage repair efficiency. As expected, nucleoside treatment increased DNA damage repair efficiency regardless of PFK2 status (Figure 3-14A). Consistently, nucleoside treatment effectively rescued the DNA damage repair deficiency caused by PFK2 overexpression (Figure 3-14B). Finally, DHEA-mediated PPP inhibition reversed the PFK2 suppression-mediated increase in DNA damage repair efficiency, and PPP inhibition was circumvented by nucleoside supplementation (Figure 3-14C). Based on these results, we conclude that nucleotide synthesis through the PPP contributes strongly to UV lesion repair. However, if sufficient nucleotide precursors are provided, then the inhibition of PFK2 is not necessary.

DISCUSSION

In this study, we report the identification of a novel p53 suppression target PFK2. In response to DNA damage-induced nucleotide shortage, p53 suppresses PFK2 expression resulting in the inhibition of glycolysis through the reduction of fructose-2,6-bisphosphate (F-2,6-BP) levels. Inhibition of glycolysis leads to accumulation of intracellular glucose-6-phosphate and culminates in the activation of the PPP (Boada, Roig et al. 2000; Perez, Roig et al. 2000; Bensaad, Tsuruta et al. 2006). Elevated PPP activity leads to increased production of ribose-5-phosphate (R5P) and nucleotide synthesis, which resolves nucleotide shortages and facilitates DNA damage repair. The primary role of PFK2 *in vivo* is to promote glycolysis and facilitate lipid synthesis (Duran, Navarro-Sabate et al. 2008; Huo, Guo et al. 2010). Our study suggests that p53 inhibits glycolysis to conserve energy and substrates for nucleotide synthesis when the nucleotide supply falls below a certain threshold as occurs during DNA damage repair in response to UV damage.

UV-induced DNA damage in skin cells due to excess sun exposure commonly leads to extensive cell death and causes redness, pain, edema, itching, peeling skin, and rash. UV-induced DNA damage from sun exposure also increases the risk of skin cancer (Gandini, Sera et al. 2005; Leiter and Garbe 2008). Our study suggests that the nucleotide supply is a rate-limiting factor for DNA damage repair in response to UV-induced DNA damage, which could be applied to everyday UV exposure. Our results also suggest that supplementation of nucleosides or suppression of glycolysis can facilitate DNA damage repair and reduce cell death in response to UV damage. Therefore, further investigation into the value of adding nucleosides and/or glucose supplements in sunscreens and in sunburn remedies could yield effective prophylactic treatments in the battle against skin cancer. Nucleosides and glucose are cheap, safe, and easily absorbed by the skin making their use easy and cost-effective.

A sufficient nucleotide supply in proliferating cells is also critical for proper cell division. Cell division in the absence of a sufficient nucleotide pool leads to replication stress-induced DNA damage (Austin, Armijo et al. 2012; Beck, Nahse-Kumpf et al. 2012). Furthermore, enforced cell proliferation in the presence of an insufficient pool of nucleotides leads to replication perturbation, DNA damage, and genome instability (Bester, Roniger et al. 2011). More importantly, an exogenous supply of nucleosides can counter replication stress, decrease replication-induced DNA damage, and prevent the transformation of cells expressing oncogenes. Our study suggests that p53 can respond to nucleotide shortage and can promote the PPP to generate more nucleotides, which relieves genome instability induced by oncogene overexpression.

The RB-E2F and MDM2-p53 pathways are disabled in most if not all human tumors. Tumorigenic viral strains including simian vacuolating virus 40 (SV40), adenovirus, and human papillomavirus (HPV) use large T antigen, E1A/E1B, and E6/E7, respectively, to inhibit the RB-E2F pathway and the Mdm2-p53 pathway simultaneously, suggesting that either of these two pathways may be sufficient for suppressing tumor development. Abnormal activation of E2F leads to premature initiation of DNA replication even in the presence of an insufficient pool of nucleotides. As mentioned above, nucleotide shortage during DNA replication can lead to replication stress, DNA damage, and genome instability, therefore, under these circumstances, the inhibition of the cell cycle through the activation of p53 becomes particularly important. Loss of p53 prevents activation of the nucleotide shortage response and increases genomic instability when E2F becomes activated. Previous studies have suggested that nucleotide shortage leads to the activation of p53, but how p53 alters the transcriptome of the cell to relieve nucleotide shortage is currently unknown. Our study demonstrates that p53 suppresses PFK2 expression in response to nucleotide shortage, which promotes nucleotide production by increasing glucose flux through the PPP. The resulting replenishment of the nucleotide supply removes the nucleotide shortage stress and allows DNA damage repair to continue.

Loss of p53 or over activated glycolysis prevents activation of the nucleotide shortage response and increases genomic instability. Inhibition of the RB-E2F pathway results in a nucleotide shortage, which in conjunction with loss of p53 results in constitutively activated glycolysis. In the context of previous reports, our data suggest that the simultaneous inhibition of the RB-E2F pathway and the p53 pathway may induce genomic instability, which could open the door to additional mutations accumulated by cancers.

Our data favor a model whereby metabolic regulation particularly concerning the production of nucleotides plays an important role in maintaining genomic integrity. Deregulated glucose metabolism weakens the foundation for genomic stability and sensitizes cells to spontaneous mutations. This idea is consistent with the Warburg effect, which describes the phenomenon whereby almost all tumor cells display a higher glycolysis rate than normal cells and show higher sensitivity to DNA damaging reagents or nucleotide synthesis inhibition. Our findings shed new light on the value of chemotherapeutic strategies that selectively target tumors expressing a dysfunctional p53 signaling axis often associated with increased glycolytic flux. Based on the widespread nature of alterations in the glycolytic pathway in tumors, we anticipate that drugs that specifically increase flux through the PPP may be effective as a general chemotherapy for a wide array of tumors.

MATERIALS AND METHODS

Chromatin immunoprecipitation assay

U2OS cells infected with adenovirus expressing p53 were subjected to chromatin immunoprecipitation (ChIP) assays according the manufacturer's instructions (EZ ChIP kit, Upstate Biotechnology).

Measurement of fructose-2, 6-bisphosphate levels

Fructose-2,6-bisphosphate levels were determined based on the activation of pyrophosphate-dependent PFK1 as previously described (Yalcin, Clem et al. 2009).

Measurement of cellular nucleotide levels

An AKTA (GE Healthcare Life Science #18-1900-26) FPLC was used to quantitate the Methanol-soluble extracts. Samples of 500uL were injected onto a Partisil 5 SAX anion-exchange column (4.6mm × 250mm; Whatman #4222-227). The nucleotides were separated with a gradient of 50% Buffer A (0.005M (NH₄)H₂PO₄ pH 3.8) and 50% Buffer B (0.25M (NH₄)H₂PO₄, 0.5M KCL pH 4.5) to 100% Buffer B for 30 min followed by an isocratic elution of 100% Buffer B for 15 min at a flow rate of 1.5ml/min. The column was allowed to re-equilibrate to initial conditions for 5 min prior to the next injection. The absorbance of the nucleotides was determined at 280nm.

Cell culture and reagents

U2OS cells were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, 100 µg/ml penicillin, and 100 µg/ml streptomycin. Cells were grown in a humidified 37°C incubator with 5% CO₂. Primary mouse embryonic fibroblast (MEF) cells were isolated on embryonic day 13.5 and were grown in a humidified 37°C incubator with 5% CO₂ and 3% O₂ to simulate endogenous oxygen concentration and prevent oxidative stress in DMEM supplied with 10% FBS and 100 µg/ml penicillin-streptomycin. 4-hydroxytamoxifen was

purchased from Sigma. Mammalian protein extraction reagent was purchased from ThermoFisher Scientific.

Plasmids and adenovirus

The AdEasy XL system (Stratagene) was used to generate adenovirus constructs according to the manufacturer's instructions. Briefly, the full-length PFK2 cDNA (Open Biosystems) was amplified by PCR, cloned into pShuttle-CMV, recombined with pADEASY-1 vector, and transfected into 293 QBT cells to generate adenovirus particles.

Microarray analysis

Total RNA was isolated from MEFs treated with 4-hydroxytamoxifen (4-OHT) for 12 and 24 h using the RNeasy kit (Qiagen). One microgram of total RNA was amplified and labeled using the low RNA input linear amplification kit (Agilent Technologies), and hybridization was performed at the University of North Carolina Genomics and Bioinformatics core facility using an Agilent 4 x 44 K mouse whole genome DNA microarray. Arrays were washed and scanned using an Agilent scanner (Agilent Technologies). Data were stored in the University of North Carolina Microarray Database. Genes that were significantly upregulated or downregulated were identified by using significance analysis of microarrays statistics.

Immunofluorescence assays

Cells grown in a monolayer were fixed with formaldehyde, permeabilized with 0.2% Triton X-100 (for endoplasmic reticulum staining), and stained with primary anti-phospho-H2A.X (S139) antibody. Cells were stained with goat anti-rabbit Rho secondary antibody (Jackson ImmunoResearch Laboratories). 4',6-diamidino-2-phenylindole (DAPI) was used for nuclear counterstaining. Immunostained cells were analyzed by using an Olympus IX-81 microscope fitted with a SPOT camera and software.

Antibodies

The following antibodies were purchased commercially: mouse anti-Mdm2 2A10 (University of North Carolina Tissue Culture and Molecular Biology Support Facility), mouse anti-actin (Neomarkers), mouse anti-p53 DO.1 (Neomarkers), mouse anti-mp53 NCL-p53-505 from Leica microsystem. Rabbit anti-PFK2 antibody (ProteinTech Group 13763-1-AP) and rabbit anti phospho-H2A.X (S139) #9718S were purchased from Cell Signaling. Rabbit anti-p21 (C-19) was generously provided by Dr. Yue Xiong (UNC).

DNA damage repair efficiency assay

Cells were treated with the caspase inhibitor QVD and various treatments for 30 minutes. UV treatment (30 J/m² for U2OS cells and 10 J/m² for MEF cells) was performed to induce DNA damage. Fresh medium containing QVD and the indicated treatment was added and incubated at 37°C. Cells were then fixed 0, 4, 20, and 48 hours later and stained for the DNA damage marker phosphorylated histone γ -H2AX (Mah, El-Osta et al. 2010) and DAPI. γ -H2AX-positive cells were counted and normalized with the total cell count as determined by DAPI staining.

Lentivirus-based shRNA and siRNA treatment

Lentivirus-based shRNA constructs were purchased from Open Biosystems for human PFK2 (TRCN0000007338 NM_004566 RHS3979-9576297, TRCN0000007340 NM_004566 RHS3979-9576299, TRCN0000007341 NM_004566 RHS3979-9576300, TRCN0000007342 NM_004566 RHS3979-9576301) and mouse PFK2 (TRCN0000025414 NM_133232 RMM3981-9592822, TRCN0000025415 NM_133232 RMM3981-9592823, TRCN0000025416 NM_133232 RMM3981-9592824, TRCN0000025417 NM_133232 RMM3981-9592825, TRCN0000025418 NM_133232 RMM3981-9592826).

Lentivirus-based shRNA cloned in the pLKO.1 vector were co-transfected into HEK293T cells along with the appropriate packaging vectors to produce infective virions.

siRNA constructs targeting human and mouse PFK2:

siPFK2a: GCTGACTCGCTACCTCAACTT
TTCGACTGAGCGATGGAGTTG

siPFK2b: TCTCCAGCCCGGATTACAATT
TTAGAGGTCGGGCCTAATGTT

FIGURES

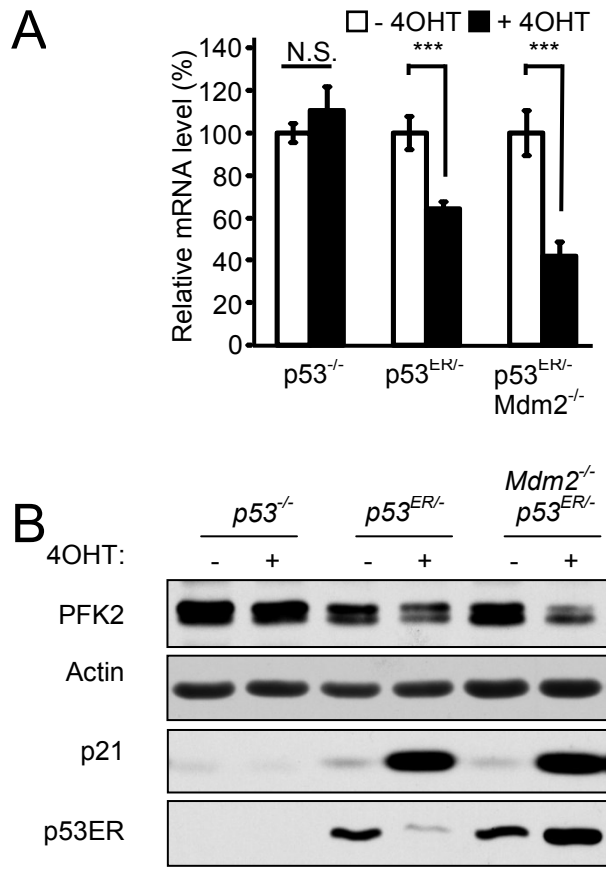


Figure 3-1. Cellular PFK2 mRNA and protein expression is suppressed upon p53 activation.

(A). $p53^{-/-}$ MEFs, $p53^{ER/-}$ MEFs and $p53^{ER/-} Mdm2^{-/-}$ MEFs were treated with or without 100 nM 4-OHT for 24 hours. PFK2 mRNA expression was assayed by qRT-PCR.

(B). WT MEFs, $p53^{-/-}$ MEFs, $p53^{ER/-}$ MEFs, and $p53^{ER/-} Mdm2^{-/-}$ MEFs were treated with or without 100 nM 4-OHT treatment for 24 hours. PFK2 protein expression was assayed by western blot.

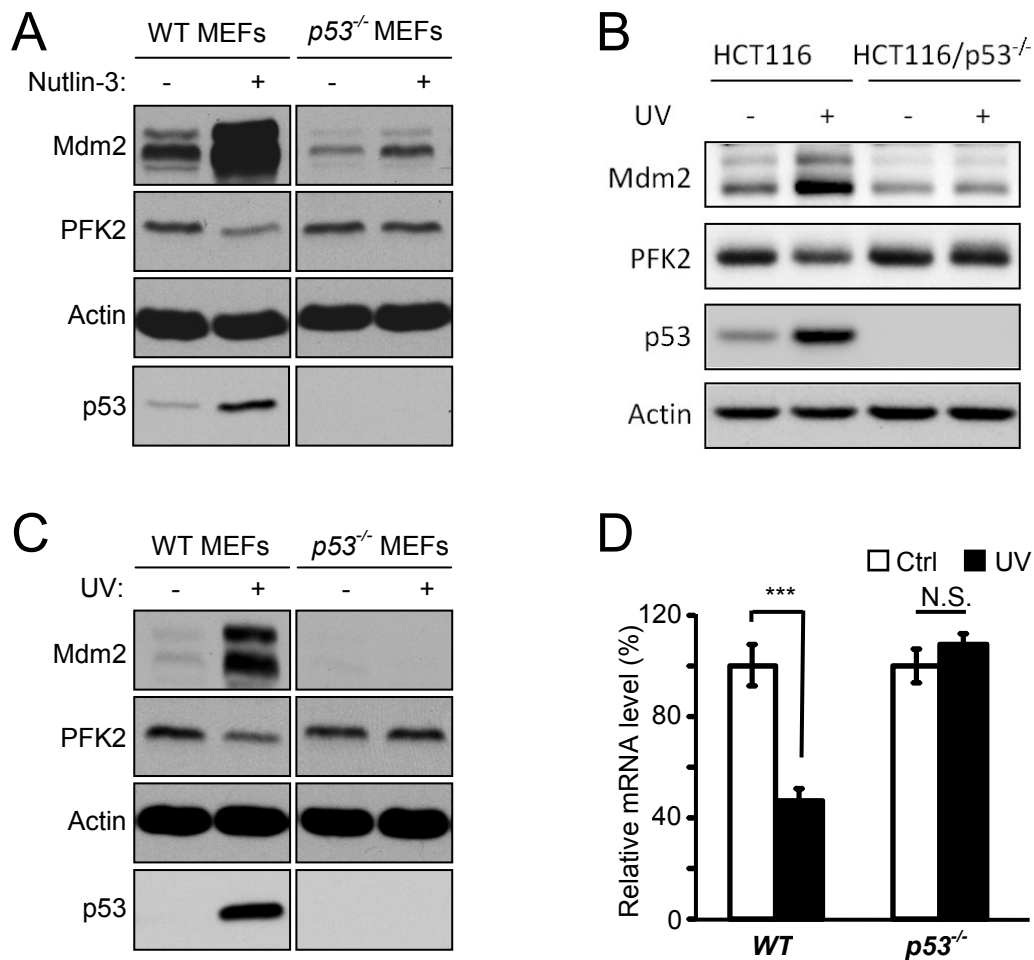


Figure 3-2. UV and Nutlin-3 induced p53 activation lead to PFK2 suppression in p53 dependent manner.

(A). *WT* and *p53*^{-/-} MEFs were treated with 10 μ M Nutlin-3 for 24 hours. PFK2 protein expression was assayed by western blot.

(B). HCT116 and HCT116/*p53*^{-/-} cells were treated with 40 J/m^2 of UV irradiation. PFK2 protein expression was assayed by Western blot 24 hours after treatment.

(C). *WT* and *p53*^{-/-} MEFs were treated with 40 J/m^2 of UV irradiation. PFK2 protein expression was assayed by western blot 24 hours after treatment.

(D). *WT* and *p53*^{-/-} MEFs were treated with 40 J/m^2 of UV irradiation. PFK2 mRNA expression was assayed by Q-PCR 24 hours after treatment.

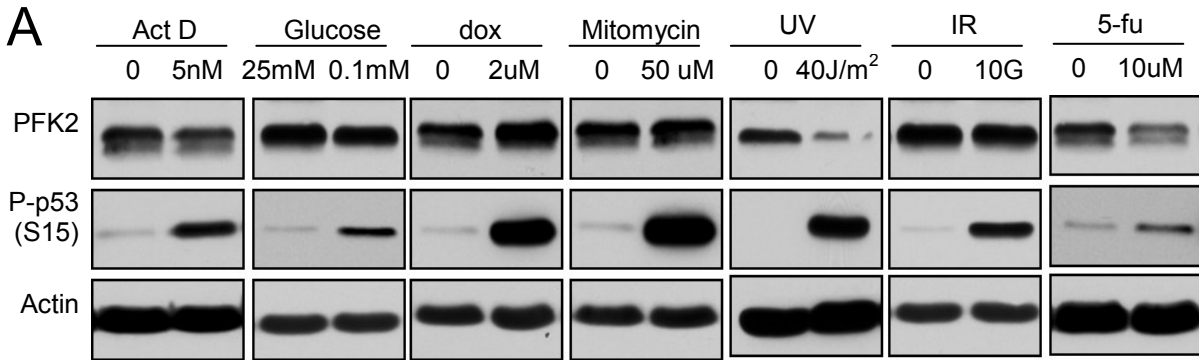


Figure 3-4. Not all stress activates p53 to lead to PFK2 suppression.

(A). *WT* MEFs were treated as indicated, PFK2 protein expression was assayed by western blot 24 hours after treatment.

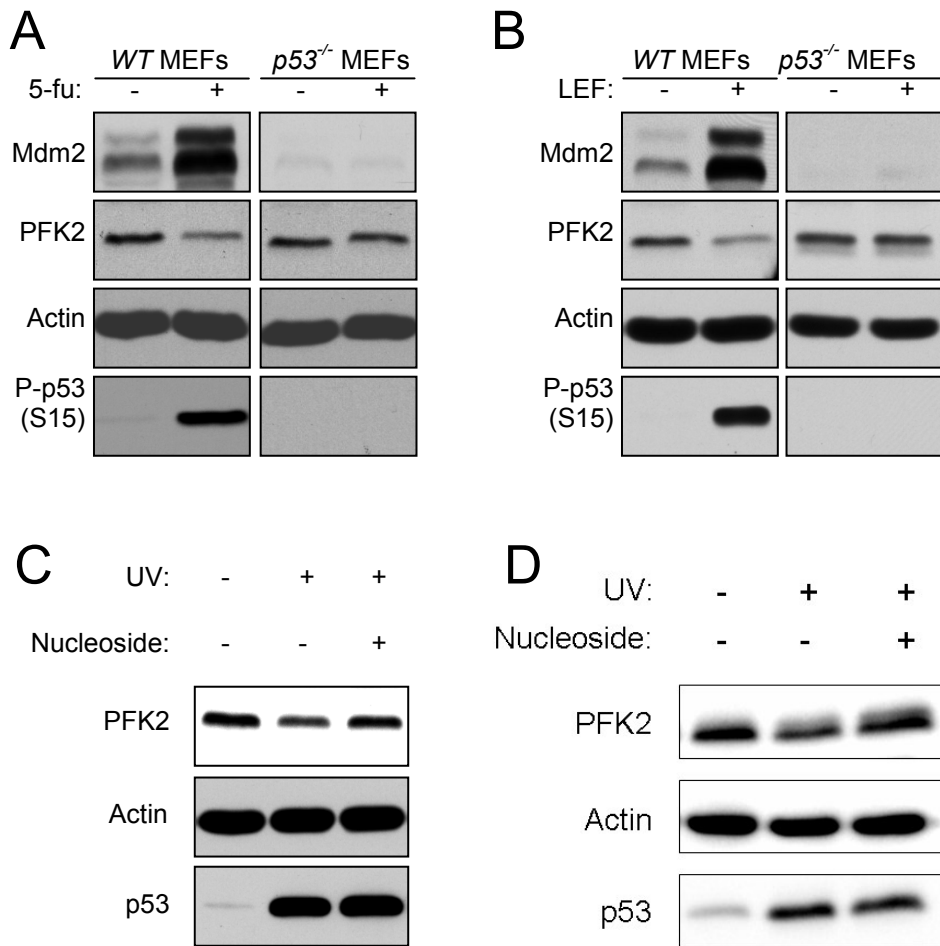


Figure 3-5. Nucleotide shortage activates p53 and suppresses PFK2 expression.

(A). *WT* and *p53*^{-/-} MEFs were treated with the pyrimidine synthesis inhibitor 5-fluorouracil (10 μ M). Cells were harvested 24 hours after treatment, and PFK2 protein expression was assayed by western blot.

(B). *WT* and *p53*^{-/-} MEFs were treated with the thymidine synthesis inhibitor leflunomide (25 μ M). Cells were harvested 24 hours after treatment, and PFK2 protein expression was assayed by western blot.

(C). *WT* MEFs were treated with 15J/m² UV and 0.2mM nucleoside as indicated, PFK2 protein expression was assayed by western blot 24 hours after treatment.

(D). U2OS cells were treated with 25J/m² UV and 0.2mM nucleoside as indicated, PFK2 protein expression was assayed by western blot 24 hours after treatment.

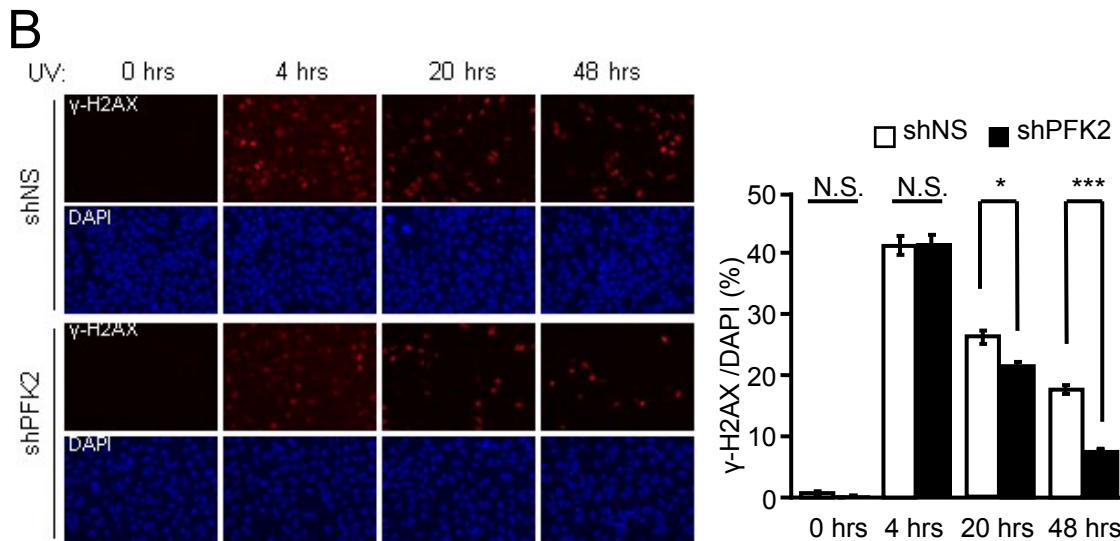
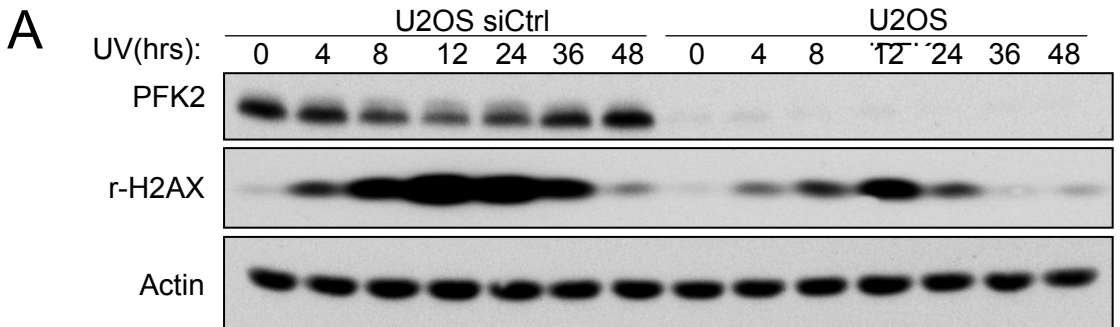


Figure 3-6. PFK2 suppression leads to increased DNA damage repair.

(A). U2OS cells were transfected with non-specific or *PFK2*-specific siRNA oligo. Cells were then UV irradiated (15 J/m^2) to induce DNA damage. Cells were collected at indicated time point and analysis by western blot.

(B). U2OS cells were infected with lentiviral particles expressing non-specific or *PFK2*-specific shRNA constructs to form stable cell lines. Cells were then treated with the caspase inhibitor QVD for 30 minutes, after which the cells were UV irradiated (25 J/m^2) to induce DNA damage. Fresh medium containing QVD was added to the cells, and the cells were incubated at 37°C . Cells were then fixed 0, 4, 20, and 48 hours after treatment, stained for γ -H2AX, and counterstained with DAPI.

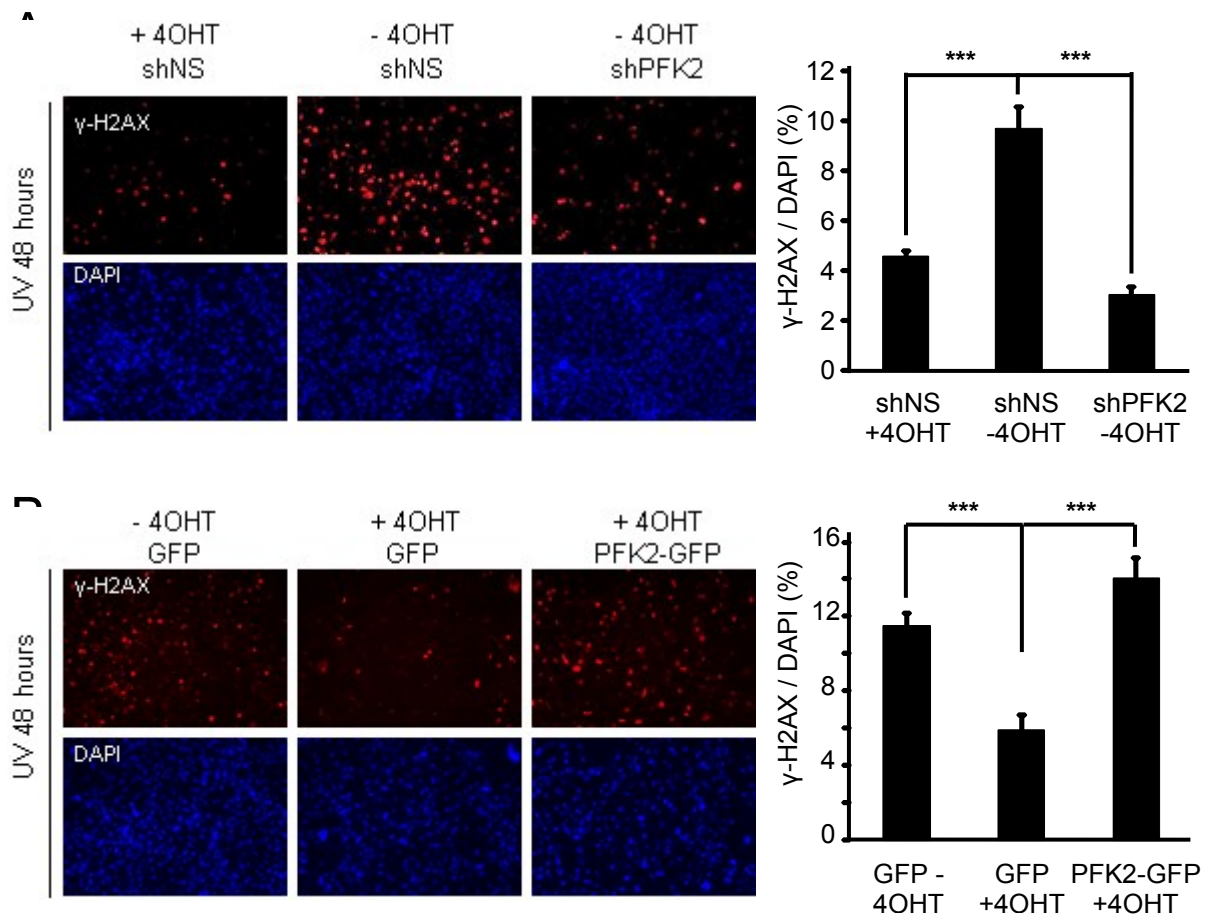


Figure 3-7. p53 promotion of DNA damage repair depends on its ability to suppress PFK2 expression.

(A). $p53^{ER/-}$ MEFs were infected with lentiviral particles expressing non-specific or *Pfk2*-specific shRNA constructs to produce stable cell lines. Cells were then treated with QVD for 30 minutes, after which the cells were treated with UV ($10\text{J}/\text{m}^2$) to induce DNA damage. Fresh medium containing QVD or QVD with 4-OHT was added after treatment, and the cells were incubated at 37°C for 48 hours. Cells were then fixed, stained for γ -H2AX, and counterstained with DAPI.

(B). $p53^{ER/-}$ MEFs were stably infected with lentiviral particles harboring *Pfk2*-GFP or GFP constructs. Then, cells were treated with QVD for 30 minutes, after which the cells were treated with UV ($10\text{J}/\text{m}^2$) to induce DNA damage. Fresh medium containing QVD or QVD with 4-OHT was added to the cells. The cells were incubated at 37°C for 48 hours. Cells were then fixed, stained for γ -H2AX, and counterstained with DAPI.

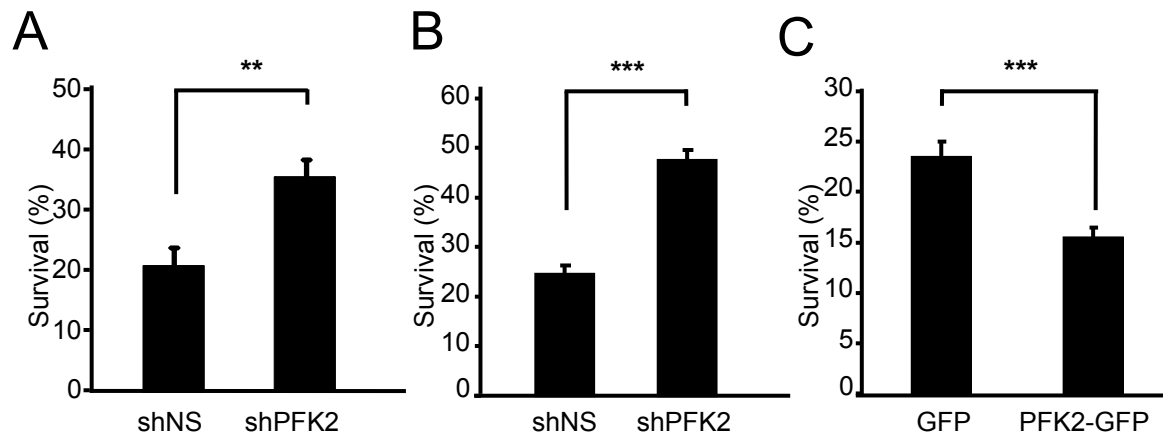


Figure 3-8. PFK2 suppression leads to increased cell survival.

(A). U2OS cells infected with lentiviral particles harboring non-specific or *PFK2*-specific shRNA constructs were irradiated with UV (40 J/cm²). Five high power magnification images of random fields were taken by microscope 24 hours after UV treatment. Surviving cells were counted manually on a computer screen using ImageJ software.

(B). *p53*^{-/-} MEFs were infected with lentiviral particles expressing non-specific or *Pfk2*-specific shRNA knockdown, after which the cells were treated with UV (40 J/cm²). Five high power magnification images of random fields were taken by microscope 24 hours after UV treatment. Surviving cells were counted manually on a computer screen using ImageJ software.

(C). U2OS cells overexpressing *PFK2* by adenoviral infection were treated with UV (40 J/cm²). Five high power magnification images of random fields were taken by microscope 24 hours after UV treatment. Surviving cells were counted manually using ImageJ software.

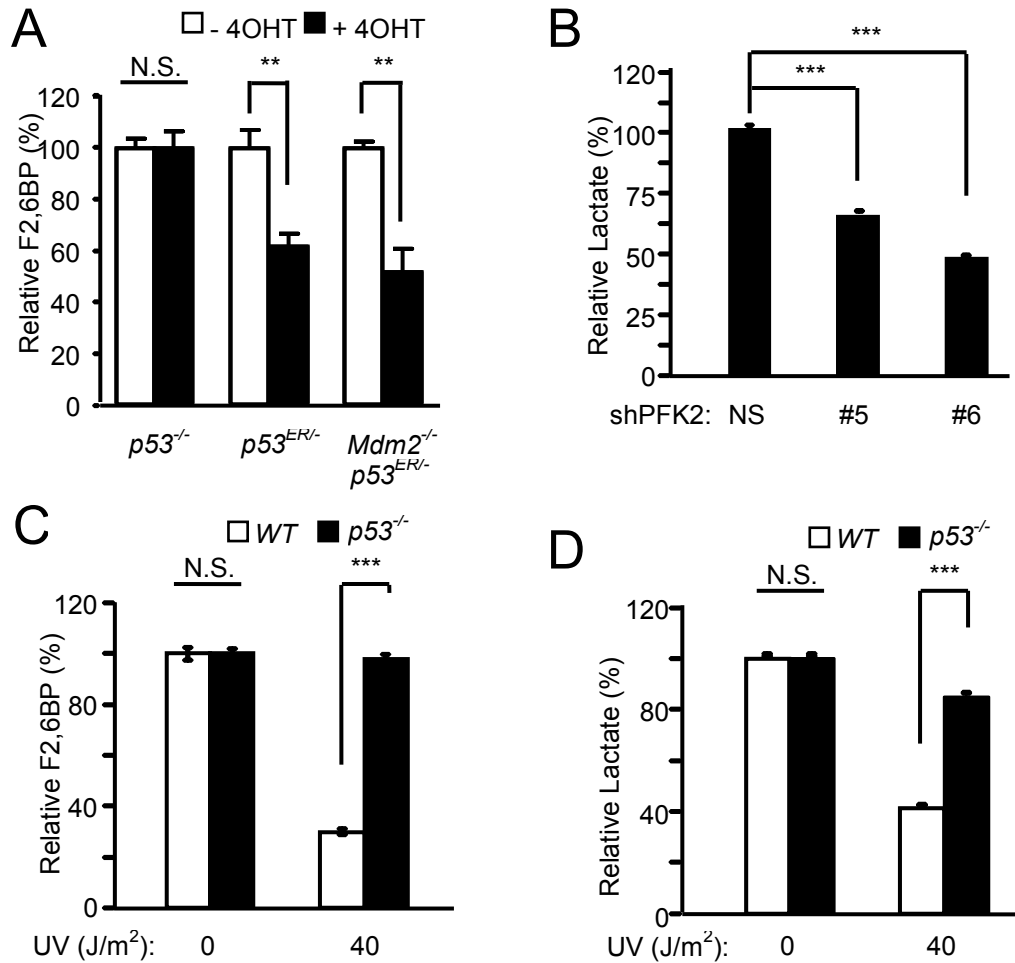


Figure 3-9. PFK2 suppression leads to decreased glycolytic flux.

(A). $p53^{-/-}$, $p53^{ER/-}$, and $p53^{ER/-} Mdm2^{-/-}$ MEFs were treated with or without 100 nM 4-OHT for 24 hours, after which intracellular F 2,6 BP levels were measured by using an F 2,6 BP detection kit.

(B). $p53^{-/-}$ MEFs were infected with lentivirus expressing control or *Pfk2* shRNA constructs. Culture media was collected, and lactate levels were measured by using a detection kit.

(C). WT and $p53^{-/-}$ MEFs were treated with UV irradiation, 24 hours after which intracellular F 2,6 BP levels were determined.

(D). WT and $p53^{-/-}$ MEFs were treated with UV irradiation, 24 hours after which the culture medium was collected and lactate levels were measured by using a lactate detection kit.

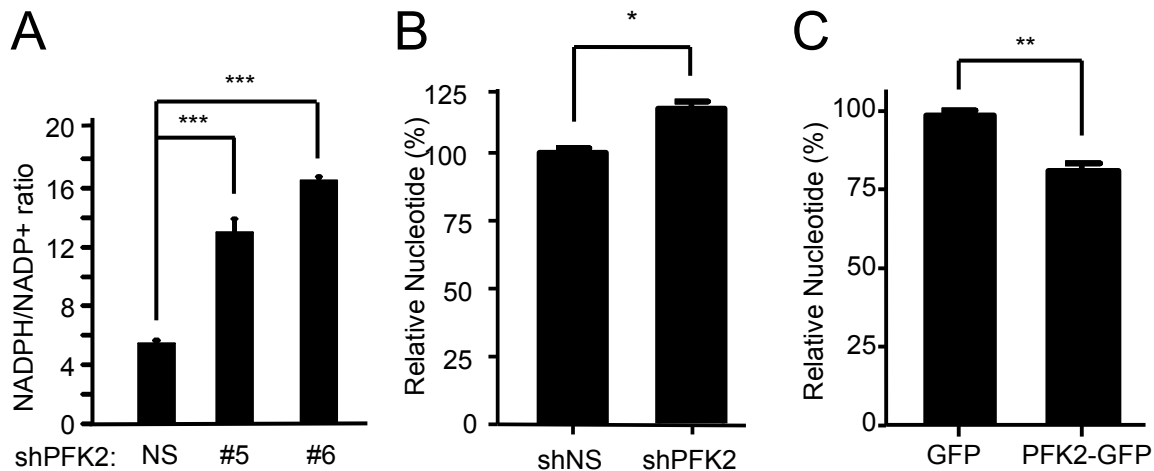


Figure 3-10. PFK2 suppression leads to decreased glycolytic flux and increased PPP activity.

(A). $p53^{-/-}$ MEFs were infected with lentiviral particles expressing control or *Pfk2*-specific shRNA constructs, after which intracellular NADPH and NADP⁺ levels were measured by using an NADPH/NADP⁺ detection kit.

(B). $p53^{-/-}$ MEFs were infected with lentiviral particles expressing control or *Pfk2*-specific shRNA constructs. Intracellular nucleotide levels were measured by HPLC.

(C). $p53^{-/-}$ MEFs were infected with lentiviral particles expressing GFP or GFP tagged PFK2. Intracellular nucleotide level were measured by HPLC

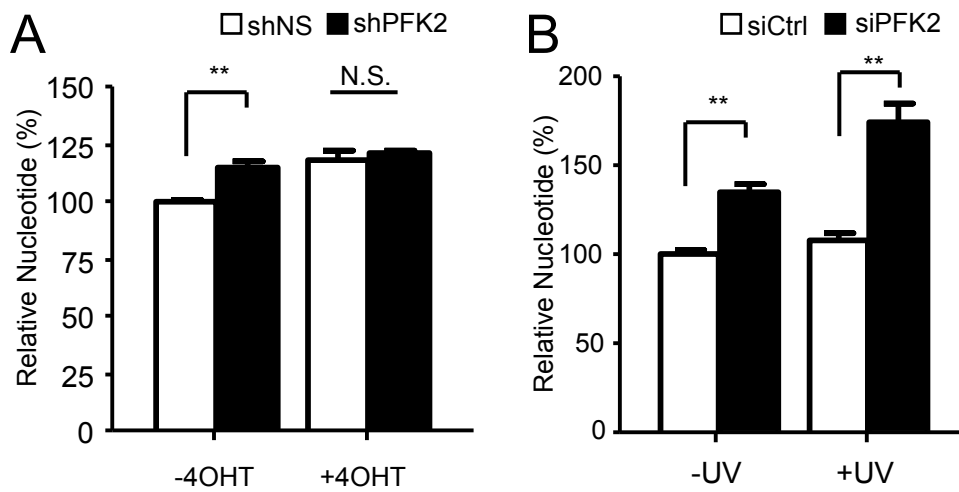


Figure 3-11. PFK2 suppression leads to increased nucleotide production.

(A) $p53^{ER-}$ MEFs were infected with lentiviral particles expressing control or *Pfk2*-specific shRNA constructs. Cells were treated with or without 4-OHT for 24 hours. Intracellular nucleotide levels were measured by HPLC.

(B) *U2OS* cells were transfected with control or *Pfk2*-specific siRNA oligos. Cells were treated with or without 25J/m^2 UV. Intracellular nucleotide levels were measured by HPLC 24 hours later.

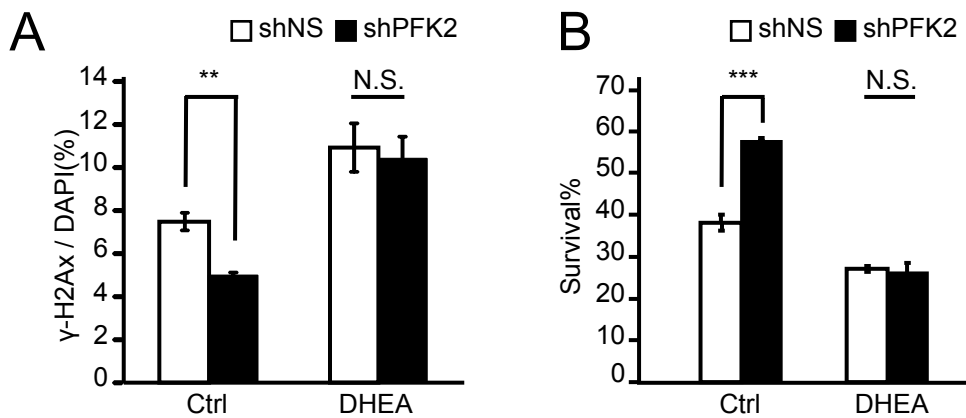


Figure 3-12. PFK2 suppression promotes DNA damage repair and cell survival in a PPP-dependent manner.

(A). U2OS cells infected with lentiviral particles harboring non-specific or *PFK2* specific shRNA constructs were treated with QVD with or without 0.25 mM DHEA for 30 minutes. Cells then received UV treatment (40 J/m^2), 48 hours after which the cells were fixed and stained for γ -H2AX and DAPI.

(B). U2OS cells stably expressing non-specific or *PFK2*-specific lentiviral shRNA constructs were treated with or without 0.25 mM DHEA for 30 minutes. Cells were then treated with UV (40 J/m^2), 24 hours after which the surviving cells were counted.

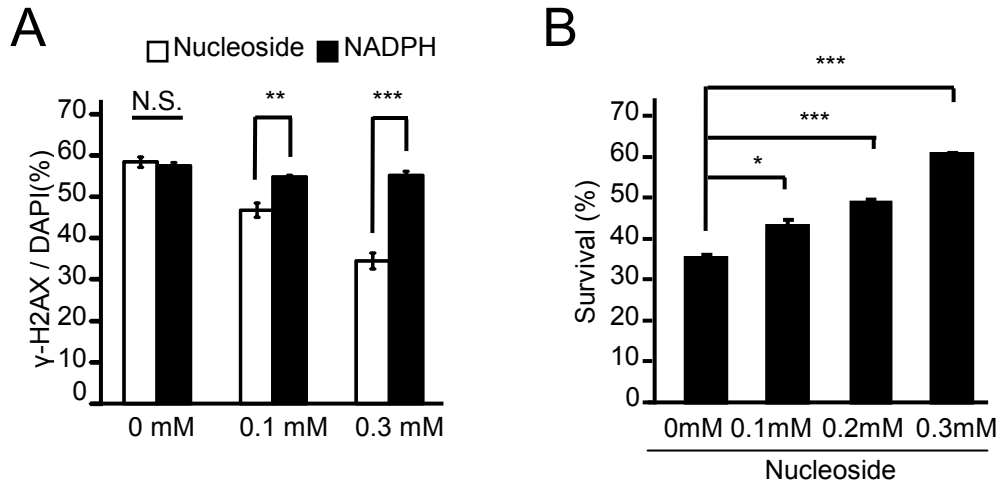


Figure 3-13. Nucleotide supply is a rate limiting factor for UV induced DNA damage repair and cell survival.

(A). U2OS cells were treated with QVD and an equimolar nucleoside mixture (0 mM, 0.1 mM, or 0.3 mM) or NADPH for 30 minutes. Then, cells were treated with UV (25 J/m²), 24 hours after which the cells were stained for γ -H2AX and counterstained with DAPI.

(B). U2OS cells were treated with a 0 mM, 0.1 mM, or 0.3 mM equimolar nucleoside mixture for 30 minutes, after which the cells were treated with UV (40 J/m²). Surviving cells were counted 24 hours after UV treatment.

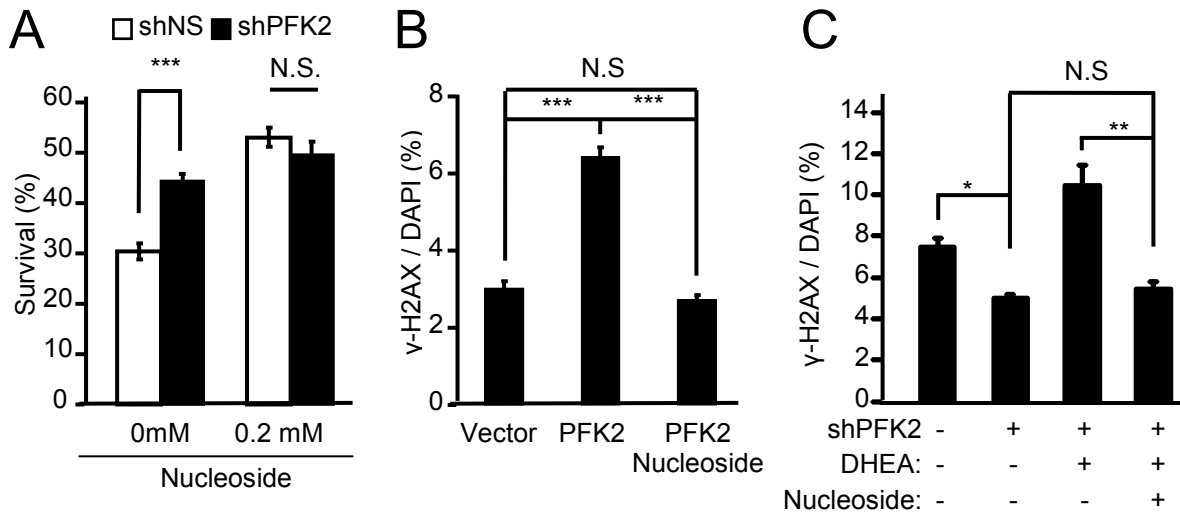


Figure 3-14. PFK2 suppression promotes DNA damage repair and cell survival in a nucleotide-dependent manner.

(A). U2OS cells stably expressing non-specific or *PFK2*-specific lentiviral shRNA constructs were treated with or without a 0.2 mM equimolar nucleoside mixture for 30 minutes. After nucleoside treatment, cells were exposed to UV (40 J/m^2), 24 hours after which the surviving cells were counted.

(B). U2OS cells stably expressing *GFP* or *PFK2-GFP* were treated with QVD with or without a 0.2 mM equimolar nucleoside mixture for 30 minutes. Cells were treated with UV (25 J/m^2), 48 hours after which the cells were fixed and stained for γ -H2AX and DAPI.

(C). U2OS cells stably expressing non-specific or *PFK2*-specific lentiviral shRNA constructs were treated with QVD with or without a 0.2 mM equimolar nucleoside mixture or with or without 0.25 mM DHEA for 30 minutes. Cells were treated with UV (25 J/m^2), 48 hours after which the cells were fixed and stained for γ -H2AX and DAPI.

CHAPTER 4. SUMMARY AND FUTURE DIRECTIONS

Mdm2 protein level is cell cycle regulated by APC/C

We have demonstrated that the APC/C E3 ubiquitin ligase complex promotes Mdm2 polyubiquitination *in vitro* and *in vivo*, and that knockdown of APC2 results in p53 independent accumulation of Mdm2 protein. These results suggest that APC/C E3 ubiquitin ligase activity contributes to Mdm2 polyubiquitination and degradation in the absence of stress.

Although the Mdm2-p53 feedback loop is well established, our data suggests that Mdm2 accumulation induced by APC2 knockdown does not necessarily lead to decreased p53 protein levels under unstressed conditions. Instead, we see a decrease in p53 accumulation in APC2 knockdown cells only after stress. Through Mdm2, knockdown of APC2 results in inhibition of p53 activation, which leads to the reduction of stress induced cell death and senescence. In light of our research, we hypothesize that the ability of Mdm2 to inhibit p53 transcriptional activity via direct binding is the primary mechanism through which Mdm2 functions to regulate p53 in the absence of stress, and it is only after stress that Mdm2 E3 ubiquitin ligase activity regulates p53 stability. This hypothesis is consistent with the observation that *Mdm2*^{Y487A/Y487A} mice survive under basal conditions, but unlike WT mice, are unable to recover from low dose IR induced DNA damage. APC2 knockdown also recapitulates human cancers with Mdm2 overexpression; in these cancers high levels of Mdm2 bind and inhibit p53 activity, rather than leading to decreased p53 protein levels.

Observations made in our study directly lead to an interesting question: why does the mitotic Cullin APC2 control Mdm2 degradation? Clonal GFP-Mdm2 stable cell lines showed uneven expression of Mdm2, which suggests that Mdm2 protein degradation is different at

different phases of the cell cycle. Serum starvation and double thymidine block experiments clearly show that Mdm2 protein levels increase at S-G2, and as suggested by nocodazole treatment (Figure 4-1A) and nocodazole synchronization, Mdm2 is degraded before the anaphase transition (Inuzuka, Tseng et al. 2010). These results suggest that Mdm2 might play an important role in G2 or mitosis. *Mdm2^{-/-};p53^{-/-}* double knockout mice develop normally, which suggests that the role of Mdm2 in the cell cycle is not essential for embryogenesis and development in the mouse. In light of the embryonic lethality observed in *Mdm2^{-/-}* mice, Mdm2 may have some kind of checkpoint function, likely involved in p53 regulation (Jones, Roe et al. 1995; Montes de Oca Luna, Wagner et al. 1995).

In G2/M, chromosome condensation occurs in the cell, which results in the generation of many double strand breaks by topoisomerase (Champoux 2001). During chromosome condensation, Mdm2 may function to temporarily inhibit p53 activation; following completion of chromosome condensation, Mdm2-mediated inhibition of p53 is released to allow for p53 checking for incompletely re-ligated double strand breaks. In light of the data presented here, this release of p53 inhibition is likely achieved by degradation of Mdm2 by APC/C. To test the hypothesis that Mdm2 accumulation during S phase and G2 phases of the cell cycle functions to inhibit premature activation of p53 by normal chromosome condensation and induced double strand breaks, we can knock down Mdm2 in cells with wild type p53 that are arrested at G1/S, or with chromosome condensation inhibited. If our hypothesis that Mdm2 levels increase in S phase and G2 phase to inhibit p53 is correct, then inhibition of p53 by Mdm2 during G1 phase is less important than in S phase or G2 phase. Therefore, we expect to see cells arrested at G1/S or with chromosome condensation inhibited, to demonstrate reduced p53 activation compared to untreated cells after Mdm2 knockdown. Replication of chromosomal DNA also involves chromosome de-condensation and the induction of strand breakage by topoisomerase activity; it is possible that Mdm2 protein levels elevate in S phase to inhibit p53 and prevent the normal native DNA strand breaks that occur during DNA replication to prematurely activate p53. If this

is the case, only cells arrested in G1/S will demonstrate reduced p53 activation upon Mdm2 knock down. Another possibility is that inhibition of chromosome condensation may only produce an intermediate reduction in p53 activation.

In summary, our study provides an alternative explanation to the Mdm2-p53 negative feedback model. We hypothesize that the feedback between Mdm2 and p53 results in oscillation of Mdm2 and p53 protein levels during the cell cycle, rather than the constant maintenance of these proteins at low levels. In our model, p53 protein levels increase as the cell cycle progresses, increased p53 protein then functions to transcriptionally activate *Mdm2*, resulting in the generation of more Mdm2 protein. Once a sufficient level of Mdm2 is produced, it binds and inhibits p53 transcriptional activity to prevent overproduction of Mdm2. However, our model predicts that elevated Mdm2 protein levels will not lead to p53 degradation in the absence of stress, but will instead allow for high levels of p53 accumulation, which remains inactive until the cell reaches a DNA damage checkpoint. Our theory is that p53 only senses DNA damage at specific checkpoints of the cell cycle, providing a potential explanation for why DNA damage and DNA damage induced phosphorylation happens in less than 30 minutes, but p53 accumulation and activation can take 4-8 hours. Although p53 phosphorylation and stabilization occurs quickly, additional time is necessary for unsynchronized cell populations to progress through the cell cycle and reach a DNA damage checkpoint, at which point p53 senses this damage and becomes active if DNA damage is detected. We predict that synchronized cells will demonstrate faster DNA damage induced p53 activation in S and G2, while slower or disrupted p53 activation will occur when cells are arrested in G1; furthermore, this theory can be easily tested by experimentation.

APC/C may mediate the tetraploidy checkpoint response to Mdm2-p53

Our study also provides a possible explanation for how p53 senses tetraploidy and mediates tetraploid G1 arrest. The oscillation of Mdm2 levels following release from double

thymidine block or nocodazole synchronization indicates that Mdm2 is likely degraded prior cyclin B1 during mitosis (Inuzuka, Tseng et al. 2010). More specifically, Mdm2 is observed at low levels after nocodazole treatment, suggesting that Mdm2 degradation likely occurs prior, or during, nocodazole-induced pro-metaphase arrest. Also, as shown in Figure 4-1A, nocodazole and Colcemid treatment leads to a decrease of Mdm2 protein levels in a proteasome dependent manner, suggesting that Mdm2 is degraded before the anaphase transition. Although reagent availability prevented us from expressly showing that CDC20 can activate APC2 mediated Mdm2 polyubiquitination, the degradation of Mdm2 before the anaphase transition suggests that it is due to APC2-CDC20, similar to the mechanism of degradation for p21, Cyclin A and NEK2A. Further experimentation to determine the binding affinity of CDC20 with Mdm2, and whether this interaction is required for Mdm2 degradation during pro-Metaphase, can provide a better understanding of the regulation of Mdm2 stability.

When cells escape the spindle checkpoint following prolonged nocodazole treatment, resulting in tetraploid cells (also referred to as mitotic catastrophe), the absence of proper mitotic exit may cause maintained APC/C activity towards Mdm2. I hypothesize that while condensed chromosome confirmation in prometaphase prevents extremely low Mdm2 protein levels from resulting in increased p53 activity in normal diploid cells, tetraploid cells generated by mitotic catastrophe progress through mitosis; if Mdm2 levels are not sufficient, the decondensation of chromosomes in tetraploid cells as they enter G1 results in uninhibited p53 activity. Tetraploid cells generated by cell fusion or inhibition of cytokinesis have normal mitotic exit and therefore will not activate p53 by degradation of Mdm2. We hypothesize that p53 cannot sense tetraploid chromosomes, and instead, p53 senses incomplete mitosis through APC/C mediated Mdm2 degradation, and in this manner arrests mitotic tetraploid cells generated in catastrophe in tetraploid G1 through transcriptional activation of p21. This hypothesis can be tested through treatment of cells wild type for p53 (such as U2OS) with nocodazole and the APC/C inhibitor proTAME (Lara-Gonzalez and Taylor 2012). We expect to

see nocodazole treated p53 wild type cells enter tetraploid S phase after mitotic catastrophe when treated together with proTAME, and arrest at tetraploid G1 without proTAME treatment. In the case that these expected results are observed, it will suggest that p53 senses catastrophe through APC/C mediated Mdm2 degradation. Mitotic catastrophe cells do not go through the regular mitotic exit process, and thus retain prometaphase APC/C activity. Uninhibited prometaphase APC/C will degrade Mdm2 in tetraploid G1 and lead to p53 activation. If we do not observe the expected results, it may be due to interference from off target effects of proTAME, or that other substrates and regulatory mechanism are involved, requiring adjustment of our original hypothesis. Detailed analysis of the degradation dynamics of APC/C substrates at specific stages of mitosis, following mitotic catastrophe, may provide additional insight into the role of APC/C activity during tetraploid G1. We expect post mitotic catastrophe cells to have high levels of substrates normally degraded during mitotic exit. Mdm2 stable cell lines may also be a useful tool to demonstrate the effect of Mdm2 level on p53 activation in mitotic catastrophe; we expect high levels of Mdm2 to prevent proper p53 activation upon mitotic catastrophe, and with that, induction of tetraploid G1 arrest. This can be tested by treating GFP, GFP-Mdm2, GFP-Mdm2C464A stable cell lines with nocodazole and assaying the ability of cells to enter tetraploid S by BRDU staining, coupled with FACS analysis.

Mdm2 may promote Metaphase/Anaphase transition

Despite the fact that Mdm2 is a well-established oncogene, the mechanism through which ectopic expression of Mdm2 leads to cell death is not currently understood. In our effort to establish stable cell lines overexpressing Mdm2, we observed that cells expressing high and medium levels of Mdm2 protein die off or fail to proliferate, and only cells expressing extremely low levels of Mdm2 survive and proliferate normally. Close examination of U2OS cells with ectopically expressed GFP-MDM2, revealed that many cells expressing a high level of GFP-Mdm2 demonstrate irregularly shaped nuclei (Figure 4-2A); expression of another GFP-tagged

nuclear protein, GFP-PFK2, did not lead to a change in nucleus shape. Furthermore, preliminary results show that cells expressing high levels of GFP-Mdm2 demonstrate a unique grape shaped nucleus fragmentation phenotype after prolonged nocodazole treatment (Figure 4-2B). Nocodazole treated cells usually demonstrate a fragmented nucleus, loosely organized around a central mono-polar centrosome. However, nocodazole treatment of GFP-Mdm2 transfected cells results in a fragmented nucleus, without empty space in the central of fragmented micronucleus. This difference in chromosome location relative to the centrosome implies that overexpression of Mdm2 causes cells to drop out of mitosis which results in differences in phase or timing.

On the other hand, Mdm2 knockdown leads to accumulation of G2/M phase cells when assayed by FACS analysis (Figure 4-3A). In addition, Mdm2 knockdown leads to an increase in cells with a rounded up morphology; further analysis by (Figure 4-3B) immunofluorescent staining suggests that these rounded up cells are mitotic (Figure 4-4A). Quantification of phase specific mitotic cells based on chromosome origin demonstrates a specific increase of the prometaphase population of cells. These results indicate that Mdm2 knockdown leads to a delay in the progression from prometaphase to metaphase, indicating a potential role for Mdm2 in promoting metaphase transition. Therefore, overexpression of Mdm2 or the failure to properly degrade Mdm2 may lead to premature exit from prometaphase before proper spindle attachment on the kinetochore occurs. On the other hand, Mdm2 loss could also lead to prolonged prometaphase and increased mitotic catastrophe. Increased mitotic catastrophe often leads to increased tetraploidy in p53 null cells; preliminary data suggests that the additional loss of Mdm2 leads to increased aneuploidy, as shown in Figure 4-5A (Galipeau, Cowan et al. 1996).

To further confirm that Mdm2 plays a role in promoting prometaphase progression, time lapse microscopy should be used to carefully examine the timing of cell rounding during mitosis in cells with Mdm2 knocked down or overexpressed. To visualize the dynamics of mitotic

chromosome organization, GFP or RFP tagged histone H2A stable cell lines can be utilized to visualize mitotic chromosomes. In response to nocodazole treatment, we expect cells overexpressing Mdm2 to spend a shorter time in prometaphase and reduced time arrested in mitosis. On the other hand, we expect cells with Mdm2 knocked down to spend a longer amount of time in prometaphase, leading to increased mitotic catastrophe.

Another interesting question to explore is whether Mdm2 E3 ubiquitin ligase activity is responsible for its role in promoting the prometaphase/metaphase transition. As overexpression of Mdm2 E3 dead mutants, including Mdm2^{C462A} and Mdm2^{Y487A}, is better tolerated in stable cell lines when compared with wild type Mdm2, we expect that using this system the loss of Mdm2 E3 ligase function will result in the loss of its activity in promoting prometaphase exit both with and without nocodazole treatment. If Mdm2 E3 dead mutants retain the ability to promote prometaphase exit, the use of Mdm2 deletion mutants, each containing a different domain of Mdm2, can be used to further identify the specific domain of Mdm2 that is responsible for this activity.

The Mdm2 and telomere binding protein (MTBP) is known to interact with the Mdm2 acidic domain (Boyd, Vlatkovic et al. 2000). MTBP suppresses cell migration and filopodia formation by inhibiting ACTN4 (Agarwal, Adhikari et al. 2013), and more importantly, MTBP overexpression results in delayed mitotic progression at metaphase (Agarwal, Tochigi et al. 2011). These studies suggest that Mdm2 might contribute to mitotic progression at metaphase through controlling MTBP stability. We can test whether MTBP stability is regulated by Mdm2 by assessing MTBP protein levels in wild type, p53^{-/-}, and Mdm2^{-/-};p53^{-/-} MEFs. To determine if MTBP is degraded during the prometaphase/metaphase transition, cell cycle synchronization and nocodazole/taxol treatments can be utilized. To further explore the role of Mdm2 E3 ligase activity in MTBP degradation during mitosis, MTBP levels can be assessed in the Mdm2 E3 dead mutants Mdm2^{C462A} and Mdm2^{Y487A}. Finally, *in vitro* and *in vivo* ubiquitin ligase assays will provide additional information to demonstrate if Mdm2 can promote MTBP ubiquitination.

Implications of APC/C mediated Mdm2 degradation in cancer diagnosis and treatment

In many cancer cell lines, low levels of APC2 correspond with high levels of Mdm2, pointing to a biologically and disease relevant connection between APC2 and Mdm2. As further demonstration of the biological implications of APC2, we also observed that low levels of APC2 correspond to attenuated p53 accumulation and activation following stress, resulting in reduced cell death and senescence. Together, these results suggest that under-expression of APC/C can contribute to Mdm2 accumulation and cancer development. APC/C is known as a mitotic Cullin, and in this role its function is necessary for cells to pass through the mitotic spindle checkpoint and proliferate. Although reduced APC/C activity results in a longer period of time in mitosis, cells maintain the ability to proliferate. Reduced APC/C activity will also lead to accumulation of Mdm2, which may result in inhibition of p53, and subsequently tumorigenesis. Since the APC/C complex contains more than twelve subunits, it is likely that not all subunits affect Mdm2 degradation in the same manner; analyzing the effect of siRNA knock down of each subunit of the APC/C complex on Mdm2 and p53 protein levels before and after DNA damage induced stress will provide useful insight into which subunit(s) may correlate with Mdm2 overexpression in cancers. Another avenue to explore is the potential role of kinases, phosphatases, and their regulators in APC/C activity, and with that, in the regulation of Mdm2 stability and Mdm2 overexpression in cancers.

Mitotic inhibitors that arrest the cell cycle in mitosis by perturbing microtubule function and activating spindle assembly checkpoints, such as taxanes and vinca alkaloids, represent one of the most important classes of cancer drugs, and are widely used in the treatment of breast, ovarian, and lung cancer (Montero, Fossella et al. 2005). Since the spindle assembly checkpoint does not suppress APC/C activity completely, inhibitors directly targeting APC/C could provide a better therapeutic method for inducing mitotic arrest (Zeng, Sigoillot et al. 2010). Unfortunately, while APC/C inhibitors are promising in theory, the APC/C inhibitor Emi1 is highly

expressed in hepatocellular carcinoma (Zhao, Tang et al. 2013), ovarian clear cell carcinoma (Gutgemann, Lehman et al. 2008; Min, Park et al. 2013), and breast carcinoma (Liu, Wang et al. 2013). Further, our study suggests that inhibition of APC/C can result in oncogenic consequences due to the accumulation of Mdm2, and the resulting inactivation of p53. Therefore, although APC/C inhibition has therapeutic potential due to its ability to induce mitotic arrest, the effect of APC/C inhibition on p53 activity and stability hinders the prospects of pharmacologic inhibition of APC/C as viable cancer drug.

Mdm2 overexpression often correlates with wild type p53 in cancer; excessive Mdm2 functions to silence p53, resulting in a lack of selection pressure to mutate p53. Mdm2 antagonists such as Nutlin-3 are proposed to treat tumor cells containing wild type p53. APC2 deficient cells, however, demonstrate higher levels of Mdm2 and therefore a higher dose of Mdm2 antagonists may be required to activate p53 in these cells. In light of the role APC/C plays in Mdm2 stability, evaluation of APC/C-Mdm2 status is an important step in determining the appropriate cancer treatment.

Microarray and gene sequencing are relatively easy and sensitive methods to screen for mutations in Mdm2 that may affect p53 stability; however, these methods are limited to the detection of Mdm2 overexpression due to gene amplification. Our study has identified the APC/C complex as an E3 ubiquitin ligase capable of regulating Mdm2 protein levels post-translationally, making the components of the APC/C complex potentially useful new targets to predict Mdm2 overexpression and p53 misregulation by microarray screening and gene sequencing.

Although our results suggest that underexpression of APC2 in cancer cell lines correlates with high levels of Mdm2 expression, and demonstrate that copy loss and underexpression of APC2 is common in human cancer, a direct correlation between Mdm2 overexpression in the absence of gene amplification and APC/C deficiency remains to be demonstrated. If APC/C complex component expression levels, or APC/C activity in cancers

that express high levels of Mdm2 in the absence of Mdm2 gene amplification, can be assessed through the combined evaluation of *Mdm2* gene copy and Mdm2 protein level by quantitative PCR, immunohistochemistry, and western blot, this will provide strong evidence to support the role of APC2 in this type of Mdm2 stabilization.

In response to nucleotide shortage, p53 suppresses glycolysis to promote nucleotide synthesis

In our study, we report the identification of a novel p53 suppression target, PFK2. In response to DNA damage-induced nucleotide shortage, p53 suppresses PFK2 expression, resulting in the inhibition of glycolysis through the reduction of fructose-2,6-bisphosphate (F-2,6-BP) levels. Inhibition of glycolysis leads to accumulation of intracellular glucose-6-phosphate and consequent activation of the PPP (Boada, Roig et al. 2000; Perez, Roig et al. 2000; Bensaad, Tsuruta et al. 2006). Elevated PPP activity leads to increased production of ribose-5-phosphate (R5P) and nucleotide synthesis, which resolves nucleotide shortages and facilitates DNA damage repair. The primary role of PFK2 *in vivo* is to promote glycolysis and facilitate lipid synthesis (Duran, Navarro-Sabate et al. 2008; Huo, Guo et al. 2010). Our study suggests that p53 inhibits glycolysis to conserve energy and substrates for nucleotide synthesis by reducing energy storage when the nucleotide supply falls below a certain threshold, as occurs during DNA damage repair in response to UV damage.

As cytoplasmic localization results in a transcriptionally inactivate form of p53, these p53 monomers can inactivate G6PD. p53 plays a role in the inhibition of PPP and nucleotide production under normal conditions, however upon activation, p53 forms tetramers, re-localizes to the nucleus, and loses its ability to inactivate G6PD (Jiang, Du et al. 2011). Furthermore, our study suggests that upon nucleotide shortage, p53 transcriptionally suppresses PFK2, temporarily promoting PPP to allow for enhanced nucleotide production. Together, these studies suggest that p53 regulates glucose metabolism to tightly control nucleotide production.

Although we showed the p53 response to nucleotide shortage results in suppression of PFK2, the specific molecular mechanism through which nucleotide shortage functions to activate p53 remains unclear. Disruption of Mdm2-p53 interaction by Mdm2 knockout and Nutlin-3 treatment can activate p53 and lead to PFK2 suppression, suggesting that Mdm2-p53 interaction may be a potential mechanism. Immuno-precipitation of cells treated with a nucleotide synthesis inhibitor and doxorubicin or 5 nM Act D can be used to test if Mdm2-p53 interaction is specifically disrupted in cells with nucleotide shortage. Alternatively, it is also possible that Mdm2 knockout and Nutlin-3 treatment can lead to nucleotide shortage in a mechanism independent of Mdm2-p53 interaction. To rule out this possibility, measurement of cellular nucleotide levels in cells with and without Mdm2 or Nutlin-3 treatment can be performed, using treatment with a nucleotide synthesis inhibitor as positive control.

It is also possible that post-translational modifications of Mdm2 or p53 specifically induced by nucleotide shortage contribute to the specificity of the p53 response. To identify changes in Mdm2 and p53 modification status, mass spec analysis of purified Mdm2 and p53 from cells treated with a nucleotide synthesis inhibitor, UV induced DNA damage, or Act D induced ribosomal stress should be performed. Common modifications found in cells treated with nucleotide synthesis inhibitor and UV induced DNA damage, but not in cells treated with Act D; mutagenesis and overexpression experiments should be utilized to further analyze the identified modifications as potentially contributing factors in connecting nucleotide shortage and the p53 pathway.

Potential use of nucleoside and glucose supplements in sunburn treatment

Excessive sun exposure results in UV-induced DNA damage in skin cells, commonly causing redness, pain, edema, itching, peeling skin, and rash, in addition to leading to extensive cell death and an increased risk of skin cancer (Gandini, Sera et al. 2005; Leiter and Garbe 2008). Our study suggests that nucleotide supply is a rate-limiting factor for DNA damage repair

in response to UV-induced DNA damage, which could be applied to everyday UV exposure. Our results also suggest that supplementation of nucleosides or suppression of glycolysis can facilitate DNA damage repair and reduce cell death in response to UV damage. Therefore, further investigation into the value of adding nucleosides and/or glucose supplements in sunscreens and sunburn remedies could yield effective prophylactic treatments in the battle against skin cancer. Nucleosides and glucose are natural metabolic intermediate products, and as such are inexpensive and safe, making their use easy and cost-effective. However, the ability of glucose to penetrate through the epidermal layer of the skin may pose a problem in the ease of use of topical treatments using glucose. Although some primary data suggests that glucose can penetrate monkey skin (Ghosn, Sudheendran et al. 2010), the ability of glucose and nucleosides to penetrate human epidermis after UV exposure remains unclear. Animal studies should be conducted to further evaluate the effectiveness of nucleoside and glucose supplementation in reducing cell death and promoting the healing of skin cells after UV exposure. Following UV treatment of nude mice, topical compounds with and without glucose and nucleosides should be used, and any areas of redness, rash, and/or peeling skin should be quantified and analyzed, along with the incidence of UV induced skin cancer development. The effect of insulin on absorption of glucose should also be evaluated. Treatment with other nucleotide analogs that are safe easily absorbed by the skin, and metabolized to ribose *in vivo* should also be assayed for maximum absorption of glucose and nucleosides. Together, this data will provide metrics to better understand the role of nucleotide supply in DNA damage repair *in vivo*

RB-E2F pathway contributes to tumor initiation by inducing nucleotide shortage

The RB-E2F and Mdm2-p53 pathways are disabled in most, if not all, human tumors. Tumorigenic viral strains such as SV40, adenovirus, and HPV use the large T antigen, E1A/E1B, and E6/E7, respectively, to inhibit the RB-E2F pathway and the Mdm2-p53 pathway

simultaneously, suggesting that either of these two pathways may be sufficient for suppressing tumor development. Since abnormal activation of E2F leads to premature initiation of DNA replication even in the presence of an insufficient pool of nucleotides, activation of p53 and the nucleotide shortage response becomes particularly important to prevent replication stress, DNA damage, and genome instability. Although recent studies using mouse models have suggested that p53-mediated upregulation of genes that promote cell cycle arrest is dispensable for its tumor suppression activity (Li, Kon et al. 2012; Valente, Gray et al. 2013), our study suggests that the function of p53 in cell cycle arrest and the nucleotide shortage response can independently suppress nucleotide shortage-induced tumorigenesis. Therefore, we hypothesize that nucleotide shortage-induced tumorigenesis will only occur when both the cell cycle arrest and nucleotide shortage response functions of p53 are disrupted. Generation of mutant mice with both overexpression of PFK2, as in PFK2 transgenic mice (Duran, Navarro-Sabate et al. 2008), and disruption of the p53 cell cycle response, as in $p53^{3KR}$ mice (Li, Kon et al. 2012) or $p21^{-/-};PUMA^{-/-};BAX^{-/-}$ mice (Valente, Gray et al. 2013), could be used to test this hypothesis; $p53^{-/-}$ mice and $p53^{+/-}$ mice should be included as positive control. We expect $PFK2;p53^{3KR}$ mice and $PFK2;p21^{-/-};PUMA^{-/-};BAX^{-/-}$ mice to demonstrate accelerated tumor development when compared with mice harboring the PFK2 transgenic gene alone, $p53^{3KR}$ alone, or $p21^{-/-};PUMA^{-/-};BAX^{-/-}$ mutations alone. The addition of UV treatment or nucleotide synthesis inhibitor can also be used to further stress these mice and accelerate tumor formation.

Loss of p53 or overactivation of glycolysis prevents activation of the nucleotide shortage response and increases genomic instability. Inhibition of the RB-E2F pathway results in a nucleotide shortage, which in conjunction with loss of p53 results in constitutively activated glycolysis. In the context of previous reports, our data suggests that the simultaneous inhibition of the RB-E2F pathway and the p53 pathway may induce genomic instability, which could open the door to additional mutations accumulated by cancers. While RB controls G1/S transition even under unstressed conditions, p53 induces cell cycle arrest by transcriptional up-regulation

of p21 only after stress (el-Deiry, Tokino et al. 1993); furthermore, induction of cell cycle arrest by p21 is dependent on RB function, and thus we expect that inhibition of will have a stronger effect than genetic knockout of p21 in tumorigenesis when combined with a PFK2 transgenic. To induce RB inhibition, a mouse model using a transgenic T121, N-terminal 121 amino acid of the large T antigen that inhibits RB family member proteins, but not p53, can be used (Pan, Yin et al. 1998). We expect mice harboring both the T121 and PFK2 transgenic genes to develop tumors faster than mice transgenic for only T121 or PFK2. Since RB inhibition induces apoptosis, additional deletion of BAX and PUMA may be needed to reduce T121 transgenic induced apoptosis.

From nucleotide shortage to nucleotide overproduction in cancer development

Despite the fact that RB is well characterized as a tumor suppressor (Nevins 2001), the correlation between RB inhibition and the induction of apoptosis remains poorly understood. One recent study indicates that RB inhibition induces nucleotide shortage, replication stress, and genome instability (Bester, Roniger et al. 2011), and in light of this we hypothesize that nucleotide shortage is the main cause of RB inhibition-induced apoptosis. This hypothesis can be explored in both cell culture and in animal studies; the use of T121 overexpression in cell lines or in T121 transgenic mice allows for RB inhibition, while overexpression of PFK in cell lines or in transgenic mice allows for upregulation of glycolysis. In both cell line and animal models, comparisons of systems with and without nucleoside supplement will be assessed for apoptosis by TUNEL or annexin V staining.

Although nucleotide shortage plays an important role in cancer initiation, insufficient nucleotide supply is not sustainable and these cells will be unable to quickly proliferate. Cells lacking sufficient nucleotide supply have a high rate of spontaneous mutation due to nucleotide shortage-induced mismatch during DNA replication. However, cells lacking sufficient nucleotide supply will not be able to survive and proliferate. Therefore, we hypothesize that mutations that

lead to increased nucleotide production or salvage will gain growth advantage and become tumorigenic. This prediction is consistent with the finding that enzymes that promote nucleotide production (such as c-myc, TYMS, RRM2 and p53R2) are frequently up-regulated in cancer cells. Also, multiple drugs which inhibit nucleotide synthesis and the nucleotide salvage pathway are widely used in anti-cancer chemotherapies, suggesting that cancer cells utilize an alternative pathway to satisfy their nucleotide needs from energy sources other than glucose. Therefore, we hypothesize that mutations that cause elevated glycolytic activity are required for the induction of nucleotide shortages and the consequent initiation of cancer, the up-regulation of nucleotide production in cancer cells, and the salvage pathway functions to compensate for the reduction in *de novo* nucleotide synthesis from glucose as a result of elevated glycolysis activity. To test this idea, mice with a SV40 large T antigen transgene should be treated with nucleoside and nucleoside salvage pathway inhibitors starting at both early and late age points; because nucleotide shortage plays an important role in cancer initiation, we expect to see nucleoside treatment started at an early age to lead to reduced tumor incidence and delayed tumor onset. However, because the nucleoside salvage pathway is required for tumor progression, mice treated with nucleoside treatment at a later age point are expected to demonstrate accelerated tumor growth. On the other hand, treatment with a nucleotide salvage pathway inhibitor will further decrease the cellular nucleotide pool; in this case, treatment with a nucleotide salvage pathway inhibitor at an early age would accelerate tumor initiation, and inhibit tumor growth when started at a later age point. However, it is also possible that treatment with a nucleotide salvage pathway inhibitor at an early age will further decrease the cellular nucleotide pool, leading to increased cell death. Because of this possibility, a detailed dosage gradient of nucleotide salvage pathway inhibitors needs to be tested to find a concentration that promotes tumor initiation, without increasing cell death.

Despite the fact that the RB-E2F and Mdm2-p53 pathways are mutated in almost all cancers, and that silencing of both pathways simultaneously is sufficient to cause cancer, the

mechanism revealed in our study suggests that the function of the RB-E2F pathway and Mdm2-p53 pathway in tumor suppression is to prevent nucleotide shortage, and with that prevent genome instability. It is the diverse combination of mutations in multiple tumor suppressor genes and oncogene that allows for the cell to adapt to different metabolic patterns in a tissue specific manner, eventually leading to immortalization, invasion, and metastasis. Therefore, restoration of the RB-E2F pathway and Mdm2-p53 pathway is unlikely to be a good therapeutic approach; instead, drugs that destroy the metabolic pathway specifically utilized by cancer cells are more likely to have a bright future. Although multiple combinations of mutations in each tissue can lead to tumorigenic consequences, and a high number of possible combinations of mutations is probably, the evolution of computer science and sequencing technology will one day make it possible to index all possible tumorigenic mutation combinations in a tissue specific manner. All tumorigenic mutation combinations will be classified by mutated pathway, and the search for efficient therapeutic solutions for each combination prioritized based on the frequency of occurrence. Mouse models that inhibit the RB-E2F pathway and Mdm2-p53 pathway simultaneously using SV40 large T antigen, E1A/E1B, E6/E7 may be a good system to generate the large amount of tumors necessary to assay for the many possible combinations of mutations. With current technologies, the cost of sequencing these tumor samples will be a limiting factor, but the continued technological advances will ideally resolve this obstacle in the near future.

APC/C and Cul1-betaTRCP may regulate the nucleotide shortage response through the Mdm2-p53-PFK2 pathway

Through our discovery of APC/C as an E3 ubiquitin ligase for Mdm2, and the role of p53 in controlling PFK2 transcription, we were surprised to also find many connections between APC/C and glucose/nucleotide metabolism both dependent and independent of the Mdm2-p53 pathway. First, in light of the PFK2 suppression observed upon p53 activation in Mdm2 knockout MEFs and following Nutlin-3 treatment, we speculate that Mdm2 degradation likely

functions in the regulation of PFK2. Second, we found that both Mdm2 and PFK2 are targeted for degradation by APC-Fzr1 and Cullin1-betaTRCP (Herrero-Mendez, Almeida et al. 2009; Tudzarova, Colombo et al. 2011). Furthermore, APC-Fzr1 promotes the polyubiquitination and degradation of a number of proteins involved in nucleotide synthesis, including ribonucleotide reductase R2 (RR2) (Chabes, Pflieger et al. 2003), thymidine kinase 1 (TK1), and thymidylate kinase (TMPK) (Ke, Kuo et al. 2005); together with the current study this suggests that APC/C may play an important role in regulating glucose metabolism and nucleotide production.

In light of the decrease of PFK2 protein levels upon nucleotide shortage, in both a p53 dependent and independent manner, we hypothesize that this decrease is the combined effect of p53-mediated decrease in PFK2 transcription and increased PFK2 degradation. Half-life assay should be performed in p53 negative cells with nucleotide shortage treatment to confirm that changes in the dynamics of PFK2 protein degradation are indeed contributing to the decrease in PFK2 protein levels. To determine if PFK2 degradation is mediated by APC2-Fzr1 or Cullin1-beta-TRCP, the effect of nucleotide shortage treatment (UV, 5-fu or Leflunomide) on PFK2 protein levels in p53 null cells with APC2/Fzr1 or Cullin1/beta-TRCP knocked down could be assessed. Also, it would be interesting to determine whether APC2/Fzr1 or Cullin1-betaTRCP-mediated degradation of Mdm2 is the upstream signal to activate p53 and suppress PFK2.

Our data favor a model whereby metabolic regulation, particularly that concerning the production of nucleotides, plays an important role in maintaining genomic integrity. Deregulated glucose metabolism weakens the foundation for genomic stability and sensitizes cells to spontaneous mutations, and is consistent with the Warburg effect, which describes the phenomenon whereby almost all tumor cells display higher glycolytic rates, and higher sensitivity to DNA damaging reagents and nucleotide synthesis inhibition than normal cells. Furthermore, our study serves to link the regulation of glucose metabolism and nucleotide metabolism, and points toward new directions in cancer prevention studies involving sunburn,

stroke, and diabetes. Finally, this study links many p53 functions, including control of tetraploidy, cell cycle arrest, apoptosis, and glucose metabolism, to one common goal: the prevention of nucleotide shortage-induced genome instability (Figure 4-6A).

FIGURES

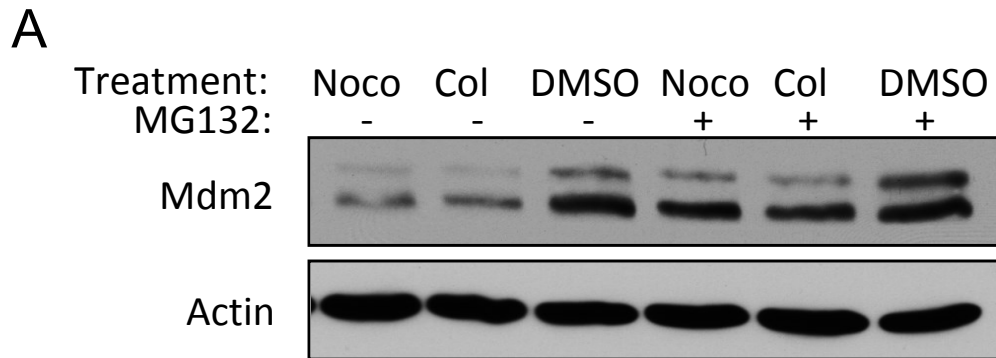


Figure 4-1. Prometaphase arrest leads to the decrease of Mdm2 protein levels in a proteasome dependent manner.

- A. U2OS cells were treated with DMSO, nocodazole or colcemid for 24 hours. Cells were then treated with DMSO or 25 μ M MG132 for four hours and analyzed by western blotting.

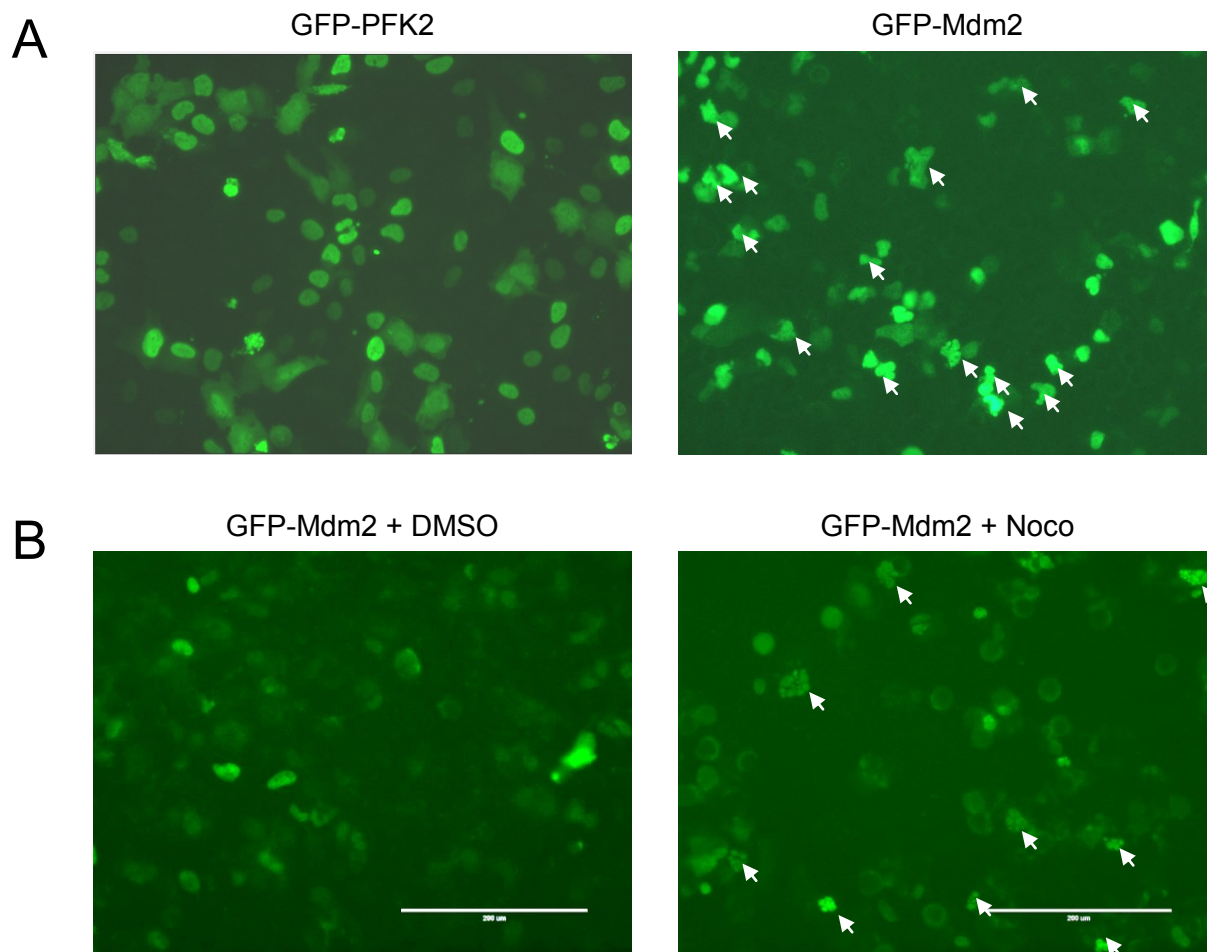


Figure 4-2. Mdm2 overexpression leads to irregular nuclear shape.

A. Transient transfection of GFP-PFK2 and GFP-Mdm2 in U2OS cells. White arrows point to cells with significant changes in nuclear shape.

B. Transient transfection of U2OS cells with GFP-Mdm2 treated with or without nocodazole, as indicated. White arrows indicate cells with grape shaped micro-nuclei.

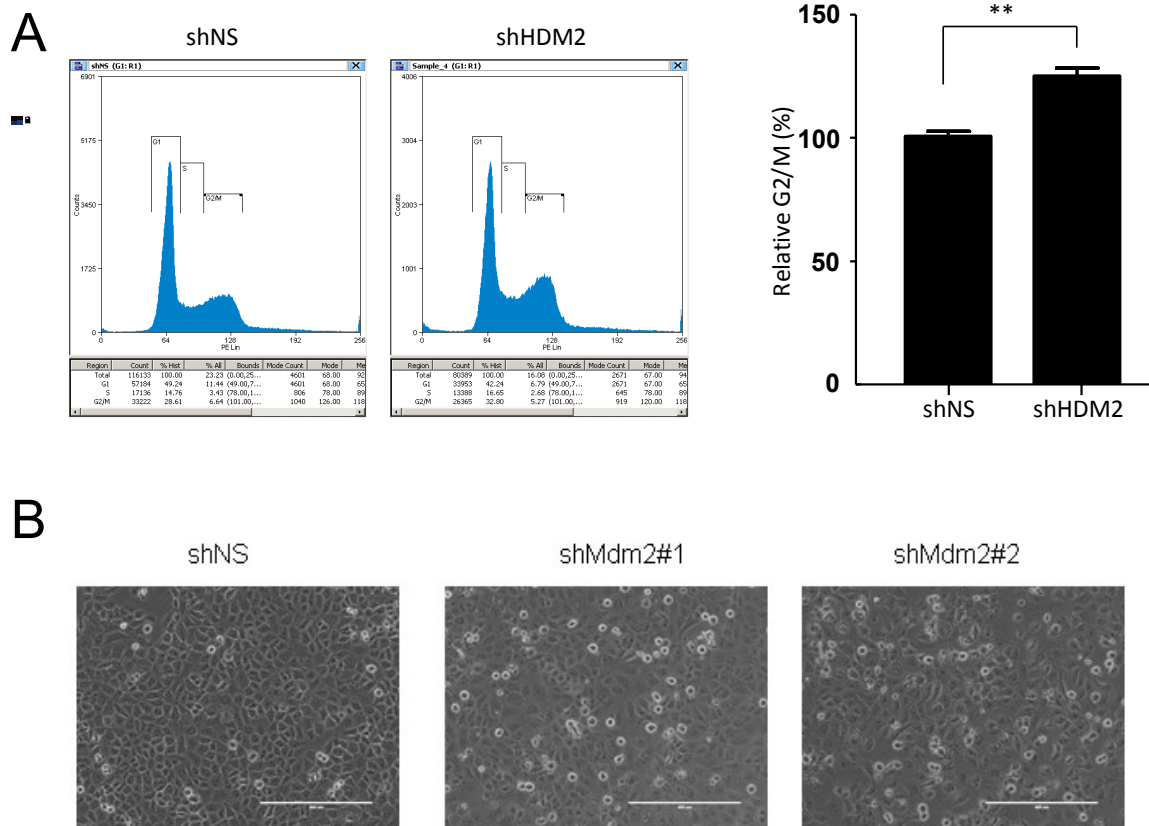


Figure 4-3. Mdm2 knock down leads to increased G2/M and rounded up cell morphology independent of p53.

A. FACS analysis of H1299 cells with infected with lentivirus based shRNA knock down of Mdm2 or a non-specific control. Cells in G/M were counted and quantified, $p=0.0011$.

B. Phase contrast images of H1299 cells infected with lentivirus based shRNA knock down of Mdm2 or a non-specific control 72 hours after infection.

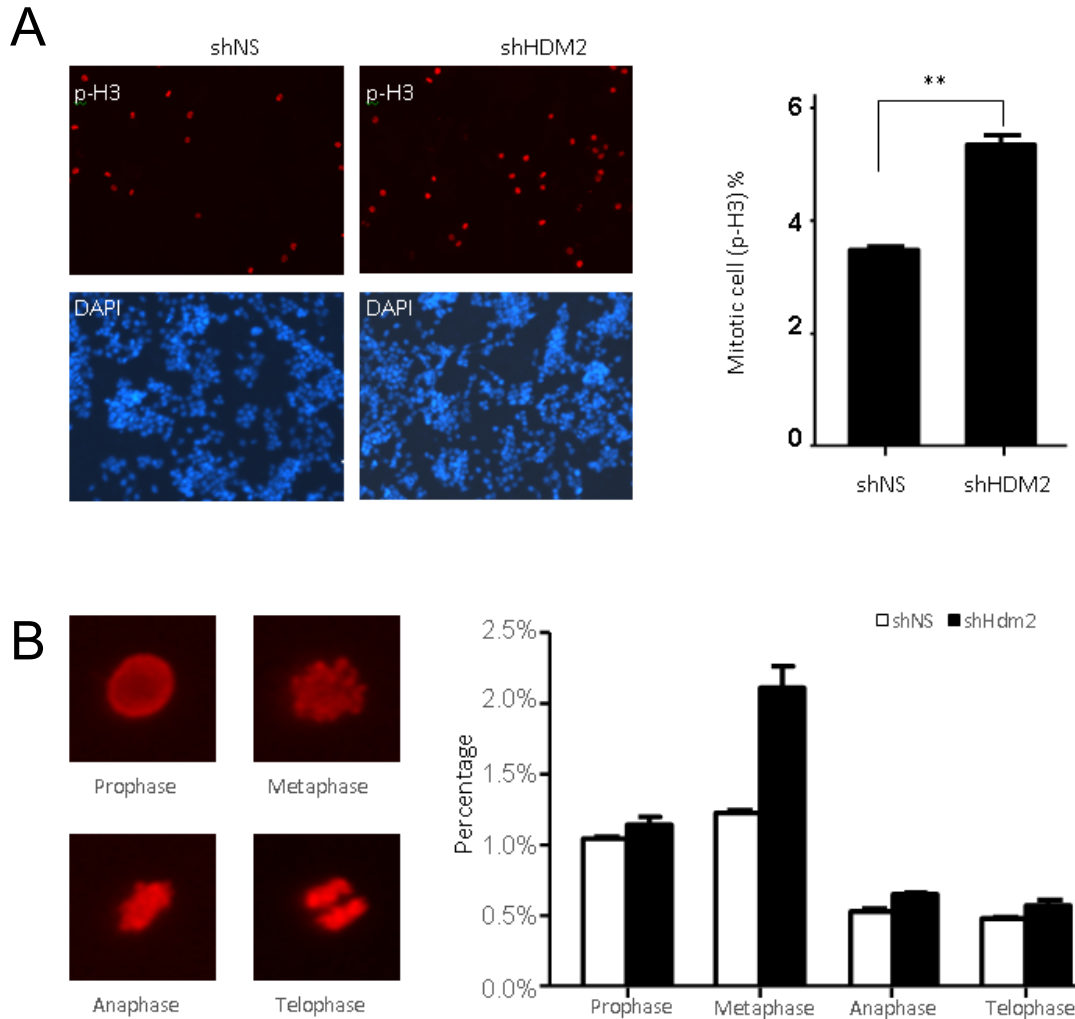


Figure 4-4. Mdm2 knock down lead to increased mitotic metaphase independent of p53.

A. Immunofluorescence staining of the mitotic marker phosphorylated Histone 3 in HCT116/p53^{-/-} cells with lentivirus based shRNA knocks down of Mdm2 or a non-specific control 72 hours after infection. p-H3 positive cells were counted and quantified, p = 0.0004.

B. Sample image of p-H3 staining of cells in difference phases of mitosis. Right panel shows the quantification and distribution of cells observed in (A) in prophase, metaphase, anaphase and telophase of mitosis.

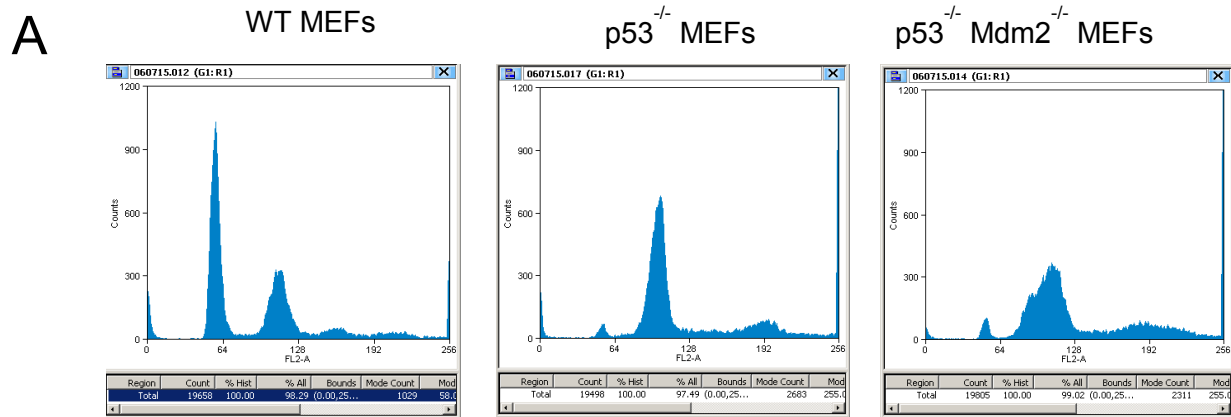


Figure 4-5. p53 knock out leads to tetraploidy and p53, Mdm2 double knock out leads to aneuploidy.

A. FACS analysis of wild type (WT) MEFs, $p53^{-/-}$ MEFs, and $p53^{-/-};Mdm2^{-/-}$ MEFs, A similar number of cells were counted for each cell type.

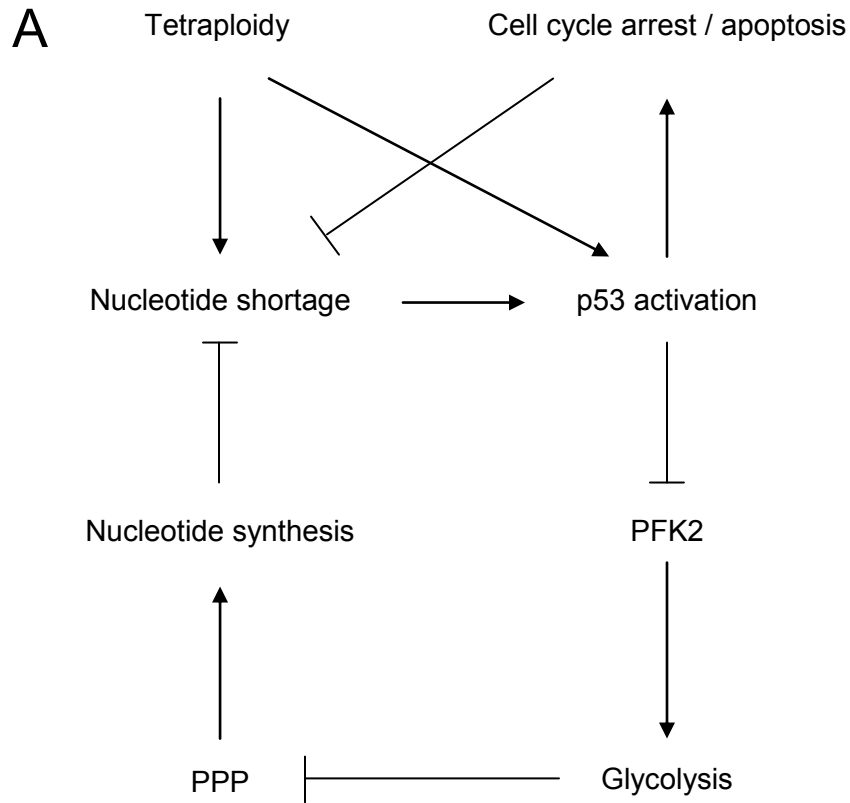


Figure 4-6. Function of p53 in tetraploidy control, cell cycle arrest, apoptosis, glucose metabolism, and nucleotide shortage response.

A. Schematic graphic demonstrating the link between the p53 functions of tetraploidy control, cell cycle arrest, apoptosis, glucose metabolism, and nucleotide shortage response.

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