

# Akt Regulation of Mdm2-p53 Signaling in Cellular Stress Responses and Tumorigenesis

A Dissertation Presented

By

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# AKT REGULATION OF MDM2-P53 SIGNALING IN CELLULAR STRESS RESPONSES AND TUMORIGENESIS

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April 25, 2019

## DEDICATION

This thesis is dedicated to my parents Maxwell and Rita Chibaya.  
You sacrificed everything and lived your lives without much to your name so you could  
pay for my education and I could be where I am today.

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## ABSTRACT

In cells undergoing stress, the p53 transcription factor is stabilized and activates the expression of numerous genes contributing to p53-mediated tumor suppression. One p53 target gene is Mdm2, which encodes an oncoprotein that binds and ubiquitinates p53 for proteasomal degradation, thus limiting the amplitude and duration of the p53-mediated stress response. Our lab recently discovered that Mdm2 phosphorylation by ATM and c-Abl regulates the DNA damage response and tumorigenesis in mice. AKT has also been found in transfection studies to phosphorylate Mdm2 at serine residues 166 and 186 (mouse S163 and S183) to alter p53 activity. However, the physiological significance of Mdm2 phosphorylation by Akt remains unknown. Therefore, I generated Mdm2<sup>S163A</sup> or Mdm2<sup>S183A</sup> mice expressing mutant Mdm2 incapable of being phosphorylated by Akt.

In contrast with our previous studies, Akt phosphorylation of Mdm2 does not alter spontaneous tumorigenesis or the DNA damage response to ionizing radiation. However, Akt phosphorylation of Mdm2-S183 (but not -S163) upregulates nuclear localization of Mdm2, destabilizes p53, and reduces p53-mediated senescence in response to elevated levels of reactive oxygen species (ROS). To examine the effects of Mdm2-S183 phosphorylation on p53 tumor suppression, I utilized three different mouse models of ROS-induced cancer. Increased levels of p53 and senescence in Mdm2<sup>S183A</sup> mice yielded reduced

tumorigenesis in an activated Ras model of lung cancer, a phorbol ester-induced skin cancer model, and a diethylnitrosamine-induced model of hepatocellular carcinoma. Since AKT is also important regulator of cell metabolism, I explored the impact of the Mdm2-S183 allele on metabolic functions. Mdm2 phosphorylation by Akt reduced glucose metabolism via glycolysis *in vitro*, and reduced insulin tolerance in mice, without altering glucose tolerance and glucose-stimulated insulin secretion. Collectively, these findings document a unique physiologic role for the AKT-Mdm2-p53 signaling axis in regulating cell growth and tumorigenesis.

## TABLE OF CONTENTS

CHAPTER I: INTRODUCTION .....	1
p53 protein: A historical perspective. ....	2
p53 protein domains .....	3
Role of p53-dependent transcription in tumor suppression .....	7
Relationship between p53 and oxidative stress .....	10
Role of p53 in glucose metabolism .....	13
Regulation of p53 by Mdm proteins.....	15
Phosphorylation of p53.....	17
Phosphorylation of Mdm2.....	19
Regulation of Mdm2-p53 signaling by Akt.....	22
Aims of this dissertation.....	24
CHAPTER II: ROLE OF MDM2-S183 PHOSPHORYLATION IN DEVELOPMENT AND STRESS RESPONSES .....	26
Introduction .....	27
Results .....	29
CHAPTER III: MDM2-S183 PHOSPHORYLATION BY AKT PROMOTES TUMORIGENESIS INDUCED BY CARCINOGEN EXPOSURE AND ONCOGENE EXPRESSION .....	53
Introduction .....	54
Results .....	60
CHAPTER IV: GENERAL DISCUSSION.....	75
General conclusions .....	97

APPENDIX: PHOSPHORYLATION OF MDM2-S183 ALTERS ASPECTS OF GLUCOSE METABOLISM .....	99
Introduction .....	<b>100</b>
Results .....	<b>106</b>
Conclusions.....	<b>111</b>
MATERIALS AND METHODS.....	113
REFERENCES .....	122



## LIST OF FIGURES

	CHAPTER I	Page
Figure 1.1	Schematic representation of the several p53 polypeptide domains.	3
Figure 1.2	Full length Mdm2 and Mdmx protein domains.	14
Figure 1.3	Proposed phosphorylation sites on hMdm2 protein sequence.	20
	CHAPTER II	
Figure 2.1	Mdm2 S163 and S183 phosphorylation is dispensable for development.	31
Figure 2.2	Mdm2 S183 phosphorylation is required for cell proliferation.	33
Figure 2.3	Normoxic conditions rescue Mdm2 <sup>S183A</sup> MEFs from senescence.	35
Figure 2.4	Mdm2 <sup>S183A</sup> MEFs are sensitive to oxidative stress.	37
Figure 2.5	Mdm2-S183 phosphorylation prevents premature senescence in MEFs.	39
Figure 2.6	Mdm2-S183 phosphorylation alters the expression of p53-target genes that mediate senescence.	42
Figure 2.7	Phosphorylation of Mdm2 at S183 by Akt promotes Mdm2 stability and degradation of p53.	44
Figure 2.8	Phosphorylation of endogenous Mdm2 at S183 by Akt promotes Mdm2 nuclear localization and p53 degradation.	46
Figure 2.9	Phosphorylation of Mdm2 at S183 does not affect p53 activation and stabilization after DNA damage.	48
Figure 2.10	Phosphorylation of Mdm2 at S183 does not alter p53-mediated cell cycle arrest induced by DNA damage <i>in vitro</i> .	50

Figure 2.11	Phosphorylation of Mdm2 at S183 does not affect DNA damage responses <i>in vivo</i> .	52
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### CHAPTER III

Figure 3.1	Phosphorylation of Mdm2-S183 promotes DEN-induced liver tumorigenesis.	61
Figure 3.2	DEN treatment triggers senescence in Mdm2 <sup>S183A</sup> livers treated with DEN.	64
Figure 3.3	Phosphorylation promotes skin tumorigenesis.	67
Figure 3.4	Mdm2-S183 phosphorylation promotes epidermal tumorigenesis.	70
Figure 3.5	Mdm2-S183 phosphorylation enhances the development of K-ras-driven tumors.	74

### CHAPTER IV

Figure 4.1	Proposed Model.	94
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### APPENDIX

App Figure 1	p53-dependent regulation of glucose metabolism.	98
App Figure 2	Schematic diagram highlighting the different stages in a glycolysis stress test.	104
App Figure 3	Mdm2 S183 phosphorylation promotes glycolysis in MEFs.	105
App Figure 4	Mdm2 S183 phosphorylation promotes insulin resistance. but not glucose tolerance and insulin release.	107

## **CHAPTER I: INTRODUCTION**

**p53 protein: A historical perspective.**

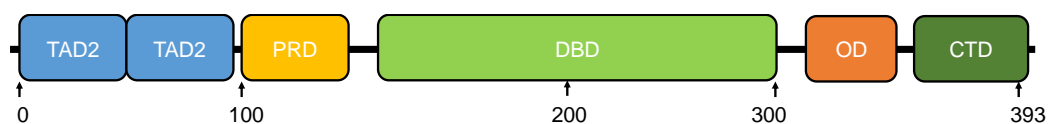
p53 was first discovered in the late 1970s as an antigen that coprecipitated with antisera against the simian virus SV40 large T antigen (Lane and Crawford, 1979, Linzer and Levine, 1979). Earlier studies suggested the protein to be an oncogene since p53 expression led to cellular transformation (Jenkins et al., 1985, Eliyahu et al., 1984). Additionally, p53 was also highly expressed in some tumors (Jenkins et al., 1985, Sarnow et al., 1982). Soon evidence began to emerge showing that various forms of p53 were expressed in tumors (Mowat et al., 1985) and that some of them, which were later shown to be mutant, possessed the ability to transform cells. However, the role of p53 in tumor suppression was demonstrated by the fact that wild type p53 cDNA suppressed the transformation of cells *in vitro* (Eliyahu et al., 1989).

The role of wild type p53 as a tumor suppressor protein was further demonstrated by studies showing that cellular stresses like DNA damage activated p53 (Maltzman and Czyzyk, 1984, Kastan et al., 1992, Hall et al., 1993). Activated p53 was able to block cell cycle progression and promoted apoptotic cell death in response to DNA damage (Lowe et al., 1993, Yonish-Rouach et al., 1991). The role of p53 as a tumor suppressor was further demonstrated by the generation of a mouse model that lacked p53 and turned out to be susceptible to tumorigenesis (Donehower et al., 1992). Further characterization of wild type p53 showed that it was a DNA binding transcription

factor (Kern et al., 1991, Funk et al., 1992, Zambetti et al., 1992), that activated the expression of genes like p21 to halt cell cycling (el-Deiry et al., 1993). Additionally, it was also demonstrated that wild type p53 activated the expression of Mdm2 (Barak et al., 1993) which is its well-established negative regulator.

### p53 protein domains

The p53 protein contains several domains that play pivotal roles in its function as a transcription factor (Figure 1). The N-terminus contains two transactivating domains (TADs), namely TAD1(amino acids 1-40) and TAD2 (amino acids 41-61). These are followed by a proline rich domain (PRD) domain (amino acids 64-92). The domain that binds to DNA (DBD) (amino acids ~100–300) spans the central region of the p53 protein peptide sequence. This is followed by an oligomerization domain (OD) (amino acids 323-355), and lastly C-terminal domain (CTD) (amino acids 364-393) which is largely unstructured.



**Figure 1.1. Schematic representation of the several p53 polypeptide domains.** The N terminus contains the two transactivation domains shown in blue, followed by the proline rich domain in yellow, then the central DNA binding domain in light green and the c-terminal is comprised of the oligomerization domain in orange and the C-terminal domain depicted here in dark green.

The function of p53 as a transcription factor requires its binding to DNA to regulate the transcription of genes. p53 monomers oligomerize and function as tetramers with direct DNA binding capacity (Friedman et al., 1993). The central DBD is crucial for p53 function since this region interacts with p53 response elements on DNA (el-Deiry et al., 1992). DNA binding enables the interaction of p53 TADs with various effector proteins to facilitate target gene expression (Qian et al., 2002). Additionally, the DBD is the most mutated region of p53 in most human cancers with several hotspot mutations that result in loss of DNA binding ability (Hollstein et al., 1991).

The two TADs are important for p53 function as a transcription factor since they add a layer of specificity, with each TAD activating a different set of p53 target genes (Brady et al., 2011). Mice expressing mutant p53 harboring inactivating mutations in both TAD1 and TAD2 do not express the vast majority of direct p53 target genes and are susceptible to tumorigenesis (Brady et al., 2011). Mice expressing inactivating mutations in either of the TADs, are able to suppress tumorigenesis, suggesting that these domains activate distinct gene sets and pathways that can mediate tumor suppression in the absence of the other.

The CTD plays an important role in regulating gene expression by mediating the scanning of DNA for p53 response elements (Tafvizi et al., 2011, Tafvizi et

al., 2008, Khazanov and Levy, 2011) and stable DNA binding (Laptenko et al., 2015). Structural determination studies have shown that the CTD is very disorganized and this facilitates the binding of a myriad of other regulatory proteins and RNAs that regulate p53-mediated transcription (Laptenko et al., 2016). In addition, this domain is subject to various post-translational modifications further adding to the complexity of the CTD's role in transcription (Mujtaba et al., 2004, Tong et al., 2015b, Tong et al., 2015a). As such, various mouse models and cell lines expressing p53 protein lacking segments of the CTD display different phenotypes in terms of p53 gene expression and tumor suppression, further highlighting complex role of this region of p53-dependent transcriptional regulation (Feng et al., 2005, Krummel et al., 2005, Simeonova et al., 2013, Hamard et al., 2013).

### **p53 mouse models**

The gene encoding the p53 transcription factor protein is often mutated in human cancer. Observations from human tumors and p53 null and mutant mice, underscores the role of p53 as a potent tumor suppressor. Mice deficient for p53 ( $p53^{-/-}$ ) or heterozygous for p53 loss ( $p53^{+/-}$ ) are susceptible to highly penetrant spontaneous tumors (Donehower et al., 1992). The tumor incidence for  $p53^{-/-}$  mice is about 4 months and all mice will succumb to cancer by 10 months of age (Donehower, 1996). As for  $p53^{+/-}$  mice, the median tumor incidence is about 18 months (Venkatachalam et al, 1998, Harvey et al., 1995).  $p53^{-/-}$  mice present

mainly with T cell lymphomas, whereas p53<sup>+/-</sup> mice display a range of tumors predominantly sarcomas and carcinomas, together with some B cell lymphomas (Jacks et al., 1994, Purdie et al., 1994). About half of p53<sup>+/-</sup> mice lose the wild type p53 allele (Venkatachalam et al., 1998), suggesting that p53<sup>-/-</sup> cells are selected for and constitute the majority of the tumor cells. Additionally, this suggests that p53 alleles act in a recessive manner and loss of both p53 alleles facilitates the development and progression of tumors. Consistently with the role of p53 as a tumor suppressor, individuals who inherit a mutant allele of p53, such as in Li Fraumeni syndrome, are predisposed to develop various types of cancer primarily sarcomas and breast cancers (Li et al., 1988). Therefore, the fact that about 50% of all human cancers harbor p53 mutations and that mice lacking fully functional alleles of p53 are susceptible to cancer, underscores the role of the p53 transcription factor protein in tumor suppression.

In human cancers, most mutations in p53 are not inherited like in the case Li Fraumeni, but are missense mutations that occur in hot spot regions such as the DBD. Most often, p53 mutants act in a gain of function manner, conferring growth advantage to cells *in vitro* and *in vivo* (Brosh and Rotter, 2009). Such hotspot mutations that alter p53's ability to bind DNA include the widely studied p53<sup>R172H</sup> and p53<sup>R270H</sup>, both of which promote cell growth and transformation both *in vitro* and *in vivo* (Brosh and Rotter, 2009). Genetically engineered mice homozygous for both the R172H and R270H alleles are highly susceptible to metastatic



tumors (Liu et al., 2000, Lang et al., 2004, Olive et al., 2004). This suggests that some full length p53 mutant proteins acquire new functions unlike those of wild type p53 to promote tumorigenesis *in vivo*. Moreover, this highlights the importance of wild type p53 DBD in mediating tumor suppression.

### **Role of p53-dependent transcription in tumor suppression**

p53 mainly exerts its function as a potent tumor suppressor through transcriptional activation of genes that control processes like cell cycle arrest, senescence, DNA damage repair, and apoptosis. Most of the p53 mutations present in human cancers are located within the DBD (Olivier et al., 2010) and often result in altered p53-mediated transcriptional activity. Central to p53-dependent transcriptional regulation of tumor suppression are consensus DNA sequences called response elements (REs). Canonical p53 REs consist of two RRRCWWGYYY decamer motifs, in which R is a purine, W is A or T, and Y is a pyrimidine with 0–13bp spacer sequences in between (el-Deiry et al., 1992). p53 binds to REs in the form of a tetramer and interact with components of the transcriptional machinery, either to activate or repress the expression of its target genes.

One of the first p53-target genes to be identified is the cyclin-dependent kinase (CDK) inhibitor p21, which triggers cell cycle arrest (Harper et al., 1993). p21 inhibits CDK-mediated hyperphosphorylation of retinoblastoma protein (Rb) and

hypophosphorylated Rb binds and inhibits E2F regulatory proteins thereby impeding cell cycle progression (Harper et al., 1993, el-Deiry et al., 1993). It is well established that p21 also mediates cellular senescence and is overexpressed in senescent human and mouse fibroblasts (Abbas and Dutta, 2009, Noda et al., 1994). p53 also activates p21 in the presence of ROS resulting in senescence (Macip et al., 2002).

p53 also triggers the expression of genes that induce apoptosis. Early studies showed that ectopic p53 expression can induce apoptosis in p53-deficient leukemic cells (Yonish-Rouach et al., 1991). Furthermore, loss of p53-mediated apoptosis in p53<sup>-/-</sup> mice was shown to promote tumorigenesis (Symonds et al., 1994). To trigger apoptosis, p53 binds to REs of various genes such as those of the pro-apoptotic Bcl2-family of proteins. In particular, p53 triggers the expression of proapoptotic gene Bax, and it was demonstrated that Bax mRNA and protein expression are greatly diminished in p53<sup>-/-</sup> mice (Miyashita et al., 1994). MEFs that are Bax deficient are refractory to p53-dependent apoptosis triggered by oncogene expression (McCurrach et al., 1997). Moreover, p53 induces the expression of pro-apoptotic BH-3 family of proteins including Puma (Nakano and Vousden, 2001) and Noxa (Oda et al., 2000). The expression of these genes among others often result in mitochondria dependent activation of caspases and apoptosis.

Cell cycle arrest, senescence, and apoptosis constitute the most studied, or rather, canonical mechanisms through which p53 target genes mediate tumor suppression. Several mouse models expressing p53 with mutations in the transactivation domains have been generated. For instance, inactivating mutations in TAD1 in mice ( $p53^{25,26}$ ) blocks the expression of p53-target genes that mediate cell cycle arrest and apoptosis in response to DNA damage but retained the ability to suppress oncogene-induced tumorigenesis (Brady et al., 2011). Surprisingly, mice with inactivating mutations in TAD2 ( $p53^{53,54}$ ) retained the ability to transactivate most genes and displayed unaltered tumor suppression (Brady et al., 2011). However, complete inactivation of both TADs in  $p53^{25,26,53,54}$  mice, inhibits p53-dependent senescence and the expression of most p53-target genes. The expression profile of  $p53^{25,26,53,54}$  mice is indistinguishable from that observed in  $p53^{-/-}$  mice. Furthermore,  $p53^{25,26,53,54}$  mice are susceptible to tumorigenesis (Brady et al., 2011, Johnson et al., 2005), suggesting that p53 transactivates specific subsets of genes crucial for tumor suppression.

Another mouse model in which three lysine residues in the DBD of p53 are mutated to arginine residues ( $p53^{3KR}$ ) also fail to undergo cell cycle arrest, senescence, and apoptosis, but are able to suppress spontaneous tumorigenesis (Li et al., 2012a). Interestingly,  $p53^{3KR}$  mice are still able to properly regulate cellular metabolism and oxidative stress responses. Lastly, cells derived from

p21<sup>-/-</sup>;puma<sup>-/-</sup>;noxa<sup>-/-</sup> mice fail to undergo cell cycle arrest, senescence, and apoptosis (Valente et al., 2013). However, unlike p53<sup>-/-</sup> mice, p21<sup>-/-</sup>;puma<sup>-/-</sup>;noxa<sup>-/-</sup> mice do not develop tumors and mount an effective DNA damage response (Valente et al., 2013).

In the aforementioned studies, p53-mediated cell cycle arrest, senescence, and apoptosis in response to DNA damage were partially or completely ablated, but tumor suppression was still maintained. This suggests that p53-mediated processes that modulate responses to acute DNA damage are not fully critical for p53-dependent tumor suppression. Additionally, these findings suggest a role for other compensatory pathways as major contributors to p53-dependent suppression of tumorigenesis. Furthermore, this also supports a role for less fully understood non-canonical p53 functions such as the regulation of oxidative stress responses and cellular metabolism briefly described below.

### **Relationship between p53 and oxidative stress**

Tight regulation of cellular oxidative stress from deregulated ROS is critical for tissue homeostasis. Oxidative stress can trigger senescence, cell cycle arrest, apoptosis (Liang et al., 2013), and ferroptosis (Jiang et al., 2015). Studies have shown that increased levels of ROS play a critical role in tumorigenesis. For instance, p53<sup>-/-</sup> mice exhibit elevated intracellular levels of ROS which can be counteracted by administration of the dietary antioxidant N-acetyl cysteine,

resulting in reduced lymphoma incidence and reduced growth of liver cancer xenografts (Sablina et al., 2005). Additionally, despite the lack of canonical p53-dependent processes thought to mediate tumor suppression in the previously mentioned p53<sup>3KR</sup> model, these mice were not susceptible to developing lymphomas as seen in p53<sup>-/-</sup> mice (Li et al., 2012a). However, these mice were able to properly regulate the production of ROS, suggesting that p53-mediated regulation of oxidative stress responses may contribute to tumor suppression.

Emerging evidence shows that p53 is involved in regulating various genes required for properly modulating the effects and levels of ROS. The main source of ROS in cells is oxidative phosphorylation in the mitochondria. p53 promotes oxidative phosphorylation but also counteracts ROS effects through the transcriptional activation of genes that mediate antioxidant activity (Liu et al., 2008). Physiological levels of oxidative stress trigger p53-dependent genes with antioxidant functions such as ALDH4, Sestrin 1&2, GLS2, GPX1, and Tigar (Hu et al., 2010, Liu et al., 2008, Budanov et al., 2004, Bensaad et al., 2006, Tan et al., 1999). Tigar alters glycolysis by inhibiting phosphofructokinase 1 which is a critical enzyme in glycolysis, thereby leading to enhanced production of NADPH which has antioxidant properties (Bensaad et al., 2006). Both GPX1 and GLS2 are powerful antioxidant enzymes that help scavenge peroxides in cells (Tan et al., 1999, Hu et al., 2010). Additionally, Sestrins 1&2 promotes the generation of peroxiredoxins, which like NADPH, exhibit immense antioxidant activity (Budanov

et al., 2004). Expression of p53-inducible Aldh4 *in vitro* lowers the levels of ROS induced by treatment with hydrogen peroxide or UV (Yoon et al., 2004).

Alternatively, in the presence of excessive oxidative stress p53 triggers the expression of pro-oxidant genes including Puma, Pig3, p21, and Bax (Liu et al., 2008). Pro-oxidant genes such as Pig3 and Puma cause apoptosis (Nakano and Vousden, 2001, Johnson et al., 1996). p21 expression in the presence of elevated levels of ROS causes senescence (Passos et al., 2010, (Macip et al., 2002). Furthermore, during chronic exposure to oxidative stress, p53 can induce more ROS (Liu et al., 2008), and this can promote the death of cells potentially harboring harmful mutations from DNA oxidation.

The seemingly cyclic relationship between p53 and oxidative stress is complex and is not yet fully understood. However, both pro-oxidant and antioxidant functions of p53 can promote clearance of potentially oncogenic cells. Tumor progression is often characterized by increased ROS levels, but cancer cells survive probably due to loss of p53 function or through other context-dependent mechanisms. Therefore, it is plausible that in different contexts, when cell cycle arrest, senescence, and apoptosis are impaired, p53-mediated regulation of redox homeostasis contributes to tumor suppression. It is also possible that all or a combination of these processes work together in unison to suppress tumorigenesis.

## **Role of p53 in glucose metabolism**

Cancer cells are known to undergo metabolic reprogramming to fulfill the higher energy demands required for rapid proliferation characteristic of cancerous cells. In particular, it is well established that cancer cells metabolize glucose mainly through aerobic glycolysis and not oxidative phosphorylation, a process known as the Warburg effect (Warburg, 1956). Since aerobic glycolysis is less efficient at ATP generation compared to oxidative phosphorylation, cancer cells typically exhibit much more elevated glucose uptake compared to untransformed cells. Additionally, utilizing aerobic glycolysis may be potentially beneficial to highly vascularized tumors since inefficient generation of ATP would not pose energy deficits due to a plentiful supply of nutrients from blood. Moreover, it is possible that glucose is not mainly required to generate ATP during nutrient abundance, but used for the biosynthesis of macromolecules like lipids, and carbohydrates. As a result, cancer cells can still survive and rapidly proliferate despite using glycolysis as a major process to generate energy.

Accordingly, several oncogenes have been shown to promote glycolysis. For instance, Akt enhances glucose uptake in cancer cell by promoting cell surface localization of glucose transporters Glut 1&4 (Cheatham et al., 1994). In addition, Akt activates mTOR by blocking its negative regulator TSC2, further promoting glycolysis (Inoki et al., 2002). However, p53 has the opposing effect as a tumor suppressor and has been shown to promotes oxidative phosphorylation

whilst inhibiting glycolysis both *in vitro* and *in vivo* (Matoba et al., 2006). This is achieved mainly through the transcriptional regulation of genes involved in glucose metabolism. p53 inhibits glycolysis by blocking the expression of glucose transporters Glut1 and Glut 4 (Kruiswijk et al., 2015, Zhang et al., 2013). In addition, p53 blocks the expression of NF- $\kappa$ B which promotes the expression of Glut3 (Kawauchi et al., 2008). In some cases, p53 also activates the expression of Tigar which reduces the levels of phosphofructokinase-1 which is a rate-limiting enzyme in glycolysis (Bensaad et al., 2009, Bensaad et al., 2006).

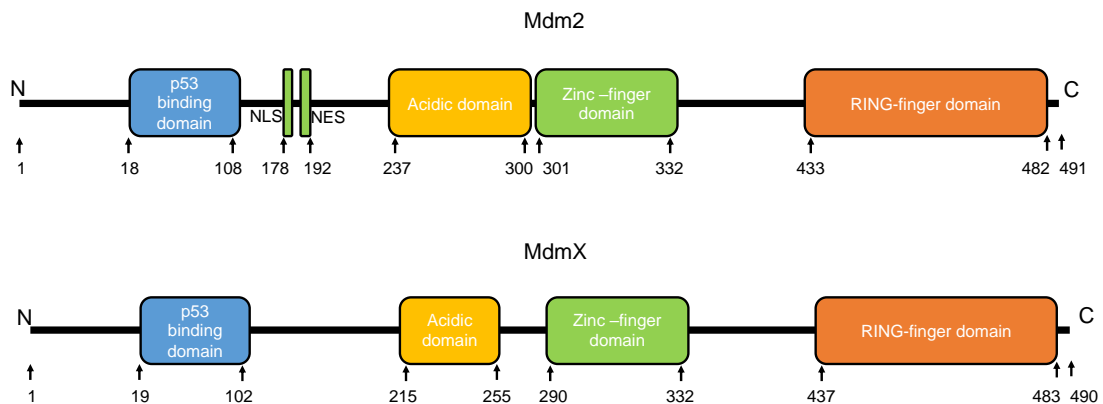
To maintain oxidative phosphorylation, p53 activates the expression of a plethora of genes. These include Sco2 and AIF which are known to promote proper functioning of the electron transport chain central to oxidative phosphorylation (Matoba et al., 2006, Stambolsky et al., 2006). Other p53 target genes include Parkin which promotes the expression of pyruvate dehydrogenase enzyme that catalyzes some of the reactions connecting glycolysis to oxidative phosphorylation (Zhang et al., 2011). The effects of Parkin on glycolysis have also been observed in some tumors (Cesari et al., 2003). Since oxidative phosphorylation is observed in quiescent tissues such as the heart and in non-transformed cells, maintenance of oxidative phosphorylation by p53 most likely contribute to tumor suppression.



## **Regulation of p53 by Mdm proteins**

Mdm2 gene is located on chromosome 10, and was first found overexpressed in mouse 3T3 cells in small extra-chromosomal nuclear bodies called double minutes (Fakharzadeh et al., 1991). Mdm2 contains an N-terminal p53 interaction domain, central acidic domain, Zinc-finger domain, and C-terminal RING domain (Figure 1.2). Mdm2 inhibits p53's transactivation activity via its N-terminal hydrophobic cleft located in the p53-binding domain (Kussie et al., 1996, Momand et al., 1992). Mdm2 physically binds p53 and blocks transactivation domains from interacting with the transcriptional machinery (Chen et al., 1993, Oliner et al., 1993). In addition, Mdm2 also engages its C-terminal RING domain responsible for its E3 ubiquitin ligase activity to ubiquitinate p53 leading to its degradation (Honda et al., 1997) .

Mdm2<sup>-/-</sup> mice exhibit embryonic lethality observed between E4.5 and E6.5 which is rescued by the concomitant loss of p53 (Jones et al., 1995, Montes de Oca Luna et al., 1995). This lethality is attributed to aberrant levels of p53-mediated apoptosis (Chavez-Reyes et al., 2003). These findings suggest that the chief role of Mdm2 during embryonic development is to restrain p53 activity. Mice expressing a hypomorphic allele of Mdm2 have increased p53-dependent apoptosis, are radiosensitive, and show defects in hematopoiesis, indicating that Mdm2-mediated inhibition of p53 is required post development in adult tissues (Mendrysa et al., 2003).



**Figure1.2. Full-length Mdm2 and Mdmx domains.** Both proteins have a p53 binding domain is located on the N-terminal followed by a central acidic domain; zinc-finger domain; and a C-terminal RING-finger domain. In Mdm2, the p53 binding domain is followed by the NLS, nuclear localization signal and NES, nuclear export signal. The numbers denote amino acid numbers

Another protein known as MdmX also negatively inhibits p53 function, but does not trigger p53 degradation since it lacks E3 ligase activity (Linares et al., 2003). However, MdmX heterodimerizes with Mdm2 via the RING domains and this enhances Mdm2-dependent ubiquitination and degradation of p53 (Linares et al., 2003). MdmX also shares sequence similarity with Mdm2 especially within the N-terminal p53-binding domain (Figure 1.2). As such, MdmX also has the ability to bind p53 and blocks p53-mediated transactivation of gene expression (Shvarts et al., 1996). Like Mdm2<sup>-/-</sup> mice, MdmX<sup>-/-</sup> mice are embryonic lethal, albeit at a later developmental stage compared to Mdm2<sup>-/-</sup> mice (that is E7.5-8.5), and this lethality is also rescued by loss of p53 (Parant et al., 2001). This implies Mdm2 and MdmX possess some independent functions. Mdm2 and MdmX genes have p53 responsive elements and can be induced by p53. Activation of

both Mdm2 and MdmX by p53 demonstrates the existence of a negative feedback loop that restores basal p53 levels post exposure to stress. Moreover, both Mdm2 and MdmX are expressed at high levels in various human tumors indicating that these proteins are oncogenes.

Some cancers do not express p53-target genes despite expressing wild type p53 suggesting that mechanisms that inhibit p53-mediated transcription are pivotal in promoting tumorigenesis. This is consistent with the observations that most tumors that harbor wild type p53 also have Mdm2 and MdmX gene amplifications (Shibagaki et al., 1995, 2008). Furthermore, some cancers do not harbor gene amplification, but have elevated protein levels of both Mdm2 and MdmX such as in retinoblastoma (Laurie et al., 2006) and melanoma (Gembarska et al., 2012). In sum, Mdm2- and MdmX-mediated inhibition of p53 is critical both during development and post development in adult tissues. Additionally, p53 inhibition due to Mdm2 and MdmX gene amplifications or protein overexpression promote tumorigenesis. This implies that modifications on Mdm2 and/or MdmX that impede their action on p53 have the potential to delay tumor development and/or progression.

### **Phosphorylation of p53**

Posttranslational modifications of p53 especially phosphorylation are thought to regulate p53 stabilization and function (Meek, 1994). *In vitro*, various kinases

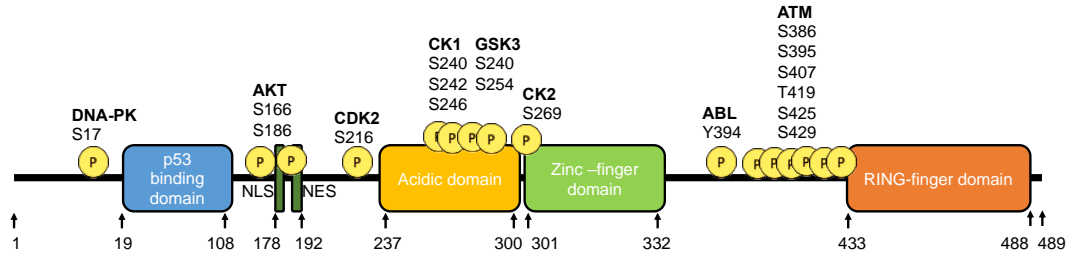
have been shown to phosphorylate p53 on numerous serine and threonine residues within the N- and C-terminal domains (Prives, 1998). N-terminal phosphorylation at conserved residues like S15 (mouse S18) and S20 (mouse S23) were proposed to stabilize p53 by inhibiting the interaction between p53 and Mdm2 (Appella and Anderson, 2001, Shieh et al., 1997). Both residues are phosphorylated following DNA damage by kinases including ATM and ATR. In particular, the phosphorylation of mouse p53 at S15 by ATM promotes p53 stabilization following DNA damage by abrogating Mdm2 inhibition of p53 *in vitro* (Shieh et al., 1997, Saito et al., 2002). This gave rise to the idea that post translational modifications on residues within the p53 N-terminus that interacts with Mdm2 are crucial for regulating p53 activation following stress.

However, loss of p53 phosphorylation on S15 mediated by ATM in mice (p53<sup>S18A</sup>) causes mild effects in apoptosis but does not alter p53 activation and stabilization along with susceptibility to tumorigenesis in mice (Toledo and Wahl, 2006, Chao et al., 2006, Sluss et al., 2004). The phosphorylation of p53 on S23, was also shown to have adverse effects of p53 function in irradiated cells *in vitro* (MacPherson et al., 2004). However, p53<sup>S23A</sup> mice also displayed mild phenotypes (Wu et al., 2002, MacPherson et al., 2004). Double mutant p53<sup>S18A,S23A</sup> mice showed a more severe defect in mounting p53-dependent apoptosis in response to DNA damage and tumorigenesis than either p53<sup>S18A</sup> or p53<sup>S23A</sup> mice (Chao et al., 2006), suggesting the two phosphorylation sites have

synergistic functions. These studies demonstrated that post translational modifications on p53 TADs shown to stabilize and activate p53 *in vitro* do not fully stabilize and activate p53 *in vivo* (Ashcroft et al., 2000, Blattner et al., 1999). Therefore, it was hypothesized that post translational modifications of other proteins that regulate p53 function contribute to p53 activation and stabilization following exposure to stress *in vivo*.

### **Phosphorylation of Mdm2**

Several phosphorylation target sites on Mdm2 have been proposed to inhibit or activate p53 function (Figure 1.3). ATM and Chk2 are activated in response to DNA damage and phosphorylate numerous serine residues on Mdm2 closer to the RING domain (Meek and Hupp, 2010). Rapid phosphorylation of Mdm2 by ATM following DNA damage result in p53 stabilization *in vitro* (Maya et al., 2001), partly by interfering with Mdm2 RING domain interactions required for Mdm2 oligomerization and the subsequent ligase activity (Cheng et al., 2009). Conversely, dephosphorylation of Mdm2 by phosphatases such as Wip1 enhances Mdm2 stability resulting in p53 ubiquitination and degradation (Lu et al., 2007).



**Figure 1.3. Proposed phosphorylation sites on hMdm2 protein sequence.** Except for phosphorylation by Akt (S166 and S186) and CK2 (S269), all the other phosphorylation events have been proposed and/or reported to diminish Mdm2-mediated p53 inhibition. Residues are numbered like in human Mdm2 protein and not all phosphorylation sites are shared between humans and mice.

Our lab generated Mdm2 phosphorylation site mutants to study the effects of ATM and c-Abl kinases on Mdm2-p53 signaling following stress. In response to DNA damage, ATM rapidly phosphorylates Mdm2 at S395 (mouse S394) prior to the accumulation of p53 *in vitro* (Maya et al., 2001). In response to DNA damage, mice expressing Mdm2 that cannot be phosphorylated at S394 (Mdm2<sup>S394A</sup>) failed to stabilize p53, trigger p53-dependent target gene expression, induce cell cycle arrest, and apoptosis (Gannon et al., 2012). As a result, Mdm2<sup>S394A</sup> mice are resistant to the effects of ionizing radiation and also more susceptible to spontaneous tumorigenesis compared with controls. Additionally, Mdm2<sup>S394A</sup> mice have an increased susceptibility to lymphomagenesis induced by Eμ-myc but not by ionizing radiation (Carr et al., 2016a), thereby demonstrating contrasting functions of Mdm2-S394 phosphorylation in tumor suppression. Conversely, mice expressing Mdm2-S394 phosphomimic (Mdm2<sup>S394D</sup>) exhibited prolonged p53 stabilization and activity in

response to DNA damage, and were not susceptible to spontaneous tumorigenesis (Gannon et al., 2012).

Another kinase that phosphorylates Mdm2 in response to DNA damage is c-Abl, which is phospho-activated by ATM (Baskaran et al., 1997, Shafman et al., 1997). Alternatively, c-Abl can also phospho-activate ATM following DNA damage (Wang et al., 2011). *In vitro*, c-Abl phosphorylation of Mdm2 promotes p53 activation and stability leading to cell cycle arrest after DNA damage (Sawyers et al., 1994, Wen et al., 1996). Additionally, c-Abl increases p53 activity by reducing Mdm2-mediated degradation of p53 (Sionov et al., 1999). Moreover, it was shown that Mdm2-Y393 phosphorylation by c-Abl enhances p53-mediated gene transcription and apoptosis in cells (Goldberg et al., 2002).

To explore the effects of c-Abl-mediated phosphorylation of Mdm2 on p53-dependent responses to stress *in vivo*, our lab generated mice expressing Mdm2 that cannot be phosphorylated at Y393 (Mdm2<sup>Y393F</sup>) (Carr et al., 2016b). In Mdm2<sup>Y393F</sup> mice, p53 stability remains unaltered following DNA damage and these mice are protected from bone marrow failure. However, Mdm2<sup>Y393F</sup> mice were susceptible to spontaneous and oncogene-induced tumorigenesis (Carr et al., 2016b). In double mutant Mdm2<sup>Y393F/S394A</sup> mice generated to study the impact of combined Mdm2-393 phosphorylation by c-Abl and Mdm2-S394 phosphorylation by ATM, tumor latency is similar to that of Mdm2<sup>S394A</sup> mice.

Additionally, the response to ionizing radiation in Mdm2<sup>Y393F/S394A</sup> mice is also similar to that observed in Mdm2<sup>S394A</sup> mice (Carr et al., 2016b). These findings suggest that the phosphorylation of Mdm2 by ATM at S394 has a stronger effect on Mdm2-p53 signaling in response to DNA damage than Mdm2-Y393 phosphorylation by c-Abl. These studies using double mutant mice imply no additive effects and this further suggests that Mdm2 phosphorylation by various stress-induced kinases can differentially alter the p53 response to stress.

### **Regulation of Mdm2-p53 signaling by Akt**

Akt is a serine/threonine kinase and there exist three conserved isoforms namely Akt 1, 2, and 3. Akt signals downstream of phosphoinositide 3-kinase (PI3K) which is lipid kinase implicated in cellular transformation and the insulin response. Akt becomes fully activated by phosphorylation at T308 by PDK1 (Mora et al., 2004, Calleja et al., 2009) and at S473 by mTORC2 (Sarbasov et al., 2005). Activated Akt phosphorylates a myriad of substrates containing minimal consensus recognition motif of R-X-R-X-X-S/T- $\phi$  (where X is any amino acid and  $\phi$  denotes a preference for large hydrophobic residues) (Alessi et al., 1996). These substrates include FOXO (Brunet et al., 1999), TSC2 (Potter et al., 2002), and GSK3 (Cross et al., 1995). Phosphorylation of proteins downstream of Akt generally inhibits their function, which often results in mTORC1 activation and enhanced cell survival, growth, metabolism, and proliferation (Georgescu, 2010). As such, mutations that render Akt constitutively active, such as somatic



oncogenic mutations in upstream EGFR (Ekstrand et al., 1992), HER2 (Holbro et al., 2003), and PIK3CA (Samuels et al., 2004), have all been implicated in promoting tumorigenesis. Comparably, inactivating mutations or loss-of-heterozygosity in tumor suppressor genes such as the PI3K inhibitor PTEN, also promote constitutive activation of Akt (Wee et al., 2008).

Transfection based studies demonstrated that Mdm2 is phosphorylated by Akt at S166 and S186 (mouse S163 and S183) (Mayo and Donner, 2001, Ogawara et al., 2002). Mdm2 phosphorylation at either residue or both enhances Mdm2 translocation to the nucleus. In addition, Her-2-neu overexpression activates Akt and this increases Mdm2-mediated p53 ubiquitination and degradation (Zhou et al., 2001). Moreover, Mdm2 phosphorylation by Akt inhibits Mdm2 auto-ubiquitination which enhances Mdm2 stability thereby promoting p53 degradation (Feng et al., 2004). Interestingly, cancer cells harboring constitutively activated Akt are sensitive to Mdm2 inhibitors (Cipriano et al., 2010). Therefore, understanding the effects of Mdm2 phosphorylation by Akt under physiological conditions is crucial, especially in the context of tumor cell signaling.

## **Aims of this dissertation**

In this dissertation I will present the work I performed investigating the physiological significance of Mdm2 phosphorylation by Akt *in vivo*. To this end, I generated two Mdm2 mutant mouse models that cannot be selectively phosphorylated at S163 and S183 on both Mdm2 alleles (Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> respectively). Since Mdm2 phosphorylation by Akt reduced Mdm2-mediated inhibition of p53 (Zhou et al., 2001, Mayo and Donner, 2001, Feng et al., 2004), I hypothesized that Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mice will express elevated levels of p53, and similar to Mdm2<sup>-/-</sup> mice, will therefore exhibit developmental defects and/or embryonic lethality. As such, my first aim in this dissertation was to examine how Mdm2 phosphorylation by Akt affects development.

Well established functions of p53 in response to stress include the induction of cell cycle arrest, senescence, and apoptosis. For that reason, the second aim of this dissertation was to study whether Akt phosphorylation of Mdm2 at endogenous levels alters cell growth and proliferation following exposure to plating stress and DNA damage. To this end, I utilized Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mouse embryonic fibroblasts (MEFs) to examine the effects on cell proliferation and p53-mediated DNA damage responses *in vitro*. Since Mdm2<sup>S183A</sup> MEFs displayed most overt phenotype under conventional cell culture conditions, I then

focused on exploring the effects of only Mdm2-S183 phosphorylation on DNA damage responses.

My third aim was to determine the impact Mdm2-S183 phosphorylation by Akt has on tumor suppression *in vivo*. I utilized three different mouse models to look at (i) DEN-induced liver cancer, (ii) DMBA/TPA-induced skin cancer, and (iii) lung cancer caused by the expression of oncogenic *K-ras*. With these models, I examined how Mdm2-S183 affects p53-dependent mechanisms known to mediate tumor suppression.

Lastly, my fourth aim was to investigate how Mdm2-S183 phosphorylation affects glucose metabolism. As mentioned previously, mice that fail to induce canonical p53 tumor suppressor functions are still able to properly regulate various aspects metabolism. Therefore, it is plausible that p53-dependent alterations in glucose metabolism may contribute to tumor suppression. To this end, I employed both *in vitro* and *in vivo* assays to examine whether Mdm2<sup>S183A</sup> mice exhibited altered glucose metabolism compared with Mdm2<sup>WT</sup> mice.

## **CHAPTER II: ROLE OF MDM2-S183 PHOSPHORYLATION IN DEVELOPMENT AND STRESS RESPONSES**

## Introduction

Mdm2<sup>-/-</sup> mice are embryonic lethal around E4.5-E6.5, and this lethality is rescued by loss of p53 (Jones et al., 1995, Montes de Oca Luna et al., 1995). This is unlike p53<sup>-/-</sup> mice which develop normally into adulthood but are more susceptible to tumorigenesis (Donehower et al., 1992). Cultured Mdm2<sup>-/-</sup> embryos show evidence of apoptosis (Chavez-Reyes et al., 2003), suggesting that loss Mdm2-mediated inhibition of p53 is necessary during mouse development. Since Mdm2 S163 and/or S183 phosphorylation by Akt reduces p53 activity in transfection based assays, I explored whether at endogenous levels, Mdm2-S183 and -S163 affects mouse development. I found that Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mice are viable, fertile, and obtained at Mendelian ratios. In addition, I also show that unchallenged Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mice are phenotypically indistinguishable from Mdm2<sup>WT</sup> mice. These findings show that Mdm2 phosphorylation at S163 or S183 is not required for development in mice.

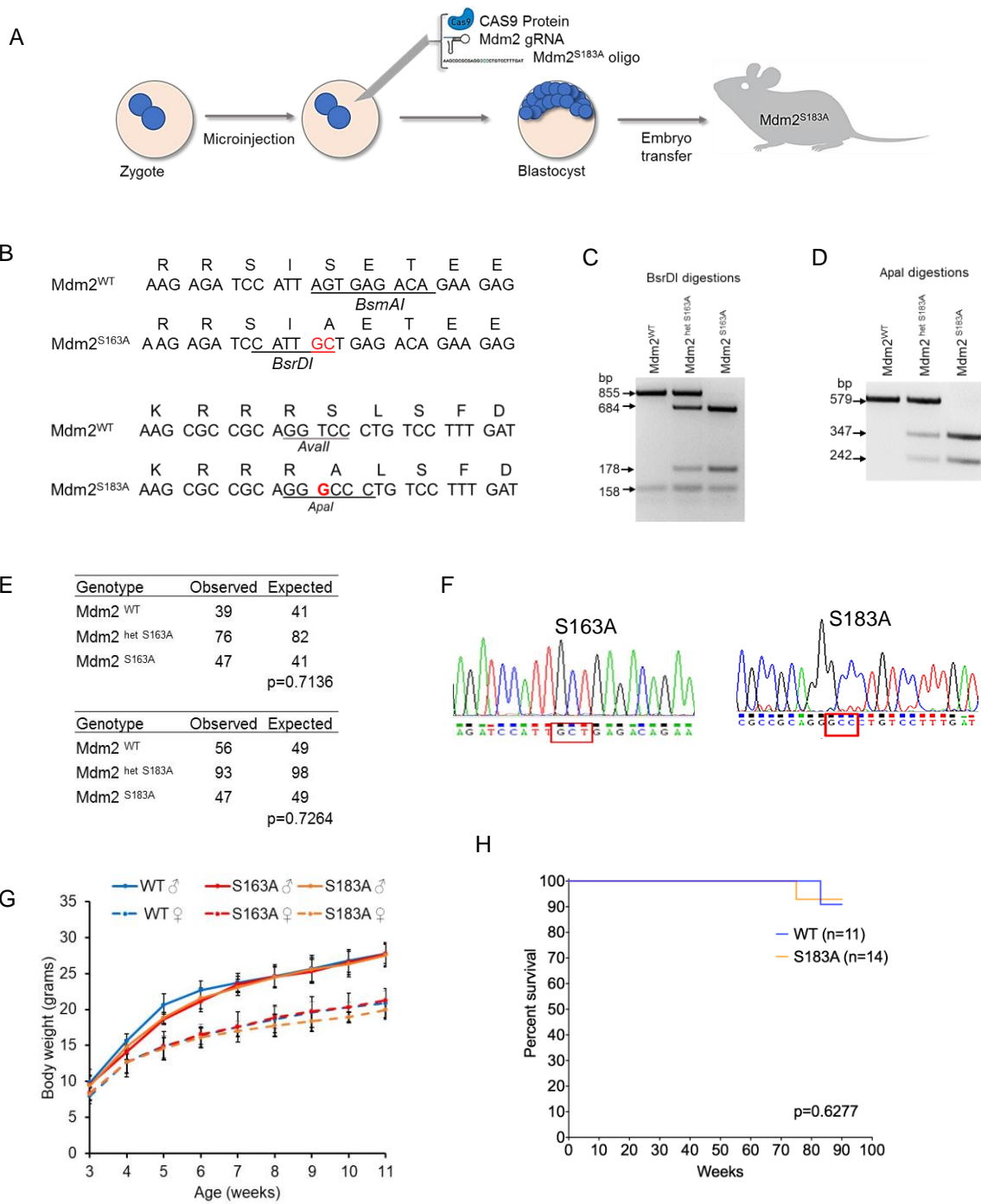
I also postulated that loss of Mdm2-S163 and -S183 phosphorylation by Akt at physiological levels increases p53 levels and activity. As a result, Mdm2<sup>S183A</sup> and Mdm2<sup>S163A</sup> MEFs will display altered p53 function following exposure to plating stress and DNA damage. I will show that Mdm2<sup>S183A</sup> MEFs, but not Mdm2<sup>S163A</sup> MEFs, failed to proliferate due to premature senescence. Mdm2<sup>S183A</sup> MEFs also expressed higher levels of p53 and p53-target genes when cultured at high oxygen and these phenotypes were reversed at low

oxygen. Furthermore, I will demonstrate that Mdm2-S183 phosphorylation at endogenous levels enhanced Mdm2 nuclear localization and stability, and lowered p53 levels. These results are consistent with what was observed in studies using transfection-based assays. To my knowledge, this is the first time showing that phosphorylation of Mdm2-S183 by Akt endogenous protect cells from p53-mediated senescence induced by oxidative stress.

Mdm2 phosphorylation by different DNA damage-induced kinases dictate the type of p53-dependent responses (Gannon et al., 2012b, Carr et al., 2016b). Therefore, I explored whether Mdm2-S183 phosphorylation by Akt affects p53-dependent DNA damage response. In this chapter, I will demonstrate that Mdm2-S183 phosphorylation does not alter p53 activation and stabilization following acute genotoxic stress. Additionally, Mdm2-S183 phosphorylation does not impact p53-mediated cell arrest *in vitro* after treatment with various DNA damage-inducing agents. Furthermore, Mdm2 phosphorylation by Akt does not alter p53-dependent radiosensitivity and DNA-damage induced apoptosis *in vivo*. My data indicates that the phosphorylation of Mdm2 does not control p53-mediated responses to DNA damage *in vitro* and *in vivo*.

## Results

To generate mice expressing Mdm2 that cannot be phosphorylated at S163 and S183, pseudopregnant mice received manipulated blastocysts from zygotes injected with Cas9 mRNA, gRNAs with complementary sequences to regions harboring S163 and S183 residues, and 100mer oligonucleotides in which S163 or S183 are substituted with alanine residues (Figure 2.1a and b). Substituting S163 and S183 with alanine residues also introduced new restriction enzyme cut sites (Figure 2.1b). Therefore, to confirm germline transmission of the intended missense mutations in the Mdm2 sequence, genomic DNA flanking the S163 and S183 was amplified using PCR followed by restriction enzyme digestion with *BsrDI* for S163A mice and *ApaI* for S183A mice (Figure 2.1b and c). Progeny from heterozygous Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> intercrosses were obtained at Mendelian ratios (Figure 2.2e), and male and female progeny were recovered at similar frequencies. In addition, homozygous Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mice were viable and fertile. Furthermore, DNA sequencing confirmed the presence of the intended mutations and absence of off target mutations in the Mdm2 sequence (Figure 2.1e). Importantly, Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mice were backcrossed to wild type mice for six and nine generations respectively, in an effort to get rid of potential off target mutations. Taken together, this data shows that Mdm2-S163 and -S183 phosphorylation is not required for development in mice.





**Figure 2.1. Mdm2-S163 and -S183 phosphorylation is dispensable for development.** (A) Schematic representation of the CRISPR-Cas9 based method used to generate of Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mice. (B) Sequences of WT and mutant alleles for Mdm2 codon 163 and 183 (C) Agarose gel electrophoresis image showing BsrDI digestion of DNA amplified by PCR using primers flanking the 163 codon of Mdm2. (D) Agarose gel electrophoresis image showing Apal digestion of DNA amplified by PCR using primers flanking Mdm2 183 codon. (E) Numbers of progeny from heterozygous intercrosses for both Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> alleles. (F) Sequencing of Mdm2 DNA regions harboring Mdm2 S163 and S183 codons. (G) Weight comparison between unchallenged Mdm2<sup>WT</sup> (n=16), Mdm2<sup>S163A</sup> (n=15) and Mdm2<sup>S183A</sup> (n=16) mice. (H) Survival of Mdm2<sup>WT</sup> (n=11) and Mdm2<sup>S183A</sup> mice (n=14).

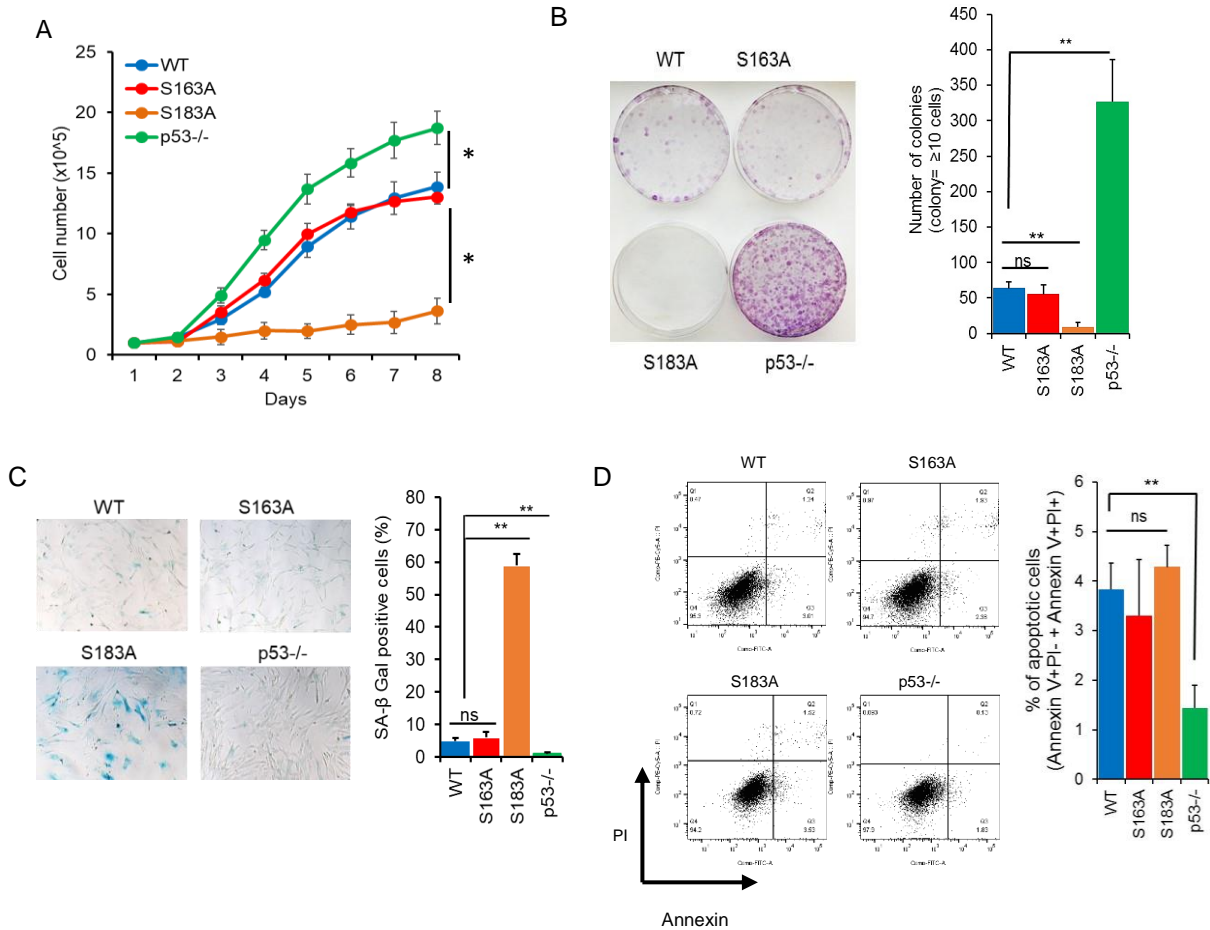
Loss of Mdm2 function in the skin in mice results in elevated levels of p53 and this triggers premature aging in the epidermis (Gannon et al., 2011).

Additionally, mice with chronically higher levels of p53 show signs of premature aging whilst being less susceptible to tumorigenesis (Tyner et al., 2002). In contrast, several transgenic mice with higher levels of p53 do not show signs of premature aging, but are resistant to tumorigenesis similar to the aging prone models (Garcia-Cao et al., 2002, Matheu et al., 2007). This suggests that p53's role in aging is influenced by the genetic background of these mice.

Next, I tested whether loss of Akt-dependent phosphorylation of Mdm2 may induce signs of premature aging such as weight loss and shortened lifespan, possibly due to elevated p53. However, unchallenged Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mice weights were comparable to Mdm2<sup>WT</sup> mice (Figure 2.1g). Additionally, there was no significant difference between the survival of Mdm2<sup>WT</sup> and that of Mdm2<sup>S183A</sup> mice (Figure 2.1h). This suggests that phosphorylation of

Mdm2 by Akt at S183 does not promote premature aging or alter longevity in unchallenged mice. Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mice did not phenocopy Mdm2<sup>-/-</sup> mice. Therefore, it is possible that p53 levels and activity in unchallenged Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice may not be significantly different.

Next, I examined whether phosphorylation of Mdm2-S163 and -S183 alters cell proliferation. To this end, I used early passage MEFs generated from Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mice and performed cell growth and proliferation assays. Surprisingly, Mdm2<sup>S183A</sup> MEFs significantly failed to proliferate compared with Mdm2<sup>WT</sup>, Mdm2<sup>S163A</sup>, and p53<sup>-/-</sup> MEFs (Figure 2.2a). Additionally, Mdm2<sup>S183A</sup> MEFs failed to form colonies following low-density stress plating (Figure 2.2 b). Intriguingly, Mdm2<sup>S183A</sup> MEFs displayed salient features of senescence which include the apparent cessation of cell growth in culture and flattened morphology. I therefore stained MEFs for senescence-associated  $\beta$ -galactosidase and the amount of cells that stained positive for senescence-associated  $\beta$ -galactosidase were significantly higher in Mdm2<sup>S183A</sup> MEFs compared with Mdm2<sup>WT</sup>, Mdm2<sup>S163A</sup>, and p53<sup>-/-</sup> MEFs (Figure 2.2c). Since these MEFs were passage 3 and Mdm2<sup>WT</sup> MEFs enters replicative senescence after about 6 passages (Groisman et al., 2006), this data demonstrated that Mdm2<sup>S183A</sup> MEFs were prone to premature senescence.

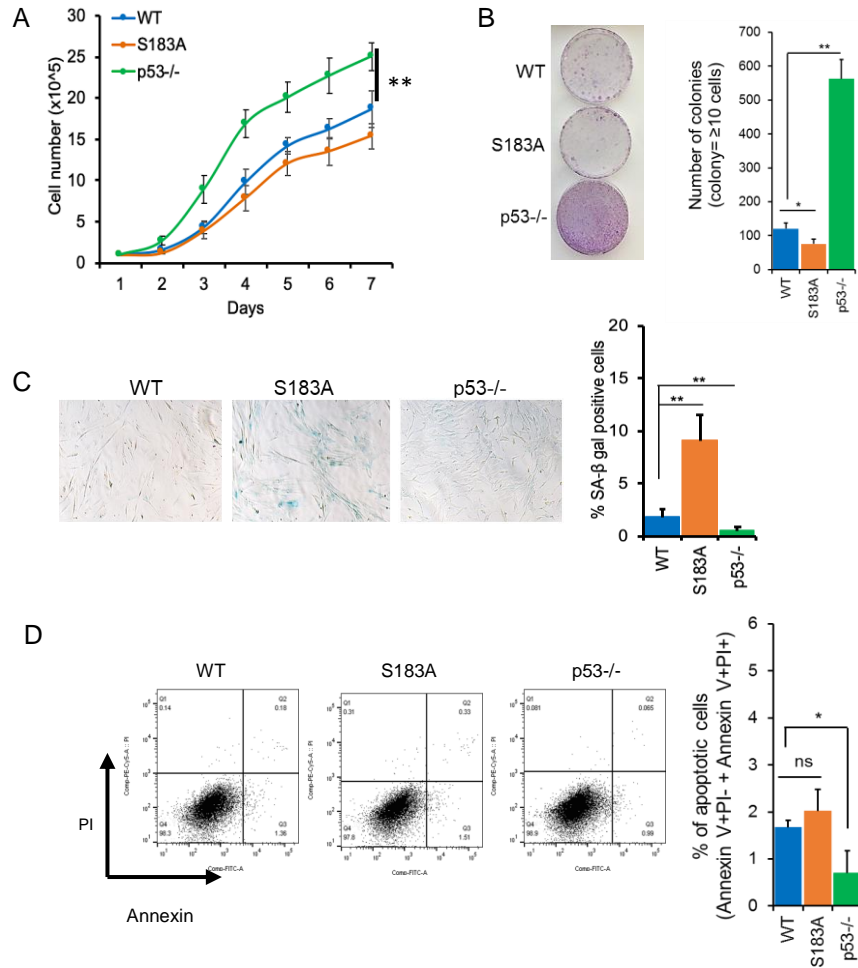


**Figure 2.2. Mdm2 S183 phosphorylation is required for cell proliferation**

(A) Proliferation of passage 3 Mdm2<sup>WT</sup>, Mdm2<sup>S163A</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> mouse embryonic fibroblasts cultured at 21% oxygen. Student's T test \*\*p<0.01 (n=3). (B) Representative images for colony formation assay (left) and colony quantification (right) performed using passage 2 Mdm2<sup>WT</sup>, Mdm2<sup>S163A</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> embryonic fibroblasts. Data is expressed as mean ± standard deviation. Student's T test \*\*p<0.01 (n=3). (C) Representative images showing results for senescence-associated β-galactosidase staining (left) and quantification (right) of positively stained passage 3 Mdm2<sup>WT</sup>, Mdm2<sup>S163A</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> mouse embryonic fibroblasts cultured at 21% oxygen. Data is expressed as mean ± standard deviation. Student's T test \*p<0.05 and \*\*p<0.01 (n=3). (D) Flow cytometry analyses (left) and quantification of apoptotic cells (right) using Annexin V and propidium iodide staining in passage 3 Mdm2<sup>WT</sup>, Mdm2<sup>S163A</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> mouse embryonic fibroblasts cultured at 21% oxygen. Data is expressed as mean ± standard deviation. Student's T test \*\*p<0.01 (n=3).

Wild type MEFs are generally resistant to apoptosis and often undergo p53-mediated G1 arrest when exposed to stress like DNA damage (Kastan et al., 1992). However, expression of adenovirus E1A oncoprotein, c-Myc, and E2F1 can trigger p53-mediated apoptosis in wild type MEFs following DNA damage (Hermeking and Eick, 1994, Debbas and White, 1993, Qin et al., 1994). Although unlikely, I explored the possibility that Mdm2<sup>S183A</sup> MEFs were failing to proliferate partly due to apoptosis triggered by plating stress. Annexin V/PI staining showed that the percentage of apoptotic cells in Mdm2<sup>S183A</sup> MEFs was not significantly different to that in Mdm2<sup>WT</sup> and Mdm2<sup>S163A</sup> MEFs (Figure 2.2d), suggesting that the observed proliferation defect in Mdm2<sup>S183A</sup> MEFs is not due to apoptosis.

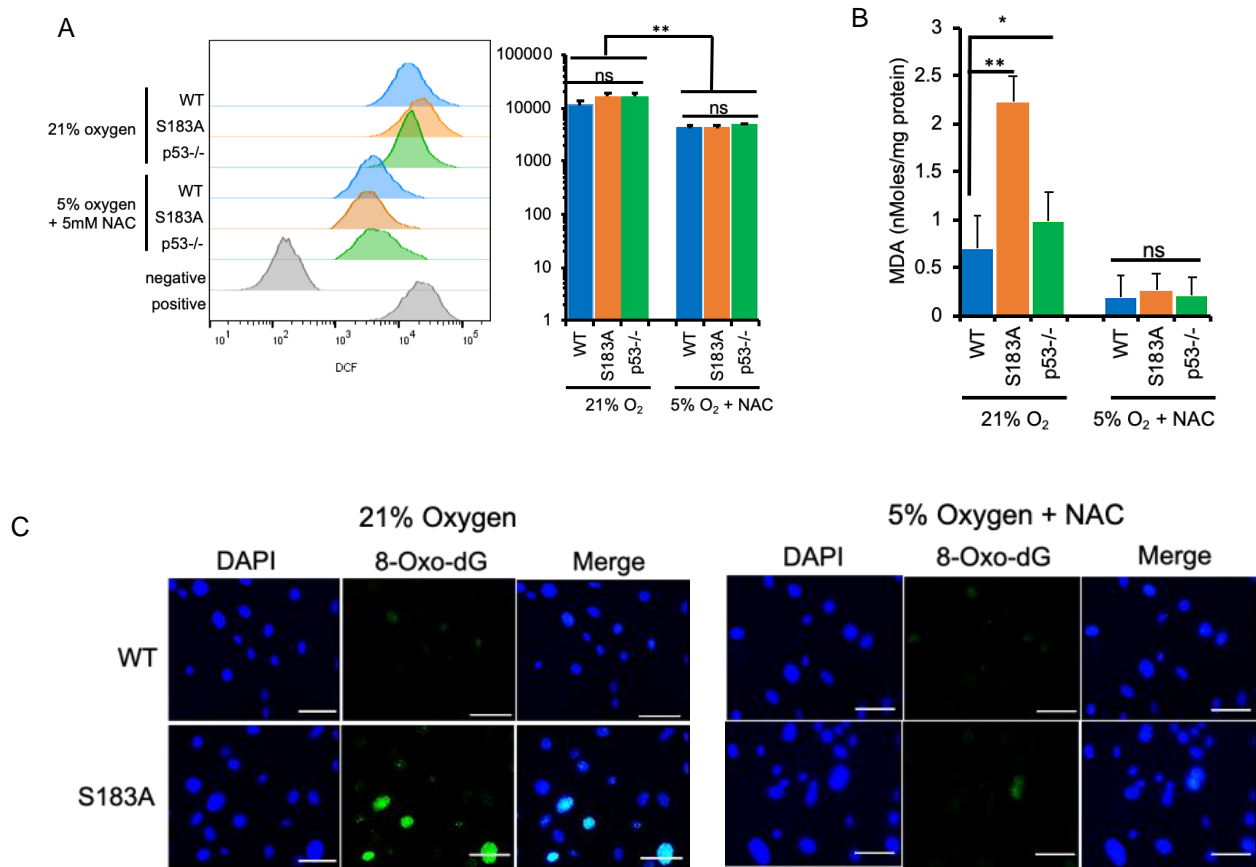
Senescence can be triggered by oncogene overexpression, telomere erosion, DNA damage from reactive oxygen species, and inflammation (McHugh and Gil, 2018). Since mice have long telomeres and MEFs used here were not transduced with oncogenes, I posited that Mdm2<sup>S183A</sup> MEFs were senescent due to oxidative stress. I cultured Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs under low oxygen conditions (5% oxygen) in the presence of a reactive oxygen species scavenger N-acetyl cysteine (NAC). Proliferation and colony formation defects observed in Mdm2<sup>S183A</sup> MEFs cultured at ~21% oxygen were rescued by culturing these MEFs at low oxygen (Figure 2.3a-b).



**Figure 2.3. Normoxic conditions rescue Mdm2<sup>S183A</sup> MEFs from senescence**  
 (A) Proliferation of passage 3 Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> mouse embryonic fibroblasts cultured at 5% oxygen in media supplemented with 5mM N-acetyl cysteine. Student's T test \*\*p<0.01 (n=3). (B) Representative images for colony formation assay (left) and Colony quantification (right) of passage 2 Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> mouse embryonic fibroblasts cultured at 5% oxygen in media supplemented with 5mM N-acetyl cysteine. Data is expressed as mean ± standard deviation. Student's T test \*p<0.05 and \*\*p<0.01 (n=3). (C) Staining for senescence-associated β-galactosidase (left) and quantification (right) of positively stained Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> mouse embryonic fibroblasts cultured at 5% oxygen in media supplemented with 5mM N-acetyl cysteine. Data is expressed as mean ± standard deviation. Student's T test \*\*p<0.01 (n=3). (D) Flow cytometry analyses (left) and quantification of apoptotic cells (right) using Annexin V and Propidium iodide staining in passage 3 Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> mouse embryonic fibroblasts cultured at 5% oxygen in media supplemented with 5mM N-acetyl cysteine. Data is expressed as mean ± standard deviation. Student's T test \*p<0.05 (n=3).

In addition, culturing Mdm2<sup>S183A</sup> MEFs at low oxygen also reduced amount of premature senescent cells by about 40% of what was observed at high oxygen (Figure 2.3c). There was no significant difference in the percentage of apoptotic cells in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs cultured at low oxygen MEFs (Figure 2.3d). Therefore, the rescue of proliferation defects in Mdm2<sup>S183A</sup> MEFs by low oxygen culture indicates that Mdm2 phosphorylation at S183 prevents premature senescence in the presence of oxidative stress.

Next, I performed a 2',7'-dichlorofluorescein diacetate (DCFDA) staining assay to test whether Mdm2<sup>S183A</sup> MEFs had higher levels of intracellular ROS when cultured under high oxygen compared to low oxygen (Figure 2.4a). DCFDA is a cell-permeant and fluorogenic dye used to determine the amount of hydroxyl, peroxy and other reactive oxygen species (ROS) in the cell. Both at high and low oxygen, the levels of ROS in Mdm2<sup>S183A</sup> MEFs were similar to those in Mdm2<sup>WT</sup> MEFs and p53<sup>-/-</sup> MEFs (Figure 2.4a). This indicates that the proliferation defect observed in Mdm2<sup>S183A</sup> MEFs cultured at high oxygen is not due to increased production of ROS.

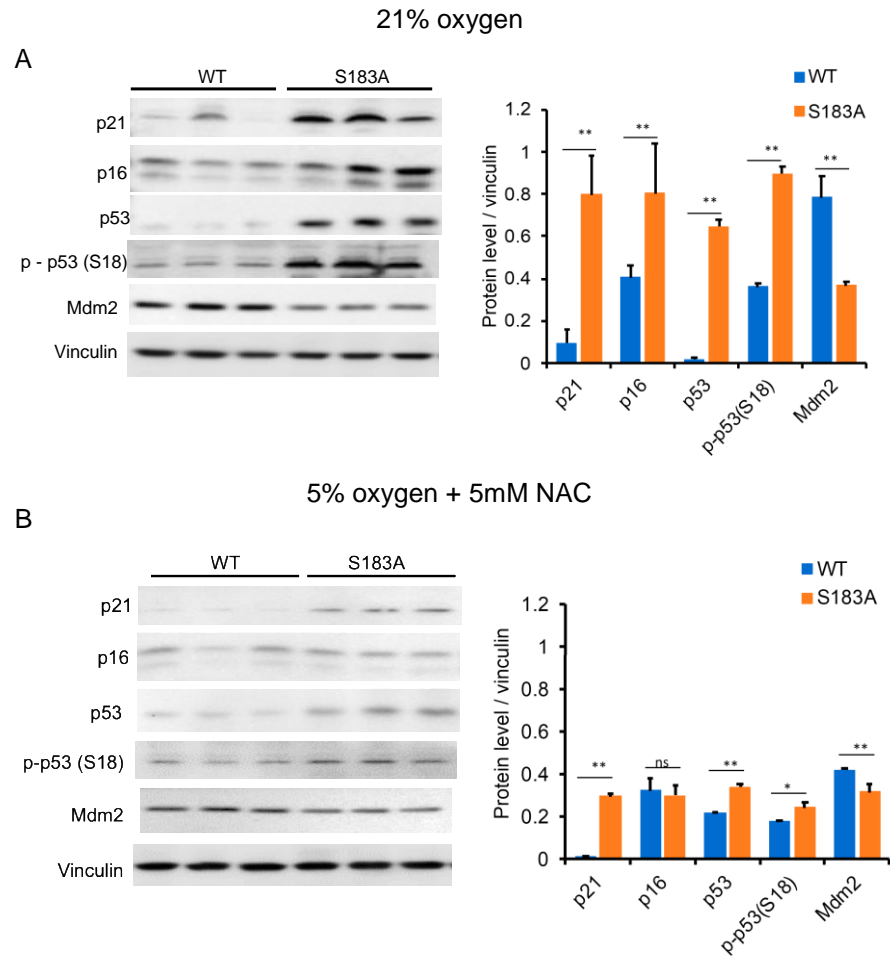


**Figure 2.4. Mdm2<sup>S183A</sup> MEFs are sensitive to oxidative stress.**

(A) Flow cytometry analyses (left) and quantification of DCF staining (right) in passage 3 Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs cultured at 21% oxygen and 5% oxygen in media supplemented with NAC. Data is expressed as mean fluorescence  $\pm$  standard deviation. Student's T test \*\*p<0.01 (n=3). (B) Lipid peroxidation assay measuring the quantity of MDA normalized to protein concentration for high and low oxygen passage 3 MEFs. Data is expressed as mean  $\pm$  standard deviation. Student's T test \*p<0.05 and \*\*p<0.01 (n=3). (C) Immunofluorescence staining for 8-Oxo-2'-deoxyguanosine in passage 3 Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs cultured either at 21% oxygen (left) or 5% oxygen (right) in media supplemented with NAC. Scale bar = 10 $\mu$ m.

Since the amount of ROS is similar between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs, it is possible that Mdm2<sup>S183A</sup> MEFs are sensitive to the levels of oxidative stress present in culture at high oxygen. Exposure to high levels of oxidative stress can cause damage to lipids and DNA in cells with compromised oxidative stress responses. Therefore I, quantified the amount of the lipid peroxidation by-product malondialdehyde (MDA) to determine whether Mdm2<sup>S183A</sup> MEFs are more susceptible to lipid peroxidation compared with Mdm2<sup>WT</sup> MEFs. The amount of MDA in Mdm2<sup>S183A</sup> MEFs was significantly higher than in Mdm2<sup>WT</sup> and p53<sup>-/-</sup> MEFs (Figure 2.4b). Additionally, the amount of 8-Oxo-2-deoxyguanosine, which is a marker of DNA damage induced by oxidative stress, was significantly elevated in Mdm2<sup>S183A</sup> MEFs compared with Mdm2<sup>WT</sup> MEFs (Figure 2.4c). These findings suggest that loss of Mdm2-S183 phosphorylation compromises oxidative stress responses in MEFs and sensitizes them to oxidative stress.



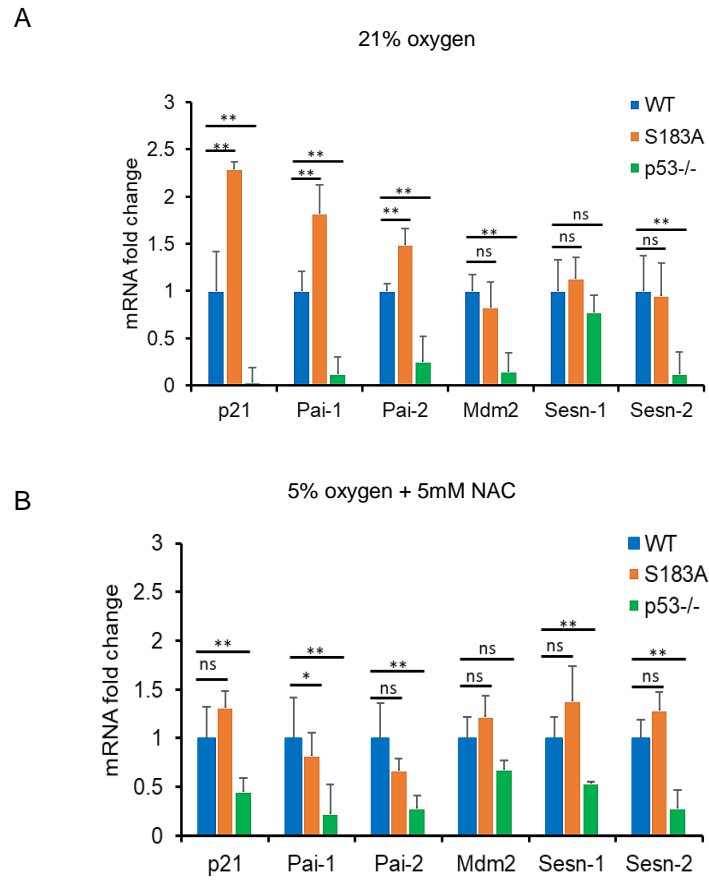


**Figure 2.5. Mdm2-S183 phosphorylation prevents premature senescence in MEFs** (A) Western blot analyses (left) and quantification of protein levels (right) in passage 3 Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> mouse embryonic fibroblasts cultured at 21% oxygen. Data is expressed as mean  $\pm$  standard deviation. Student's T test \*\* $p < 0.01$  (n=3). (B) Western blot analyses (left) and quantification of protein levels in (right) passage 3 Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> mouse embryonic fibroblasts cultured at 5% oxygen in media supplemented with 5mM N-acetyl cysteine. Data is expressed as mean  $\pm$  standard deviation. Student's T test \* $p < 0.05$  and \*\* $p < 0.01$  (n=3).

Phosphorylation of Mdm2 at S163 and/or S183 enhances Mdm2-mediated degradation of p53 in overexpression studies (Zhou et al., 2001, Mayo and Donner, 2001). To determine whether p53 levels are altered in Mdm2<sup>S183A</sup> MEFs, I performed western blot analysis using protein lysates extracted from MEFs cultured at either low and high oxygen. At high oxygen, the levels of p53 and activated p53 protein were significantly elevated in Mdm2<sup>S183A</sup> MEFs compared with Mdm2<sup>WT</sup> MEFs (Figure 2.5a). In contrast, Mdm2 protein levels were significantly lower in Mdm2<sup>S183A</sup> MEFs compared to Mdm2<sup>WT</sup> MEFs cultured at high oxygen. This suggests that phosphorylation of Mdm2 at S183 increases Mdm2 stability and degradation of p53 in the presence of oxidative stress.

In MEFs cultured at low oxygen in the presence of ROS scavenger N-acetyl cysteine, p53 and activated p53 (p-p53 (S18)) protein levels were still significantly higher in Mdm2<sup>S183A</sup> than in Mdm2<sup>WT</sup> MEFs (Figure 2.5b). However, the differences in levels of p53 and p-p53 (S18) between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> was significantly smaller at low oxygen compared to high oxygen, about 2-fold for p53 protein at low oxygen compared to 6-fold difference at high oxygen. The same trend was observed for activated p53. Additionally, Mdm2 protein levels were also still significantly higher in Mdm2<sup>WT</sup> MEFs than in Mdm2<sup>S183A</sup> MEFs, with this difference being more pronounced at high oxygen than at low oxygen as well. These results indicate that the effects of Mdm2-S183 phosphorylation on p53 expression and activation are diminished at low oxygen.

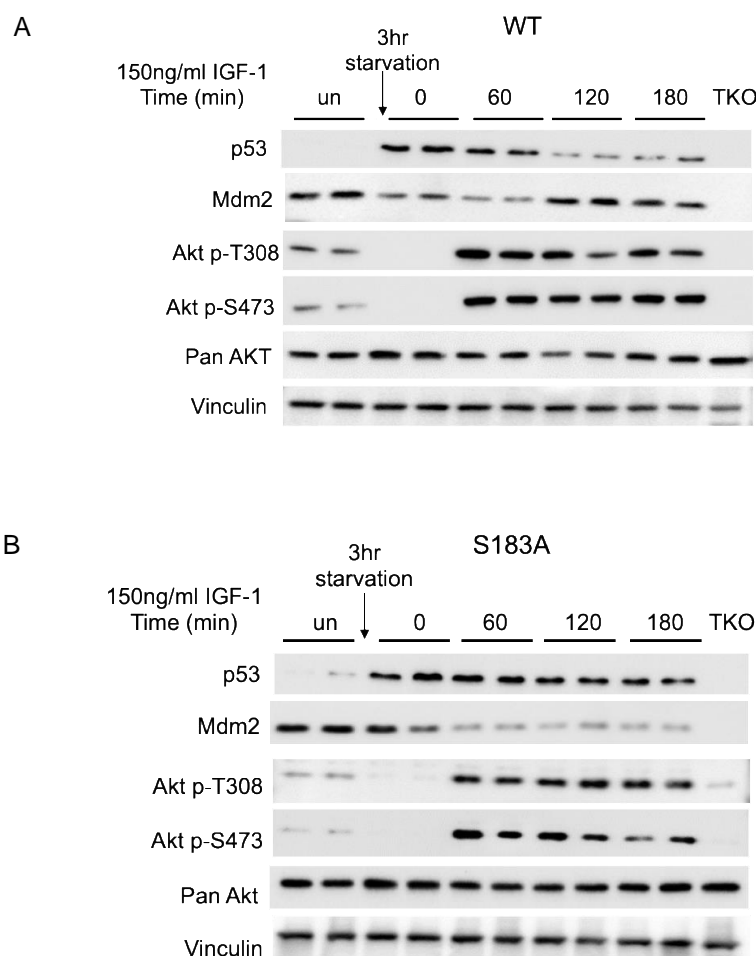
p21 is known to mediate p53-dependent senescence (Macleod et al., 1995). As a result, p21 protein and mRNA expression are often used as markers for p53-mediated senescence. Another frequently used senescence marker is the CDK4/6 inhibitor p16. In high oxygen MEFs, both p21 and p16 protein levels were significantly elevated in Mdm2<sup>S183A</sup> MEFs than in Mdm2<sup>WT</sup> MEFs (Figure 2.5a). However, at low oxygen, there was a significant difference in p21 protein levels, but not p16, between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs (Figure 2.5b). These results suggest that phosphorylation of Mdm2-S183 prevents premature senescence likely mediated by p53 in early passage MEFs cultured at high oxygen.



**Figure 2.6. Mdm2-S183 phosphorylation alters the expression of p53-target genes that mediate senescence.** (A) Relative mRNA expression levels for genes known to mediate senescence in passage 3 Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> mouse embryonic fibroblasts cultured at 21% oxygen. Data is expressed as mean  $\pm$  standard deviation. Student's T test \*\*p<0.01 (n=3). (B) Relative mRNA expression levels for genes known to mediate senescence in passage 3 Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> mouse embryonic fibroblasts cultured at 5% oxygen in media supplemented with 5mM N-acetyl cysteine. Data is expressed as mean  $\pm$  standard deviation. Student's T test \*p<0.05 and \*\*p<0.01 (n=3).

In addition to p21 and p16, several other genes controlled by p53 such as Sestrins and Pai-1 can mediate senescence. Therefore, I performed RT-qPCR to quantify the mRNA levels of these genes in low and high oxygen MEFs. At high oxygen, Mdm2<sup>S183A</sup> MEFs expressed significantly higher levels of p21, Pai-1, and Pai-2 genes than Mdm2<sup>WT</sup> MEFs (Figure 2.6a). At low oxygen, there were no significant differences in the mRNA levels of senescence-mediating genes in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs (Figure 2.6b). These results further support a role for Mdm2 phosphorylation at S183 in preventing p53-mediated premature senescence by inhibiting the induction of a subset of p53-target genes that mediate senescence.

Mdm2 phosphorylation by Akt enhances Mdm2 translocation to the nucleus and enhance p53 degradation in overexpression studies (Mayo and Donner, 2001, Zhang et al., 2018). Therefore, I performed western blot analysis using low oxygen MEFs stimulated with IGF-1 to determine whether phosphorylation of endogenous Mdm2 by Akt alters Mdm2-p53 signaling (Figure 2.7). In both Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs, stimulation with IGF-1 activated Akt as shown by the increase in Akt S473 and T308 phosphorylation (Figure 2.7a-b). In Mdm2<sup>WT</sup> MEFs, activation of Akt led to a reduction in p53 levels which was accompanied by an increase in Mdm2 levels (Figure 2.7a). In contrast, activation of Akt did not alter p53 levels in Mdm2<sup>S183A</sup> MEFs which remained elevated whilst Mdm2 levels declined. These findings suggest that activation of Akt enhanced Mdm2 stability and promotes degradation of p53.

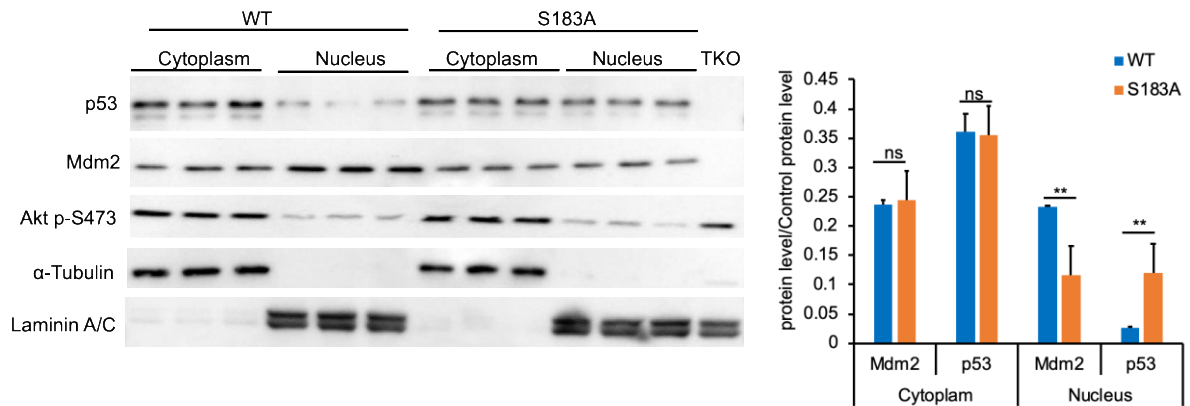


**Figure 2.7. Mdm2-S183 phosphorylation by Akt promotes Mdm2 stability and 53 degradation. (A)** Western blot analyses for passage 2 Mdm2<sup>WT</sup> embryonic fibroblasts cultured at 5% oxygen with N-acetyl cysteine. Cells were either left untreated, or nutrient starved for 3 hours and stimulated with 150ng/ml IGF-1 followed by protein extraction at the indicated time points. Triple knockout cells (TKO) are deficient for Mdm2, MdmX, and p53. **(B)** Western blot analyses for passage 2 Mdm2<sup>S183A</sup> mouse embryonic fibroblasts cultured at 5% oxygen with N-acetyl cysteine. Cells were either left untreated, or nutrient starved for 3 hours and stimulated with 150ng/ml IGF-1 followed by protein extraction at the indicated time points. Triple knockout cells (TKO) are deficient for Mdm2, MdmX, and p53.

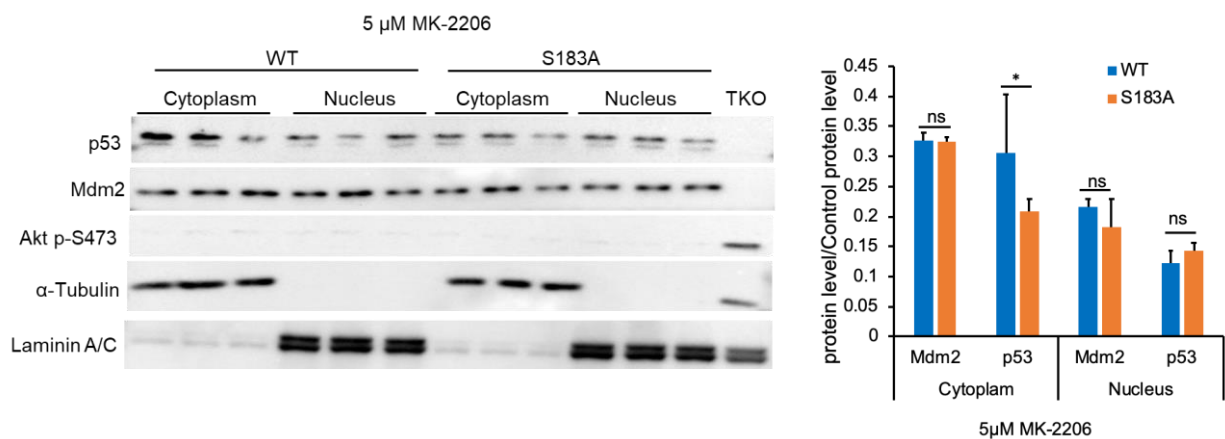
To determine whether activation of Akt alters the localization of Mdm2 and affects p53 levels, I performed western blots on nuclear and cytoplasmic protein lysates extracted from MEFs cultured at low oxygen and stimulated with IGF-1 for 2 hours (Figure 2.8). Mdm2 protein levels were significantly higher in the nucleus of Mdm2<sup>WT</sup> MEFs than in Mdm2<sup>S183A</sup> MEFs (Figure 2.8a), indicating that Akt activation promotes Mdm2 nuclear localization at endogenous levels. Additionally, stimulation of IGF-1 reduced p53 protein levels in the nucleus in Mdm2<sup>WT</sup> MEFs but not in Mdm2<sup>S183A</sup> MEFs. These findings demonstrate that Mdm2-S183 phosphorylation by Akt at endogenous levels promotes Mdm2 nuclear localization and p53 degradation.

To confirm that Akt phosphorylates Mdm2, alter Mdm2 localization and affect p53 levels, I blocked Akt activation using the Akt inhibitor MK-2206 and repeated the western analyses in figure 2.8a. There was no significant difference between the nuclear Mdm2 protein levels in Mdm2<sup>WT</sup> MEFs and Mdm2<sup>S183A</sup> MEFs (Figure 2.8b). This suggests that inhibition of Akt activation, which I infer to block Mdm2-S183 phosphorylation, diminishes Mdm2 nuclear translocation. Furthermore, the levels of nuclear p53 in Mdm2<sup>WT</sup> MEFs was comparable to those in Mdm2<sup>S183A</sup> MEFs, implying that Akt-mediated phosphorylation of Mdm2-S183 enhances Mdm2-dependent degradation of p53 in Mdm2<sup>WT</sup> MEFs.

A



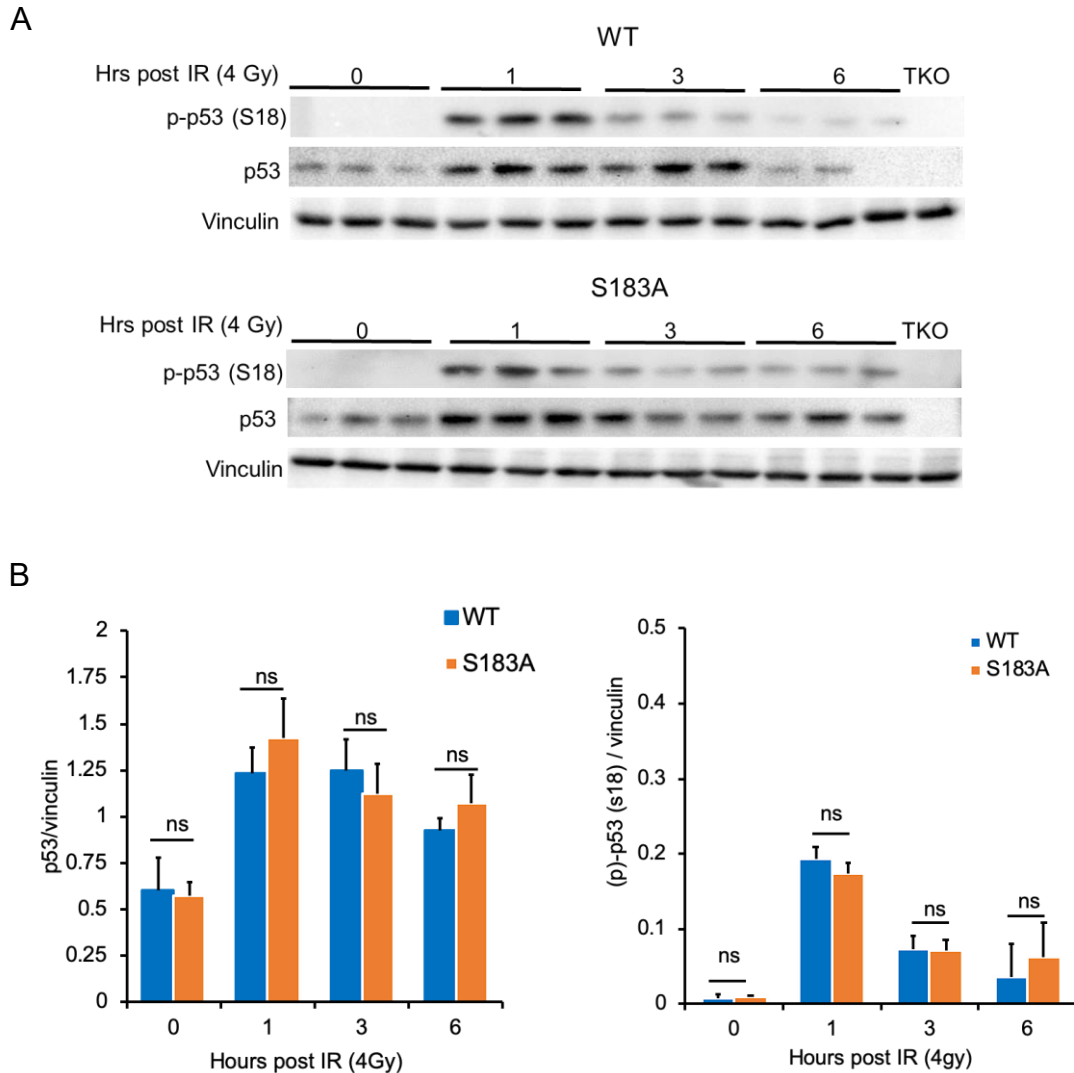
B



**Figure 2.8. Mdm2-S183 phosphorylation by Akt promotes Mdm2 nuclear localization and p53 degradation.** (A) Western blot analyses (left) and quantification of protein levels (right) for nuclear and cytoplasmic proteins extracted 2 hours post IGF-1 treatment from passage 2 Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mouse embryonic fibroblasts cultured at 5% oxygen with N-acetyl cysteine. Student's T test \*p<0.05 and \*\*p<0.01 (n=3). (B) Western blot analyses (left) and quantification of protein levels (right) for nuclear and cytoplasmic proteins extracted 2 hours post IGF-1 treatment in the presence of an Akt inhibitor MK-2206 (5 μM) from passage 2 Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mouse embryonic fibroblasts cultured at 5% oxygen with N-acetyl cysteine. Student's T test \*p<0.05 and \*\*p<0.01 (n=3).



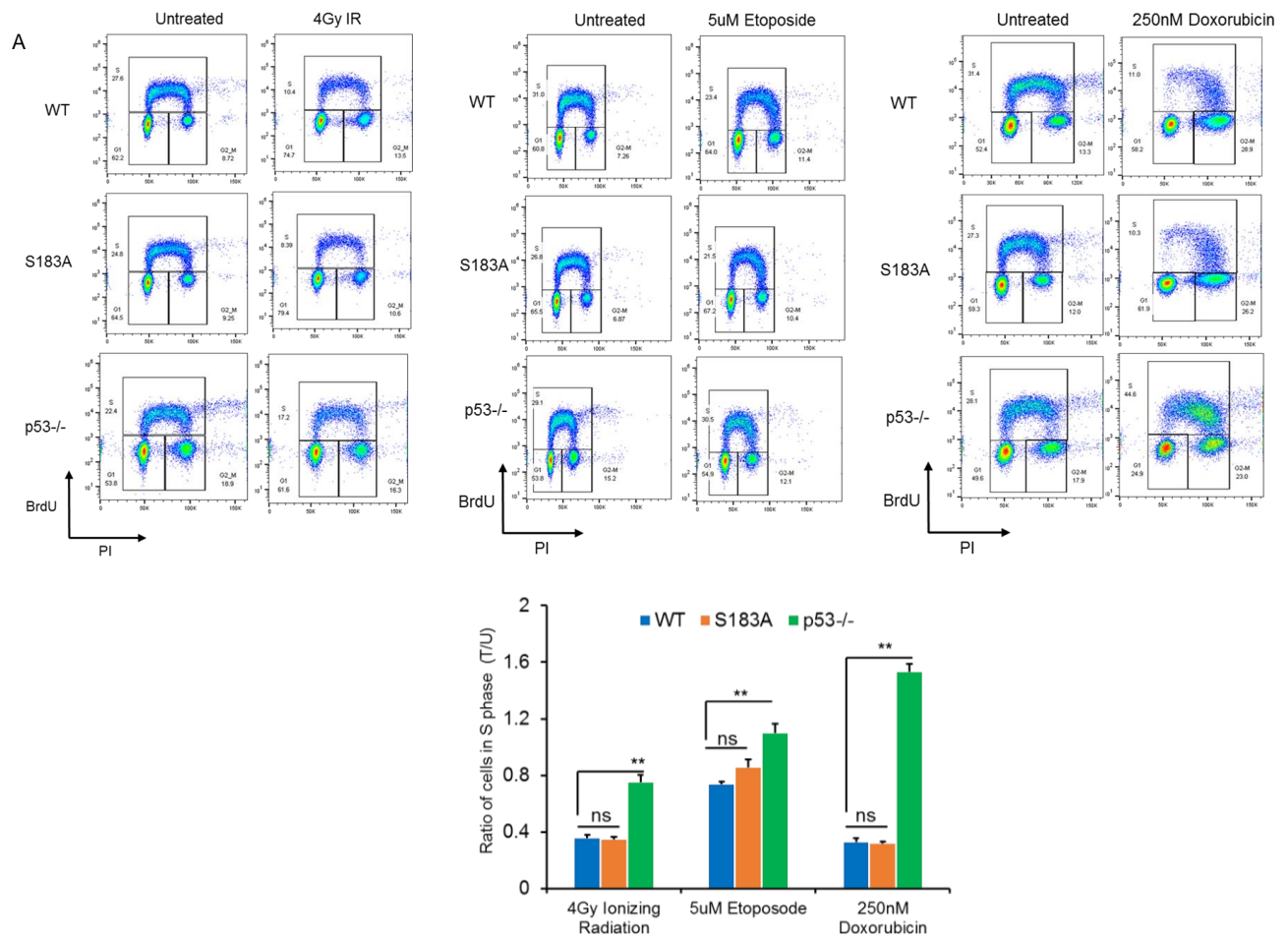
Having established that Akt activation can affect Mdm2-p53 signaling at endogenous levels, next, I explored whether Mdm2 S183 phosphorylation influences DNA damage responses mediated by p53. Exposure to DNA damage inducing agents such as ionizing radiation activates and stabilizes p53, which is a critical effector of DNA damage responses which include DNA repair and cell cycle arrest (Williams and Schumacher, 2016). As expected, p53 and activated p53 protein levels were elevated in low oxygen Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs following treatment with 4Gy of ionizing radiation (Figure 2.9a). However, there was no significant difference in the levels of p53 and activated p53 protein in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs (Figure 2.9a and b). This data demonstrated that Mdm2 S183 phosphorylation does not affect p53 activation and stabilization following DNA damage.



**Figure 2.9. Phosphorylation of Mdm2 at S183 does not affect p53 activation and stabilization after DNA damage.** (A) Western analyses of protein lysates collected from passage 2 Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs cultured at 5% oxygen in NAC supplemented media and treated with 4Gy IR for the indicated time points. Triple knockout cells (TKO) are deficient for Mdm2, MdmX, and p53. (B) Quantification of p53 and p-p53 (S18) relative to the loading control protein vinculin for westerns in (A). Data is expressed as mean  $\pm$  standard deviation. Student's T test \* $p < 0.05$  and \*\* $p < 0.01$  ( $n = 3$ ).

To examine DNA damage-induced cell cycle arrest, which is also mediated by p53, I performed cell cycle analysis on low oxygen MEFs treated with various DNA damage inducing agents (Figure 2.10). The percentage of cells in S phase was not significantly different between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs treated with ionizing radiation, etoposide, and doxorubicin (Figure 2.10a). As expected, p53<sup>-/-</sup> MEFs failed to undergo cell cycle arrest after DNA damage. This data suggests that Mdm2-S183 phosphorylation does not influence DNA damage-induced cell cycle arrest mediated by p53.

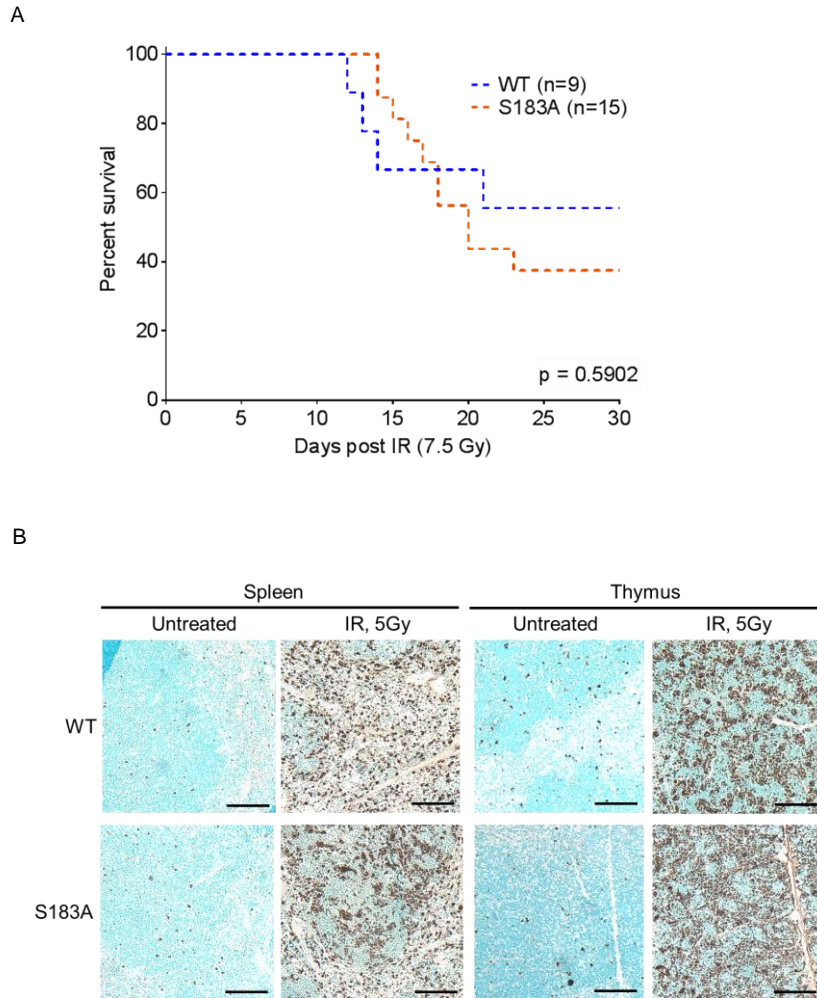
Mice exposed to acute ionizing radiation succumb to bone marrow failure (Gudkov and Komarova, 2003) (Komarova et al., 2004). This is mediated by p53-dependent apoptosis in bone marrow and other radiosensitive tissues such as the spleen and thymus. p53<sup>-/-</sup> mice do not succumb to hematopoietic failure since the cells are resistant to apoptosis. To determine whether Mdm2 phosphorylation will affect p53-mediated DNA damage responses *in vivo*, cohorts of Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice were treated with a sublethal dose of ionizing radiation (7.5Gy) and monitored for survival. There was no significant difference in survival between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice (Figure 2.11a), suggesting that Mdm2-S183 phosphorylation does not influence sensitivity to ionizing radiation in mice.



**Figure 2.10. Phosphorylation of Mdm2 at S183 does not alter p53-mediated cell cycle arrest induced by DNA damage *in vitro*.**

(A) Representative flow cytometry results for cell cycle analyses (top panel) in Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs cultured at 5 % oxygen with NAC and exposed to the indicated DNA damaging agents and pulsed with BrdU and stained with PI. (B) The % of cells in S phase is quantified (bottom panel) and presented as mean  $\pm$  standard deviation. Student's T test \* $p < 0.05$  and \*\* $p < 0.01$  ( $n = 3$ ).

To ascertain whether Akt-mediated phosphorylation of Mdm2 affects apoptosis in radiosensitive tissues following exposure to ionizing radiation , I performed TUNEL staining on spleen and thymus tissues harvested from Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice following exposure to ionizing radiation (5Gy). There was no difference in the amount of apoptosis in spleen and thymus of Mdm2<sup>WT</sup> mice compared with Mdm2<sup>S183A</sup> mice (Figure 2.11b). Therefore, phosphorylation of Mdm2 by Akt at S183 is not required for mediating p53-dependent DNA damage responses *in vivo* in mice.



**Figure 2.11. Phosphorylation of Mdm2 at S183 does not affect DNA damage responses *in vivo*.** (A) Survival of Mdm2<sup>WT</sup> (n=9) and Mdm2<sup>S183A</sup> (n=15) mice treated with a sub lethal dose of ionizing radiation (7.5Gy). (B) TUNEL staining of thymus and spleen harvested from Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice 2 hours after treatment with ionizing radiation (5Gy). Scale bars are 200  $\mu$ m.

**CHAPTER III: MDM2-S183 PHOSPHORYLATION BY AKT PROMOTES  
TUMORIGENESIS INDUCED BY CARCINOGEN EXPOSURE AND  
ONCOGENE EXPRESSION**

## Introduction

p53 deficient mice develop highly penetrant spontaneous tumors, underscoring the role of p53 as a potent tumor suppressor protein (Donehower et al., 1992). Post translational modifications on Mdm2 such as phosphorylation of S394 by ATM and S393 by c-Abl, have an impact on p53-dependent tumor suppression. For instance, Mdm2<sup>S394A</sup> mice are more susceptible to Eμ-myc-driven lymphomagenesis and spontaneous tumorigenesis, but less susceptible to lymphomas induced by ionizing radiation compared to Mdm2<sup>WT</sup> mice (Gannon et al., 2012a, Carr et al., 2016a). Additionally, Mdm2<sup>Y393F</sup> mice are more susceptible to spontaneous and oncogene-induced tumorigenesis (Carr et al., 2016c). However, Mdm2<sup>S394A/Y393F</sup> mice develop spontaneous tumors at a frequency similar to that observed in Mdm2<sup>S394A</sup> mice, thereby suggesting that ATM phosphorylation of Mdm2 plays a more prominent role and there are no synergistic effects on tumor suppression.

In chapter II, I established that phosphorylation of endogenous Mdm2 at S183 by Akt increases Mdm2 stability and Mdm2 nuclear localization. As a result, p53 levels and activity are reduced and this protects cells from p53-dependent premature senescence induced by oxidative stress. However, Mdm2-S183 phosphorylation does not mediate p53-dependent DNA damage response *in vitro* and *in vivo*. To explore whether Mdm2 phosphorylation at S183 affects aspects



of p53-mediated tumor suppression, I utilized three different mouse models in which oxidative stress plays a critical role in promoting tumorigenesis.

First, I explored the role of Mdm2-S183 phosphorylation in tumorigenesis utilizing the carcinogen diethylnitrosamine (DEN) to induce liver cancer. Biotransformation of DEN produces DNA alkylating metabolites resulting in damage to various macromolecules which contribute to hepatocarcinogenesis (Verna et al., 1996). DEN also triggers the accumulation of ROS which causes DNA alkylation, lipid peroxidation, and protein oxidation, necessary for hepatocarcinogenesis (Sanchez-Perez et al., 2005, Cerutti et al., 1994). Infant mice express high levels of DEN alkylating enzymes, with males having higher and more prolonged expression than females, and therefore more susceptible to DEN-induced carcinogenesis (Rao and Vesselinovitch, 1973). Development of hepatocellular carcinoma (HCC) is also prevalent in males in both humans and rodents. DEN induces HCC in about 100% of male mice compared to about 30% in female mice (Nakatani et al., 2001, Li et al., 2012). This gender disparity in HCC formation is partly due to estrogen-mediated inhibition of IL-6 production in females but not in males (Naugler et al., 2007). IL-6 regulates hepatic responses to systemic inflammation and promotes hepatocyte proliferation (Cressman et al., 1996). Accordingly, this sexual dimorphism in DEN-induced liver cancer is abolished in IL-6 knockout mice (Naugler et al., 2007). In addition, androgen signaling in males activates Foxa1/2-mediated biosynthesis and replication of

DNA and cell proliferation in male mice, whilst these processes are inhibited by estrogen in female mice (Li et al., 2012). Intriguingly, loss of Foxa1/2 reverses the gender dimorphism in DEN-induced hepatocarcinogenesis, further supporting the notion that sexual differences in susceptibility to HCC are primarily driven by sex-dependent hormonal signaling pathways. Therefore, I administered DEN only in infant male mice for my liver cancer studies in order to obtain more consistent results unconfounded by sexual differences.

The administration of DEN activates p53 in hepatocytes and mesenchymal cells (Lim, 2002), and this is thought to promote the clearance of damaged cells. DEN-treated mice with conditional deletion of p53 in hepatic stellate cells (HSC-p53 $\Delta\Delta$ ) developed more tumors compared with controls (Lujambio et al., 2013). HSC-p53 $^{+/+}$  mice displayed p53-driven senescence-associated secretory phenotype (SASP) which triggered macrophages to an antitumorigenic M1 fate. In contrast, HSC-p53 $\Delta\Delta$  mice polarized macrophages towards a pro-tumorigenic M2 phenotype which enhanced the clearance of senescent HSCs and enhanced cell proliferation. This suggests that p53 expression in the liver promotes senescence to suppress DEN-induced liver tumorigenesis. Here, I will discuss my findings showing that phosphorylation of Mdm2-S183 promotes DEN-induced liver tumorigenesis in mice, partly through the inhibition of p53-dependent senescence.

Secondly, I took advantage of the well-established 7,12-dimethylbenz[a]-anthracene (DMBA)/12-O-tetradecanoylphorbol-13-acetate (TPA)-driven cancer model to explore the role of Mdm2-S183 phosphorylation in carcinogen-induced skin tumorigenesis. Single application of the carcinogen DMBA causes mutations in several genes in keratinocyte stem cells in the bulge region of hair follicles, with *H-ras* being the most prominent DMBA target followed by *K-ras* (Ise et al., 2000, Brown et al., 1990, Morris, 2004). Two-weeks after the initiating dose of DMBA, tumor formation is promoted by the twice-weekly application of TPA. This promotion step using TPA triggers epidermal hyperplasia and selective clonal expansion of the mutant stem cells (Karen et al., 1999, Hennings et al., 1987). Additionally, TPA treatment also triggers the generation of ROS which causes oxidative damage and inflammation (Perchellet and Perchellet, 1989, Nakamura et al., 1999). Furthermore, TPA treatment also leads to additional genetic lesions and aneuploidy (Abel et al., 2009). Moreover, tumor promotion with TPA causes p53 mutations and p53 loss of heterozygosity during the malignant stages when papillomas progress to squamous and spindle cell carcinoma (Ruggeri et al., 1991, Burns et al., 1991).

However, there is evidence using DMBA/TPA mouse models suggesting that p53<sup>-/-</sup> mice develop fewer and smaller papillomas compared with p53<sup>+/-</sup> and p53<sup>+/+</sup> mice (Kelly-Spratt et al., 2004, Kemp et al., 1993). Additionally, the expression of oncogenes such as *H-ras* on a p53<sup>-/-</sup> background also failed to

accelerate the formation of skin lesions suggesting the epidermis is somehow resistant to neoplastic transformation which is often accompanied by p53 loss (Greenhalgh et al., 1996). In contrast, mice with cre-mediated keratinocyte-specific p53 knock-out (p53<sup>EKO</sup>) were more susceptible to DMBA/TPA-induced skin cancer, suggesting that the expression of p53 in epidermal keratinocytes promotes tumor suppression (Page et al., 2016). These studies highlight discrepancies in determining the role of p53 in tumor suppression during skin carcinogenesis and this may be influenced by the level of p53 expression or context such as genetic background in mice. Since Mdm2<sup>S183A</sup> MEFs display elevated levels of endogenous p53 when cultured in the presence of oxidative stress compared with Mdm2<sup>WT</sup> MEFs, I explored whether loss of Mdm2-S183 phosphorylation will enhance p53-mediated tumor suppression in DMBA/TPA treated mice.

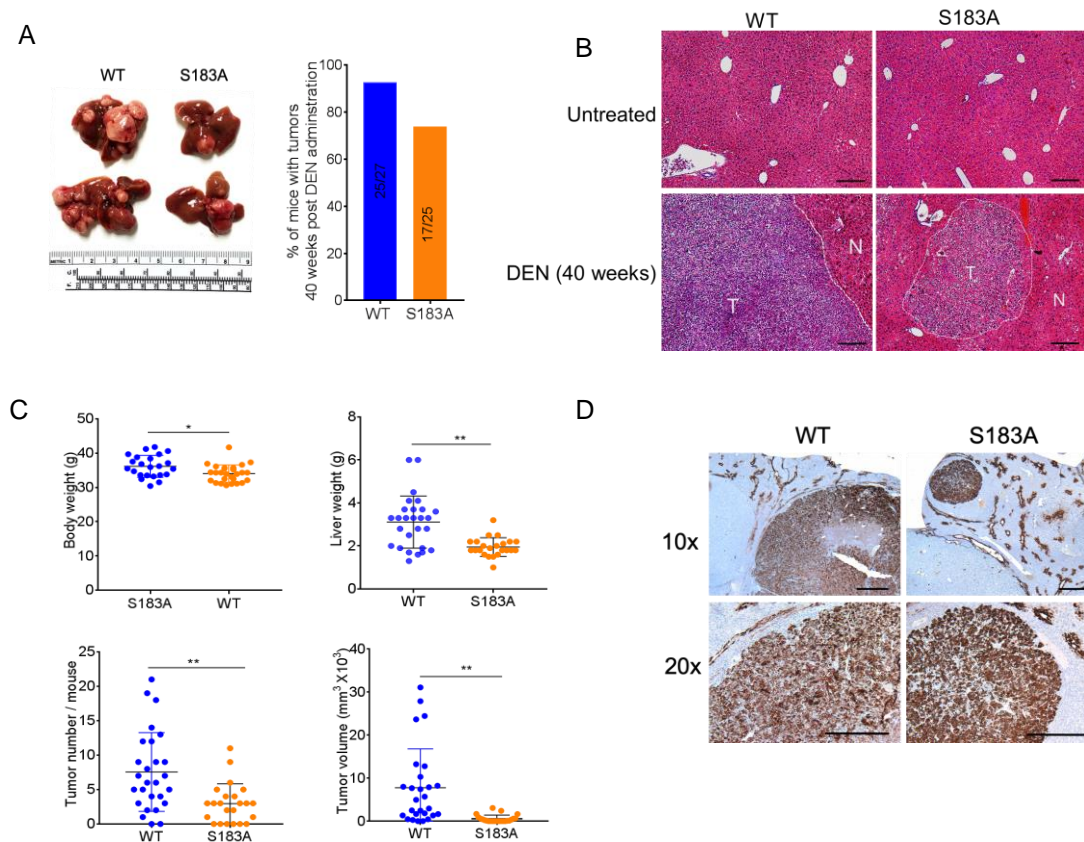
Lastly, I also used the *K-ras*-induced lung cancer model to determine whether Mdm2-S183 phosphorylation affects tumorigenesis primarily driven by the expression of an oncogene. In MEFs, the expression of p53 and oncogenic *K-ras*<sup>G12V</sup> triggers cell senescence (Ferbeyre et al., 2002, Serrano et al., 1997, Lin et al., 1998). There is evidence to suggest that this senescence is independent of p21 expression (Castro et al., 2004), implying that additional compensatory signals can modulate the outcome of p53 activation in the presence of oncogenic Ras. Additionally, the expression of oncogenic *K-ras*

activates signaling pathways and trigger ROS generation which promotes malignant transformation (Park et al., 2014), and ROS-induced premature senescence (Kodama et al., 2013, Weyemi et al., 2012). However, endogenous *K-ras*<sup>G12D</sup> expression does not induce senescence but increases cell proliferation in MEFs and developmental defects in mice (Tuveson et al., 2004). This may suggest that endogenous Ras expression potentially inhibits the activation of downstream effector pathways or the levels were not sufficient to trigger effector pathways that mediate senescence.

In contrast, loss of NF1 in human cells, which allows endogenous Ras expression, inhibits downstream effector pathways but these cells senesce after several passages (Courtois-Cox et al., 2006), thereby ruling out downstream effector pathway silencing. Additionally, the notion that endogenous Ras levels are not sufficient to trigger senescence was challenged by findings showing that preneoplastic pancreatic lesions driven by endogenous levels of oncogenic *K-ras* are positive for various senescence markers, and display activation of downstream effectors in pathways governed by Ras expression (Ji et al., 2009). These contrasting findings suggest that the type of response from activation of endogenous Ras may be context dependent. I hypothesized that Mdm2-S183 phosphorylation promotes lung tumorigenesis by lowering p53-mediated senescence induced by the expression of endogenous levels of oncogenic *K-ras*.

## Results

*Diethylnitrosamine-induced liver cancer:* Two-week old Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> male mice were injected with DEN and sacrificed after 40 weeks. Upon dissecting the mice, 93% of Mdm2<sup>WT</sup> mice presented with outwardly visible tumors on the liver compared with about 61% of Mdm2<sup>S183A</sup> mice (Figure 3.1a). In addition, Mdm2<sup>WT</sup> mice had significantly larger and high number of tumors compared with Mdm2<sup>S183A</sup> mice (Figure 3.1b-c). Consistently, the liver weights and volumes were significantly higher in Mdm2<sup>WT</sup> mice than in Mdm2<sup>S183A</sup> mice. Tumors in both Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice also stained positive for glutamine synthetase which is one of the markers for liver cancer, suggesting that these liver lesions are indeed tumors. Collectively, these data suggest that Mdm2-S183 phosphorylation increases susceptibility to DEN-induced liver tumorigenesis.



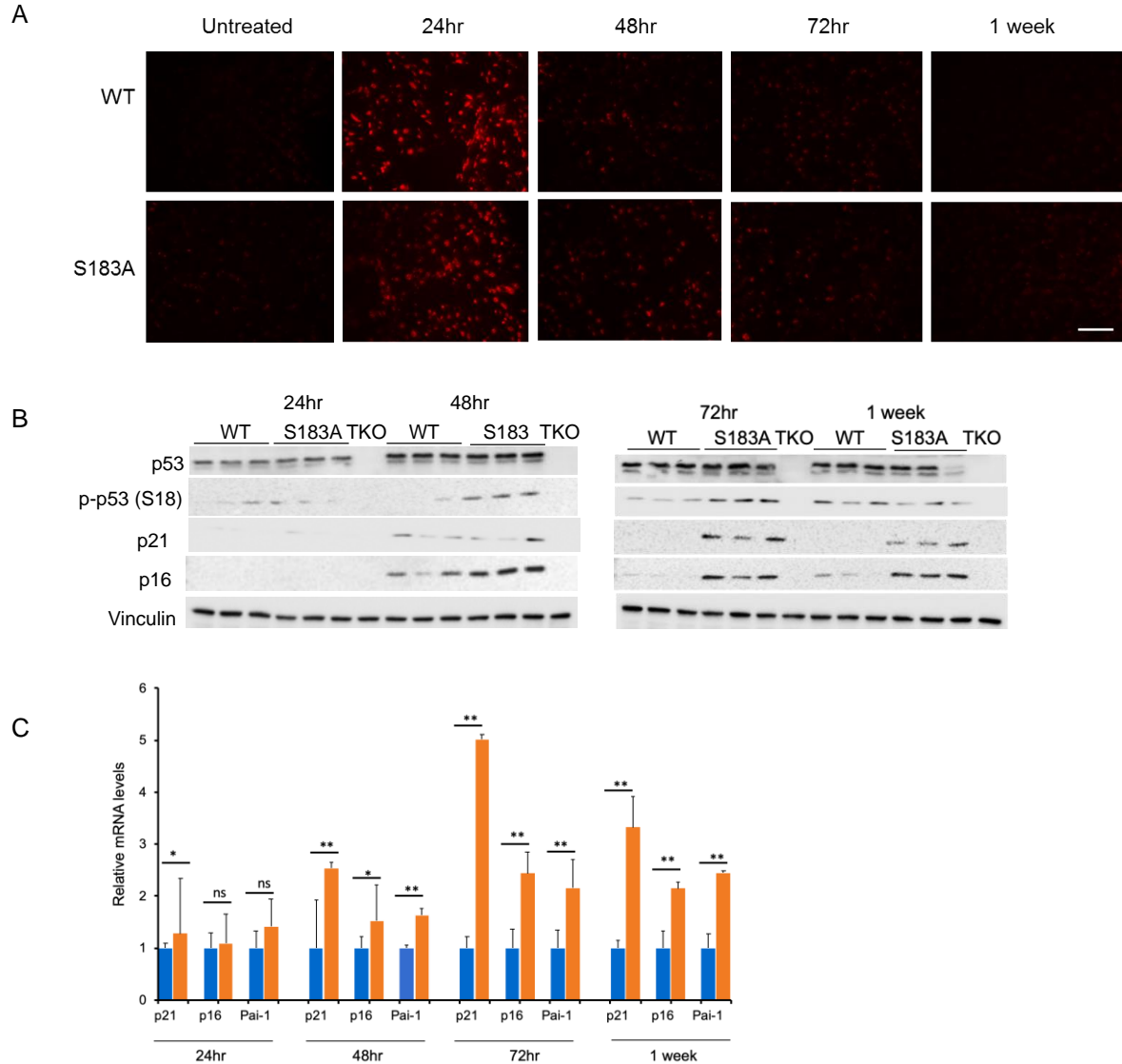
**Figure 3.1. Phosphorylation of Mdm2-S183 promotes DEN-induced liver tumorigenesis.** (A) Representative images showing the tumor burden in  $\text{Mdm2}^{\text{WT}}$  (n=27) and  $\text{Mdm2}^{\text{S183A}}$  (n=25) mice sacrificed 40 weeks after DEN was administered at 2 weeks of age. Percentage of  $\text{Mdm2}^{\text{WT}}$  and  $\text{Mdm2}^{\text{S183A}}$  mice with outwardly visible liver nodules at the time of sacrifice (Right panel). (B) Liver sections from untreated and DEN-treated  $\text{Mdm2}^{\text{WT}}$  (n=27) and  $\text{Mdm2}^{\text{S183A}}$  (n=25) tumor-bearing mice were stained with H&E. scale bar = 50 $\mu\text{m}$ . (C) Body weights, liver weights, tumor numbers, and tumor volumes for both  $\text{Mdm2}^{\text{WT}}$  (n=27) and  $\text{Mdm2}^{\text{S183A}}$  (n=25) 40 weeks after the administration DEN. Student's T test \*p<0.05 and \*\*p<0.01. (D) Higher and lower magnification of glutamine synthetase-stained liver sections from 42 week old  $\text{Mdm2}^{\text{WT}}$  and  $\text{Mdm2}^{\text{S183A}}$  DEN-treated mice (n=3). Scale bar = 100 $\mu\text{m}$ .

The administration of carcinogens such as DEN triggers the generation of ROS which can cause DNA adducts through oxidative alteration and strand breakage potentially giving rise to cancer causing mutations (Ziech et al., 2010). DEN-induced oxidative stress also disrupts antioxidant function in the liver and this is thought to contribute to early histopathological alterations observed in DEN treated mice (Paula Santos et al., 2014). I therefore determined whether there was a difference in ROS generation between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> livers following DEN injection by staining fresh liver cryosections with a redox sensitive fluorescent probe dihydroethidium (DHE). The oxidized form of DHE, 2-hydroxyethidium, intercalates with DNA and displays red fluorescence in the nucleus. I found that ROS was generated at comparable levels in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice with a time dependent decrease which I observed over the course of a week (Figure 3.2a). The fact that there were no differences in the amount of ROS in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> livers following DEN injection is in line with my findings in MEFs (Figure 2.6). This suggests that Mdm2-S183 phosphorylation does not alter DEN-induced generation of ROS.

I established that Mdm2<sup>S183A</sup> MEFs were more susceptible to p53-mediated senescence than Mdm2<sup>WT</sup> MEFs when exposed to oxidative stress (Figure 2.4C). Similarly, p53 expression suppresses tumor development and progression in hepatic stellate cells by triggering senescence-associated secretory phenotype in livers of DEN-treated mice (Lujambio et al., 2013). To



examine whether DEN-induced oxidative stress triggers p53 expression and p53-dependent senescence in mouse livers, I performed western blot analysis using protein lysates extracted from livers of DEN-treated mice (Figure 3.2b). The protein levels of activated p53 and the senescence marker p16 were increased in livers of DEN-treated Mdm2<sup>S183A</sup> mice relative to Mdm2<sup>WT</sup> mice. Additionally, p21 which is often used as marker of p53-mediated senescence was also elevated in livers of Mdm2<sup>S183A</sup> mice compared with Mdm2<sup>WT</sup> mice. Furthermore, the gene expression levels of senescence marker p16 and p53-dependent senescence markers p21, and Pai-1 were significantly elevated in Mdm2<sup>S183A</sup> livers than in Mdm2<sup>WT</sup> livers after DEN administration (Figure 3.2c). These findings suggest that loss of Mdm2-S183 phosphorylation promotes p53-mediated senescence in the livers of Mdm2<sup>S183A</sup> exposed to DEN, potentially contributing to the suppression of liver cancer.



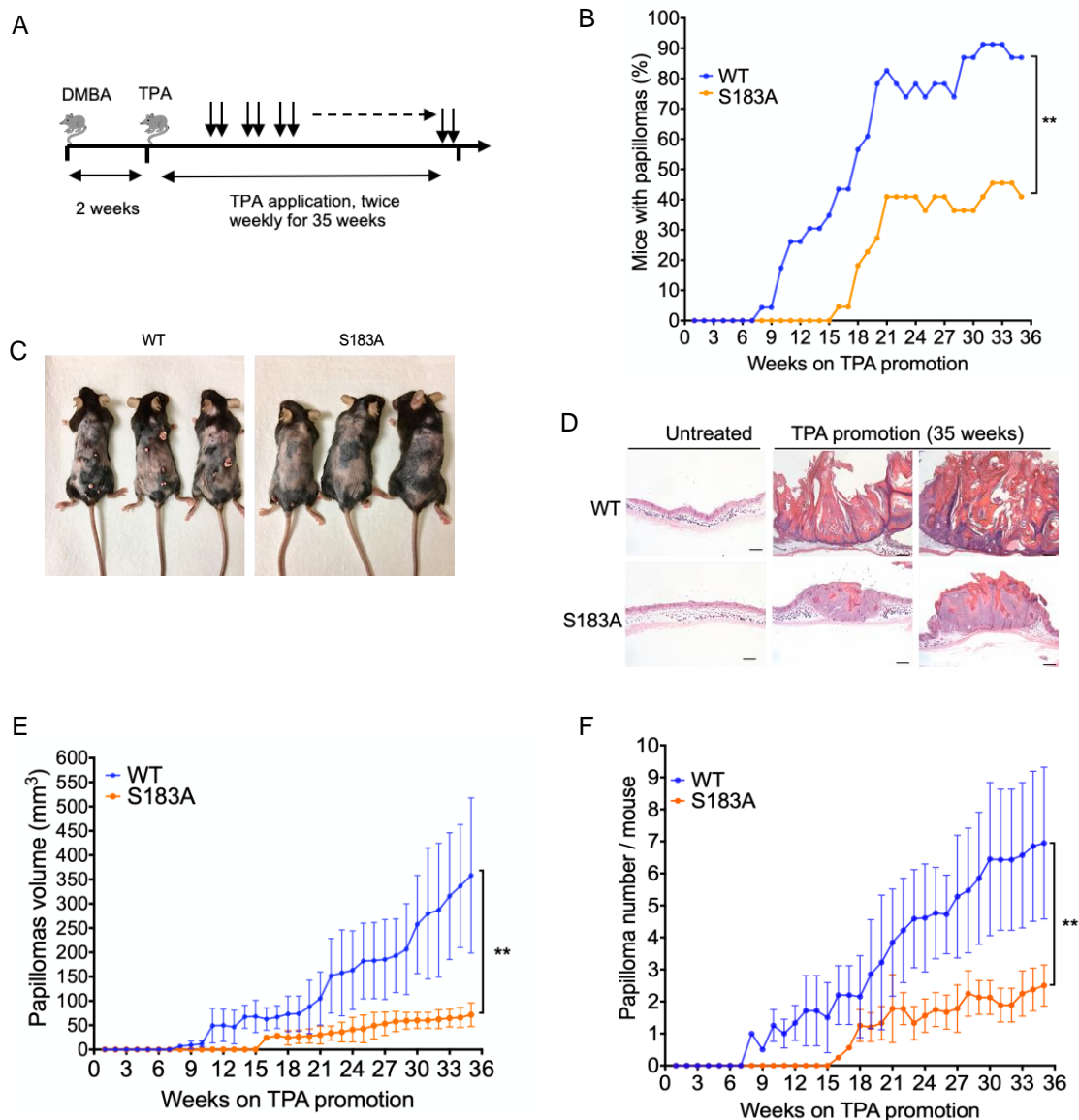
**Figure 3.2. DEN treatment triggers senescence in Mdm2<sup>S183A</sup> livers treated with DEN.** (A) Representative images of dihydroethidium staining in liver sections from Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> male mice at various time points post DEN administration (n=3). Scale bar is 20 $\mu$ m. (B) Western blot analysis of protein lysates from liver tissues harvested from DEN-treated Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice at the indicated time points (n=3). (C) mRNA expression levels for senescence genes in livers of young DEN-treated Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice (n=3). Data is expressed as mean  $\pm$  standard deviation. Student's T test \*p<0.05 and \*\*p<0.01.

*DMBA/TPA-driven skin tumorigenesis:* To investigate whether Mdm2-S183 phosphorylation affects tumorigenesis in yet another model of carcinogenesis, I utilized the two stage DMBA/TPA to induce the initiation and promotion of epidermal tumors. DMBA causes mutations in genes such as *H-ras* in keratinocytes and TPA promotes the clonal expansion of mutant cells and loss of tumor suppressors such as p53, giving rise to skin tumors (Abel et al., 2009). There is evidence showing that p53 expression in the skin epidermis inhibits tumor formation in mouse epidermis (Lyle et al., 2014) (Martinez-Cruz et al., 2008). Additionally, grafting of keratinocytes deficient for p53 but expressing *H-ras* gives rise to malignant transformation which is increased by the loss of one allele of p53 (Weinberg et al., 1994). These studies together with the one using conditional deletion of p53 in keratinocytes mentioned in the introduction of this chapter, underscores the role of p53 in suppressing DMBA/TPA-induced skin tumorigenesis.

However, there is contrasting evidence using DMBA/TPA models showing that the expression of p53 and *H-ras* promote papilloma formation but reduces malignant progression (Kemp et al., 1993). Additionally, expression of Ras oncogenes activate p19<sup>Arf</sup> which inhibits Mdm2 thereby activating p53-dependent growth arrest (Kelly-Spratt et al., 2004). Furthermore, in p19<sup>Arf</sup> deficient mice subjected to the DMBA/TPA protocol, the rate of papilloma growth was higher than in wild type and p53<sup>-/-</sup> mice. This further suggests that p53 dependent and

independent functions regulate tumor suppression in the epidermis. Additionally, various ways of activating p53 in the epidermis may have different effects on tumor suppression.

Since MEFs expressing Mdm2 that cannot be phosphorylated at S183 exhibited elevated levels of p53 compared with wild type MEFs in the presence of oxidative stress, I hypothesized that Mdm2<sup>S183A</sup> mice will be less susceptible to epidermal tumorigenesis induced by DMBA/TPA. I postulated that when Mdm2<sup>S183A</sup> mice are exposed to oxidative stress and oncogene activation caused by the topical application of DMBA/TPA, the levels of p53 activation and function will exceed those observed in Mdm2<sup>WT</sup>. As a result, this would activate downstream p53 effector pathways that mediate tumor suppression, such as the p53-dependent senescence observed in Mdm2<sup>S183A</sup> MEFs.



**Figure 3.3. Mdm2-S183 phosphorylation promotes skin tumorigenesis.**

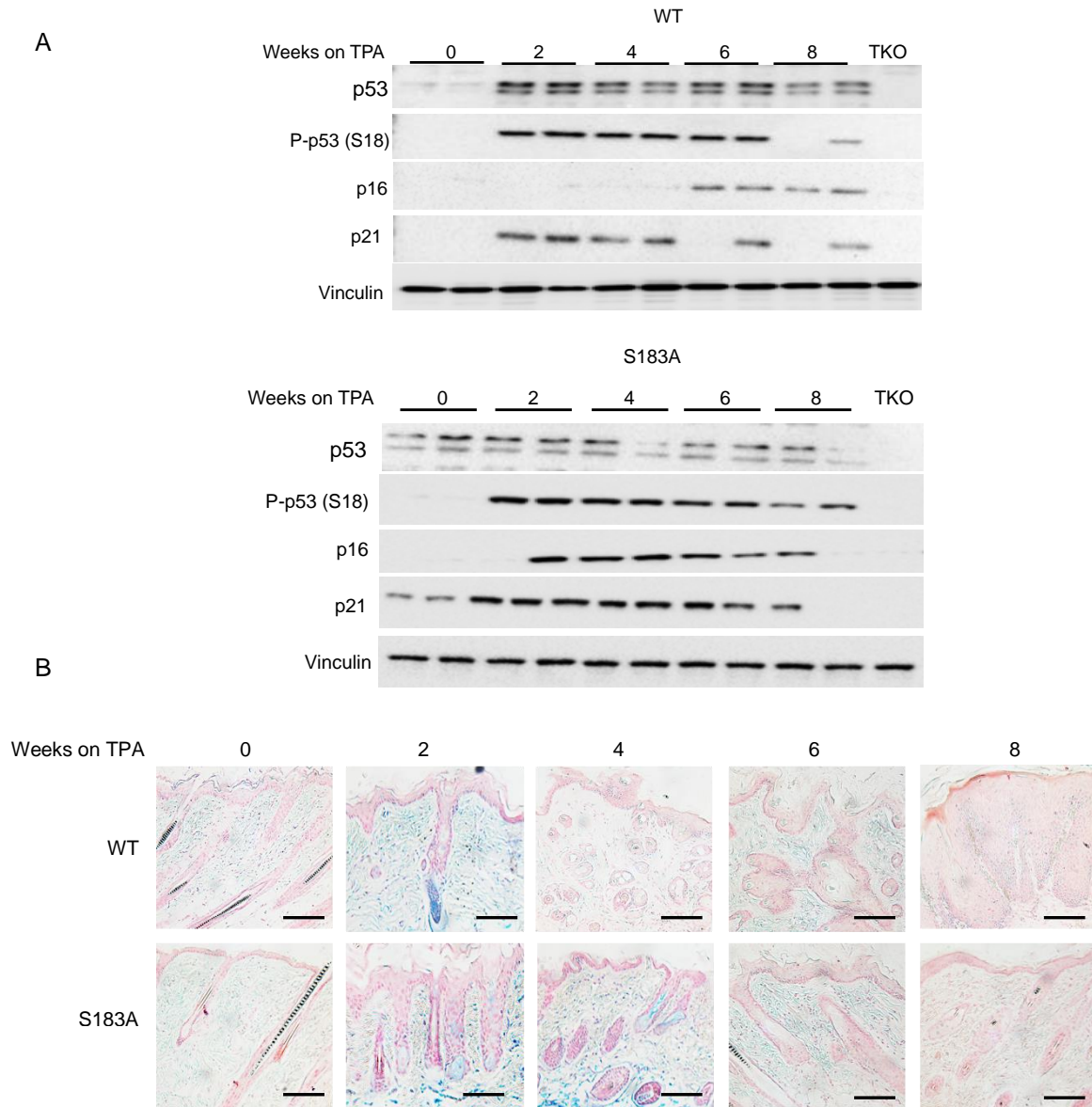
(A) Schematic illustration of DMBA/TPA application timing of skin carcinogenesis protocol. (B) Percentage of Mdm2<sup>WT</sup> (n=23) and Mdm2<sup>S183A</sup> (n=22) mice with papillomas. (C) Representative photographs of Mdm2<sup>WT</sup> (n=3) and Mdm2<sup>S183A</sup> (n=3) mice with papillomas after 32 weeks of TPA promotion. (D) Representative H&E images of papillomas isolated from Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice after 32 weeks of TPA treatment. Scale bar = 500um. (E) Papilloma volume per mouse in Mdm2<sup>WT</sup> (n=23) and Mdm2<sup>S183A</sup> (n=22) mice. Data is expressed as mean  $\pm$  standard deviation. (F) Number of papillomas per mouse in Mdm2<sup>WT</sup> (n=22) and Mdm2<sup>S183A</sup> (n=23) mice. Data is expressed as mean  $\pm$  standard deviation. Two way Anova and paired Student T tests \*\*p<0.01.

Tumor initiation in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice was achieved by the topical application of DMBA in two-month old mice. Two weeks post DMBA administration, I began twice-weekly applications of TPA to promote tumor formation for up to 35 weeks (Figure 3.3a). Mdm2<sup>S183A</sup> mice were significantly less susceptible to papilloma development, with the first tumors in Mdm2<sup>S183A</sup> mice appearing 2 months after the first papillomas appeared in Mdm2<sup>WT</sup> mice (Figure 3.3b). About 86.9% of Mdm2<sup>WT</sup> mice developed at least one or more papillomas compared to 40.9% of Mdm2<sup>S183A</sup> mice (Figure 3.3b). Furthermore, Mdm2<sup>S183A</sup> mice also exhibited significantly smaller tumors compared to Mdm2<sup>WT</sup> mice as shown the papilloma images, histological staining, and papilloma volumes (Figure 3.3c-e). Moreover, Mdm2<sup>WT</sup> mice had significantly higher number of tumors compared with Mdm2<sup>S183A</sup> mice (Figure 3.3f). Taken together, these findings indicate that Mdm2-S183 phosphorylation enhances epidermal tumor formation in mice exposed to topical carcinogens.

Next, I performed western blots using protein lysates extracted from the skin of mice in the first few weeks of TPA promotion before Mdm2<sup>WT</sup> starts presenting with papillomas to determine the expression of senescence markers (Figure 3.4a). In Mdm2<sup>WT</sup> mice, DMBA treatment led to a minor induction of p53 expression and no p21 protein expression (Figure 3.4a). In contrast, DMBA treatment induced the expression of both p53 and p21 proteins in Mdm2<sup>S183A</sup> mice with very minimal expression of activated phospho-p53 (Figure 3.4a).

However, there were no differences in the levels of p53 and phospho-p53 protein expression between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice during the TPA promotion period. Nonetheless, p21 and p16 protein levels were elevated in Mdm2<sup>S183A</sup> mice compared with Mdm2<sup>WT</sup> mice, with some of the Mdm2<sup>WT</sup> mice losing both p21 and p16 expression around 6 weeks, that is two weeks before the emergence of papillomas (Figure 3.4a and b). This demonstrates that Mdm2-S183 phosphorylation inhibits p53 activation during the initiation stages with DMBA and reduces p53-dependent senescence during TPA promotion.

Furthermore, I stained for SA- $\beta$  galactosidase in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice skin to determine whether there was any evidence of senescence during TPA promotion. Indeed, there was positive staining for SA- $\beta$  galactosidase in the skin of both Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice, with prolonged SA- $\beta$  galactosidase staining in Mdm2<sup>S183</sup> (Figure 3.4b). These findings point to a role of p53-mediated senescence in contributing to tumor suppression in Mdm2<sup>S183A</sup> skin treated with DMBA/TPA. In sum , the data suggests the phosphorylation of Mdm2-S183 promotes epidermal tumorigenesis partly by diminishing p53-mediated senescence triggered by topical application of carcinogens in mice.



**Figure 3.4. Mdm2-S183 phosphorylation promotes epidermal tumorigenesis.** (A) Western analysis of protein extracts from Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice skin treated with TPA for the indicated time in weeks (n=3). (B) Senescence-associated-β-galactosidase staining in skin of Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice treated with TPA for the indicated time in weeks. Senescence-associated-β-galactosidase stained sections were counterstained with nuclear fast red (n=3). Scale bar =20 μm.



*Oncogenic K-ras driven lung cancer:* The expression of p53 together with oncogenic Ras triggers senescence *in vitro* (Serrano et al., 1997, Ferbeyre et al., 2002). Senescence induced by the expression of oncogenic Ras is thought to impede cellular transformation. However, there are discrepancies regarding the role of endogenous levels of oncogenic Ras in inducing senescence. MEFs derived from *K-ras*<sup>G12D</sup> mice in which Ras is expressed at endogenous levels displayed enhanced proliferation and susceptibility to transformation (Tuveson et al., 2004). Additionally, expression of activated B-Raf (*B-raf*<sup>V600E</sup>) which is downstream of Ras, failed to trigger senescence *in vitro* (Pritchard et al., 1995). In contrast to these findings, *in vivo* expression of oncogenic *K-Ras*<sup>GV12</sup> and *B-raf*<sup>V600E</sup> triggers senescence in premalignant adeno carcinoma (Collado et al., 2005, Dankort et al., 2007). Additionally, endogenous levels of *K-ras*<sup>GV12</sup> induced senescence in PanIN lesions (Ji et al., 2009). In the aforementioned *in vivo* studies, tumors progressed to malignant cancer upon the loss of p53, underscoring the role of p53-mediated senescence in suppressing tumor progression driven by the expression of endogenous oncogenic Ras.

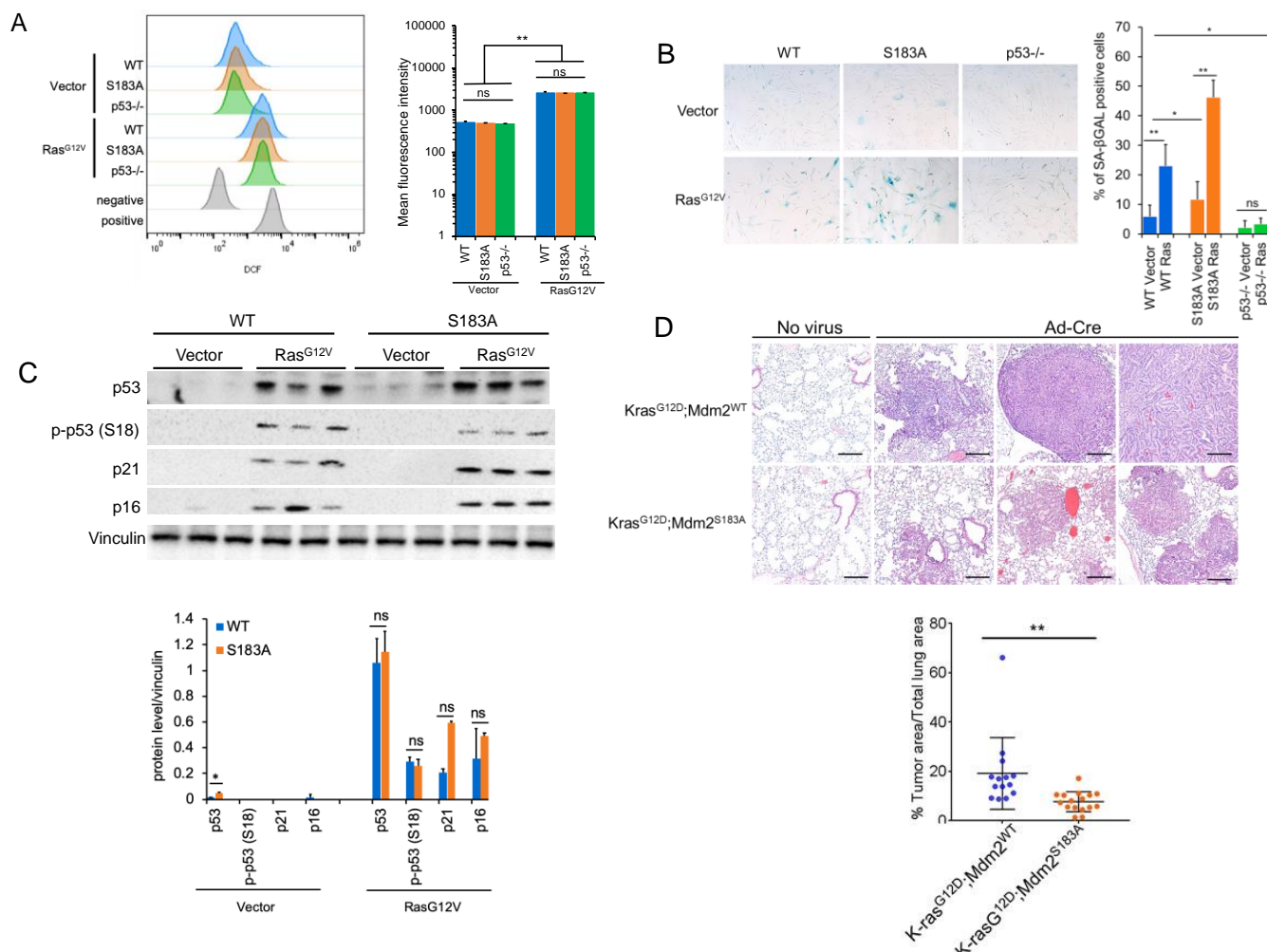
Additionally, it is well established that oncogenic Ras expression triggers the generation of reactive oxygen species (Lee et al., 1999, Colavitti and Finkel, 2005). Therefore, I postulated that blocking Mdm2-S183 phosphorylation, which led to increased p53-mediated senescence in the presence of oxidative stress in MEFs, suppresses lung tumorigenesis driven by the expression of endogenous

oncogenic Ras. First, I transduced Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs with an empty retroviral vector (Vector) or its derivative expressing oncogenic Ras (*K-ras*<sup>G12V</sup>) and stained cells with DCFDA to determine the levels of ROS. There were no significant differences in the level of ROS among Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs when transduced with vector (Figure 3.6a). Similarly, there were also no significance differences in the level of ROS among Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs when transduced with *K-ras*<sup>G12V</sup> (Figure 3.5a). However, ROS levels were significantly elevated in Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs transduced with oncogenic K-ras than in Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs transduced with an empty vector, suggesting that expression of oncogenic Ras promotes ROS accumulation.

Secondly, I stained vector- and *K-ras*<sup>G12V</sup>-transduced MEFs for senescence associated  $\beta$ -galactosidase to measure senescence. The percentage of cells that stained positive for SA- $\beta$  galactosidase was significantly increased in Mdm2<sup>S183A</sup> MEFs expressing *K-ras*<sup>G12V</sup> compared with their Mdm2<sup>WT</sup> counterparts and no effect was observed in p53<sup>-/-</sup> MEFs (Figure 3.5b). In addition, I extracted protein lysates from Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs transduced with either vector and *K-ras*<sup>G12V</sup> and performed western blot analysis. *K-ras*<sup>G12V</sup> triggered the expression of p53 and phospho-p53 at comparable levels in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs. However, Mdm2<sup>S183A</sup> MEFs had higher protein levels of senescence markers p21 and p16 than Mdm2<sup>WT</sup> MEFs (Figure 3.5c). This

indicates that Mdm2-S183 phosphorylation reduces the amount of p53-mediated senescence induced by endogenous levels of oncogenic K-ras.

Next, I examined the effects of Mdm2-S183 phosphorylation on lung cancer induced by the expression of oncogenic *K-ras*. To this end, 8-12 week old K-ras<sup>G12D</sup>;Mdm2<sup>WT</sup> and K-ras<sup>G12D</sup>;Mdm2<sup>S183A</sup> mice were infected with aerosolized adenoviral CMV-Cre and were sacrificed after 7.5 months. K-ras<sup>G12D</sup>;Mdm2<sup>WT</sup> and K-ras<sup>G12D</sup>;Mdm2<sup>S183A</sup> mice both presented with lung tumors (Figure 3.5d). I found that tumor volumes in K-ras<sup>G12D</sup>;Mdm2<sup>S183A</sup> mice were significantly lower than in K-ras<sup>G12D</sup>;Mdm2<sup>WT</sup> mice (Figure 3.5d). These findings indicate that Mdm2-S183 phosphorylation promotes Ras-induced lung tumorigenesis, potentially by altering p53-dependent senescence.



**Figure 3.5. Mdm2-S183 phosphorylation enhances the development of K-ras-driven tumors.** (A) Flow cytometry analyses (left) and quantification of DCF staining (right) in Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs cultured at 5% oxygen in media supplemented with NAC and transduced with pBabe (vector) or its derivative expressing oncogenic Ras (G12V). Data is expressed as mean fluorescence  $\pm$  standard deviation. Student's T test \*\*p < 0.01 (n = 3). (B) Representative images showing results for senescence-associated  $\beta$ -galactosidase staining (left) and quantification of positively stained Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mouse embryonic fibroblasts (right) cultured at 5% oxygen and transduced with pBabe (vector) or its derivative expressing oncogenic Kras(G12V). Data is expressed as mean  $\pm$  SD. Student's T test \*p < 0.05 and \*\*p < 0.01 (n = 3). (C) Western blot analyses of Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mouse embryonic fibroblasts transduced with pBabe (vector) or its derivative expressing oncogenic Ras (G12V) (top). Quantification of bands on western blot relative to the loading control protein, vinculin (bottom) (n = 3). (D) Representative images of H&E stained lungs from Kras<sup>+G12D</sup>;Mdm2<sup>WT</sup> (n = 14) mice and Kras<sup>+G12D</sup>;Mdm2<sup>S183A</sup> (n = 16) mice 250 days after the administration of Ad-CMV-cre (top). Quantification of area occupied by the tumors in Kras<sup>+G12D</sup>;Mdm2<sup>WT</sup> and Kras<sup>+G12D</sup>;Mdm2<sup>S183A</sup> mice (bottom). Data is expressed as mean  $\pm$  SD. Student's T test \*\*p < 0.01.

## **CHAPTER IV: GENERAL DISCUSSION**

### **Brief recap of findings from Mdm2 phosphorylation mutant mice**

It is beginning to emerge that post translational modifications of Mdm2 play pivotal roles in regulating p53 functions in response to stress. Modifications on various Mdm2 residues seem to influence distinct downstream p53 functions, suggesting the existence of a layered and fine-tuned regulation of Mdm2-p53 signaling. In our lab we have focused on investigating the role played by Mdm2 phosphorylation in regulating p53-dependent stress responses. We have developed several knock-in mouse models which express Mdm2 that cannot be phosphorylated by c-Abl (Mdm2<sup>Y393F</sup> mice), ATM (Mdm2<sup>S394A</sup> mice), or both kinases ( double mutant Mdm2<sup>Y393F;S394A</sup> mice) and S394 phosphomimic (Mdm2<sup>S394D</sup>) mice. Previous studies using the aforementioned models paved way for the work presented in this dissertation.

Briefly, Mdm2-S394 phosphorylation by ATM promotes p53 stability and activation following DNA damage (Gannon et al., 2012b). As such, loss of Mdm2-S394 phosphorylation, modelled in Mdm2<sup>S394A</sup> mice, leads to diminished p53 activation and stability, and radioresistance in mice. These findings demonstrate that phosphorylation of Mdm2-S394 influences p53-mediated DNA damage responses *in vivo*. Mdm2<sup>S393A</sup> mice are more prone to spontaneous tumorigenesis compared with Mdm2<sup>WT</sup> and Mdm2<sup>S394D</sup>. Furthermore, Mdm2<sup>S394A</sup> mice display accelerated lymphomagenesis driven by the expression of Eμ-myc (Carr et al., 2016a). In contrast, these mice have decreased lymphomagenesis

resulting from exposure to ionizing radiation. These findings imply contrasting effects of Mdm2-S394 phosphorylation by ATM on tumor suppression.

On the other hand, Mdm2-Y393 phosphorylation by c-Abl does not alter p53 stability, activation, and p53-dependent apoptosis following DNA damage. However, Mdm2<sup>Y393F</sup> show increased bone marrow repopulation, recover from radiation exposure more rapidly, and are therefore radioresistant (Carr et al., 2016b). Additionally, Mdm2<sup>Y393F</sup> mice are more susceptible to spontaneous and Eμ-myc driven tumorigenesis, suggesting that Mdm2-Y393 phosphorylation alters tumor suppression in ways that cannot be extrapolated from p53 expression and activity following DNA damage in Mdm2<sup>Y393F</sup> mice. Furthermore, Mdm2<sup>Y393F/S394A</sup> mice display phenotypes resembling those of Mdm2<sup>S394A</sup> mice, suggesting no additive effects of these two phosphorylation events. Based on these aforementioned studies, it is becoming clear that Mdm2 phosphorylation differentially alters p53 responses following exposure to stress. This is critical in therapeutic development, since this provides alternative avenues to obtain multiple desirable responses.

*In vitro* transfection-based studies demonstrated that Mdm2 phosphorylation by Akt at S163 and S183 enhances Mdm2 nuclear localization thereby increasing Mdm2-mediated degradation of p53 (Mayo and Donner, 2001, Zhou et al., 2001). In this dissertation, I presented data from my studies

investigating the functional relevance of Mdm2 phosphorylation at S163 and S183 by Akt at endogenous levels. I mainly focused on determining how Mdm2-S183 phosphorylation affects processes subject to control by p53-dependent pathways such as development, cell proliferation, DNA damage response, tumorigenesis, and glucose metabolism.

### **Mdm2-S183 phosphorylation in development**

Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mice were viable, fertile, and were obtained at Mendelian ratios (Figure 2.1), suggesting that Mdm2 phosphorylation by Akt is dispensable for development. This is in contrast to what is observed in Mdm2<sup>-/-</sup> mice that are embryonic lethal and are rescued by the concomitant loss of p53 (Jones et al., 1995, Montes de Oca Luna et al., 1995). Embryonic lethality in Mdm2<sup>-/-</sup> embryos is attributed to elevated p53-dependent apoptosis (Chavez-Reyes et al., 2003). *In vitro*, Mdm2 phosphorylation at either S163 or S183 reduces p53 levels and activity (Mayo and Donner, 2001, Zhou et al., 2001). Therefore, I proposed that Mdm2<sup>S183A</sup> and Mdm2<sup>S163A</sup> mice may exhibit embryonic lethality due to increased p53 activity. Alternatively, if they are not embryonic lethal, I postulated that Mdm2<sup>S183A</sup> and Mdm2<sup>S163A</sup> mice may show signs of premature aging. However, culturing Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs at low oxygen with NAC (Figure 2.3), conditions which probably closely resemble those present in utero, exerts minimal effects on p53 expression, activation and cell proliferation. Therefore, it is plausible that loss of Akt-mediated



phosphorylation of Mdm2 in Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> embryos does not increase p53 levels and activity, contrary to what is observed in Mdm2<sup>-/-</sup> embryos, hence the absence of an embryonic lethal phenotype.

There is evidence showing that elevated levels of p53 promote premature aging in mice (Tyner et al., 2002). The conditional expression of non-functional Mdm2 lacking exons 11-12 (Mdm2<sup>Δ11-12</sup>) in the skin epidermis using K5-cre leads to pre-mature aging phenotypes, elevated p53 activity and senescence in the skin (Gannon et al., 2011). I postulated that loss of Mdm2-S163 or Mdm2-S183 phosphorylation promotes premature aging phenotypes due to increased p53 activity. However, there were no significant differences in body weights (Figure 2.1g), longevity (Figure 2.1h), and physical appearance between controls and Mdm2 mutant mice. The fact that unchallenged Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice are phenotypically indistinguishable suggests that loss of Akt-mediated phosphorylation of Mdm2 does not promote premature aging in mice. These findings are consistent with my findings in MEFs showing that at low oxygen, Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs express slightly different levels of p53, but this difference was small enough to not affect cell proliferation and has minimal effects on p53-mediated senescence (Figure 2.2 and 2.5). As mentioned before, these low oxygen cell culture conditions potentially closely mimic what happening *in vivo*, and this may account for the similar phenotypes between unchallenged Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice.

Mdm2<sup>S183A</sup> MEFs proliferated at low oxygen but failed to do so at high oxygen implying that Mdm2-S183 phosphorylation sensitizes cells to oxidative stress present in conventional cell culture incubators not adjusted for oxygen levels (Figure 2.3). Mdm2<sup>S183A</sup> mice displayed significantly elevated levels of p53 at high oxygen compared to low oxygen, suggesting that Mdm2-S183 phosphorylation lowers p53 levels in the presence of oxidative stress. As a future direction, it is important to examine the effects of Mdm2 phosphorylation on development and maybe aging in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice treated with paraquat, a widely used herbicide and ROS inducer in mice (Castello et al., 2007). This may include the administration of paraquat in pregnant Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice to study resulting developmental phenotypes. In addition, the administration of paraquat starting at a young age in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice would probably activate p53 expression more in Mdm2<sup>S183A</sup> mice than with Mdm2<sup>WT</sup> mice, and this may cause premature aging. Additionally, simple culture of Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> embryos *in vitro* at the different oxygen tensions, could shed some light on the role of Akt-Mdm2 signaling in development. Furthermore, generation of double mutant mice (Mdm2<sup>S163A;S183A</sup>) may provide more information of the role of Mdm2 phosphorylation by Akt in development. It is conceivable that in Mdm2<sup>S183A</sup> mice, phosphorylation of S163 compensates for the loss of S183 phosphorylation and vice versa.

### **Mdm2-S183 phosphorylation in cell proliferation**

At endogenous levels, the phosphorylation of Mdm2-S183, but not Mdm2-S163, is required for normal cell proliferation in MEFs (Figure 2.2 and 2.3). Additionally, Mdm2<sup>S183A</sup> MEFs were more prone to premature senescence compared with Mdm2<sup>WT</sup> and Mdm2<sup>S163A</sup> MEFs, hence they failed to proliferate (Figure 2.2). Moreover, Mdm2-S183 residue is also more conserved than S163 (Lane et al., 2011). Interestingly, the proliferation defect in Mdm2<sup>S183A</sup> MEFs was rescued by culturing these cells at low oxygen in the presence of reactive oxygen species scavenger, N-acetyl cysteine (NAC) (Figure 2.3). This suggests that Mdm2-S183, but not Mdm2-S163, phosphorylation protects cells from premature senescence induced by oxidative stress. It will be useful to also cross Mdm2<sup>S183A</sup> mice with p53<sup>-/-</sup> mice to further determine that senescence observed in Mdm2<sup>S183A</sup> is indeed p53-dependent. These proliferation assay results are in contrast to findings using Mdm2<sup>Y393F</sup> and Mdm2<sup>S394A</sup> MEFs which exhibited normal proliferation kinetics similar to Mdm2<sup>WT</sup> MEFs (Gannon et al., 2012a, Carr et al., 2016c). This further reinforces the notion that different Mdm2 phosphorylation events have contrasting functions.

Mdm2<sup>S183A</sup> MEFs cultured at high oxygen expressed elevated levels of p53 which were accompanied by reduced Mdm2 protein levels compared with Mdm2<sup>WT</sup> MEFs (Figure 2.5). Additionally, activation of Akt using IGF-1 led to increased p53 expression in Mdm2<sup>S183A</sup> than in Mdm2<sup>WT</sup> MEFs. This increase in

p53 levels in Mdm2<sup>WT</sup> was accompanied by a decrease in Mdm2 levels. This data suggests that phosphorylation of Mdm2-S183 stabilizes Mdm2 in the presence of oxidative stress and promotes p53 degradation. However, Mdm2 phosphorylation following stress like DNA damage generally destabilizes Mdm2 (Gannon et al., 2012a, Meek and Hupp, 2010, Carr et al., 2016a), potentially through ubiquitin-mediated degradation. For instance, ATM phosphorylates and promotes nuclear localization of casein kinase I (CKI) (Wang et al., 2012). Phosphorylation of Mdm2 by CKI at multiple serine residues in response to DNA damage facilitates its interaction with SCF (beta-TRCP) which ubiquitinates Mdm2 and promotes its degradation (Inuzuka et al., 2010). This suggests that phosphorylated Mdm2 is a target for E3 ligases following exposure to stress.

Since Mdm2-S183 phosphorylation enhances Mdm2 localization into the nucleus (Figure 2.8), it is plausible that this protects some of the Mdm2 from E3 ligases, hence the increased stability observed in Mdm2<sup>WT</sup> MEFs than in Mdm2<sup>S183A</sup> MEFs. Reduced Mdm2 stabilization in Mdm2<sup>S183A</sup> MEFs may also be due to altered levels of other post translational modifications that promote Mdm2 stabilization such as sumoylation (Buschmann et al., 2001), or decreased expression of Mdm2 deubiquitinating enzymes such as HAUSP (Meulmeester et al., 2005). Therefore, the mechanism governing how Mdm2-S183 phosphorylation alters Mdm2 protein levels still need to be elucidated. This may include determining the levels of Mdm2 ubiquitination in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup>

MEFs. In addition, looking at the presence or expression levels of other proteins or modifications that can enhance Mdm2 degradation may shed some light on the effects of Mdm2-S183 phosphorylation on Mdm2 stability. Furthermore, pulse-chase experiments on nuclear and cytoplasmic lysates from high oxygen Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs can also reveal how Mdm2 phosphorylation by Akt alters Mdm2 stability.

My findings showing that Mdm2-S183 phosphorylation promotes cell proliferation in MEFs in the presence of oxidative stress (Figures 2.2 and 2.3), are consistent with the well-established role of Akt in promoting cell growth, survival, and proliferation. Findings from over expression studies *in vitro* showed that Mdm2 phosphorylation by Akt reduces p53 levels and activity, and this is thought to be one of the ways through which Akt promotes cell survival. PI3K/Akt signaling promotes oxidative metabolism in the mitochondria critical for promoting cell survival and this contributes to the generation of ROS (Schieber and Chandel, 2014). ROS can activate the PI3K/Akt pathway (Ushio-Fukai et al., 1999, Okoh et al., 2013), suggesting the existence of a cyclical relationship between ROS and the PI3K/Akt pathway. High levels of ROS can activate p53's prooxidant functions resulting in senescence and/or apoptosis (Liu et al., 2008). Therefore, it is conceivable that ROS promotes Mdm2-S183 phosphorylation by Akt and the subsequent reduction in p53 activity contribute to cell proliferation and survival under oxidative stress. To further understand the

effects oxidative stress on Akt/Mdm2/p53 signaling axis, I still have to ascertain the activation of Akt by ROS in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> MEFs cultured at high oxygen compared to those at low oxygen or low oxygen treated with hydrogen peroxide.

Moreover, there is also a possibility that other kinases similar to Akt may phosphorylate these residues. For instance, serum and glucocorticoid regulated kinase 1 (Sgk1) has been shown to phosphorylate human Mdm2-S166 and -S188 (Amato et al., 2009). IGF-1 experiments in Chapter II show that Akt can potentially phosphorylate Mdm2 and affect p53 levels. Therefore, it is possible that Sgk1 also phosphorylates these residues and affect p53 levels and function. Experiments to determine these kinases may include pull-down of wild type and single or double mutant Mdm2 and perform western blot analyses with currently non-existent antibodies against Mdm2 phospho-S163 and phospho-S183. Additionally, *in vitro* kinase assays with either wild type and single or double mutant Mdm2 with various kinases that have phosphorylation motifs present in Mdm2 can further shed some light on the type of kinases that modifies the residues in question.

Another alternative will be to perform a screen to determine the phosphatase responsible for dephosphorylating these serine residues. For instance, overexpression of specific phosphatases may cause phenotypes

similar to those observed in Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> MEFs. It is possible that different phosphatases influence the phosphorylation status of these two residues hence the different phenotypes observed. Alternatively, Mdm2-S183 may be preferentially phosphorylated compared with Mdm2-S163 and pull-down and mass spectrometry analyses may determine the phosphorylation status regarding the context and levels. Also, generating Mdm2 phosphomimic mice models in which either Mdm2-S163 and/or Mdm2-S183 are substituted with aspartic acid will allow me to further demonstrate that the effects observed in Mdm2 mutant MEFs used in my studies are due to phosphorylation. Additionally, if Mdm2<sup>S183D</sup> MEFs phenotypically resemble Mdm2<sup>WT</sup> MEFs, this will further demonstrate that the observed proliferation defects and the effects on p53 levels and function are due to the mutation in Mdm2.

### **Mdm2-S183 phosphorylation in DNA damage responses**

Emerging evidence shows that Mdm2 phosphorylation is critical in regulating the DNA damage response. Mdm2-S394 phosphorylation by ATM promotes p53 stabilization and activity in response to DNA damage (Gannon et al., 2012a). However, Mdm2-Y393 phosphorylation does not affect p53 stabilization and activity following DNA damage, but promotes bone marrow repopulation thereby causing radioresistance in mice (Carr et al., 2016c). Similarly, there was no significant difference in p53 stabilization and activation, p53-dependent apoptosis, and cell cycle arrest between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup>

MEFs in response to DNA damage (Figure 2.9-2.10). This indicates that Mdm2-S183 phosphorylation does not affect p53-mediated responses to DNA damage from ionizing radiation, etoposide, and doxorubicin examined here. It is plausible that Akt-mediated phosphorylation of Mdm2-S163 following DNA damage compensates for loss of Mdm2-S183 phosphorylation. This may explain the comparable levels of p53 activation and stability, and cell cycle progression in Mdm2<sup>S183A</sup> MEFs compared with controls. However, Mdm2<sup>S183A</sup> MEFs exhibited more 8-Oxo-2'-deoxyguanosine adducts than Mdm2<sup>WT</sup> MEFs, suggesting that Mdm2<sup>S183A</sup> MEFs are more prone to oxidative DNA damage (Figure 2.6). It seems the effects of Mdm2-S183 phosphorylation in DNA damage response are dependent on the type of stress signals causing the damage. In addition, it is also possible that the time cells are exposed to the stress causing the DNA damage affects the type of response. Time spent under exposure to genotoxic agents in low oxygen MEFs used to study the DNA damage response is different from the chronic exposure to oxidative stress experienced by high oxygen MEFs. Furthermore, ionizing radiation and the genotoxic drugs examined here induce double stranded breaks whilst oxidative stress often induce DNA base oxidation and alkylation.

It will also be informative to generate and use the double mutant Mdm2<sup>S163/S183A</sup> mice to further characterize the effects of Mdm2 phosphorylation in the DNA damage response. The proposed model will eliminate the



confounding effects of Mdm2-S163 phosphorylation in potentially influencing the observed results in Mdm2<sup>S183A</sup> MEFs and mice following DNA damage. Additionally, if reagents were available, it will be important to confirm Mdm2 phosphorylation following DNA damage and exposure to oxidative stress.

### **Mdm2-S183 phosphorylation in tumorigenesis**

Here, I have shown that Mdm2<sup>S183A</sup> mice were less susceptible to DEN-induced tumorigenesis compared with Mdm2<sup>WT</sup> mice (Figure 3.1). In addition, there was no difference in the amount of DEN-induced ROS between DEN Mdm2<sup>S183A</sup> mice and Mdm2<sup>WT</sup> mice (Figure 3.2). Since Mdm2<sup>S183A</sup> livers expressed significantly higher levels of p53-dependent senescence markers compared with controls, I concluded that Mdm2-S183 phosphorylation promotes DEN-induced hepatocellular carcinoma (HCC) by inhibiting p53-dependent senescence triggered by ROS and DEN-induced DNA damage.

A recent study showed that CD44 expression induced by DEN administration enhances Akt-mediated phosphorylation of Mdm2 leading to reduced p53-dependent senescence and apoptosis in response to DEN-induced DNA damage (Dhar et al., 2018). This promotes the proliferation of damaged cells that will act as HCC progenitors. These findings are consistent with the data presented here showing that Mdm2-S183 phosphorylation by Akt diminishes p53-dependent senescence and promotes liver tumorigenesis (Figure 3.1 and 3.2). In Mdm2<sup>S183A</sup> mice treated with DEN, it is plausible that the observed p53-

mediated senescence, facilitates the clearance of potential HCC progenitors together with reduced proliferation and increased cell death. However, since I did not observe differences in DNA damage induced by DEN in Mdm2<sup>S183A</sup> and Mdm2<sup>WT</sup> mice, it will be informative to examine the effects of Akt-mediated phosphorylation of Mdm2 on DEN-induced compensatory proliferation and cell death.

Moreover, It is also possible that both Mdm2-S163 and -S183 phosphorylation promote DEN-induced liver tumorigenesis but through slightly different mechanisms. Hence, it is important to determine the effects of Mdm2-S163 phosphorylation in DEN-mediated liver tumorigenesis in Mdm2<sup>S163A</sup> mice. Additionally, determining whether the suppression of DEN-induced liver cancer in double mutant mice (Mdm2<sup>S163A/S183A</sup>) is comparable to those of CD44<sup>-/-</sup> mice will also shed some light on the individual contribution of each phosphorylation site in promoting liver tumorigenesis.

Mdm2-S183 phosphorylation also promotes skin neoplasia following exposure to carcinogens DMBA and TPA (Figure 3.3-3.4). Overall, Mdm2<sup>S183A</sup> mice exhibited reduced papilloma incidence, volume, and number compared with Mdm2<sup>WT</sup> mice. Similar to DEN-liver cancer model, there was evidence of p53-dependent senescence which likely contributes to tumor suppression in Mdm2<sup>S183A</sup> mice. Administration of TPA is known to cause oxidative stress

(Perchellet and Perchellet, 1989, Perchellet et al., 1988). Therefore, it is conceivable that in Mdm2<sup>S183A</sup> MEFs, elevated levels of ROS from the administration of TPA triggered p53 activation and the expression of senescence regulators like p21. Notably, some mice lost these senescence markers a few weeks before the presentation of papillomas, suggesting that loss of senescence potentially facilitates the progression from epidermal hyperplasia to papilloma formation. Additionally, unlike Mdm2<sup>S183A</sup> mice, there was barely any positive staining for senescence in skin of Mdm2<sup>WT</sup> mice after a month on TPA promotion, further implying that p53-mediated senescence contributes to tumor suppression. It is also important to note that p53 protein expression was maintained even to the point when Mdm2<sup>WT</sup> mice started presenting with papillomas. This may suggest the presence of additional pro-tumorigenic mutations or activation of other pathways that may affect downstream p53 signaling.

Susceptibility to DMBA/TPA-induced tumorigenesis depends on the genetic background in mice (Boutwell, 1964). This stems mainly from differences in metabolizing the tumor promoting agents as well as their mode of action in promoting tumorigenesis (Slaga, 1983). More sensitive models develop papillomas sooner and progress to squamous cell carcinomas in about 20 weeks, which may not occur in C57BL/6 mice since they are the least sensitive (DiGiovanni et al., 1993, DiGiovanni et al., 1991). Since p53 is lost during these malignant stages, backcrossing the Mdm2 mutation to another strain more

sensitive to DMBA/TPA tumorigenesis will be more effective at delineating the role of Mdm2 phosphorylation by Akt on p53-mediated tumor suppression. PI3K/Akt pathway is also activated by the administration of DMBA and TPA (Suzuki et al., 2003). It is plausible that Mdm2-S183 phosphorylation, which increases Mdm2 nuclear localization and stability, may also hasten the loss of p53 to promote malignant tumor growth. Moreover, DMBA/TPA-induced tumors are primarily papillomas and there is no human equivalency. Therefore, sensitive strains that progress to spindle and squamous cell carcinoma which is equivalent to human squamous cell carcinoma will provide more therapeutically relevant information regarding the role of Akt-signaling to Mdm2 in epithelial cancers.

Oncogenic K-ras activate many pathways including the PI3K/Akt pathway to promote cell growth, proliferation, and survival. Oncogenic K-ras cooperates with p53 to induce senescence (Serrano et al., 1997) (Ferbeyre et al., 2002). Senescence is often observed during the early stages of K-ras driven tumorigenesis and contributes to tumor suppression (Pylayeva-Gupta et al., 2011). I found that oncogenic Ras expression triggered the accumulation of ROS (Figure 3.5a), and Mdm2<sup>S183A</sup> MEFs were more prone to p53-mediated senescence triggered by oncogenic Ras expression (Figure 3.5b-c). Furthermore, phosphorylation of Mdm2 at S183 increased susceptibility to oncogenic K-ras-driven lung tumorigenesis in mice (Figure 3.5d). These findings suggest that oncogenic-ras expression triggers ROS accumulation and elevates

p53 expression giving rise to senescence which likely contributed to enhanced tumor suppression observed in K-ras<sup>G12D/+</sup>, Mdm2<sup>S183A</sup> mice.

Oncogenic mutations in various isoforms of Ras also have distinct functional consequences in cancer (Haigis et al., 2008). There is evidence showing that mutations at the three hot spots in K-ras confer different oncogenic potencies, suggesting that G12 mutations may have dissimilar effects compared with G13 and Q61 mutations (Hunter et al., 2015, Ihle et al., 2012). Therefore, the effect of Mdm2-S183 phosphorylation in tumor suppression may be differentially altered depending on the type of K-ras mutation or the isoform of oncogenic Ras being expressed. Since Mdm2<sup>S183A</sup> MEFs expressing *K-ras*<sup>G12V</sup> exhibited more senescent cells compared with Mdm2<sup>WT</sup> MEFs, future work may focus on determining the presence and amount of senescence during the early stages of lung cancer in K-ras<sup>G12D/+</sup>;Mdm2<sup>WT</sup> and K-ras<sup>G12D/+</sup>;Mdm2<sup>S183A</sup> mice. This will provide information on whether the effects of Mdm2-S183 phosphorylation on tumorigenesis are similar in cells expressing either the G12V or G12D mutations. Nonetheless, findings from the DMBA/TPA model in which H-ras (Brown et al., 1990) and K-ras mutations (Huang and Balmain, 2014) play pivotal roles in tumor initiation, were consistent with my findings using the K-ras lung cancer model. This implies that the effects of Mdm2-S183 phosphorylation may be similar in cancer models expressing different isoforms of Ras.

In most carcinogen-induced cancer models, including the ones examined here, the immune cells play a pivotal role in facilitation tumor formation through inflammation. Therefore, it will be important to generate tissue specific Mdm2<sup>S183A</sup> transgenic mice in which these immune cells express wild type Mdm2 since the effects of Mdm2-S183A mutation on other critical cells involved in tumor formation is unknown. In addition, tissue specific expression may be more physiologically relevant since most cancer causing mutations are somatic and not germline. Therefore, despite the Mdm2<sup>S183A</sup> mutation not being cancer causing in my studies, it will be more informative to understand its effects in a tissue specific context in which other cells, such as immune cells, express wild type Mdm2.

Mdm2-S183 phosphorylation may exert different effects in suppressing tumorigenesis induced by other oncogenes such as Myc and Beta-catenin. There is evidence suggesting that expression of Myc promotes the accumulation of ROS and reduces p53-mediated DNA damage responses (Vafa et al., 2002). Expression of oncogenes, such as Myc, can trigger the loss of p53 or the negative regulator of Mdm2 known as Arf (Zindy et al, 1998). Therefore, it is possible that in tumor models driven by the expression of Myc, Mdm2<sup>S183A</sup> mice will exhibit enhanced tumor suppression through senescence facilitated by Myc-induced increase in ROS accumulation and Mdm2-S183A protein expression.

There is evidence demonstrating that oxidative stress can activate Wnt and Beta-catenin signaling (Funato et al., 2006). In contrast, other studies have shown that oxidative stress inhibits the downstream effects of Wnt and Beta-catenin signaling by increasing the activity of FOXO transcription factors to reduce cell proliferation allowing for oxidative DNA damage repair (Essers et al., 2005). The discrepancy in these studies may be due to the level of oxidative stress as well as the context. I have demonstrated that high levels of oxidative stress promote senescence in Mdm2<sup>S183A</sup> MEFs and tumor suppression in Mdm2<sup>S183A</sup> mice with carcinogen- and oncogene-induced tumors. Therefore, loss of Mdm2-S183 phosphorylation may also promote tumor suppression in mice with cancers driven by Wnt and Beta-catenin signaling, for example if there is an increase in the expression of FOXO transcription factors. In this case, tumor suppression in Wnt and Beta-catenin-driven tumors in Mdm2<sup>S183A</sup> mice may be mediated through senescence or other mechanisms including apoptosis.

## **Potential therapeutic implications**

ROS accumulation induced by the activation of PI3K/Akt pathway triggers Nrf2 nuclear localization (Menegon et al., 2016). Nrf2 is a transcription factor central to ROS detoxification and it activates the expression of antioxidant genes such as glutathione S-transferase and superoxide dismutase (Kansanen et al., 2013). Inhibition of PI3K/Akt signaling in BRCA1-deficient tumors using BKM120 or overexpression of PTEN sensitizes these tumors to elevated levels of ROS (Gorrini et al., 2014). Therefore, it is plausible that temporary inhibition of Mdm2-S183 phosphorylation in cancer cells expressing hyperactive Akt together with the administration of chemotherapeutic agents that perturb the redox balance such as Nrf2 inhibitor Brusatol, can further sensitize cells to oxidative stress. This can manifest as senescence and/or apoptosis thereby slowing down the development and/or progression of tumors with hyperactive PI3K/Akt signaling.

In addition, sensitizing cells to ROS is critical especially in breast cancers that are resistant to PI3K/AKT signaling inhibitors. Withdrawal of PI3K/Akt inhibitors causes Akt-independent metabolic rewiring characterized by massive generation of ROS and profound proliferation defects, all of which are rescued by the administration of ROS scavengers such as NAC (Dermitt et al., 2017). In this case, blocking Mdm2-S183 phosphorylation during the drug withdrawal period could sensitize these cells to senescence and halt the potential expansion of cancer cells resistant to PI3K/Akt inhibitors such as GDC-0941.



Most tumors disrupt p53 signaling and previous studies have shown that p53 reactivation can cause tumor regression in mice (Xue et al., 2007, Ventura et al., 2007). For instance, p53 reactivation in lung carcinomas triggers senescence and immune clearance of tumorigenic cells (Xue et al., 2007). Additionally, p53 reactivation triggers apoptosis in lymphomas and senescence in sarcomas (Ventura et al., 2007). A significant percentage of tumors express functionally inert wild type p53 due to Mdm2 gene amplification like in the case of most human sarcomas (Oliner et al., 1992). Therefore, it is conceivable that progression of tumors may be halted by treatment of small molecule inhibitors that block Mdm2-S183 phosphorylation combined with agents that perturb redox homeostasis.

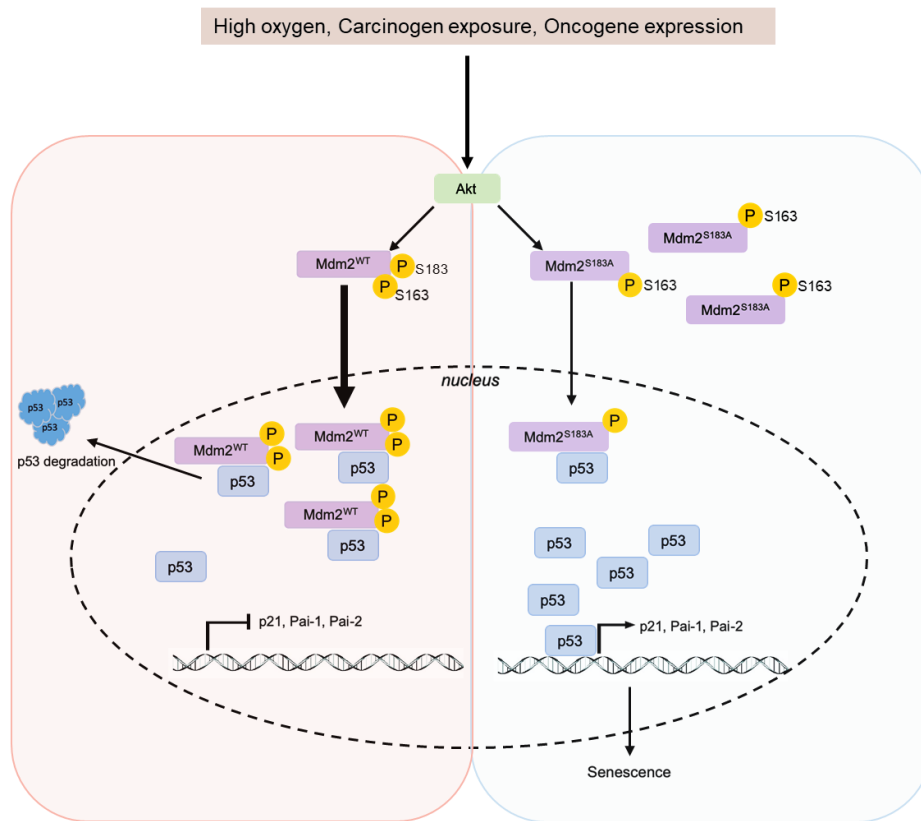
Efforts to reactivate p53 by blocking Mdm2-S183 phosphorylation may have to be temporary. Inhibition of Mdm2-S183 phosphorylation in order to reactivate p53, especially in highly metabolic tumors, may also affect other normal tissues which are more prone to p53 induced-apoptosis such as the intestines and spleen. Additionally, Mdm2-mediated inhibition of p53 is required even in adult tissues. For instance, loss of Mdm2 function in mice expressing inducible p53 (p53<sup>ERTAM KI</sup>) causes the ablation of radiosensitive tissues and acute lethality in adult mice (Ringshausen et al., 2006). Since blocking Mdm2-S183 phosphorylation does not get rid of Mdm2, it is possible that this will not

affect normal tissues as is the case with other Mdm2 inhibitors such as MI-219 which selectively triggers apoptosis in tumor cells (Shangary et al., 2008). MI-219 still activates p53 in normal tissues, but at low levels that are not toxic and this is regulated through mechanisms that are yet to be elucidated. Furthermore, It is also important to consider the p53-independent effects of Mdm2 inhibition since Mdm2 binds to other effector proteins. My findings showing that Mdm2-S183 phosphorylation prevents p53-mediated premature senescence triggered by oxidative stress will also be useful in potential therapeutic efforts for other disease states in which p53 and ROS play central roles including diabetes, cardiovascular disease, and aging.

Activation of Akt allows cells to overcome the G2/M cell cycle checkpoint induced by DNA damage (Kandel et al., 2002). Akt is activated by ionizing radiation in some glioblastoma cell lines and inhibition of both EGFR and PI3K radiosensitizes these cells suggesting that Akt activation from radiation therapy promotes radioresistance (Li et al., 2009). Constitutive Akt activation is also observed in cisplatin resistant lung cancer, ovarian cancer, and glioma cell lines (Winograd-Katz and Levitzki, 2006). Activation of Akt in response to ionizing radiation reduces p53 levels in cell lines (Boehme et al., 2008). Therefore, it is also possible that Akt signaling following DNA damage promotes drug resistance in tumors by abrogating p53-mediated DNA damage responses such as cell cycle arrest and apoptosis. Since some therapeutic agents trigger ROS-mediated

apoptosis, combination therapies with compounds that inhibit Mdm2-S183 phosphorylation in tumors with hyperactive Akt may promote cell death by exacerbating oxidative DNA damage.

## General conclusions



**Figure 4.1. Proposed Model.** In the presence of oxidative stress from exposure to high oxygen, carcinogens, and oncogene expression, Mdm2-S183 phosphorylation by Akt promotes Mdm2 nuclear localization and enhances p53 degradation. As a result, this promotes cell proliferation by reducing p53-mediated senescence

In this dissertation, I have shown that Mdm2-S183 phosphorylation by Akt at endogenous levels promotes normal cell proliferation under oxidative stress.

Mdm2-S183 phosphorylation enhances Mdm2 nuclear localization and stability resulting in diminished p53 levels and activity. Therefore, loss of this phosphorylation site increases p53 levels and activity thereby triggering premature senescence likely mediated by p53 in the presence of oxidative stress.

The effects of Mdm2-S183 phosphorylation by Akt on p53-dependent responses are different from those exerted by ATM- and c-Abl phosphorylation of Mdm2, but with some shared similarities in terms of promoting tumor suppression. This reinforces the notion that Mdm2 post translational modifications are critical in influencing the type of p53-dependent responses to stress. This is important when thinking about therapeutic interventions such as p53 reactivation cancer. The type of stress contributing to different disease states, will require distinct ways of activating p53 through Mdm2 in order to obtain the desired therapeutic outcomes.

**APPENDIX: PHOSPHORYLATION OF MDM2-S183 ALTERS ASPECTS OF  
GLUCOSE METABOLISM**

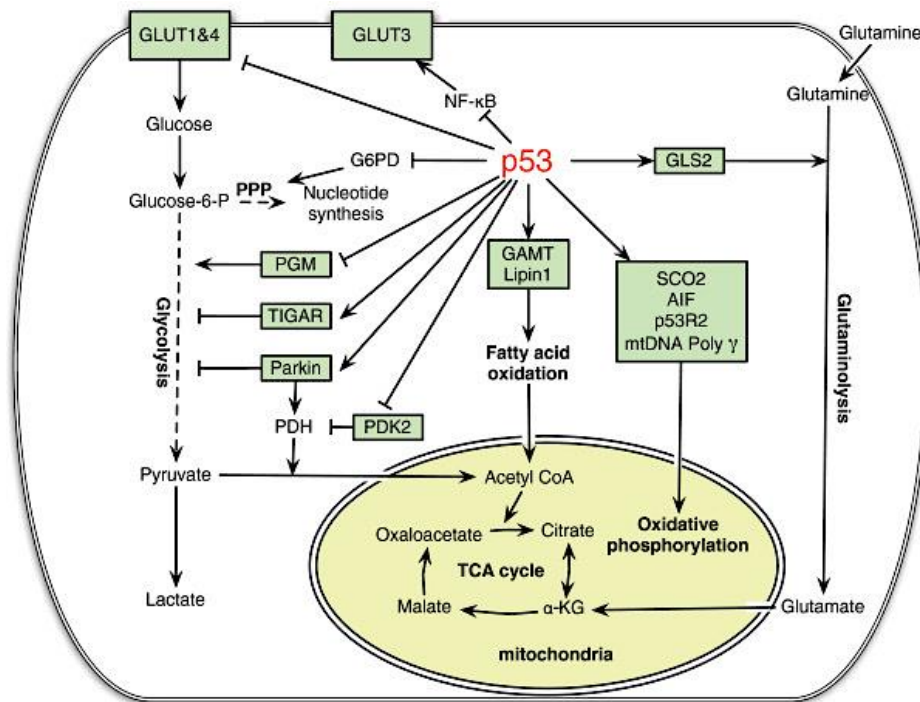
## Introduction

### *Role of p53 in glycolysis and mitochondrial respiration*

p53 has been shown to play pivotal roles in metabolic pathologies such as cancer and diabetes (Vousden and Ryan, 2009). Additionally, p53 generally favors an increase in oxidative phosphorylation whilst reducing glycolysis. Most cancer cells primarily depend on glycolysis for energy generation and p53 transcriptionally activates genes that inhibit this pathway, suggesting that p53-dependent regulation of glucose metabolism is consequential to tumor suppression. p53-dependent inhibition of glycolysis is mainly through the transcriptional regulation of metabolic genes.

A plethora of metabolic genes are known to be activated by p53 as briefly discussed in chapter 1 and summarized in appendix figure 1. Briefly, p53 controls cellular glucose uptake by inhibiting the expression of glucose transporters Glut 1,3 and 4 (Kruiswijk et al., 2015, Zhang et al., 2013) . Additionally, p53 also promotes mitochondrial respiration by activating the expression of key enzymes that control the flux of metabolites into the mitochondria such as PDK2 and GLS2 (Hu et al., 2010, Suzuki et al., 2010, Contractor and Harris, 2012). p53 also inhibits glycolysis by shuttling glycolytic intermediates into the pentose phosphate pathway which is important for nucleotide synthesis by triggering the expression of Tigar (Li et al., 2014). On the other hand, p53 activates the expression of genes that promote oxidative phosphorylation such as Sco2 and Parkin. SCO2

regulates the function of cytochrome oxidase complex and Sco2 deficient human cells that express wild type p53 display enhanced glycolysis that is similar to what is observed in p53<sup>-/-</sup> MEFs (Matoba et al., 2006). Furthermore, Parkin expression promotes oxidative phosphorylation by enhancing the expression of pyruvate dehydrogenase which is a crucial enzyme in pathways that generate acetyl-CoA which is a major substrate for mitochondrial respiration Acetyl-coA (Zhang et al., 2011).



**Appendix figure 1: p53-dependent regulation of glucose metabolism.** p53 blocks cellular glucose uptake by inhibiting glucose transporters Glut 1,3 and 4. It inhibits glycolysis by triggering the expression of genes such as Tigar and Parkin, both of which play critical roles in promoting glycolysis. On the other hand, p53 promotes oxidative phosphorylation by activating genes such as SCO2 and interacts with mtDNA Poly γ required to maintain the proper mitochondrial function. This image was directly adapted from (Liang et al., 2013)

### *Role of p53 in diabetes*

The two main forms of diabetes are characterized by either insulin deficiency due to impaired function or death of pancreatic beta islet cells (Type I) or diminished response to insulin due to inefficient insulin absorption by tissues such as muscles, liver and adipose tissue (Type II). Type II diabetes is often characterized by both insulin resistance and impaired beta-cell function (Cavaghan et al., 2000). Previous work has shown that p53 influences several aspects of diabetes.

There is evidence to show that p53 is involved in the pathophysiology of diabetes, particularly in promoting insulin resistance. The idea that p53 activation affects insulin function came from a study showing that p53 negatively regulates expression of the insulin receptor (Webster et al., 1996). In addition, p53 and p53-target genes are activated in the adipose tissues of obese and insulin intolerant mice (Yahagi et al., 2003). Various studies have been done using both *in vitro* and *in vivo* models to determine the role of p53 in molecular events governing the development of diabetes. For instance, p53 expression in adipose tissues of mice with type 2 diabetes-like disease under high fat diet promoted ROS generation, senescence and inflammation in the adipose tissue (Minamino et al., 2009). Loss of p53 ameliorated this phenotype and provided protection against insulin resistance. In addition, embryonic lethality in Lig4<sup>-/-</sup> mice caused by defective non-homologous end joining is rescued by the concomitant loss of



p53 (Tavana et al., 2010). However, Lig4<sup>-/-</sup>;p53<sup>-/-</sup> mice are susceptible to lymphomagenesis which is abrogated by the expression of apoptosis deficient p53 mutant (p53<sup>R172P</sup>), but this results in acute diabetes and death. p53<sup>R172P</sup> still retains the ability to trigger senescence and cell cycle arrest, suggesting that p53-mediated senescence and apoptosis in the adipose tissue and pancreas promotes the development of diabetes. Furthermore, loss p53's E3 ubiquitin ligase arf-bp1 in pancreatic beta cells led to diabetes and death in transgenic mice by 12 months and this phenotype was rescued by the loss of p53 (Kon et al., 2012). Taken together, these studies indicate that p53 activation in the adipose tissue and in the pancreas can trigger apoptosis or senescence. This potentially alters insulin production and sensing resulting in diabetes-like phenotypes including insulin resistance.

Mdm2 inhibition using nutlin-3a activates p53-target genes known to enhance gluconeogenesis (Goldstein et al., 2013), suggesting that p53 promotes gluconeogenesis. Additionally, in a mouse model of diabetes and diet-induced obesity (A<sup>y</sup> mice), p53 expression activates gluconeogenic genes in the liver (Minamino et al., 2009). Consistently, p53<sup>-/-</sup> mice exhibit reduced ability to trigger gluconeogenesis compared with wild type mice following starvation and this is mediated by p53-induced expression of pantothenate kinase-1 (Wang et al., 2013). Insulin suppresses gluconeogenesis in healthy individuals but fails to do

so in some cases of type 2 diabetes. Therefore, promotion of gluconeogenesis by p53 may contribute to insulin resistance in humans and rodents.

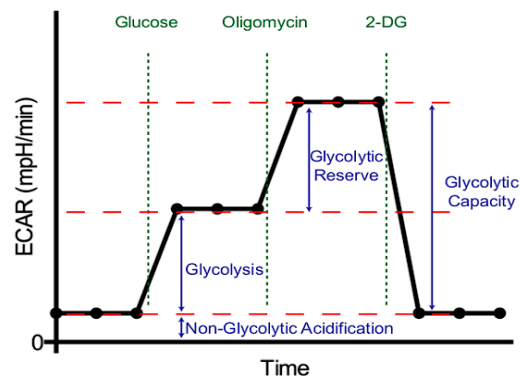
The effects of p53 in influencing insulin function is also observed in other insulin-responsive tissues like the skeletal muscles. p53 is expressed in skeletal muscles of aged rodents and is thought to trigger senescence often present in old muscle cells (Zwetsloot et al., 2013). Consistently, insulin signaling is impaired in muscle cells due to p53-dependent senescence triggered by ceramide (Jadhav et al., 2013). Additionally, in a rat model of Type II diabetes, exercise training lowered p53 and TIGAR levels in skeletal muscles, consequently resulting in reduced levels of ROS and diminished insulin resistance (Qi et al., 2011). p53 is also activated in endothelial cells of blood vessels in diabetic patients where it enhances endothelial senescence (Orimo et al., 2009). It has been established that dysfunctional insulin signaling in endothelial cells compromises insulin-mediated capillary recruitment thereby reducing blood flow and glucose uptake by skeletal muscles (Clark et al., 2003). Accordingly, in a mouse model of diet-induced obesity, p53 expression is elevated in endothelial cells and this triggers insulin resistance which is ameliorated by endothelial cell-specific p53 deficiency (Yokoyama et al., 2014).

Given that p53 alters cellular glucose metabolism by favoring oxidative phosphorylation instead of glycolysis, I posited that Mdm2-S183 phosphorylation

by Akt, which I have shown to lower p53 levels (Figure 2.7-2.8), promotes glycolysis in MEFs and glucose intolerance in mice. In addition, phosphorylation of Mdm2-S183 enhanced p53-mediated senescence in the presence of oxidative stress. Therefore, since p53 expression can induce senescence and cell death in the pancreas and adipose tissues, I also hypothesized that blocking Mdm2-S183 phosphorylation may promote insulin resistance whilst reducing glucose-stimulated insulin release.

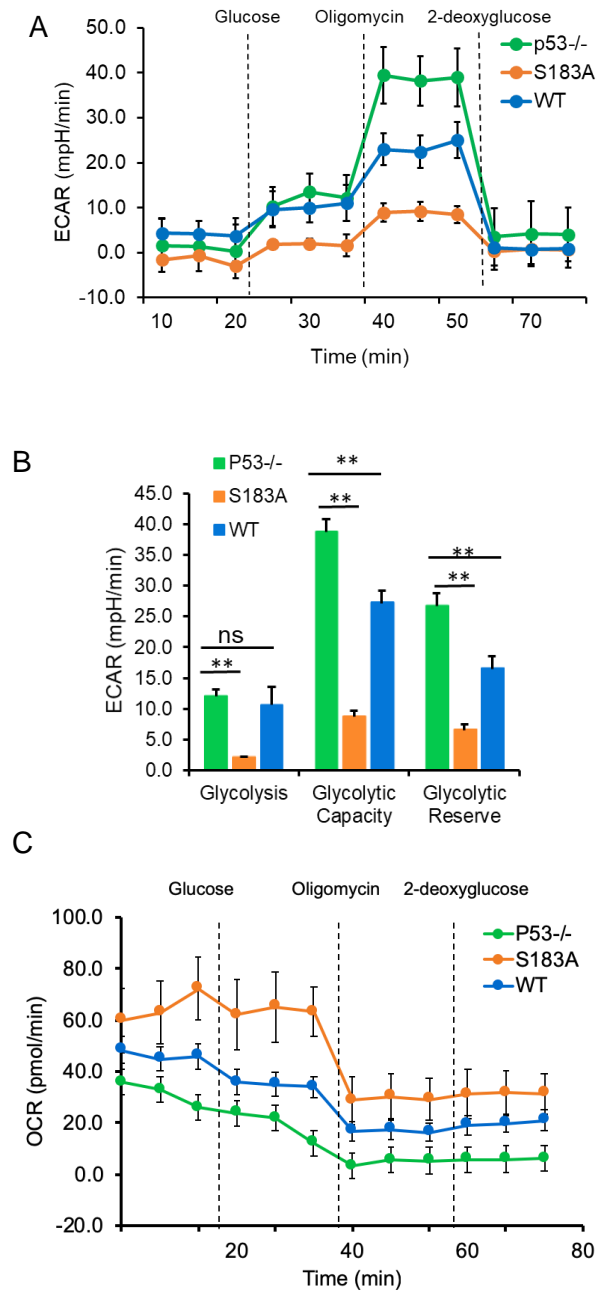
## Results

To evaluate whether Mdm2-S183 phosphorylation affects cellular glucose metabolism, I used the extracellular flux analyzer (Seahorse XF<sup>e</sup> 96) to perform glycolysis stress test (Appendix Figure 2) to measure glycolytic pathway capacity in Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs. Glycolysis produces lactate and H<sup>+</sup>, both of which are the major sources of media acidification during glycolysis stress test. First, the addition of glucose activates glycolysis leading to an increase in extracellular acidification rate (ECAR). Second, the addition of oligomycin, which inhibits ATP generation through mitochondrial respiration, causing a massive second increase in ECAR (glycolysis reserve). Lastly, the addition of 2-deoxyglucose (2-DG) totally abolishes glycolysis thereby bringing ECAR down to levels similar to those present in starved cells at the beginning of the experiment (glycolytic capacity). An increase in glycolysis is usually accompanied by a decrease in oxygen consumption rate (OCR) arising from the decrease in oxidative phosphorylation.



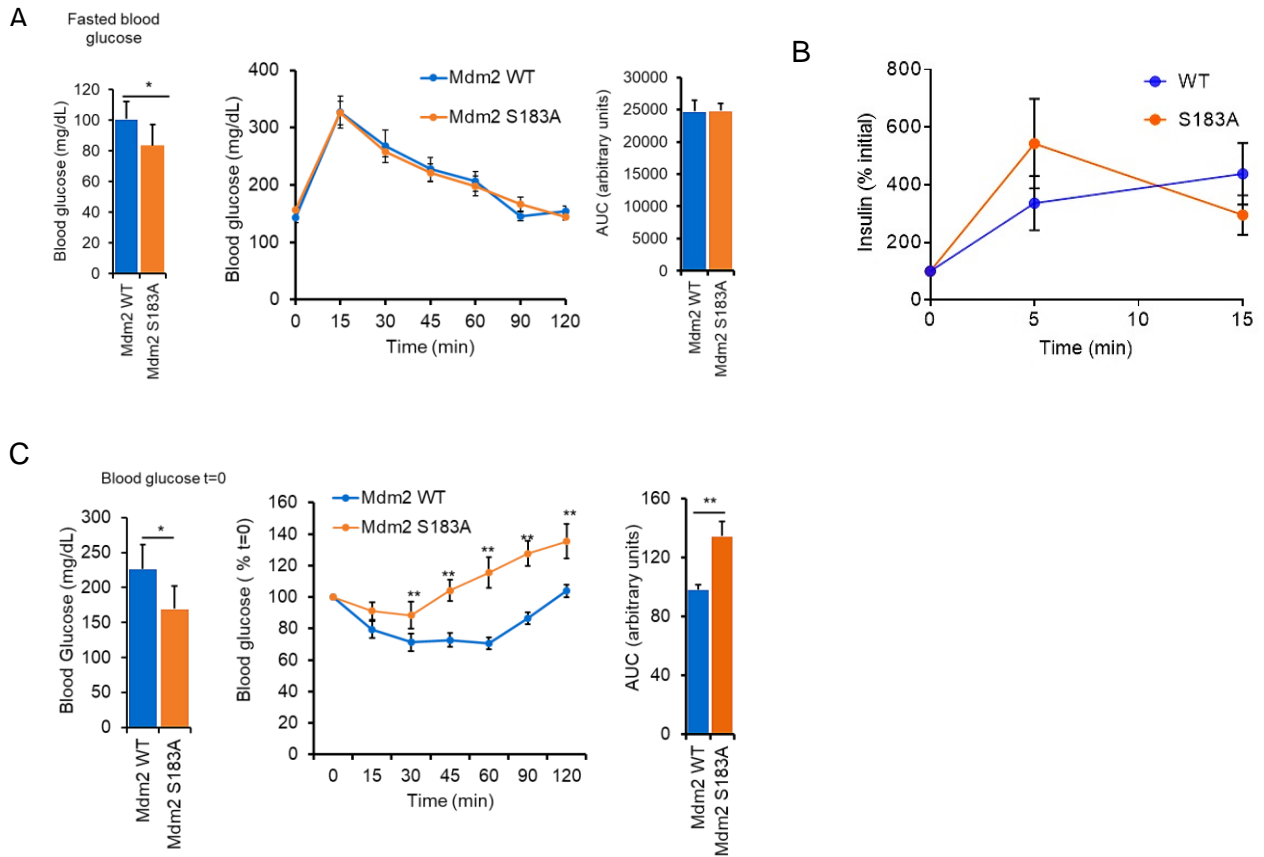
**Appendix Figure 2.** Schematic diagram highlighting the different stages in a glycolysis stress test. Adapted from (Traba et al., 2016)

The glycolysis was significantly reduced in Mdm2<sup>S183A</sup> MEFs compared with Mdm2<sup>WT</sup> and p53<sup>-/-</sup> MEFs (Appendix Figure 3a-b). In addition, Mdm2<sup>S183A</sup> MEFs exhibited the lowest glycolytic capacity and glycolytic reserve, significantly different to the levels present in Mdm2<sup>WT</sup> and p53<sup>-/-</sup> MEFs. Accordingly, the reduction in glycolysis observed in Mdm2<sup>S183A</sup> MEFs also corresponded with elevated oxygen consumption rates which were significantly higher than in Mdm2<sup>WT</sup> and p53<sup>-/-</sup> MEFs, presumably from oxidative phosphorylation (Appendix Figure 3c). The oxygen consumption rate was lowest in p53<sup>-/-</sup> MEFs suggesting that loss of p53 reduces oxidative phosphorylation consistent with previous work in HCT116 cells (Matoba et al., 2006). This demonstrates that Mdm2 phosphorylation, which leads to slightly elevated levels of p53 in low oxygen MEFs (Figure 2.5 b), reduces oxidative phosphorylation and promotes the metabolism of glucose via glycolysis.



**Appendix Figure 3. Mdm2-S183 phosphorylation promotes glycolysis in MEFs.** (A) Assessment of glycolysis, glycolytic capacity, and glycolytic reserve in low oxygen Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs expressed as extracellular acidification rate (ECAR). (B) Quantification of glycolysis, glycolytic capacity, and glycolytic reserve in low oxygen Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs. (C) Assessment of oxygen consumption rates from glycolysis stress test performed in low oxygen Mdm2<sup>WT</sup>, Mdm2<sup>S183A</sup>, and p53<sup>-/-</sup> MEFs. (n=3 MEF lines per genotype)

Next, I explored the role of Mdm2-S183 phosphorylation in glucose homeostasis *in vivo*. To this end, I performed glucose tolerance tests in Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice. I found that there was no significant differences in glucose tolerance between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice (Appendix Figure 4a). Glucose uptake from the blood is regulated by insulin. Therefore, I examined the effects of Mdm2-S183 phosphorylation on glucose-stimulated insulin release. I found that there was no significant difference in glucose-stimulated insulin release between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice (Appendix Figure 4b). These results indicate that Mdm2-S183 phosphorylation does not impact glucose tolerance and glucose-stimulated insulin release in mice. Furthermore, I performed an insulin tolerance test and Mdm2<sup>S183A</sup> mice displayed significant insulin insensitivity compared with Mdm2<sup>WT</sup> mice (Appendix Figure 4c). This suggests that phosphorylation of Mdm2-S183 prevents insulin resistance in mice.



#### Appendix Figure 4. Mdm2 S183 phosphorylation promotes insulin resistance but not glucose tolerance and glucose-stimulated insulin release.

(A) Glucose tolerance test performed in Mdm2<sup>WT</sup> (n=8) and Mdm2<sup>S183A</sup> (n=7) mice fasted overnight and injected intraperitoneally with glucose (1g/kg). Data represents measurements of blood glucose concentration at the indicated time points. (B) Glucose-stimulated insulin release in Mdm2<sup>WT</sup> (n=10) Mdm2<sup>S183A</sup> (n=9) mice fasted overnight and injected intraperitoneally with glucose (2 mg/g). Data represents insulin concentration expressed as a percentage of time zero. (C) Insulin tolerance test in Mdm2<sup>WT</sup> (n=8) and Mdm2<sup>S183A</sup> (n=9) mice fed ad libitum and injected intraperitoneally with insulin (0.75 mU/g). Data represents measurements of blood glucose concentration at the indicated time points. Student's T tests and ANOVA with Fisher's tests \*p<0.05 and \*\*p<0.01.



## Conclusions

Preliminary studies examining the effects of Mdm2-S183 phosphorylation in glucose metabolism *in vitro* showed that Mdm2<sup>S183A</sup> MEFs displayed reduced glycolysis and glycolytic capacity compared with Mdm2<sup>WT</sup> MEFs. It is possible that slightly elevated p53 protein levels in Mdm2<sup>S183A</sup> MEFs triggered the expression of genes that promote oxidative phosphorylation and/or genes that block glycolysis. This may account for the diminished glycolysis observed in Mdm2<sup>S183A</sup> mice. Experiments exploring whether Mdm2-S183 phosphorylation affects glucose metabolism *in vivo* demonstrated that this phosphorylation event did not influence glucose tolerance as well as glucose-stimulated insulin release. It is possible that p53 levels were not different between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice since I would expect oxidative stress to be way lower *in vivo* than in low oxygen culture. In that case, the expression of p53-target genes supposed to promote the glucose uptake will be comparable and therefore no difference will be observed in glucose tolerance and glucose-stimulated insulin release between Mdm2<sup>WT</sup> and Mdm2<sup>S183A</sup> mice. Additionally, the phosphorylation of S163 may compensate for the loss of S183 and as a result, Mdm2<sup>S183A</sup> mice will behave more like Mdm2<sup>WT</sup> mice. These findings suggests that Mdm2-S183 phosphorylation promotes glucose metabolism via glycolysis *in vitro*, but does not influence glucose homeostasis *in vivo*.

Insulin tolerance tests revealed that Mdm2-S183 phosphorylation regulates insulin sensitivity in mice. Mdm2<sup>S183A</sup> mice exhibited significant insulin resistance compared with Mdm2<sup>WT</sup> mice. However, it is possible that Insulin resistance observed in Mdm2<sup>S183A</sup> mice may not due to defective beta islet cell function since Mdm2<sup>S183A</sup> mice are capable of releasing insulin at comparable levels with Mdm2<sup>WT</sup> mice. Therefore, insulin insensitivity in Mdm2<sup>S183A</sup> mice mostly likely stems from altered insulin sensitivity in other peripheral tissues. Since the mice used in this study harbor whole body Mdm2-S183A knock-in mutations, it is difficult to determine the mechanism governing the observed insulin intolerance in Mdm<sup>S183A</sup> mice. For future studies, it will be more informative to generate mice with tissue specific Mdm2-S183A or both Mdm2 S163A and S183A mutations in tissues involved in insulin sensing.

Loss of Mdm2-S183 phosphorylation may trigger p53 activation in some cancers that display high levels of ROS. Increased glycolysis observed in Mdm2<sup>S183A</sup> MEFs can be useful in in designing therapies for cancer. Small molecules capable of inhibiting Mdm2-S183 phosphorylation may trigger senescence and/or reduce glycolysis to the detriment of cancer cell survival.

## MATERIALS AND METHODS

### **Animal studies**

*Generation and genotyping of Mdm2<sup>S163A</sup> and Mdm2<sup>S183A</sup> mice.* Mdm2 mutant mice were generated by injecting a mixture of Cas9 mRNA (50ng/μl), sgRNA (25ng/μl), and single stranded oligonucleotides (100 ng/μl) into zygotes at the pronuclear stage. Resulting modified embryos were transferred into pseudo pregnant mice at 2.5 dpc and heterozygous mice obtained after three weeks and were later crossed to WT C57BL/6J mice acquired from The Jackson Laboratory for 9 generations. The intended missense mutations substituting serine to alanine on Mdm2 codon 163 and 183 introduced a BsrDI and Apal restriction enzyme cut site, respectively. Therefore, to determine the presence of anticipated knock-in alleles, we performed genomic PCR to amplify regions harboring S163 and S183 codons followed by BsrDI and Apal restriction enzyme digestions, respectively. Digested PCR products were subjected to gel electrophoresis to confirm the genotypes. Additionally, we performed DNA sequencing of the regions harboring the 2 Mdm2 codons to further confirm the presence of the intended mutations and absence of off target mutations in the Mdm2 sequence.

Mdm2 -S163 gRNA	5' AGGAGATCCATTAGTGAGACAGG 3'
Mdm2-S183 gRNA	5' GATCAAAGGACAGGGACCTGCGG 3'
Mdm2 -S163 donor oligo	5' AAACCTTCATCTTCTGATTTAATTTCTAGACT GTCTACCTCATCTAGAAGGAGATCCATTGCTGA GACAGGTACATAGGGACATTATGTGACATACTA AACAGTTGCTTGGTGTTCATTTT 3'
Mdm2-S183 donor oligo	5' TAATCTATAGAAGAGAACACAGATGAGCTAC CTGGGGAGCGGCACCGGAAGCGCCGCAGGGC CCTGTCCTTTGATCCGAGCCTGGGTCTGTGTGA GCTGAGGGAGATGTGCAGCGGCGGCAGC 3'
<b>Mdm2 S163A genotyping</b>	
Forward Primer	5' CCAGCATGCAAATGACTCTTG 3'
Reverse primer	5' CTGGATTAAAGATGAGAGCCAC 3'
<b>Mdm2 S183A genotyping</b>	
Forward Primer	5' GAGCTTTATGTGTAGCTGAACAG 3'
Reverse primer	5' CAGTGCGCACAGAGCCCTG 3'

*In vivo mice experiments.* For the DNA damage experiments, mice were exposed to ionizing irradiation using a cesium-137 source (Gammacell 40) and were either monitored for survival or sacrificed at various time points to harvest tissues. To induce hepatocellular carcinoma, 2-week-old mice male were injected intraperitoneally with a single dose of 25mg/Kg diethylnitrosamine (DEN) (Sigma N0258) diluted in glyceryl trioctanoate (Sigma T9126). DEN treated mice were sacrificed after 40 weeks to determine tumor presence and burden. For the skin carcinogenesis experiments, 400nM of 7,12-dimethylbenz[a]-anthracene (DMBA) (sigma D3254) dissolved in acetone was topically applied to 8 week old mice. Two weeks after DMBA initiation, tumor formation was promoted by topical application of 10nM 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma P8139) dissolved in acetone, twice weekly for up to 35 weeks. To study Ras-induced

lung tumorigenesis, 8-week-old mice were intranasally intubated with 125 $\mu$ L of  $2.5 \times 10^7$  PFU of purified Adeno-Cre virus (University of Iowa, Gene Transfer Vector Core) and sacrificed after 7.5 months to harvest lung tissues for immunohistochemical analyses. Glucose tolerance, insulin tolerance, and pyruvate challenge tests. To determine insulin and glucose tolerance, mice were fed a standard chow diet for 8 wks. Glucose tolerance tests were performed by intraperitoneal injection of 1g/kg glucose (VWR 4908-06) and measurement of blood glucose concentration at various time points. To measure glucose-stimulated insulin secretion, mice were fasted overnight and were injected intraperitoneally with glucose (2 g/Kg) followed by blood insulin concentration measurements at various time points. Insulin in plasma was measured by multiplexed ELISA using a Luminex 200 machine (Millipore). To determine insulin resistance, mice fed ad libitum were injected intraperitoneally with insulin (0.75 mU/g) followed by measuring blood glucose concentration at various time points. All animal procedures complied with guidelines set by Institutional Animal Care and Use Committee at the University of Massachusetts Medical School.

### **Cell Culture and *In vitro* treatments**

MEFs were generated from E13.5 embryos and cultured in DMEM supplemented with 1% penicillin-streptomycin (Thermo 15140122), 1%  $\beta$ -mercaptoethanol (sigma M3148), and 15% fetal bovine serum (Hyclone SH 30071.03). Low oxygen MEFs were cultured in a hypoxic incubator (5% oxygen)

in media further supplemented with 5mM N-acetyl cysteine (Sigma A9165) and deoxygenated by bubbling with trigas (5% CO<sub>2</sub>, 5% O<sub>2</sub>, Balance N<sub>2</sub>) (Airgas).

To stain for senescence associated  $\beta$ -galactosidase, MEFs were fixed with 4% paraformaldehyde and incubated overnight with staining solution (5mM potassium ferricyanide; 5 mM potassium ferrocyanide; mM MgCl<sub>2</sub>; 150mM NaCl; 1mg/ml X-gal) dissolved in 40mM citrate/sodium phosphate buffer at pH 6. To assay for colony formation, 2x10<sup>4</sup> low and high oxygen MEFs were cultured for 10 days, fixed with cold methanol for 10 minutes and stained with 0.05% crystal violet dissolved in 1% methanol.

To determine the levels of ROS, high and low oxygen MEFs were subjected to DCFDA staining assay following manufacture's protocol (Abcam 113851). Briefly, 1.5 x 10<sup>5</sup> passage 3 high and low oxygen MEFs were stained with 25uM DCFDA for 1 hour at 37°C. DCF fluorescence intensity was measured using flow cytometry Ex/Em = 485/535 nm.

To determine the percentage of cells in S phase following DNA damage, passage 2 MEFs cultured at low oxygen with NAC were exposed to 4 Gy ionizing radiation (Gammacell 40), 250nM doxorubicin hydrochloride (Sigma D1515), and 5 $\mu$ m Etoposide (Sigma E1383) overnight, and pulse labeled with 50 $\mu$ M BrdU (Sigma B5002) for 3hrs. Treated MEFs were then fixed in 70% ethanol and

incubated with anti-BrdU antibody (BD 347583) and Propidium Iodide (Sigma P4170) followed by flow cytometry analyses. Resulting data was analyzed using FlowJo software.

To assay for lipid peroxidation, we used lipid peroxidation microplate assay (Sigma MAK085) according to manufacturer's instructions. Briefly, passage 3 high and low oxygen MEFs were collected, lysed, and the amount of lipid peroxidation was determined by calorimetric measurement of MDA at 532nm from reacting 50ug whole cell lysate with thiobarbituric acid. The amount of MDA was normalized to the amount of protein.

For measuring apoptosis, we used the Dead Cell Apoptosis Kit with Annexin V FITC and PI, for flow cytometry (Thermo V13242) and analyzed the data to quantify both early and late apoptotic cells using Flowjo software. Flow cytometry was performed by the Flow Cytometry Core at UMASS Medical School.

### **Immunoblotting**

Whole cell and tissue protein lysates were made using NP-40 lysis buffer (50 mM; Tris-HCl; pH 7.5; 150 mM NaCl; 0.5% NP-40; 20% Glycerol) supplemented with protease inhibitor cocktail (Roche 11873580001) and phosphatase inhibitor cocktail (Roche 4906845001). Lysates (50µg) were

subjected to SDS-PAGE and transferred to PVDF membranes. Proteins were detected using antibodies against vinculin (Sigma V9131), p53 (Leica NCL-L-p53-CM5p), phospho-p53 (S15) (CST 9284), p21 (Novus NBP2-29463), Mdm2 (Novus NBP1-02158), phospho-Akt (S473) (CST 9271), phospho-Akt (T308) (CST 9275), Akt (pan) (CST 2920) and p16 (Santa Cruz sc-377412) in 5% non-fat milk. Membranes were developed using Clarity Western ECL Substrate (BioRad 1705060) and imaged using Chemidoc Molecular Imaging System (BioRad). Quantification of western blot band intensity was done using Image J software.

### **Immunofluorescence**

Passage 3 high and low oxygen MEFs were fixed in 4% paraformaldehyde, permeabilized with 0.2% Triton-X100 in PBS for 15 min, treated with 200ug/ml RNase A (Sigma R4875) at 37 °C for 1 hour, and blocked in 1% bovine serum albumin with 0.2% Triton-X100 in PBS for 30 minutes. Fixed MEFs were subsequently incubated overnight at 4 °C in primary 8-Oxo-2'-deoxyguanosine (ab206461) antibody followed by secondary Alexa Fluor488-conjugated goat anti-mouse antibody (Abcam 150113) for 1 hour. Cells were mounted with ProLong™ Diamond Antifade Mountant with DAPI (Thermo P36962) to stain for nuclear DNA.



To determine ROS production following DEN injection in two-week-old mice, dihydroethidium (DHE) (Thermo D1168) was used to measure ROS production in 5µm frozen cryosections incubated with 0.1 mM DHE dissolved in HEPES-Tyrode buffer solution (132 mM NaCl, 4 mM KCl, 1 mM CaCl<sub>2</sub>, 0.5 mM MgCl<sub>2</sub>, 9.5 mM HEPES, and 5 mM glucose) for 15 min at room temperature. Images were captured using Zeiss Axioplan 2 microscope (Zeiss, Thornwood, NY, USA).

### **Gene Expression**

To quantify gene expression levels using qPCR, RNA was isolated from high and low oxygen Passage 3 MEFs or mouse tissues using RNeasy Plus Mini Kit (Qiagen 74134) and cDNAs were generated using SuperScript™ III Reverse Transcriptase (Invitrogen 18080093). qPCR was done using Power SYBR™ Green PCR Master Mix (Applied Biosystems 4367659) with StepOnePlus Real-Time PCR System (Applied Biosciences). The comparative CT method ( $2^{-\Delta\Delta CT}$ ) was used to determine fold differences between the target gene and the reference gene Rplp0. Sequences for primers were as follows;

p21 Forward p21 Reverse	5' CTGAGCGGCCTGAAGATT 3' 5' ATCTGCGCTTGGAGTGATAG 3'
Mdm2 Forward Mdm2 Reverse	5' AGTCTCTGGACTCGGAAGATTA 3' 5' CTGTATCGCTTTCTCCTGTCTG 3'
Rplp0 Forward Rplp0 Reverse	5' CTGAGTACACCTTCCCACTTAC3' 5' CTCTTCCTTTGCTTCAGCTTTG 3'
Sestrin-1 Forward Sestrin -1 Reverse	5' ACTGCGTCTTTGGCATCA 3' 5' CATCCTACGGGTCGTCTTCT 3'
Sestrin-2 Forward Sestrin -2 Reverse	5' TTCAGGGAGAAGCGTTTGC 3' 5' ACACACTCTATCACTGGCACC 3'
Pai-1 Forward Pai-1 Reverse	5' TGCATCGCCTGCCATTG 3' 5' GGACCTTGAGATAGGACAGTGCTT 3'
Pai-2 Forward Pai-2 Reverse	5' CACCACAGGGGGATTATTTG 3' 5' GGATGCGTCCTCAATCTCAT 3'

## Histology

Mouse tissues were fixed in 10% buffered formalin for overnight and embedded in paraffin, sectioned and stained with Hematoxylin and Eosin (HE). To assay for cell death in vivo, fixed tissues were stained with TUNEL using *In situ Cell Death Detection Kit* (Roche 11684817910) according to manufacturer's protocol. Paraffin embedded livers from DEN treated mice were incubated with glutamine synthetase antibody (Abcam 73593) to stain liver tumors,  $\gamma$ -H2A.X (phosphor S139) (Abcam 11174) to detect DNA damage, and the aforementioned TUNEL kit to assay for cell death.

To stain skin tissue from DMBA/TPA treated mice for senescence associated  $\beta$ -galactosidase, skin sections were briefly fixed 4% paraformaldehyde for 5 minutes and incubated for 4 hours at 37 °C in the dark in a staining solution (5mM potassium ferricyanide; 5mM potassium ferrocyanide; 2mM MgCl<sub>2</sub>; 150mM NaCl; 1mg/ml X-gal) dissolved in 40mM citrate/sodium phosphate buffer at pH 6. The tissues were subsequently washed in PBS and fixed overnight in 10% buffered formalin and embedded in paraffin. Sections were counterstained with Nuclear Fast Red and images were taken using a light microscope (Zeiss) outfitted with Axiocam camera.

Lungs from mice that inhaled purified Adeno-Cre virus to activate mutant K-ras expression were sacrificed after 7.5 months and lungs perfused for 5 minutes and fixed overnight with 10% formalin. Lungs were embedded in paraffin and sections were stained with HE. The area occupied by tumors were calculated using ImageJ software.

### **Statistical analysis**

Statistical analyses were performed using either Microsoft Excel or GraphPad Prism Software to perform Student 's T tests and ANOVA with the Fisher's tests. Log Rank tests were used to determine p values for all survival curves and  $\chi^2$  tests were used to determine the p values for Mendelian ratios.

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