THE P53-NMNAT2 FEEDBACK REGULATORY LOOP: MECHANISMS UNDERLYING P53 ACTIVATION

by

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To people dedicated to science.

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ABSTRACT

The tumor suppressor p53 acts as a master transcription factor that controls hundreds of effecter genes in response to various cellular stresses. The flexibility of p53 to regulate its target genes with distinct functions (growth arrest, DNA repair, apoptosis etc.) is largely conferred by extensive and dynamic posttranslational modifications of the protein, including phosphorylation, acetylation, methylation, ubiquitination, sumoylation, neddylation and ADP-ribosylation. Recent evidence suggests that acetylation is indispensable for p53 activation. A major regulator of p53 acetylation, and hence p53 function, is a group of Class III histone deacetylases known as Sirtuins (SIRTs), that utilize nicotinamide adenine dinucleotide (NAD^+) as substrate to catalyze the removal of acetyl groups from p53, resulting in the "silencing" of p53 activity. In an effort to determine whether a feedback loop exists whereby p53 is involved in the regulation of NAD^+ metabolism, nicotinamide adenylyltransferase 2 (Nmnat2), a key NAD^+ synthetase, was identified to be a novel target gene of p53, from which two transcript variants are expressed in human (TV1 and TV2). Two putative p53 response elements within the first intronic region of human *Nmnat2* gene were also identified that can actively drive the expression of luciferase reporter gene in a p53-dependent manner. Most importantly, data suggests that Nmnat2, like SIRTs, is involved in the regulation of p53-mediated apoptosis and protein acetylation upon DNA damage. Furthermore, Nmnat2 isoforms exert opposite functions on SIRT-mediated deacetylation of p53. Specifically, ectopic expression of Nmnat2 TV2 isoform promotes p53 acetylation after DNA damage, whereas ectopic expression of Nmnat2-TV1 isoform suppresses it. Manipulation of SIRT activities by either RNA interference or specific inhibitors modifies p53 acetylation status the same way Nmnat2-TV2 isoform does. Collectively, the results suggest the existence of a p53-Nmnat2 feedback loop, whereby p53 can regulate its own activity positively or negatively, depending on the nature and extent of DNA damage.

LIST OF ABBREVIATIONS USED

5-FU	5-Fluorouracil
Acetyl-CoA	Acetyl Coenzyme A
ADP	adenosine diphosphate
ADP-HPD	adenosine diphosphate (hydroxymethyl)pyrrolidinediol
Apaf	apoptotic protease activating factor
AIF	apoptosis inducing factor
AMP-K	5' adenosine monophosphate-activated protein
ARF	alternate reading frame
ART	ADP-ribose transferases
ATM	ataxia telangiectasia mutated
ATP	adenosine triphosphate
ATR	ataxia telangiectasia and Rad3-related
Bak	Bcl-2-antagonist/killer
Bax	Bcl-2-associated X
Bcl-2	B-cell lymphoma 2
BCL-XL	B-cell lymphoma like X
BER	base-excision repair
BH3	Bcl-2-homology domain 3
BIRC5	baculoviral IAP repeat containing 5
BSA	bovine serum albumin
Caspase	cysteinyl aspartic acid-protease
DBC1	deleted in breast cancer 1

CD	cluster of differentiation molecule
Cdc	cell-division cycle
ChIP	Chromatin Immunoprecipitation
CIP1	Cdk-interacting protein
CPS-1	carbamoyl phosphate synthetase 1
Cy	Cyanine
DAPI	4', 6-diamidino-2-phenylindole
DDB2	DNA damage-binding protein 2
DMEM	Dulbecco's modified Eagle's medium
DR5	Death Receptor 5
CO_2	carbon dioxide
ddH2O	double-distilled or distilled-deionized water
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DNA-PKcs	DNA-dependent protein kinase catalytic subunit
DNase	deoxyribonuclease
DRAM	p53-induced modulator of autophagy
DYRK2	dual-specificity tyrosine-phosphorylation-regulated kinase 2
ER	endoplasmic reticulum
ERK	extracellularsignal-regulated kinase
EDTA	ethylenediaminetetraacectic acid
FACS	fluorescence-activated cell sorting
FBS	fetal bovine serum

g	gram(s)
G6PDH	glucose-6-phosphate dehydrogenase
GDH	glutamate dehydrogenase
GGR	global genomic repair
GLUT	glucose transporter
GRP75	glucose regulated protein 75
GTP	guanosine triphosphate
GSH	reduced glutathione
GSR	glutathione reductase
GSSG	glutathione disulfide
h	hour(s)
H2O	water
HA	Hemagglutinin
HAT	histone acetyltransferase
HCl	hydrochloride acid
HDAC	histone deacetylase
HIC1	hypermethylated in cancer 1
HIPK-2	homeodomain-interacting protein kinase-2
HPV	human papillomavirus
HR	homologous recombination
HRP	horseradish peroxidase
HSV-TK	herpes simplex virus-thymidine kinase
IgG	immunoglobulin G

kDa	kilodalton
KD	knock-down
L (l)	litre(s)
M	molar; moles per liter
MAPK	mitogen-activated protein kinase
mg	milligram(s)
min	minute(s)
ml	millilitre(s)
mM	millimolar
MDM2	murine double minute 2
MEF	mouse embryonic fibroblast
MIC-1	macrophage inhibitory cytokine 1
MMLV	moloney murine leukemia virus
MMR	mis-match repair
MOMP	mitochondrial outer membrane permeabilization
MSCV	murine stem cell virus
mTOR	mammalian target of rapamycin
NAD^+	nicotinamide adenine dinucleotide
Nam	nicotinamide
Nampt	nicotinamide phosphoribosyltransferase
NER	nucleotide-excision repair
NF-kB	nuclear factor kappa B
NHEJ	non-homologous end-joining

NMN	nicotinamide mononucleotide
Nmnat	nicotinamide mononucleotide adenylyltransferase
NADPH	nicotinamide adenine dinucleotide phosphate
nM	nanomolar(s)
OD	optical density
P53BS	p53-binding site
P53RE	p53 responsive element
p53AIP1	p53-regulated apoptosis-inducing protein 1
pADPr	poly ADP ribose
PAI-1	plasminogen activator inhibitor 1
PAR	poly ADP ribose
PARG	poly(ADP-ribose) glycohydrolase
PARP	poly (ADP-ribose) polymerase
PCR	polymerase chain reaction
PERP	p53 apoptosis effector related to PMP-22
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PGM	phosphoglycerate *mutase*
pH	logarithmic unit measuring acidity
PI	propidium iodide
PI3K	phosphatidylinositol 3-kinase
PIG3	p53-inducible gene 3
PLB	passive lysis buffer

PML	promyelocytic leukaemia
PPP	pentose phosphate pathway
PPARγ	peroxisome proliferator-activated receptor γ
PRAK	p38-regulated/activated protein kinase
PTM	posttranslational modification
PTEN	Phosphatase and tensin homolog
PUMA	p53 upregulated modulator of apoptosis
p-value	probability (of incorrectly rejecting the null hypothesis)
PVDF	polyvinylidene fluoride
R	purine
rDNA	ribosomal DNA
RNase	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
RT	room temperature
RT-qPCR	real-time quantitative PCR
s or sec	second(s)
SCO2	synthesis of cytochrome c oxidase 2
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	standard error of the mean
SIRT	sirtuin (silent mating type information regulation 2, homolog)
TBST	tris buffered saline with Tween-20
TCA cycle	tricarboxylic acid cycle

TCR	transcription-coupled repair
TEMED	tetramethylethylenediamine
TIGAR	tp53-induced glycolysis and apoptosis regulator
TUNEL	terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling
TV	transcript variant
UTR	untranslated region
XPC	xeroderma pigmentosum group C
Y	pyrimidine

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CHAPTER 1 INTRODUCTION

1.1 p53 Acts As A Master Transcription Factor

1.1.1 Overview

The proliferation and differentiation of cells needs to be tightly regulated, ensuring their appropriate physiological functions. Failure to do so can lead to diseases such as cancer. To adapt to dynamic environmental changes such as genotoxic insults, oncogenic activation and availability of nutrients/oxygen, cells have evolved sensitive and elaborate mechanisms to switch their gene transcription patterns. One of such core regulatory loops is activation of the tumor suppressor p53, a master transcriptional regulator that modulates the expression of hundreds of effecter genes [1, 2]. Products of p53 effecter genes play roles in cellular processes such as cell-cycle arrest, apoptosis, senescence, metabolism, autophagy and DNA repair [3-6]. For example, by inducing the expression of $p21^{Waf1/Cip1}$, a cyclin-dependent kinase inhibitor, p53 rapidly responds to DNA damage and oncogenic activation, causing reversible cell-cycle arrest [7, 8]. If problems persist, p53 initiates an apoptotic program by inducing target genes such as *puma*, *bax*, *perp*, *fas, DR5/killer, noxa* and *p53AIP1*, and/or directly triggers mitochondrial outer membrane permeabilization (MOMP) [9-13]. Alternatively, depending on the context of stress and cell type, p53 can induce permanent cell growth arrest or senescence [14, 15]. The importance of p53 in tumor prevention is manifested by the fact that about half human cancers bear direct p53 gene mutations and the rest manage to inactivate the p53 pathway [16, 17]. Animal studies demonstrated that p53

knock-out mice spontaneously develop tumors of distinct tissues with 100% penetrance [18, 19]. An ancestral function of p53 is to trigger programmed cell death, also known as apoptosis, in response to DNA damage, thereby keeping the integrity of the germ line [20-22]. It is thought that p53 has acquired the ability during evolution to respond to oncogenic stimuli, and functions as a safeguard against neoplasia [21].

1.1.2 Transcriptional Regulation By p53

Most cancer-derived p53 mutations have been found within the DNA-binding domain (DBD), underscoring the main role of p53 as a sequence-specific DNA-binding protein [23, 24]. To date over 125 protein-coding genes and noncoding RNAs have been shown to be direct transcriptional targets of p53 [6]. p53 target genes contain sequence-specific responsive elements (RE) to which p53 binds, leading to activation of their transcription. p53 was originally characterized as a transcriptional activator. Further research expanded p53's functions to include transcriptional repression [25]. p53, like other transcription factors, has a modular protein domain structure [3, 26-28] (Figure 1). There are two transcriptional activation domains (TAD) within the N-terminus of p53 protein, TAD1 and TAD2, which span amino acid residues 1-40 and 41-80, respectively. A proline-rich domain (PRD) is located C-terminal to TAD2 and was originally proposed to play a role in protein-protein interactions based on the presence of PxxP motifs that resemble Src homology 3 (SH3)-domain-binding regions [29]. Although gene knock-in mice with p53 containing proline-to-alanine mutations at putative protein interaction sites appear normal, complete deletion of the PRD abolishes the tumor-suppressive function of p53 [30]. The central DNA-binding domain spans residues 100-300. p53 gene mutations in tumors can either alter residues essential for direct contact with the protein's response

elements (contact mutation) or cause mis-folding of the domain (structural mutation). “Hot-spot” mutations of p53 have been found in the DNA-binding domain, including R175, G245, R248, R249, R273 and R282 [17, 23, 31]. Those p53 mutations not only disrupt DNA-binding but also confer new capacities to p53 thus turning p53 into an oncoprotein, a phenomenon known as “gain-of-function”, which is relevant to increased tumor invasiveness and metastasis [3, 23, 31-33]. p53 binds to its response elements as a tetramer through its tetramerization domain comprising residues 325-356. At the very C-terminus of p53 protein lies a basic, lysine-rich domain containing residues 363-393. Lysines within the C-terminus of p53 (CTD) are subject to extensive post-translational modifications that could modulate p53 protein stabilization and sequence-specific DNA-binding [34-36].

p53 tetramers bind as dimers of dimers to sequence-specific p53 response elements, which are classically defined as two DNA half sites of RRRCWWGYYY (where R is a purine, W is adenine or thymine and Y is a pyrimidine) with a spacer of 0-13 base pairs between half sites [6, 27, 37-39]. p53 REs are most commonly found in the promoter at varying distances upstream (e.g. *p21, noxa*) from the transcription initiation site. Some p53 REs (e.g. *mdm2*, *pcna*) are found to be located very close (within ~300 bp) to the transcription initiation site, or within early intronic sequences (*e.g. puma, pig3* microsatellite RE), but can even be found within exons (*e.g. miR-34a*) [6].

Regarding the discrete locations of p53 REs, one important question is: How does p53 locate its target sequence within highly condensed chromatin? One clue may be the fact that p53 can bind to DNA with two distinct DNA-binding domains, the central DBD and the highly basic C-terminal domain (CTD). The DBD is capable of recognizing

specific p53 Res, whereas the CTD of p53 has been shown to render p53 the ability to linearly diffuse on naked DNA [40-42]. Whether such sliding contributes to p53's binding site localization remains to be determined but it remains as an appealing possibility. The CTD of p53 can positively regulate p53-binding to unique DNA structures including single-stranded DNA overhangs, hemicatenated DNA, minicircular DNA, and supercoiled DNA [40, 43-45]. However, a carboxy-terminally truncated p53 (p53ΔC30) is markedly impaired in binding to chromatinized DNA templates *in vitro* [46] and to p53 target promoters *in vivo* [47], indicating that the CTD of p53 facilitates p53 promoter binding in the context of chromatin.

p53 is involved in two steps of gene transcription. First, p53 stimulates transcription initiation of RNA polymerase II-transcribed genes. Upon DNA damage, p53 is involved in the recruitment of the histone variant H2A.Z, which is required for full activation of the p21 gene [48]. Histone methyltransferases (HMTs) PRMT1 and CARM1, that cooperate with p300/CBP in a p53-dependent fashion, facilitate transcription on the *gadd45* gene after UV radiation [49]. p53 also promotes histone acetylation. Histone acetyltransferases (HATs) such as p300/CBP, PCAF, GCN5, or TIP60, are recruited after p53 has bound to its recognition site to acetylate histones within the vicinity of p53 RE [50-55]. p53 can direct pre-initiation complex (PIC) assembly on target gene promoters. This process involves the ordered recruitment of histone methyltransferases (HMTs), histone acetyltransferase (HATs), and other cofactors in the vicinity of the p53 response element. Subsequently, RNA polymerase II and its associated transcription factors (TFs) can bind to the transcription start site [56-63]. Under certain conditions, p53 can also modulate transcription elongation through

interaction with various elongation factors [64]. During elongation, RNA polymerase II activity is facilitated by several elongation factors that function to repress the stalling and pausing of RNA polymerase. It has been reported that p53 interacts physically or functionally with several of these factors, including cdk9, FACT, various components of the mediator complex, and ELL [65-68]. p53 and its upstream signaling pathways have been reported to be involved in the stimulus- and locus-specific control of transcription elongation. For example, DNA-PK is implicated in inhibiting the elongation of *p21* RNA but not *puma* RNA, in response to chromium exposure [69], whereas after replication stress, *p21* RNA elongation is inhibited in a Chk1-dependent fashion [70]. The regulation of transcription elongation is emerging as a key layer of control in fine-tuning p53-regulated transcription. p53 has also been shown to repress a wide range of target genes. Several mechanisms of p53-mediated repression of transcription include: First, p53 can directly bind to its RE and recruit co-repressors; Second, p53 can induce a repressor protein thereby indirectly repressing some genes; Third, binding of p53 to its RE may occlude the binding of other transcription factors; Fourth, p53 can bind to other transcription factors and repress them by recruiting HDACs [6, 71].

1.1.3 Regulation Of p53: Posttranslational Modifications

The diversity of p53 function is attributed to the flexibility of the protein to be modified by upstream regulators that sense various cellular stress signals, for example, DNA damage [13, 72]. There are about fifty sites within the p53 polypeptide that are subject to extensive posttranslational modifications (PTMs), including phosphorylation, acetylation, ubiquitination, sumoylation, methylation, ADP-ribosylation and neddylation [73] (Figure 1). It is worth noting that de-modifying enzymes exist to balance p53-

mediated DNA damage responses [74-78]. For example, histone lysine acetyltransferases (HATs) such as P300 and CBP acetylate p53 and promote its transcriptional activity. On the other hand, histone deacetylases (HDACs) catalyze the removal of acetyl groups from p53, resulting in the "silencing" of p53 activity [74]. Protein p53 is kept at a low levels in cells under physiological conditions. The mouse double minute (MDM2) oncoprotein, a ubiquitin E3 ligase, constantly ubiquitinates p53 and promotes its degradation through cytoplasmic proteasomal machinery [79]. Interestingly, the *mdm2* gene is a direct target of p53 transcription such that p53 protein levels is controlled in a negative feedback manner [79, 80]. MDM2 binds to amino-terminal transactivation domain 1/2 (TAD1/2) of p53 and ubiquitinates a cluster of six lysine residues within carboxyl-terminal of the protein [79-81]. TAD1 and TAD2 comprise amino acids 1-40 and 41-80, respectively, both of which contain multiple serine and threonine residues that can be phosphorylated by upstream kinases from diverse cellular pathways. Phosphorylation of p53 in the TAD domains results in the release of p53 from MDM2 and stabilization of the protein [82]. MDM2 not only degrades p53 by the ubiquitination-proteasome pathway, but also blocks its transcriptional activity because acetylation, critical for p53's function, occurs at the same set of lysine residues targeted by MDM2 [81, 83-85]. Several lines of evidence suggest a complex sequential and inter-dependent regulation between phosphorylation and acetylation events [86].

Although a large body of evidence supports the view that phosphorylation events taking place in TAD1 and TAD2 are essential for p53 activation, gene knock-in mice expressing p53 with amino-terminal serines substituted with alanines show no increased susceptibility to cancer [87]. It is likely that these mutations in TAD domains

simultaneously disrupt p53-MDM2 interaction and facilitate acetylation of p53, thereby preserving its tumor suppression activity. If this is the case, activation of p53 can be achieved by mechanisms where phosphorylation of p53 is dispensable. Indeed, the tumor suppressor protein ARF, activated by oncogenic signals, binds to MDM2 and unleashes p53 functions [88, 89]. Importantly, there is no detectable phosphorylation of p53 following induction through the oncogene-ARF pathway, although acetylation of p53 occurs during this process [88]. It is thus possible that the TAD domain of p53 serves as an antenna for incoming signals relayed from upstream stress sensors (e.g. DNA damage-activated protein kinases), disrupting p53-MDM2 interaction, and primes p53 for further modifications such as acetylation. Indeed, activation of p53 can be achieved by blocking negative regulators such as MDM2 and HDAC deacetylases, mechanisms that do not necessarily depend on TAD phosphorylation.

Protein p53 is the first non-histone protein identified to be acetylated by a group of histone lysine acetyltransferases such as P300, CBP, PCAF, and TIP60/hMOF, which play important regulatory roles in chromatin assembly, gene transcription and DNA repair [58, 90-92]. Seven lysine residues (K305, K370, K372, K373, K381, K382, and K386) within the carboxyl-terminus of human p53 are acetylated by P300 and CBP [93-95]. The same HATs also acetylate K164 in the central DNA-binding domain (DBD) of p53 and this acetylation event is related to cell-cycle regulation [96]. Acetylation of p53 by PCAF at K320, located in the tetramerization domain, is important for p53 nuclear localization [97]. K120, acetylated by the MYST HATs family members TIP60/hMOF, has been shown to regulate p53-dependent apoptosis, but not cell-cycle arrest [98-100]. Strikingly, mutation of all eight lysines (K120/164 in DBD, K370, K372, K373, K381,

K382 and K386 in carboxyl-terminus) to arginines abolishes p53's ability to induce p21 but not MDM2 [96]. Of particular interest is the observation that acetylation events within the DBD (e.g. K120 for apoptosis, K164 for cell-cycle arrest) have profound impacts on the regulation of the promoter binding selectivity of p53 toward its target genes. Also deducible from these studies, these acetylation events (e.g. K120) need to be tightly controlled to avoid inappropriate activation of p53's apoptotic function under conditions favoring cell survival. This safeguarding mechanism possibly acts through HDACs that negatively regulate p53 in such way that a p53 response is fine-tuned. If this is true, then inhibition of HDACs under pro-apoptotic conditions will be necessary for p53-triggered cell death.

1.2 Cellular Pathways Regulated By p53

p53 is activated by diverse cellular stress signals through protein stabilization and posttranslational modifications (discussed above). Once activated, p53 binds to DNA and stimulates the transcription of either protein-coding or non-coding genes (e.g. microRNAs and intergenic lincRNAs) [6, 27, 101, 102]. Through its downstream genes, p53 regulates cell life in many aspects (Figure 2).

1.2.1 Cell-Cycle Arrest

The cell has cell-cycle checkpoint mechanisms to ensure the fidelity of cell division. At each phase of the cell cycle, cellular processes (e.g. DNA replication) are verified to be accurately completed before progression into the next phase. The role of p53 in regulating cell-cycle arrest has been extensively studied and both upstream regulators and downstream effectors of p53 have been identified [103]. p53 regulates

cell-cycle arrest at both G1/S and G2/M. p53 induces G1 cell-cycle arrest primarily through transactivation of the cyclin-dependent kinase inhibitor $p21^{Waf1/Cip1}$. In mouse embryonic fibroblasts (MEFs), DNA damage activates upstream kinases ATM/ATR, leading to p53-dependent G1 arrest. Targeted gene knock-out of $p21^{Waf1/Cip1}$ compromises the G1/S check-point in MEFs, to an extent less than that caused by p53 inactivation ([104, 105]. p53 induces G2/M cell-cycle arrest by perturbing the function of cyclin B1 and cdc2, which constitute the maturation-promoting factor (MPF) driving the cell-cycle from G2 to mitosis. p53 can either transcriptionally repress the expression of cdc25c, a phosphatase that activates cyclin B1 and cdc2 complex [106], or activates the expression of the 14-3-3 sigma gene, which prevents nuclear localization of cyclinB1 and cdc2 after DNA damage [107, 108]. Interestingly, gene deletion of either $p21^{Waf1/Cip1}$ or 14-3-3 sigma in human colon cancer HCT116 cells resulted in enhanced cell death in response to DNA damage, suggesting that p53-mediated apoptosis in this cell line is inhibited by cell-cycle arrest [107].

1.2.2 Apoptosis

Among the many functions of p53, controlling apoptosis is the most extensively studied. A p53-dependent apoptotic program was first documented by the observation that thymocytes from p53 gene knock-out mice are resistant to ionizing radiation-induced apoptosis [109, 110]. Thereafter, it was reported that oncogene activation and certain DNA-damaging agents can activate p53 leading to apoptosis [111]. These studies established the role of p53 in an intrinsic fail-safe mechanism that prevents cellular transformation. As more p53 downstream effectors have been implicated in apoptosis than other processes, the complexity of p53-controlled apoptosis is getting revealed.

Evidence accumulated from the past indicates that the apoptotic function of p53 is tightly controlled and determined by both quantitative and qualitative factors, including tissue type, signal intensity and the nature of stress [112-118]. p53-mediated apoptosis involves both transcription-dependent and transcription-independent function of p53. On the one hand, once activated, p53 is capable of transactivating a large group of genes that are involved in regulating apoptosis, including *bax*, *p53AIP1*, *perp*, *DR5/Killer*, *fas/CD95*, *Pig3*, BH3-only protein *noxa* and *puma* [6, 7, 28, 32, 119]. On the other hand, p53 can induce apoptosis in a transcription-independent manner. Specifically, in response to death signals (e.g. DNA damage), p53 translocates to mitochondria and induces mitochondrial outer membrane permeabilization (MOMP), resulting in the release of pro-apoptotic factors from mitochondrial intermembrane space. Mechanisms of action of p53-induced MOMP may include interaction with Bcl2 and Bcl-X_L as a derepressor, or directly activating Bak/Bax like BH3-only proteins [9, 120-126]. The pro-apoptotic gene p53-upregulated modulator of apoptosis (*puma*) is worth noting for its role in coordinating the transcription-dependent and transcription-independent mechanisms of p53-induced apoptosis [127]. Specifically, in response to cellular stresses, p53 transactivates the *puma* gene. PUMA then translocates to mitochondria, where it binds to the Bcl-X_L protein, releasing p53 to activate Bax [127]. These findings indicate that a full activation of p53-induced apoptosis is synergized between transcription-dependent and transcription-independent events of the p53 network, in which PUMA might play a critical role. Indeed, it is the only p53 target gene whose inactivation leads to a similar apoptotic defect as p53 inactivation in response to IR in mouse lymphocytes [128]. However, *puma* knock-out mice are not tumor prone, indicating that multiple p53 downstream pathways need to be

inactivated in order to initiate tumorigenesis. This might also suggest that p53 protein itself is critical for apoptosis induction.

1.2.3 Senescence

Normal cells lack endogenous telomerase and consequently suffer from telomere erosion that limits their proliferative potential, a process known as replicative senescence. Cellular senescence is a permanent form of cell-cycle arrest, which was originally described in normal human fibroblasts by Hayflick [129]. It was proposed that telomere erosion beyond a certain limit can trigger a cellular DNA damage response and activation of the ATM/ATR-p53 pathway, which leads to growth arrest [130, 131]. Senescence is an important and evolutionarily conserved tumor-suppressive mechanism that acts as a natural barrier to cellular transformation, which renders cells able to replicate with unlimited passages (i.e. immortalized) [132-136]. The p53 and pRb pathways are critical for the initiation and maintenance of senescence in human and mouse cells. In mouse embryonic fibroblasts (MEFs), disruption of p53 alone is sufficient to overcome senescence. p53 gene knock-out MEFs are immortalized and mice are highly susceptible to spontaneous tumor formation [135, 137, 138]. p53-dependent senescence can be induced by a wide spectrum of stress signals, including telomere erosion, oncogenic activation, oxidative stress, inhibition of HDAC, protein misfolding and DNA-damaging agents. DNA damage caused by sub-lethal doses of radiation, chemotherapeutic drugs and telomere shortening can induce senescence primarily through the p53-p21 pathway. Deficiency of DNA repair caused by inactivating genes such as DNA ligase IV induces premature aging in mice and senescence of MEFs [139, 140]. Interestingly, simultaneous inactivation of p53 can rescue these phenotypes,

suggesting the critical role of p53 in DNA damage-triggered cellular senescence [139, 140]. $p21^{Waf1/Cip1}$ is important for both DNA damage-induced senescence and transient growth arrest. The determinant factor for the decision between these outcomes remains largely elusive. It has been proposed that efficient DNA repair may inhibit the p53–p21 pathway-mediated senescence, allowing cell-cycle progression, whereas irreparable DNA lesions provoke the ATM/ATR–p53–p21 DNA damage response, maintaining the senescent phenotype [141]. However, one question still remains as to the decision making between senescence and apoptosis upon severe DNA damage. In contrast to the role of p53 in inducing cell-cycle arrest, mechanisms of activation of p53-mediated senescence are not fully understood. In addition to p21, there are other p53 effector genes involved in induction of senescence, including plasminogen activator inhibitor-1 (PAI-1) and MIC-1, a cytokine of the TGF-β family [142-145]. The tumor suppressor ARF (p14ARF in human; p19ARF in mouse), encoded by the *INK4a–ARF* locus, stimulates p53 activity by sequestering MDM2 (HDM2 in human). MDM2 is an E3 ubiquitin ligase that targets p53 for proteasome-mediated degradation. Therefore ARF acts to prevent the MDM2-mediated negative regulation of p53 [146, 147]. The ARF-MDM2-p53 regulatory pathway represents an important tumor suppressive mechanism in response to oncogenic signals. In human cells the role of ARF and p53 is more complicated. For example, ARF and p53 are critical regulators of E2F-induced senescence but not RAS-induced senescence in human primary fibroblasts [148-150]. Another important activator of p53 is the promyelocytic leukaemia (PML) tumor suppressor. PML has been implicated in replicative senescence and in premature senescence in response to oncogenic RAS [151]. PML stabilizes p53 through acetylation by activating CBP/p300 acetyltransferase [152].

Oncogenic RAS-induced senescence also activates PRAK (p38-regulated/activated protein kinase), which phosphorylates p53 on Ser37. In addition, p38 can phosphorylate p53 on Ser33 and Ser46 during RAS-induced senescence [153].

1.2.4 DNA Repair

In eukaryotes, there are five major DNA repair mechanisms including: nucleotide-excision repair (NER), base-excision repair (BER), mis-match repair (MMR), non-homologous end-joining (NHEJ) and homologous recombination (HR) [154-157]. p53 is involved in these cellular DNA repair processes mostly through both transcription-dependent and transcription-independent mechanisms, except HR, in which p53 functions only in a transcription-independent manner. The most versatile type of DNA repair is NER, which includes global genomic repair (GGR) and transcription-coupled repair (TCR) [158, 159]. By NER, cells can fix damaged bases and disrupted base pairings caused by ultraviolet light (UV) or oxidative damage. Inactivation of p53 in human cells can cause reduced repair of UV-induced DNA damage [160-162] . p53 regulates the transcription of p48-DDB2 and XPC [163, 164]. p48-DDB2 is the key downstream gene responsible for the transport of XPC to sites of DNA damage in irradiated cells [165, 166]. Besides, p53 may function as a chromatin-accessibility factor in NER and this function of p53 is independent of transcription [158]. It has been shown that, in response to localized sub-nuclear UV radiation, p53 can initiate chromatin relaxation, which subsequently extends over the whole genome, thereby facilitating the GGR system for detecting lesions [158]. Interestingly, p53 also recruits p300/CBP to NER sites to promote acetylation of histone H3, which is the key event in p53-dependent chromatin relaxation [158]. Inhibition of p53 in human cells also impairs cellular TCR [163, 167].

However, the role of p53 in TCR is probably dependent on the nature of UV light. In response to UVC (wavelength 254 nM), p53 is dispensable for TCR [168]. The p53 response depends on its sub-cellular localization in regard to the site of DNA lesions, cell-cycle phase, the dose and duration of DNA damage. Therefore, when DNA damage is low, the latent p53 might interact with cellular DNA repair machinery, alone or together with other repair proteins, promoting genomic integrity. However, if accumulated DNA lesions exceed the capacity of basal DNA repair, upstream pathways are activated and stabilize p53 protein, leading to transactivation of genes such as p21 to induce growth arrest allowing cells time to correct lesions. p53 probably is also involved in this step by interacting with a different set of DNA repair factors. If DNA damage persists or is irreparable, p53 can induce cell death in a transcription-dependent or transcription-independent manner. Therefore, p53 serves as the "guardian of the genome" [169], ensuring the integrity of genome [170].

1.2.5 Energy Metabolism And Antioxidant Defense

The p53 tumor suppressor plays a key role in maintaining genetic stability. However, as p53 gene knock-out mice develop normally, it was initially concluded that p53 functions are dispensable under physiological condition. Recent studies suggest additional important roles of p53 in regulating the homeostasis of energy metabolism, coordination of biosynthesis, and cellular anti-oxidation against reactive oxygen species (ROS), which serve as preventive mechanisms against transformation, because cellular damages generated naturally and constantly by physiological processes constitute the major threats leading to cancer and aging process.

p53 can control cellular aerobic respiration and glycolysis. In normal cells, glucose is the major source of energy. Glycolysis, an anaerobic process in the cytoplasm, generates two molecules of pyruvate and two molecules of ATP from one glucose molecule. Aerobic mitochondrial respiration instead produces nearly 30 molecules of ATP consuming one glucose molecule. However, despite its high energy efficiency, mitochondrial aerobic respiration is a slow process compared to glycolysis, which serves as a fast source of energy. Therefore, glycolytic production of ATP may be beneficial under certain conditions, where rapid release of energy and/or massive biosynthesis is required [171]. The balance between glycolysis and mitochondrial energy metabolism is subject to tight regulation [172]. Recent studies suggest that the p53 tumor suppressor has multiple roles in coordinating energy metabolism with growth condition and proliferation status of cells [173, 174]. Inactivation of p53 causes cells to depend more on glycolysis for energy production, due to impaired mitochondrial functions [175-178]. Although the gross amount of ATP in p53 −/− cells does not change, p53 +/+ cells produce three times more ATP from mitochondrial respiration than from glycolysis. In contrast, p53 −/− cells produce three times more ATP from glycolysis than from mitochondrial respiration, suggesting that mitochondrial ATP synthesis depends on p53 functions [176]. The *SCO2* gene is the p53 target responsible for this process, and encodes a copper chaperon protein required for the assembly of mitochondrial cytochrome c oxidase complex IV [133, 176, 179]. AIF, encoding a protein required for the assembly of mitochondrial respiratory complex I, might be another effector gene regulated by p53 [180, 181]. p53 also regulates glucose catabolism at multiple levels. By repressing transcription of *Glut* 1 and *Glut* 4 genes, which encode glucose transporters [182], p53 can inhibit cellular glucose

uptake. However, p53 also stimulates cellular glucose catabolism by transactivating the *Hexokinase II* gene [183], which converts glucose to glucose-6 phosphate and serves as a key enzyme in cytoplasmic glycolysis. Furthermore, phosphoglycerate mutase (PGM), an enzyme that functions at the late-stage of glycolysis, is also regulated by p53 [184]. p53 is able to repress the expression of PGM through a posttranscriptional mechanism [184], adding another levels of complexity to the regulation of glycolysis by p53. The *tigar* gene encodes a protein homologous to the bis-phosphatase domain of 6-phosphofructo-2-kinase, which converts glucose-6-phosphate to fructose-2,6-bis-phosphate, an inhibitor of fructose-1,6-bis-phosphatase [185, 186]. Fructose-1,6-bis-phosphatase is a regulatory enzyme in the biosynthesis of glucose. Thus, this finding suggests that TIGAR can stimulate glucose synthesis. On the other hand, fructose-2,6-bisphosphate is also an allosteric activator of 6-phosphofructo-1-kinase, a key enzyme of glycolysis. Therefore TIGAR can also block glycolysis and divert glucose catabolism toward the Pentose Phosphate Pathway (PPP). Moreover, glucose-6-phosphate dehydrogenase (G6PDH), which catalyzes a rate-limiting step in the PPP, is also regulated by p53 [187, 188]. Enhanced PPP activity results in increased synthesis of nucleotides that are needed for DNA repair, and generation of NADPH, which is essential for cellular anti-oxidation. Therefore, p53 can exert a pro-survival function by diverting glycolysis toward the PPP that enhances cellular DNA repair and anti-oxidation capacity [171]. Like many metabolic enzymes, p53 is affected by cellular energy status in a feedback manner. For example, it has been reported that ADP can bind to the p53 tetramer and promote its DNA-binding activity [189], while ATP, GTP and NAD^+ act in opposite ways [189, 190]. Accordingly, p53 might also function as a sensor for cellular energy status.

Cancer cells switch their energy metabolism from oxidative phosphorylation to aerobic glycolysis, a phenomenon known as the "Warburg effect" [191]. The switch from oxidative phosphorylation to glycolytic fermentation is a characteristic of many rapidly dividing cells, such as lymphocytes, hematopoietic cells, embryonic cells and cells of other types [192, 193]. The high glycolytic rate provides certain advantages for proliferating cells. First, although the yield of ATP is low, the rate of ATP production during glycolysis is much higher compared to that from oxidative phosphorylation [194]. Second, proliferating cells require intermediates for biosynthesis, including NADPH, citrate and glycerol for lipids, and ribose sugars for nucleotides, while energy might not be an issue. p53 could stimulate oxidative phosphorylation and production of ATP through up-regulation of the *SCO2* gene. Therefore, disruption of the p53 pathway in cancers may result in uncontrollable proliferation due to dysregulated cellular energy metabolism.

Organisms living in aerobic conditions consume oxygen not only for energy production. Oxygen can be metabolized into reactive oxygen species (ROS) that are highly reactive intermediates capable of modifying numerous biological substrates. Oxidation of cellular macromolecules such as lipids, proteins and nucleic acids damages cellular structures and represents a major threat, leading to aging and other diseases. Several cellular anti-oxidation mechanisms exist to regulate metabolism and homeostasis of ROS. Glutathione peroxidase (GPX) is essential for rapid elimination of large amounts of ROS. Exposure of cells to high levels of ROS leads to oxidative stresses, which induce the p53 response, resulting in the inhibition of cell proliferation, premature senescence or apoptosis [174, 195]. Therefore, p53 restricts further proliferation of (or eliminates) cells

exposed to hazardous oxidative environments. Approximately one third of the genes that are highly responsive to hydrogen peroxide treatment represent transcriptional targets of p53 [196]. Also, p53 induces other genes, such as the quinine oxidoreductase homologue *PIG3*, whose products increase intracellular ROS and sensitize cells to oxidative stress [197]. These observations suggest that p53 may facilitate apoptotic cell death through the oxidative degradation of mitochondrial components, as the induction of apoptosis itself is accompanied by the massive release of ROS [197]. It has been reported that abrogation of p53 functions resulted in a substantial increase of intracellular ROS. Similar increases of ROS were observed in tissues of p53 −/− mice [198, 199]. The increases of ROS in p53-deficient cells correlated with down-regulation of p53 effector genes such as *gpx1, sestrin1* and *sestrin2*, indicating that latent p53 is sufficient for maintaining functions of these genes under physiological condition [198, 199]. As discussed above, *Tigar* encodes a homologue of the bis-phosphatase domain of 6-phosphofructo-2-kinase which diverts glycolysis toward the pentose phosphate pathway. Stimulation of the PPP promotes the production of NADPH, leading to up-regulation of reduced glutathione. This additionally contributes to the anti-oxidant activity of p53 [200]. Consequently, inactivation of p53 in cancer cells leads to elevated intracellular ROS levels. However, p53 loss also makes these highly proliferating cells tolerant to aberrant ROS levels, which results in increased rates of genetic instability.

1.2.6 Autophagy

Macroautophagy (autophagy), a self-eating process, can be triggered by nutrient deprivation and provides cells with nutrients for their survival. The process of autophagy consists of formation of autophagosomes and double-membrane vesicles that engulf

certain volumes of the cytoplasm, including portions of endoplasmic reticulum, endosomes and mitochondria. The autophagosomes fuse with lysosomes to form autolysosomes, in which the engulfed structures are digested and nutrients are recycled [201]. Defective mitochondria and cell death-induced MOMP release certain free molecules and ROS that can stimulate autophagy, leading to the removal of these unwanted masses [202-204]. So, autophagy promotes viability under certain conditions. However, under other conditions, autophagy promotes apoptosis [205]. Autophagy is a tightly regulated process, which is negatively regulated by the mTOR pathway [206, 207]. Confusingly, it seems that p53 has dual mechanisms to regulate autophagy. First, p53 can activate autophagy through inhibition of the mTOR pathway [208]. Second, p53 can induce autophagy through its effector gene *DRAM* [209]. Ectopic over-expression of *DRAM* results in increased clonogenicity, suggesting autophagy may have a pro-survival role [210, 211]. On the other hand, DRAM was shown to be required for p53-induced apoptosis, suggesting there probably is an inter-regulation between autophagy and apoptosis [209]. Cytoplasmic p53 has been shown to be a strong inhibitor of autophagy [212, 213]. Therefore, p53 modulates autophagy in both transcription-dependent and transcription-independent manner. Regarding the role of p53 in regulating mitochondrial biogenesis, base excision repair of mitochondrial DNA and induction of *AIF* and *SCO2* genes [175, 176, 214], it is likely these functions of p53 are co-regulated. Apoptosis-inducing factor (AIF) is a NADH oxidase in the inner membrane of mitochondria, which facilitates assembly of the mitochondrial respiratory complex I [180]. Similarly, the product of SCO2 gene also participates in oxidative phosphorylation by promoting the assembly of mitochondrial respiratory complex IV [133, 154, 173, 176, 179]. It has been

proposed that deficiency in these p53-regulated genes may compromise mitochondrial functions, leading to autophagy [215].

1.2.7 Dual Mechanisms Of p53 Functions

As discussed above, p53 is a multi-functional protein. As for the cell, p53 exerts two facets of protector or killer [3, 200, 216] (Figure 3). The function of p53 in the regulation of processes such as DNA repair, cell-cycle arrest, anti-oxidation and maintenance of energy metabolism casts p53 as a protector, ensuring genetic stability and a balanced cellular environment. However, under situations where propagation of the cell is harmful or potentiates malignant transformation, p53 acts as a killer to eliminate incipient cells from the population, by inducing apoptosis or senescence. The mechanism of decision-making between p53's protective and destructive functions remains a challenging issue in the field of p53 study. It is widely accepted that under mild stresses, which are manageable to the cell's check-point or surveillance system, p53 co-ordinates cellular processes to promote cell survival. Under certain conditions such as severe DNA damage and sustained oncogenic activation, p53 is active to induce the suicide program, which irreversibly leads to elimination of the cell. Establishment of p53 as a critical regulator sitting in the center of a network formed by various inter-connected pathways suggests that this protein is capable of adapting to dynamic changing environments. This notion also implies that multiple feedback regulatory loops exist in the cell to fine-tune p53 functions. In other words, the consequence of p53 activation might determine the up-coming p53 responses, positively or negatively. Several lines of evidence presented in this thesis might support such a notion.

1.3 THE REGULATION OF NAD^+ METABOLISM

1.3.1 The Salvage Biosynthesis Of NAD^+

Nicotinamide adenine dinucleotide (NAD^+) and its phosphorylated and reduced forms, $NADP^+$, NADH and NADPH, have central roles in cellular metabolism and energy production as redox co-enzymes. Cellular NAD^+ consumption is intrinsically linked to signaling pathways that control gene expression, Ca^{2+} mobilization, cell death and aging. Although *de novo* synthesis of NAD^+ from amino acids (i.e. tryptophan and aspartic acid) exists across all organisms, in mammals the salvage pathway for cellular NAD^+ synthesis is essential to meet the demand from non-redox NAD^+-consuming proteins such as SIRTs and PARPs [217]. NAD^+ precursors (i.e. nicotinamide, nicotinic acid and nicotinamide riboside), are either derived from nutrients or released from performed reactions (e.g. deacetylation and poly ADP-ribosylation) [218]. There are two steps in the synthesis of NAD^+ from nicotinamide precursor. First, nicotinamide (Nam) is condensed with 5-phosphoribosyl-1-pyrophosphate to generate nicotinamide mononucleotide (NMN), catalyzed by nicotinamide phosphor-ribosyltransferase (Nampt). Second, NMN is then condensed with ATP to form NAD^+, catalyzed by nicotinamide nucleotide adenylyltransferase (Nmnat) 1, 2 and 3 (Figure 4) [218]. Nampt, the rate-limiting enzyme, is thought to maintain cellular NAD^+ levels and not surprisingly, affects activities of NAD^+-consuming proteins such as SIRTs [219, 220]. Unlike the ubiquitous cellular localization of Nampt, Nmnat proteins are compartmentalized, with Nmnat1 in the nucleus, Nmnat2 in the cytoplasm and Nmnat3 in mitochondria, indicating their specialized and localized regulatory functions [221-224]. Cellular NAD^+ is partitioned into reduced (NADH), phosphorylated ($NADP^+$) and reduced, phosphorylated (NADPH)

pools, in addition to the NAD^+ pool. Each pool resides differentially in membrane-bound compartments and is partially sequestered from free NAD^+ by binding to proteins.

1.3.2 NAD^+ As A Signaling Molecule

Pioneering work by Otto Warburg and co-workers in the 1930s led to the identification of nicotinamide adenine dinucleotides NAD (H) and NADP (H). $NADP^+$ was discovered as the co-factor of glucose-6-phosphate dehydrogenase, whereas NAD^+ turned out to be the obligatory cofactor of fermentation. Later studies revealed that NAD and NADP play indispensable roles in cellular oxidative and reductive reactions, with the NAD^+/NADH couple primarily driving oxidative reactions and the $NADP^+$/NADPH couple driving reductive reactions. NAD (H) and NADP (H) are predominantly bound to cellular proteins. The concentrations of free NAD^+, NADH, $NADP^+$, and NADPH are much lower than the total concentrations, as determined in protein-free tissue extracts [225]. As co-factors, NAD^+ and NADH have hydride-accepting and hydride-donating capacities in metabolic reactions catalyzed by key enzymes of the glycolytic pathway and the respiratory chain, and in the redistribution of the electron equivalents generated from these catabolic pathways into *de novo* biosynthesis of macromolecules. In the last decade, it has become clear that NAD^+ not only acts as a coenzyme in oxidative and reductive reactions, but also serves as a substrate for non-redox reactions. Three major families of enzymes can cleave NAD^+ in mammals: 1) ADP-ribose transferases (ARTs) and poly (ADP-ribose) polymerases (PARPs); 2) cADP-ribose synthases; 3) Sirtuins or SIRTs (class III histone deacetylases). ARTs and PARPs consume NAD^+ to create an ADP-ribosyl protein modification and/or to form the ADP-ribose polymer, PAR. PARP has complex roles in cell survival, DNA repair and cell death. cADP-ribose synthases are a

pair of ecto-enzymes also known as the lymphocyte antigens CD38 and CD157, which produce the Ca^{2+}-mobilizing second-messenger cADP-ribose from NAD^+ [226-228]. Sirtuins (SIRTs), homologues of yeast silent information regulator 2 (Sir2), are enzymes that function primarily in removing the acetyl moiety from lysine on histones and other proteins [229]. SIRT is also known as class III histone deacetylases (HDACs), or class III protein lysine deacetylases. SIRT binds to two substrates: a protein or peptide that contains an acetylated lysine and NAD^+ [230]. SIRT positions the acetyl moiety to attack the ribose C1 carbon of the ADP-ribose moiety of NAD^+, producing acetylated ADP-ribose, nicotinamide and the deacetylated lysine on protein [231]. ART, PARP, CD38 and SIRT enzymes all contain a nicotinamide-product site that can be occupied in the presence of substrates and enzyme intermediates [232-234]. Therefore, all these NAD^+ consumers are sensitive to inhibition by nicotinamide. Because of this type of product inhibition, the salvage and/or elimination of nicotinamide are crucial steps in NAD^+ metabolism, which is primarily fulfilled by salvage NAD^+ biosynthesis. These NAD^+-consuming enzymes not only indirectly affect NAD^+ bioavailability but also have a major impact on energy metabolism, cell survival, and aging. These facts have led to the hypothesis that NAD^+-consuming enzymes, mostly SIRTs, might act as energy sensors through NAD^+ and, consequently, trigger appropriate adaptive responses. Cellular processes affected by NAD are illustrated in Figure 5.

1.3.3 Class III NAD^+-Dependent Histone Deacetylase SIRTs

SIRTs are a family of NAD^+-dependent protein deacetylases with similarity to the yeast silent information regulator 2 (Sir2), which remove acetyl groups of lysine residues on histones and other proteins, releasing nicotinamide, O-acetyl ADP ribose, and

the deacetylated substrate [229]. It has been shown that providing yeast cells with extra copies of Sir2 gene increases their life span by 30%, whereas ablation of the Sir2 gene reduces their life span by 50% [235]. This finding in yeast was further confirmed in metazoans such as *Caenorhabditis elegans* and *Drosophila melanogaster*, which also live longer with extra copies of Sir2 homologs [236, 237]. Considering the NAD^+ dependence of Sir2 for its deacetylase activity, it was hypothesized that Sir2 could act as a metabolic sensor, coordinating cellular gene transcription [238]. Indeed, several lines of evidence showed that Sir2 is a critical mediator in the calorie restriction (CR)-caused extension of yeast life span [239, 240]. In mammals, there are seven homologs of Sir2, namely SIRT1–7, which are ubiquitously expressed and share a conserved catalytic core comprising 275 amino acids [241, 242]. Consistent with a crucial role of yeast Sir2 in the regulation of chromatin remodeling and gene expression, SIRT1, SIRT 6, and SIRT 7 are nuclear proteins, enriched in the nucleoplasm, heterochromatin, and nucleolus, respectively [243, 244]. SIRT2 is mainly localized in the cytoplasm, but undergoes nucleocytoplasmic shuttling, and is thus also involved in the regulation of gene transcription [244]. During mitosis, SIRT2 contributes to chromatin condensation, therefore regulating cell-cycle progression [245]. Other SIRTs (SIRT 3, SIRT 4, and SIRT 5) are localized predominantly in mitochondria [243, 246, 247]. In addition to their distinct sub-cellular localizations, mammalian SIRTs display different enzymatic activities. Indeed, SIRT1 and SIRT5 seem to act exclusively as deacetylases [248, 249]. SIRT4 and SIRT6 act as mono-ADP-ribosyl transferase [250, 251]. SIRT2 and SIRT3 display both activities [248, 252]. SIRT7 has been proposed to act as a deacetylase [253].

SIRT activity is sensitive to intracellular NAD^+ levels and energy metabolism changes induced by experimental manipulations. However, physiological concentrations of NAD^+ rarely fluctuate more than 2-fold, and thus it is uncertain whether SIRT is indeed regulated by NAD^+ concentrations in cell [254-257]. While SIRTs can be stimulated by NAD^+, competition for NADH binding inhibits their catalytic activities, but at ranges of around millimolar [258, 259]. Regarding the inter-convertible nature of NAD^+ and NADH, it is possible that cellular NADH levels might affect SIRT activities indirectly [260]. As discussed above, nicotinamide can noncompetitively bind to SIRTs, acting as a potent inhibitor [229, 261, 262]. The *in vivo* relevance of this inhibition, however, needs to be further investigated because the salvage NAD^+ biosynthesis can efficiently recycle cellular nicotinamide. Due to the difficulty of quantifying cellular NAD^+ (free and bound) and nicotinamide, the impact of these metabolites on SIRT activity are not completely clarified. Therefore, it is not clear whether the link between intracellular NAD^+ levels and SIRT activity is correlative or causal.

SIRT1 is the best characterized mammalian SIRT. SIRT1 can be activated in response to energy stress, such as fasting, exercise, or low glucose availability, which lead to increased intracellular NAD^+ levels [254, 255]. SIRT1 critically regulates the activity of a number of transcription factors and cofactors by modulating their acetylation status, including the peroxisome proliferator-activated receptor γ (PPARγ), PPARγ coactivator-1α (PGC-1α), p53, and the FOXO family of transcription factors, all of which are key regulators in cell metabolism [263].

SIRT2 was first identified as a tubulin deacetylase [248]. It has been shown that ectopic expression of SIRT2 in glioma cells decreases colony formation, implying that

SIRT2 could be a tumor suppressor [264]. This hypothesis could be supported by the observation that SIRT2 controls mitotic exit in the cell-cycle and delays cell-cycle progression through mitosis [265]. Furthermore, SIRT2 is involved in the mitotic checkpoint in early metaphase that prevents chromosomal instability and the formation of hyperploid cells [266]. Interestingly, SIRT2 might inhibit adipogenesis by deacetylating FOXO1, which then inactivates the pro-adipogenic nuclear receptor PPARγ through protein interaction [244].

The mitochondrial SIRT3 is related to adaptive thermogenesis, mitochondrial function, energy homeostasis, and cellular survival during genotoxic stress [252, 267, 268]. SIRT3 might affect cellular energy production because MEFs from SIRT3 knock-out mice have lower intracellular ATP levels than do wild-type MEFs [267]. SIRT3 seems to interact with and deacetylate subunits of complex I of the mitochondrial respiratory chain [267]. This might cause defective mitochondrial functions. Also, SIRT3 might affect energy metabolism by deacetylating other mitochondrial proteins, including glutamate dehydrogenase (GDH) and acetyl-CoA synthetase 2 [269]. Interestingly, upon massive cytosolic NAD^+ depletion, mitochondrial NAD^+ levels are reported to be maintained, suggesting that the transfer of NAD equivalents between the cytosol and mitochondria is tightly regulated during stress [268, 270]. This could be due to the fact that mitochondria contain all the necessary enzymes required for salvage NAD^+ synthesis, including Nampt and Nmnat3, thereby maintaining mitochondrial NAD^+ levels and NAD^+-dependent activities. This also suggests that NAD^+ production might be confined to specific sub-cellular compartments to achieve activation of specific SIRTs, triggering appropriate physiological responses.

SIRT4 modulates activities of target proteins through ADP-ribosylation instead of deacetylation [250]. For instance, GDH can be ADP-ribosylated and inhibited by SIRT4 [250]. Like SIRT3 and SIRT4, SIRT5 is a mitochondrial protein and has deacetylase activity. Not much is known about SIRT5 targets. The identification of carbamoyl phosphate synthetase 1 (CPS-1) as a target of SIRT5 in liver provided the first clue of the possible biological functions of this SIRT [271]. SIRT6 possesses both ADP-ribosylation activity and deacetylase activity. SIRT6 can deacetylate histones and DNA polymerase β, a DNA repair enzyme, suggesting that SIRT6 is involved in genomic stability and DNA repair [251, 272]. Interestingly, SIRT6 −/− mice display an aging phenotype, indicating an essential role of SIRT6 in maintaining organ integrity during aging [272]. SIRT7 is localized in the nucleolus and is thought to be a component of the RNA polymerase I (Pol I) transcriptional machinery [273]. SIRT7 positively regulates transcription of ribosomal DNA (rDNA) during elongation [273, 274]. As a consequence, depletion of SIRT7 stops cell proliferation and triggers apoptosis [273]. Interestingly, SIRT7 could directly control p53 acetylation in cardiomyocytes [253]. This might indicate that SIRT7 is relevant to tumorigenesis. Indeed, the tumorigenic potential of several cell lines inversely correlates with SIRT7 expression [275]. Future research will bring some light in our understanding of SIRT7 functions.

1.3.4 PARP And CD38 Are Modulators Of Intracellular NAD^+ Levels

Chambon *et al*. first described in 1963 that addition of NAD to liver nuclear extracts stimulated ADP-ribosylation activities (i.e. synthesis of poly ADP-ribose) [276]. Unlike deacetylation catalyzed by SIRTs, which use NAD^+ as a substrate for removal of acetyl moieties from target proteins, poly ADP-ribosylation attaches ADP-ribose to an

amino acid acceptor (e.g. glutamic acid) [277]. The half-life of poly ADP-ribose is very short due to cellular poly(ADP-ribose) glycohydrolase *(*PARG*)* activities, indicating that poly ADP-ribosylation is a highly dynamic process [278]. In higher eukaryotes, poly and mono ADP-ribosylation are catalyzed by ADP-ribosyl transferases [279]. Specifically, poly ADP-ribosylation reaction is catalyzed by PARPs. The PARP proteins are the most abundant ADP-ribosyl transferases. The PARP superfamily of proteins includes 17 members that share a conserved catalytic domain [280]. PARP1 and PARP2 have been widely studied because they account for almost all PARP activities in the cell [281]. Targets of PARP1 and PARP2 are involved in the maintenance of chromatin structure and DNA metabolism. In response to DNA damage, PARP1 accounts for the most cellular PARP activity, whereas PARP2 only accounts for 5–10% [277, 282, 283]. It has been shown that the catalytic activity of PARPs seems to be regulated by binding to DNA breaks through their DNA-binding domains and that DNA-binding stimulates the catalytic activity of PARP by more than 500-fold [284]. Therefore, PARP proteins play important roles in DNA damage responses. It has been reported that NAD^+ availability affects the length of poly ADP-ribose polymers (free or attached) but not the activity of PARPs [285]. Despite being insensitive to cellular NAD^+ levels, PARP proteins are thought to be the major NAD^+-consuming enzymes, forcing the cell to continuously synthesize NAD^+ from *de novo* or salvage pathways to maintain cellular activities [286-289]. DNA-damaging agents can stimulate a robust PARP activity and a concomitant decrease in cellular NAD^+ levels to 10–20% of their normal levels within a few minutes [286, 290]. Considering that ATP is required for NAD^+ synthesis, depletion of cellular

NAD^+ pools by PARPs under severe genotoxic conditions may cause a cellular energy crisis and consequent cell death [291].

The predominant form of Nmnat proteins in mammals, Nmnat1, is a nuclear protein, suggesting that nuclear NAD^+ synthesis might be required to compensate for the high rates of NAD^+ consumption caused by PARP activation [292]. PARP1 and Nmnat1 are reported to interact with each other and it was proposed that an inter-regulation between these two proteins may exist [293, 294]. The fact that activation of PARPs in response to DNA damage could consume a high amount of cellular NAD^+ brings up one possibility: PARPs and SIRTs might compete for intracellular NAD^+ pools. In myocytes, PARP activity induced by H_2O_2 depletes the cellular NAD^+ pool and down-regulates SIRT1 deacetylase activity [295]. Adding NAD^+ directly to culture medium or over-expressing NAD^+ biosynthetic enzymes was able to maintain cell viability but only in the presence of SIRT1 [295]. In this experiment, hyper-acetylation of p53 was also observed after H_2O_2 treatment, probably due to decreased SIRT1 activity [295]. Therefore, PARPs critically modulate cellular NAD^+ levels in response to DNA damage, consequently determining SIRT activity. Interestingly, activation of SIRT1 with resveratrol, a SIRT activator, can decrease PARP1 activity [296]. Furthermore, knocking out SIRT1 greatly stimulates PARP1 activity [296]. These experiments strongly suggest that cellular SIRT and PARP proteins affect each other by competing for the NAD^+ supply.

The cADP-ribose synthases CD38 and CD157 are a pair of ectoenzymes [297]. CD38 is a type II glycosylated protein with a single transmembrane domain near its N terminus [298]. CD38 and CD157 are multi-functional enzymes that use NAD^+ as a substrate to generate second messengers, such as cADP-ribose, which regulate calcium

mobilization [298-301]. Similar to PARPs, the catalytic activity of CD38 may not be limited by intracellular NAD^+ levels [302]. However, the stoichiometry of the reaction catalyzed by CD38 requires a massive amount of NAD^+, around 100 molecules, to yield a single cADP-ribose [303, 304]. Indeed, research identified CD38 as one of the main cellular consumers for NAD^+ in mammalian tissues and confirmed CD38 as a critical regulator of cellular NAD^+ levels [304]. CD38 −/− mice displayed up to a 30-fold increase in intracellular NAD^+ levels, which is much greater than physiological increases in NAD^+ levels upon fasting or CR, which do not exceed 2-fold changes [254, 256]. Consequently, CD38 depletion stimulates SIRT1 activity through up-regulation of intracellular NAD^+ levels [305]. Therefore, CD38 may serve as a pharmacological target for stimulating SIRT activity.

1.4 Link Between p53 And NAD^+ Metabolism

1.4.1 p53 And Cellular NAD^+ Synthesis

NAD^+ and $NADP^+$, together with their reduced forms NADH and NADPH, are essential regulatory molecules in cellular metabolism and other processes such as gene transcription and anti-oxidation [306, 307]. NADH is a coenzyme involved in metabolic pathways such as the citric acid cycle (also known as the tricarboxylic acid cycle/TCA cycle). It serves as the electron carrier in the electron-transition chain during mitochondrial oxidative phosphorylation, a major source of the cellular energy currency ATP. When the cellular ATP/AMP ratio, the index of cellular energy status, falls below a threshold, AMP-activated kinase (AMP-K) phosphorylates a broad range of protein targets including p53, SIRT1 and mammalian target of rapamycin (mTOR), enabling

cells to adjust their gene transcription and protein synthesis rates [255, 308, 309]. Thus, the NAD^+/NADH ratio reflects the redox state and metabolic activities of the cell, indirectly affecting the activities of cellular energy sensors. Oxidative reactions in proliferating cells (e.g. cancer cells) constantly generate reactive oxygen species (ROS) such as free radicals and peroxide that, if not neutralized properly, will damage cellular components (e.g. genomic DNA). ROS can be reduced by anti-oxidant enzymes through the conversion of glutathione to its oxidized form glutathione disulfide (GSSG). On the other hand, GSSG is constitutively reverted to reduced glutathione (GSH) by glutathione reductase (GSR) that requires the coenzyme NADPH for reducing equivalents [310-312]. Therefore, the levels of GSH can be used as an indicator of cellular anti-oxidation capacity. As discussed above, p53-induced TIGAR diverts cellular glycolysis toward the pentose phosphate pathway that generates considerable amounts of NADPH [185, 313]. Interestingly, p53 transactivates glutathione peroxidase 1 (*gpx1*), an antioxidant enzyme that scavenges hydrogen peroxide and organic hydroperoxides and hence protects cells from ROS [314]. Therefore, p53 is implicated in the regulation of cellular metabolism and anti-oxidation. In view of cancer metabolism, inactivation of p53 not only promotes glycolysis but also renders cancer cells refractory to increased cellular levels of ROS. Also advantageous to cancer cells is the accelerated accumulation of genetic mutations due to aberrant high levels of ROS, resulting from hyper-proliferation.

Regarding the fact that p53 is involved in the regulation of glycolysis and other metabolic processes such as mitochondrial energy generation, all of which affect cellular NAD^+/NADH and $NADP^+$/NADPH ratios, it is tempting to ask one question: Does p53 directly regulate NAD^+ biosynthesis? One would expect that modulation of the NAD^+

biosynthesis pathway should affect activities of cellular proteins, particularly NAD^+-dependent histone deacetylases (SIRTs) and the poly (ADP-ribose) polymerase family of protein (PARPs), whose activation depends on the availability of NAD^+ and its derivatives (i.e. $NADP^+$, NADH, NADPH) (Figure 5).

1.4.2 p53 And SIRTs

SIRT proteins, also known as Sirtuins, resemble members of a family of deacetylases that utilize NAD+ as a substrate to catalyze the removal of acetyl moieties from target proteins [315]. There are seven SIRTs (1-7) identified so far in human, with SIRT1/2 in both nucleus and cytoplasm, SIRT 3,4,5 in mitochondria and SIRT6,7 exclusively in the nucleus [243]. Distinct sub-cellular localization of SIRTs, parallel to that of Nmnat proteins, implies that their activities are likely closely linked. SIRT1 can deacetylate p53 at residue K382 *in vitro* and *in vivo* and is considered as a negative regulator of p53, hence a tumor promoter [249]. Hypermethylated in cancer 1 (HIC1), a p53-inducible transcriptional repressor, directly binds to the SIRT1 promoter and represses its transcription, thus forming a positive feedback circuit of p53-HIC1-SIRT1-p53 [316]. At the protein levels, deleted in breast cancer 1 (DBC1) protein interacts with SIRT1 and inhibits its deacetylase activity, leading to hyperacetylation of p53 and enhanced p53 activities [317, 318]. These results suggest that p53 can modulate SIRT1 activity. However, it has also been shown that SIRT1 promotes DNA repair and hence genomic stability upon DNA damage, indicating that SIRT1 might be a tumor suppressor [319-321]. Indeed, doubly heterozygous mice (i.e. p53+/- and SIRT1+/-) spontaneously develop tumors in multiple organs at 5 months of age whereas only a few heterozygous mice with p53+/- or SIRT1+/- genotypes grow tumors in the same period of time,

suggesting that SIRT1 functions in concert with p53 to suppress tumor growth [322]. Interestingly, over-expression of SIRT1 in p53 gene knock-out mice largely suppresses their spontaneous tumor growth, presumably by promoting genomic stability [319]. Nonetheless, it is clear that SIRT1 and p53 activities are closely linked, although the mechanisms are more complicated than expected. SIRT2, first identified as a tubulin deacetylase, has been shown to regulate mitotic cell-cycle progress and can deacetylate p53 at K320 *in vitro* [323, 324]. However, whether SIRT2 indeed targets p53 at K320 in the cell has no supportive evidence. As discussed above, p53 has been shown to be a target of SIRT7 *in vitro* [253]. More experiments are needed to address the inter-regulation between these two complicated networks, both of which are crucial regulators for diverse cellular processes such as gene transcription, DNA repair/genomic stability, aging, metabolism and cell death, key events relevant to cancers.

1.5 Figure s And Legends

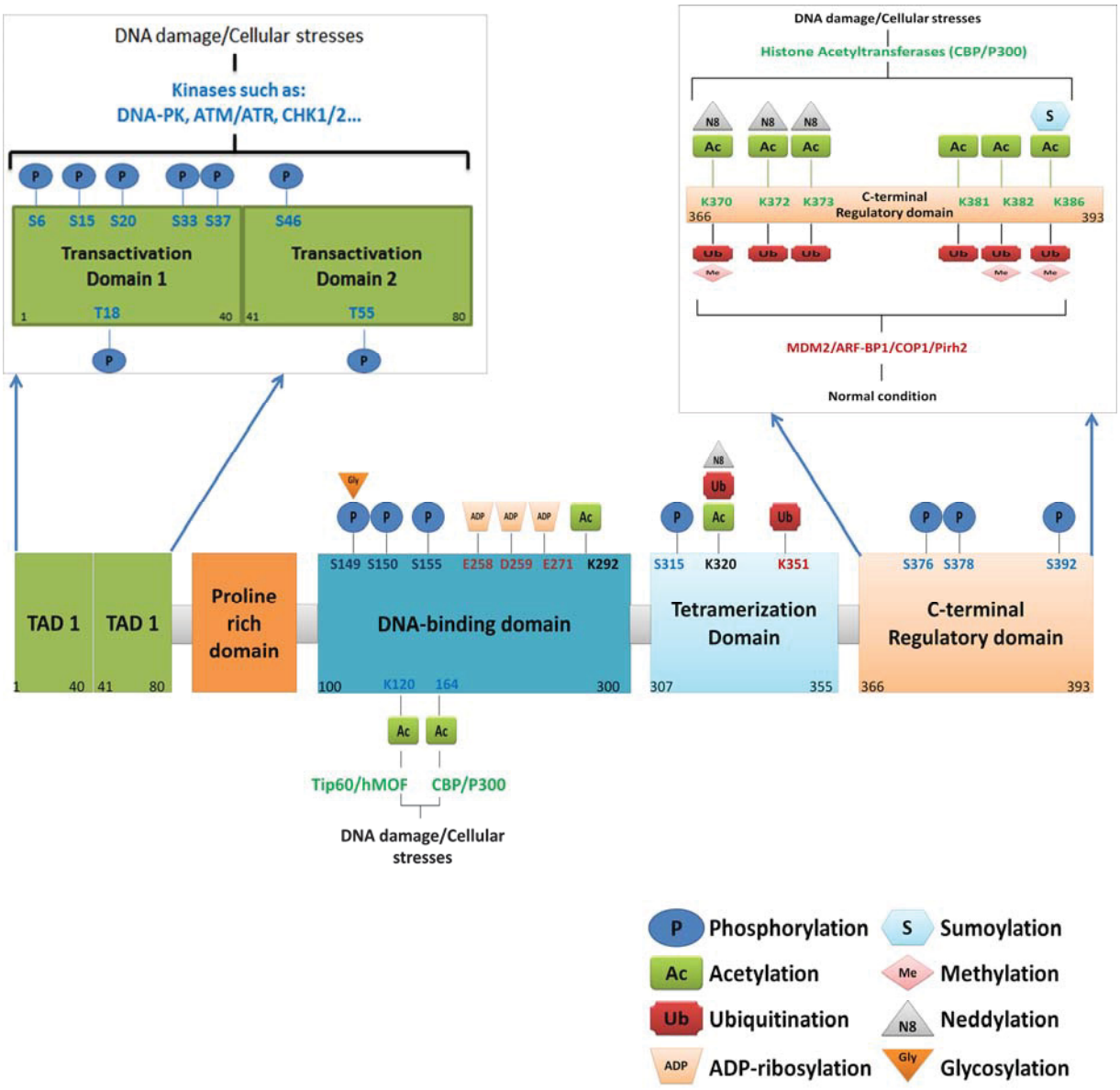


Figure 1. Schematic of p53 protein structure and posttranslational modifications. See text for details.

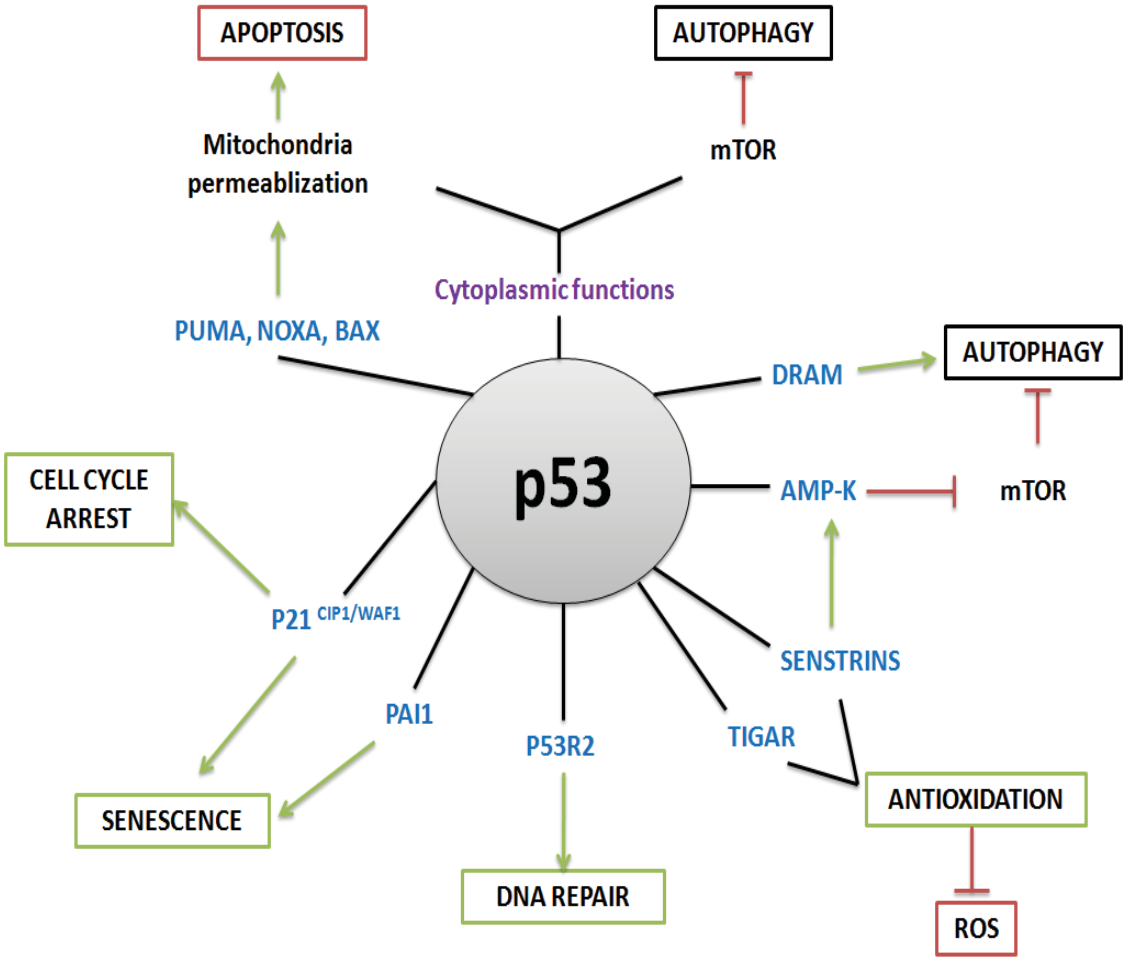


Figure 2. p53-regulated cellular pathways. See text for details.

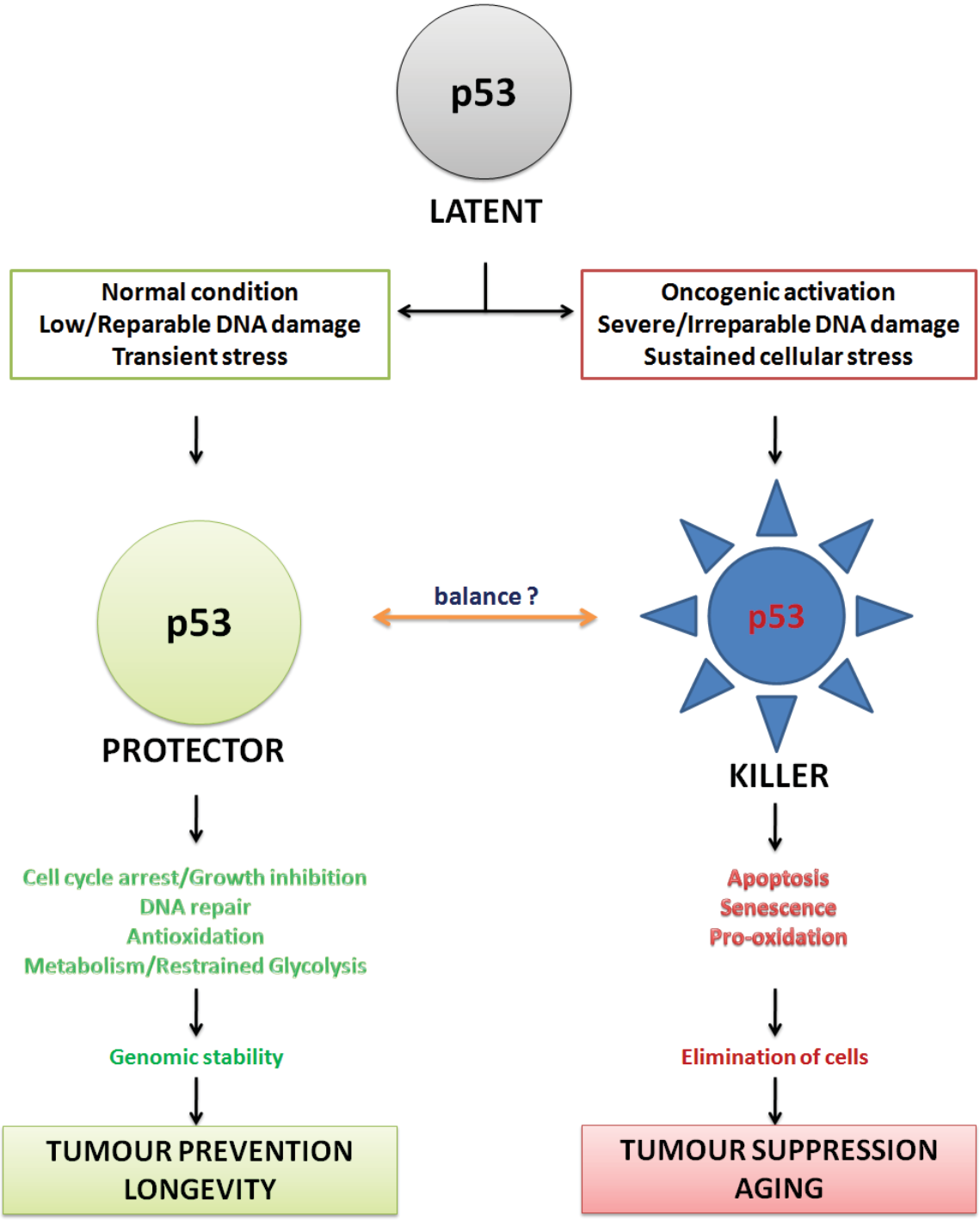


Figure 3. Dual mechanisms of p53 functions in tumor suppression and aging. See text for details.

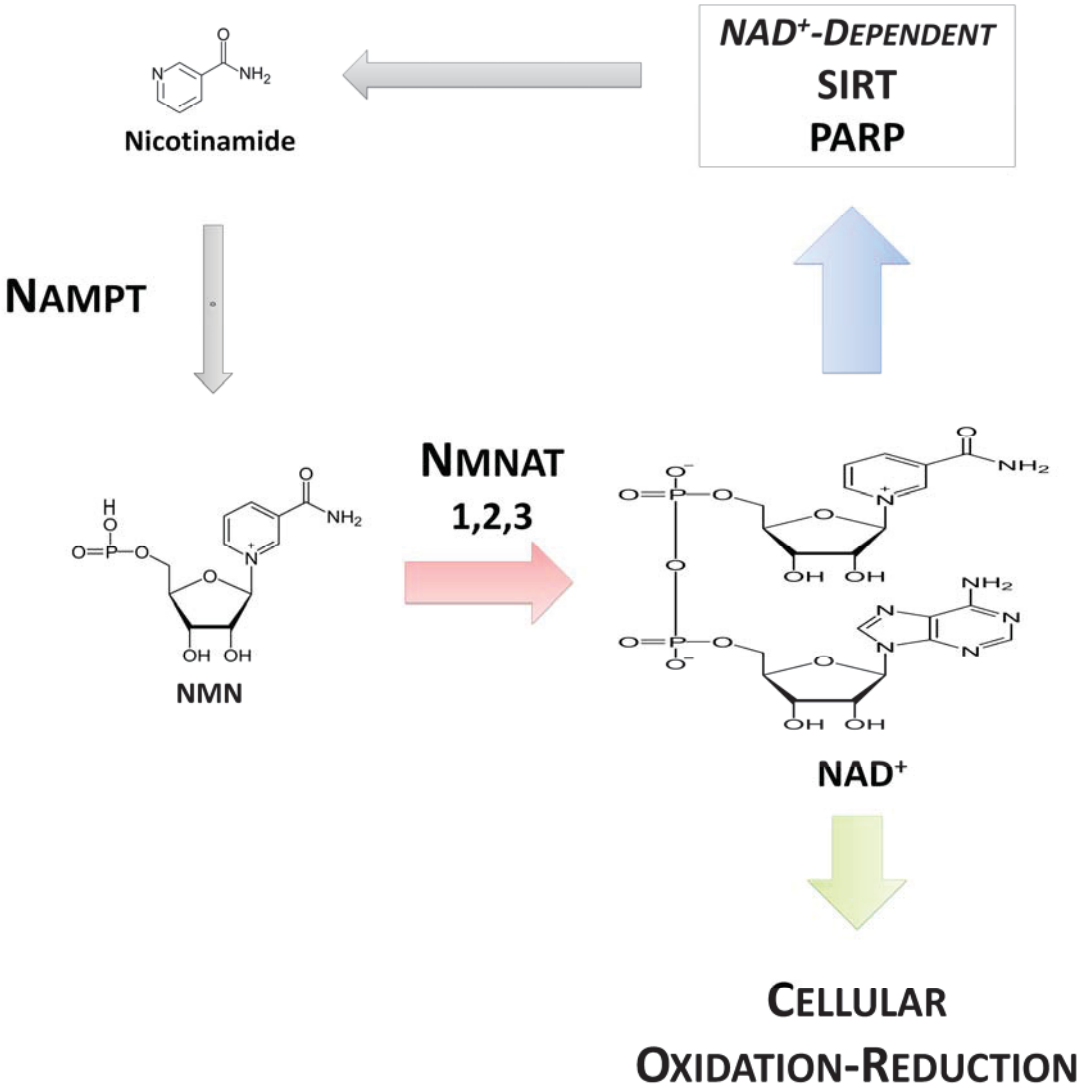


Figure 4. The non-redox recycling of NAD^+. See text for details.

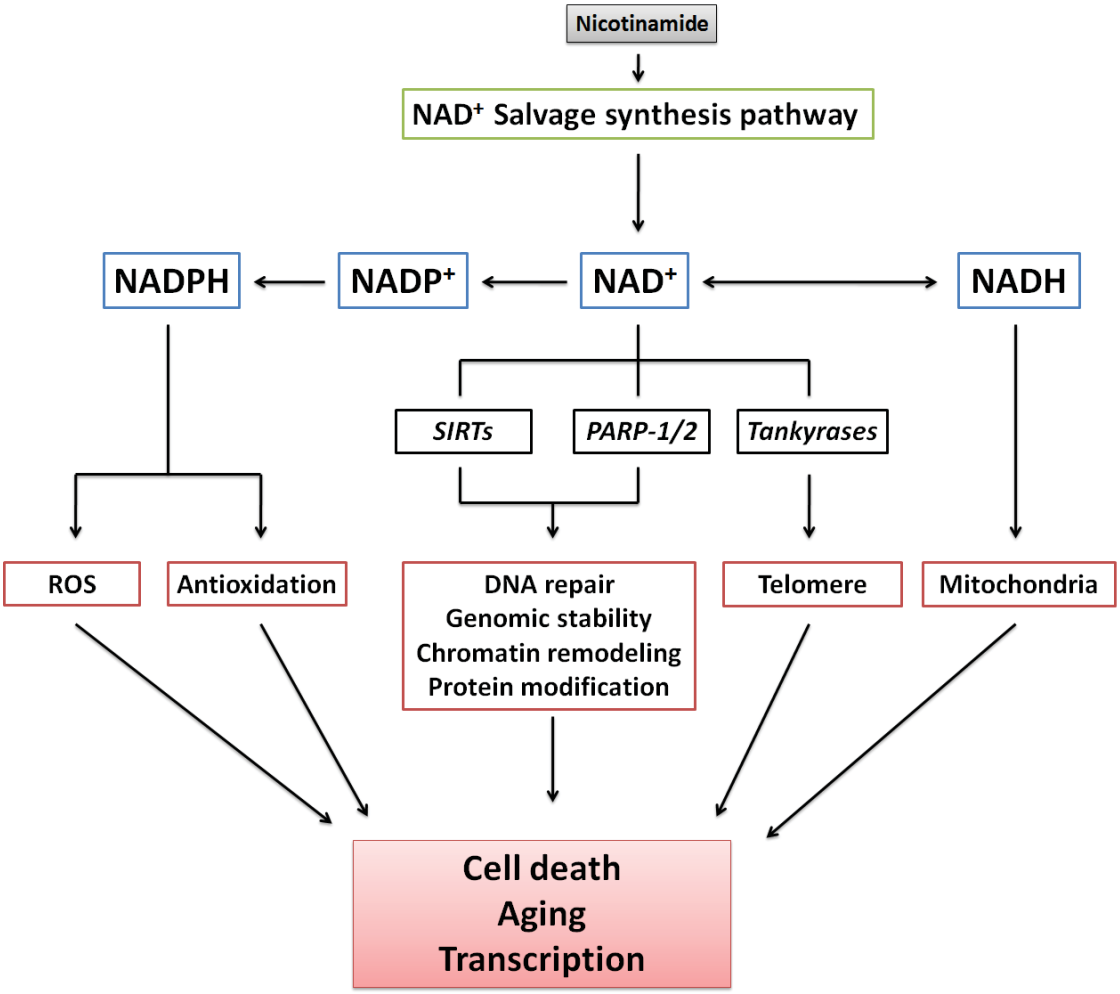


Figure 5. Cellular pathways affected by NAD^+ and its derivatives. See text for details.

CHAPTER 2 MATERIALS AND METHODS

2.1 CELL CULTURE AND TREATMENT

All human cell lines utilized in this thesis were either purchased from American Type Culture Collection (ATCC) or maintained in Dr. Patrick Lee's laboratory. U2OS human osteosarcoma cell line and Saos-2 human osteosarcoma cell line were cultured in McCoy's 5A medium (Invitrogen, CA). Hs68 human foreskin fibroblast cell and 293T cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, CA). H1299 human lung cancer cell line was grown in RPMI 1640 medium. All culture media were supplemented with 10% fetal bovine serum (FBS) (Invitrogen, CA) and anti-mycotics and antibiotics (Invitrogen, CA). Cells were grown in a humidified incubator with 5% CO_2 at 37 °C. U2OS, 293T and H1299 cell lines were split every 2~3 days by trypsinization and one fifth was passaged. Saos-2 was normally sub-cultured once a week. Hs68 cells were split every 4~5 days. Cell culture medium was changed at least every 2~3 days.

In UV-irradiation experiments, cells were washed with PBS and irradiated as a monolayer with UV-C rays on a UV cross-linker (50, 100 or 250 Joule/m^2; Stratagene). Pre-warmed culture medium containing reagents specified in each individual experiment was added immediately after irradiation. For ionizing irradiation, cells were kept in culture dishes without removing medium.

2.2 Antibodies And Reagents

Antibodies used in western blotting and immunofluorescence are listed in Table-1. Information of DNA-damaging agents (chemicals) and inhibitors are included in Table-2.

2.3 Sub-G1 And Cell-Cycle Analysis By DNA Content

Cells were collected at desired time-points post DNA damage by trypsinization and washed twice with cold phosphate buffered saline solution without Ca^{2+} or Mg^{2+} (PBS). After each wash, cells were recovered by centrifugation (500 x g, 5 min, and 4°C). After final wash, cells were resuspended at 2 x 10^6 cells in 1 ml ice-cold PBS. 3 ml 95% ethanol was added slowly (dropwise) to cell suspension with gentle vortexing. Cells were fixed at 4°C to - 20°C for at least 2 hours or stored up to few weeks. Before FACS, cells were centrifuged at 500 x g for 10 min and cell pellet was resuspended in 10 ml cold PBS for washing. Cells were washed twice then resuspended in 500 µl PI/Triton X-100 staining solution (0.1 % (v/v) Triton X-100 in PBS with 20 µg/ml RNase A (DNase free) and 50 µg/ml propidium iodide). Single-cell suspension was made by gentle pipetting then incubated at room temperature for at least 30 minutes or stored at 4°C overnight (protected from light). Samples were run on flow cytometer within 24 hours to avoid cell breakdown. Flowcytometer was set to FL2-A and FL2-W as linear. Data was analyzed with FCS express software.

2.4 Chromatin Immunoprecipitation

U2OS cells were treated with Actinomycin D (10 nM) or Nutlin-3 (10 µM) for 48 hours then cross-linked with formaldehyde solution. In a fume hood, cell cross-linking

was performed by adding 37 % formaldehyde stock to culture medium to reach 1% final concentration (815 µl to 30 ml medium). Cells were incubated for 10 minutes at room temperature. Then, cross-linking was stopped by adding glycine to medium to 0.125 M final concentration. Medium containing formaldehyde was removed and cells were washed twice with PBS. Cross-linked cells were collected by scraping and transferred to 15 ml tube. Cell pellet was washed twice with 15 ml cold PBS. After final wash, cells were resuspended thoroughly in 1ml PBS and transfered to micro-centrifuge tube and spun down at 12,000 g for 30 seconds. To lyse cells, 250 µl lysis buffer (50 mM Tris-HCl, pH8.0; 5 mM EDTA, pH 8.0; 1% SDS; protease inhibitor cocktail (Sigma)) was added to cell pellet. Cell lysate was sonicated with 15 pulses using a sonicator. Sonication was optimized to generate chromatin DNA fragments of about 500 base pairs. Sonicated cell lysate was spun down at 12,000 g for 10 minutes then supernatant was transferred a new microcentrifuge tube. Protein concentration was measured with MicroBCA assay (Pierce) and adjusted to 4 µg/ml. 10% of total cell lysate was saved as input for PCR. Cell lysate was then diluted with IP dilution buffer (Tris-Cl pH 8.0; 200 mM sodium chloride; 0.01 % SDS; 1% Triton-X100; 5 mM EDTA; protease inhibitor cocktail) to decrease SDS concentration to 0.25% (i.e. 1:3). Cell lysate was pre-cleared with 20 µl Dynabeads (pre-blocked with 1 % BSA and sheared salmon sperm DNA) at room temperature with rotation. Pre-cleared lysate was split into two tubes. Protein G-Dynabead pre-bound with antibodies (control IgG and anti-p53 (DO1)) was added to lysate for immunoprecipitation of p53. Reactions were incubated overnight with rotating at 4 °C. The next day, Dynabeads were washed thoroughly with washing buffers , twice with WS1 (5 mM EDTA; 50 mM Tris-Cl pH 8.0) and twice with WS2 (100 mM Tris-Cl

pH8.0; 500 mM LiCl; 1% NP40; 1% deoxycholate; 5mM EDTA). Elution of protein and DNA from Dynabeads were achieved by incubating beads with 100 µl IP elution buffer at room temperature for one hour. Then protein-DNA complex was decross-linked by adding 18 µl decross-linking buffer (6 µl 20% SDS and 12 µl 1 M $NaHCO_3$) and incubated overnight at 65°C. Samples were further treated with RNase A (200 µg/ml) for 30 minutes at room temperature to destroy any RNA. Immunoprecipitated chromatin DNA fragments were recovered with Quickspin PCR purification kit (Qiagen) according to instructions. Recovered DNA fragments were then used as templates for PCR amplification of target p53 REs. PCR primers used for each p53-binding site in Nmnat2 gene or for $p21^{Waf1/Cip1}$ and MDM2 genes are listed in Table-3.

2.5 IMMUNOPRECIPITATION, SDS-PAGE AND WESTERN BLOTTING

Protein immunoprecipitation (IP) was conducted for enrichment of p53 protein from U2OS cells. p53 protein was immunoprecipitaed with anti-p53 antibody (DO1, Santa Cruz) from whole cell lysate prepared from U2OS cells over-expressing Nmnat2-TV1, Nmnat2-TV2 or empty. In general, cells were damaged with Actinomycin D and collected at indicated time-points. Cells were lysed with 1% SDS buffer (50 mM Tris-Cl pH 8.0; 150 mM sodium chloride; 1% SDS; protease inhibitor cocktail) followed by 3 minutes boiling at 95°C. Protein concentrations were measured with MicroBCA assay and adjusted to 2 µg/ml. For IP, SDS concentration in the sample was reduced to 0.2 % by adding RIPA buffer (50mM Tris-HCl pH8.0; 150 mM sodium chloride; 1% NP-40; 0.5% sodium deoxycholate). Protein G-Dynabeads were pre-bound with either anti-p53 antibody (DO1) or control IgG in the presence of 1% BSA in RIPA buffer and used for immunoprecipitaion. 500 µg of diluted whole cell extract was used for each IP.

Reactions were allowed overnight at 4°C with rotating. Proteins bound to Dynabeads were eluted with 20 µl of 1% SDS sample buffer by boiling at 95°C for 5 minutes. 10 µl of elutes were resolved with SDS-PAGE and transferred to PVDF membrane for western blotting. Anti-acetyl lysine antibody and anti-acetylated p53 (site-specific) were used to detect acetylation on p53 protein. Antibodies information is listed in Table-1.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) based on Tris-glycine buffer was employed in most cases for separating proteins of interest. Gels were poured with various concentrations according to the size of target proteins. For detection of cellular poly ADP-ribose (PAR), 5% SDS-PAGE gel was used because of the linearity of the molecule. Otherwise, SDS-PAGE and western blotting was performed following standard protocols.

2.6 LENTIVIRAL INDUCIBLE P53 EXPRESSION VECTOR

The lentiviral inducible gene expression system was constructed by modifying the TRIPZ-Tet On vector from Openbiosystems. TRIPZ vector was originally designed for inducible expression of shRNAmir. To convert it into a gene expressing vector, the shRNA cassette was replaced with p53 open reading frame. Briefly, TRIPZ vector was digested with AgeI and MluI restriction enzymes and the vector backbone was recovered. p53 coding sequence was PCR amplified from IMR-90 cell (human lung fibroblast with wild-type p53 gene) cDNA with forward primer containing AgeI linker sequence and reverse primer containing MluI linker sequence. PCR product was digested with AgeI and MluI then ligated with pre-cut TRIPZ vector. Cloning was confirmed by DNA sequencing. The new p53 inducible vector was named TPZ-p53-Tet On and used for generating lentiviruses. H1299 human lung cancer cell (p53 null) was infected with TPZ-

p53-Tet On as described in section 2.8 in this thesis. Primers used for amplification of p53 are listed in Table-3.

2.7 RETROVIRUS-MEDIATED RNAI AND GENE EXPRESSION

RNA interference was delivered with retroviral vectors for most cell lines used in this thesis. There were two retrovirus-based shRNA expression systems being used. For gene knock-down in Hs68 human fibroblast cell, the pSUPER-Retro (Oligoengine) vector was used. However, for gene knock-down in other cell lines, the SMP system from Openbiosystems was used. These two systems utilize different promoters for driving the expression of shRNA. pSUPER-Retro has a human H1 RNA polymerase III promoter, whereas SMP vector has a CMV-based RNA polymerase II promoter. For each target gene, at least three shRNA sequences were designed and cloned into either pSUPER-Retro or SMP. p53 knock-down in Hs68 cells was approached with pSUPER-Retro. Knock-down of Nmnat2, Nmant1, Nmnat3, SIRT1, SIRT2 and p53 in U2OS cells was approached with SMP vectors.

For cloning pSUPER-Retro vectors, DNA oligos (forward and reverse) were annealed in a thermocycler (95°C for 3 minutes, 75°C for 3 minutes, and decrease by 1 °C/minutes to room temperature). pSUPER-Retro vector was cut with BglII and XhoI as directed by the instruction. Annealed oligos were digested with BamHI and XhoI then ligated into pSUPER-Retro. For cloning SMP vectors, the shRNA mir sequences were from Openbiosystem but cloned with PCR. Oligos containing each shRNA sequence were PCR amplified using SMP-Forward and SMP-Reverse primers, which contain XhoI and EcoRI restriction site, respectively. PCR products and SMP vector were digested with XhoI and EcoRI and ligated together. Constructs were confirmed by DNA

sequencing. Retroviruses were made according to protocols described in the next section. Primer sequences and shRNA sequences are listed in Table-3.

Gene overexpression of Nmnat2-TV1 and Nmnat2-TV2 was approached with MSCV retroviral vector (Clontech). First, MSCV vector expressing p53 was constructed. Briefly, p53 gene was PCR cloned into MSCV with a forward primer containing XhoI restriction site and a reverse primer containing one copy in-frame coding sequence of HA tag, which is separated form p53 coding sequence by a restriction site of NotI. The reverse primer also contains an EcoRI restriction site at its end. PCR product and MSCV vector were cut with Xho I and EcoR I and ligated together. The recombinant (MSCV-P53HA) was used for cloning of Nmnat2 isoforms, Nmnat1 and Nmnat3 via XhoI and NotI sites. Nmnat 1, Nmnat2-TV1, Nmnat2-TV2 and Nmnat3 were PCR amplified from cDNA prepared from U2OS cell. XhoI restriction site and NotI restriction site were incorporated into each forward and reverse primer, respectively. PCR products and MSCV-p53HA vector were digested with XhoI and Not I then ligated together. All constructs were confirmed by DNA sequencing. MSCV retroviruses were generated with protocol described in the next section and used to infect U2OS cells for establishing stable cell lines expressing each Nmnat protein. Primers used for gene cloning are listed in Table-3.

2.8 Generation Of Retrovirus And Lentivirus

To generate infectious retroviruses and lentiviruses, a general protocol was followed. This protocol was developed based on transfection of 293T cell line with three plasmids of MD2.G, pHIT60 and retroviral vectors (MSCV, SMP and pSUPER-Retro). For lentivirus production, the similar procedure was followed except pPAX (Addgene)

vector was used instead of pHIT60. Briefly, 293T cells were seeded in 10 cm dishes one day before transfection such that they reached 70~80% confluence upon transfection. Thirty minutes before transfection, cell culture medium was changed to 8 ml fresh medium without antibiotics. Chloroquine was added to the medium at a final concentration of 25 μM. DNA solution A was prepared by mixing 14 μg retroviral vector, 8 μg of MD2.G and 8 μg of HIT60 in nuclease-free water, then 86.8 μl 2 M calcium solution was added to the total volume of 700 μl. Meantime, 2X HBS solution was thawed and warmed up to room temperature. The equal volume of 2X HBS solution was added dropwise with vortexting to DNA solution A. The mixture was incubated at room temperature for 10 minutes then gently added to cell culture medium. The cell culture dish was swirled to ensure an even distribution of DNA complex. 6~12 hours post transfection, DNA containing medium was removed and cells were washed with PBS, 8 ml complete medium was added to culture dishes. Cells were returned to incubator and virus-containing medium was collected 24 hours later, followed by continuous collection with 12 hours-intervals until cells lost viability (normally after 72 hours). Collected medium was pooled and used for infection of target cell lines.

For infection, U2OS, H1299 and Hs68 cells were seeded in 6 cm dishes. Virus-containing medium was centrifuged at 500 g for 5 minutes to sediment cell debris then filtered through a 0.45 μM filter with a 50 ml syringe. Polybrene was added to the medium to a final concentration of 8 μM. 2 ml Virus-containing medium was mixed with equal volume of complete medium and added to cell. Cells were infected for 24 hours then put back to regular medium for recovery. Puromycin or blasticidin was added to cell culture medium at 2 μg/ml and 5 μg/ml, respectively. Mock-infected cells were used as a

control for monitoring cytotoxicity of drugs. Once selection was finished, cells were kept growing in low concentrations of puromycin (0.2 μg/ml) or blasticidin (0.5 μg/ml).

2.9 RNA Extraction And Quantitative PCR

Total RNA was extracted from U2OS cells with Trizol reagent (Invitrogen) following manufacturer's instruction. To recover highly pure RNA, U2OS cells were first dissolved in Trizol then RNA-containing phase was mixed with ethanol and loaded on RNeasy RNA purification kit (Qiagen). On-column DNase digestion was performed to destroy residual DNA contamination. 2 μg total RNA was used for cDNA synthesis in a reaction of 20 μl with MMLV reverse transcriptase (Invitrogen). cDNA products were then diluted with nuclease-free water to 100 μl. 2 μl of diluted cDNA was used for either semi-quantitative or real-time quantitative PCR reaction unless specified. For Nmnat2 transcripts, 5 μl of diluted cDNA was used for each PCR reaction. Semi-quantitative PCR was conducted with Taq DNA polymerase with 30 cycles (95 °C for 3 minutes; 55 °C for 45 seconds; 72°C for 1 minute). After 20 cycles, 5 μl aliquot of 50 μl PCR reaction was taken every 2 cycles such that a linear range of PCR was ensured. 4 μl was loaded on 1.5 % agrose-TAE gel for separation. PCR primers used for detecting Nampt, Nmnat1, Nmnat2, Nmnat3 and p53 are listed in Table-3.

For real-time quantitative PCR, 2 μl of cDNA was used in each reaction except for Nmnat2, for which 5 μl of cDNA was used. PCR reactions were set up with Quanti-fast Sybr-green qPCR kit (Qiagen) and run on Stratagenee MX 300 thermocycler using conditions specified by Qiagen. Data analysis was done with MXpro (Stratagene) software. mRNA levels were normalized to the actin internal control. PCR primers used for RT-qPCR for each gene are listed in Table-3.

2.10 DUAL LUCIFERASE REPORTER ASSAY

To examine the functionality of p53 responsive elements identified in human Nmnat2 gene, both wild-type and mutant p53-binding sites (BS#1 and BS#2) were cloned upstream of the firefly luciferase gene. NF-kB luciferase reporter vector was digested with Mlu I and BglII to remove NF-kB responsive sequence. Forward and reverse oligos (about 60 bases) containing Nmnat2 p53-binding sites (20 bases) with extra linker sequences of MluI (forward oligo) and BglII (reverse oligo) were annealed according to protocols described earlier. P53BS#1 and p53BS#2, either wild-type or mutant, were cloned into luciferase reporter vector (NFkB RE removed) and used for transfection. All constructs were confirmed by DNA sequencing.

U2OS cells, control or p53 knock-down, were transfected with luciferase reporter plasmids carrying individual p53BS with Lipofectamine 2000 following standard procedure (Invitrogen). Specifically, U2OS cells were seeded in 6-well plate with equal numbers. 1 μg firefly luciferase reporter plasmid was used for each transfection, together with 20 ng renilla luciferase reporter plasmid (TK-Renilla luciferase, Promega) as an internal control for transfection. Twenty four hours post transfection, cells were either mock treated or treated with Actinomycin D (10 nM) or Nutlin-3 (10 μM). Forty-eight hours later, cells were lysed in 6-well plate with PLB buffer from dual luciferase reporter assay kit (Promega). Protein concentration was measured for each sample and 10 μl from 500 μl lysate was used for reaction according manufacturer's instruction. Firefly luciferase activities were measured with the GloMax luminometer (Promega) and values were normalized to renilla luciferase activity measured in the same reaction. These values

were further normalized to protein concentration of each sample and presented as relative amount change to non-treated cells.

2.11 NAD^+/NADH Quantification Assay

To measure cellular NAD^+, U2OS cells with control, p53, Nmnat1, Nmnat2 and Nmnat3 shRNA were treated with Actinomycin D (10 nM) and harvested at indicated time-points. Cells were lysed and processed with the NAD^+/NADH quantification kit (Biovision) following manufacturer's protocol.

2.12 Immunofluorescence Microsopy Of p53 Protein In Hs68 Cells

Hs68 (control or p53-KD) cells were grown on 4-well chamber slides (LabTek). Cells were washed twice then fixed in 3.7% paraformaldehyde for 10 minutes. Permeabilization was carried out for 5 min with 0.1% Triton X-100 in PBS at room temperature. After washing 3 times with PBS, cells were blocked with 1% BSA (Fisher Scientific) in PBS containing 0.05% Tween-20 for 30 minutes, and then incubated with primary antibodies for 1 h at room temperature or overnight at 4°C followed by secondary antibodies for 30 minutes at room temperature. Slides were examined under a Axiovert fluorescence microscope or LSM 510 confocal microscope (Zeiss). Images were collected and processed with Adobe Photoshop software. For p53 staining, rabbit polyclonal anti-p53 antibody (FL-393, Santa Cruz) was used at 1:400. For cytochrome c staining, a mouse monoclonal antibody (6H2, Santa Cruz) was used at 1: 200. For Grp75, a rabbit polyclonal antibody (H-155, Santa Cruz) was used at 1: 400. Donkey anti-rabbit or mouse secondary antibodies are conjugated with either Cy2 or Cy3 and used at 1: 500.

TABLE-1. ANTIBODY INFORMATION

Antibody	Working concentration	Vendor
p53, DO1	1 : 2000 **(W.B.)**	Santa Cruz (sc-126)
p53, DO7	1 : 2000 **(W.B.)**	Santa Cruz (sc-47698)
p53, FL393	1 : 1000 **(W.B.)**	Santa Cruz (sc-6243)
HA probe	1 : 1000 **(W.B.)**	Santa Cruz (sc-805)
Actin	1 : 4000 **(W.B.)**	Santa Cruz (sc-47778)
Cytochrome C	1 : 1000 **(W.B.)**	Santa Cruz (sc-13561)
Grp75	1 : 1000 **(W.B.)**	Santa Cruz (sc-13967)
Poly-ADP-ribose	1 : 500 **(W.B.)**	Santa Cruz (sc-56198)
Poly-ADP-ribose	1 : 500 **(W.B.)**	Alexis/Enzo life Science (10H)
SIRT1	1 : 1000 **(W.B.)**	Abcam (ab32441)
SIRT2	1 : 1000 **(W.B.)**	Abcam (ab51023)
Nmnat2	1 : 250 **(W.B.)**	Abcam (ab56980)
p53, acetyl-K120	1 : 500 **(W.B.)**	Abcam (ab78316)
p53, phospho-serine 15	1 : 1000 **(W.B.)**	Cell signaling (cat#9284)
p53, phospho-serine 46	1 : 500 **(W.B.)**	Cell signaling (cat#2521)
p53, acetyl-K382	1 : 1000 **(W.B.)**	Cell signaling (cat#2525)
Acetyl-lysine	1 : 1000 **(W.B.)**	Cell signaling (cat#9441)
Acetyl-Histone 2A (Lysine5)	1 : 2000 **(W.B.)**	Cell signaling (cat#2576)
Acetyl-Histone 3 (Lysine9)	1 : 2000 **(W.B.)**	Cell signaling (cat#9671)
Cleaved caspase 3	1 : 1000 **(W.B.)**	Cell signaling (cat#9661)
p53, acetyl-K320	1 : 200 **(W.B.)**	Upstate/Millipore (cat#06-915)
p53, acetyl-K373	1 : 200 **(W.B.)**	Upstate/Millipore (cat#06-916)

Table-2. Reagents

Chemicals	Stock Solution	Vendor (Cat#)
Actinomycin D	2 mM in DMSO	Sigma (A1410)
Camptothecin	10 mM in DMSO	Sigma (C9911)
Adriamycin /Doxorubicin hydrochloride	1 mg/ml in DMSO	Sigma (D1515)
5-Fluorouracil	100 mg/ml in DMSO	Sigma (F6627)
Etoposide	50 mM in DMSO	Sigma (E1383)
Cycloheximide solution	100 mg/ml in DMSO	Sigma (C4859)
Puromycin	10 mg/ml in ddH_2O	Sigma (P7255)
LY294002	5 mg/ml in DMSO	Sigma (L9908)
Wortmannin	50 mg/ml in DMSO	Sigma (W1628)
SIRT1 Inhibitor III	10 mM in DMSO	Calbiochem (566322)
SIRT2 inhibitor (AGK2)	20 mM in DMSO	Calbiochem (566324)
SIRT2 inhibitor (AGK7)	20 mM in DMSO	Calbiochem (566326)
DNA-PK Inhibitor II	5 mM in DMSO	Calbiochem (260961)
ADP-HPD	5 mM in ddH_2O	Calbiochem (118415)
Doxycycline	5 mg/ml in ddH_2O	Clontech
Geneticin /G418	100 mg/ml in ddH_2O	Invitrogen (10131035)
Blasticidin S HCl	5 mg/ml in ddH_2O	Invitrogen (R21001)

TABLE-3. LIST OF PRIMERS

NAME	ID	SEQUENCES
RT p53	F	CACATGACGGAGGTTGTGAG
RT p53	R	TGGTACAGTCAGAGCCAACCT
RT P21	F	GACTCTCAGGGTCGAAAACG
RT P21	R	GCCAGGGTATGTACATGAGGA
RT MDM2	F	GTGAATCTACAGGGACGCCAT
RT MDM2	R	CTGATCCAACCAATCACCTGAA
RT Tigar	F	GCAGCTGCTGGTATATTTCTGA
RT Tigar	R	CACTTAGCGCTTTGCCTTCT
RT Pig3	F	ACCGCCTTCCAGCTGTTAC
RT Pig3	R	CAGCTGCTCCAAGCTTTTCT
RT Gadd45a	F	AGGAATTCTCGGCTGGAGAG
RT Gadd45a	R	ATCTCTGTCGTCGTCCTCGT
RT Nmnat1	F	TCATCATGGCAGAACTTGCT
RT Nmnat1	R	TGGCACAGCTTTTGTTTTTG
RT Nmnat3	F	CCCTGCAAATAGCAGCTACC
RT Nmnat3	R	TGAGAAGCTGCGAGGTCTTT
RT Nmnat2-TV1	F-1	TGCAGACACAACGAGACACA
RT Nmnat2-TV1	F-2	AGAGAGGCAACCCCTAGACC
RT Nmnat2-TV1	F-3	ATGACCGAGACCACCAAGAC
RT Nmnat2-TV1	*F-4*	*ATGACCGAGACCACCAAGAC*
RT Nmnat2-TV2	F-1	AGCTGGAGGTAGTGGAGCAA
RT Nmnat2-TV2	F-2	CAGCTGCACCACTTTGTGTT
RT Nmnat2-TV2	F-3	AGCACAGGGCTGCAAGTAGT
RT Nmnat2-TV2	*F-4*	*TGGAAATCCAGGAACTAGAGGA*
RT Nmnat2	*R-1*	*CCATAGGAGTCGTGGACAGG*
RT Nmnat2	R-2	ATCCCGCCAATCACAATAAA
RT Nmnat2	R-3	GGTCCACCCTGATCCAATC
Chip Primer BS#1	F	GCTAAGGGCCAGAGAGGTTT
Chip Primer BS#1	R	GTGGCAGAGGCAAGATTCA
Chip Primer BS#2	F	GTGCCAGACACTGGACAAGA
Chip Primer BS#2	R	CCCTTCAAAATCCAACTTGC
MSCV-Nmnat1	F	ATGCCTCGAGCCGCGGGCCACCATGGAAAATTC CGAGAAGACTGA
MSCV-Nmnat1	R	GCGGCCGCTGTCTTAGCTTCTGCAGTGT
MSCV-Nmnat3	F	ATGCCTCGAGCCGCGGGCCACCATGTACCAGGT CATCCAGGGTAT
MSCV-Nmnat3	R	GCGGCCGCGCTTGTCTTGCCCTCA GTGCT
MSCV-Nmnat2-TV1	F	CTGACTCGAGATCGATGCCACCATGACCGAGAC CACCAAGAC
MSCV-Nmnat2-TV2	F	CTGACTCGAGATCGATGCCACCATGGAAATCCA GGAACTAGAGGA
MSCV-Nmnat2	R	CTGATCTAGACTAGCCGGAGGCAT TGATGT

NAME	ID	SEQUENCES
MSCV-Nmnat2-HA	R	GCGGCCGCGCCGGAGGCATTGATGT
TPZ-p53	F	GTCAGAACCGGTTTAATTAAGCCACCATGGAGG AGCCGCAGTCAGATCC
TPZ-p53	R	TGAGACACGCGTATCGATTCAGTCTGAGTCAGG CCCTTCTG
pSUPER- Retro-control	F	GATCCCCTTCTCCGAACGTGTCACGTTTCAAGAG AACGTGACACGTTCGGAGAATTTTTC
pSUPER- Retro-control	R	TCGAGAAAAATTCTCCGAACGTGTCACGTTCTCT TGAAACGTGACACGTTCGGAGAAGGG
pSUPER- Retro-p53	F	GATCCCCGACTCCAGTGGTAATCTACTTCAAGA GAGTAGATTACCACTG GAGTCTTTTTC
pSUPER- Retro-p53	R	TCGAGAAAAAGACTCCAGTGGTAATCTACTCTC TTGAAGTAG ATTACCACTGGAGTCGGG
SMP-Nmnat1	98550	AGGAAAGAAGTTGTGATCTGTTTAGTGAAGCCA CAGATGTAAACAGATCACAACTTCTTTCCC
SMP-Nmnat2	100026	CGCTTGGAGAGCCATAAGGAAATAGTGAAGCCA CAGATGTATTTCCTTATGGCTCTCCAAGCAT
SMP-Nmnat2	100027	AGCCACTGTTAGGTTTCCTTTATAGTGAAGCCAC AGATGTATAAAGGAAACCTAACAGTGGCC
SMP-Nmnat2	231883	AGGTCATCGACTACATCCTCAATAGTGAAGCCA CAGATGTATTGAGGATGTAGTCGATGACCG
SMP-Nmnat3	213981	CGGATAGACATTTATCAAAGAATAGTGAAGCCA CAGATGTATTCTTTGATAAATGTCTATCCT
SMP-p53	93615	CGGAGGATTTCATCTCTTGTATTAGTGAAGCCAC AGA TGTAATACAAGAGATGAAATCCTC CA
SMP-Control	LacZ	CGCTACACAAATCAGCGATTTATAGTGAAGCCA CAGATGTATAAATCGCTGATTTGTGTAGCG
SMP-SIRT1	20111	CGCAGCTAAGAGTAATGATGATTAGTGAAGCCA CAGATGTA ATCATCATTACTCTTAGCTGCT
SMP-SIRT2	20091	AGCCATCTTTGAGATCAGCTATTAGTGAAGCCA CAGATGTA ATAGCTGATCTCAAAGATGGCC

CHAPTER 3

NICOTINAMIDE NUCLEOTIDE ADENYLYLTRANSFERASE 2 (NMNAT2) IS A DIRECT TARGET GENE OF P53 AND REGULATES P53 FUNCTIONS IN A FEEDBACK MANNER UPON DNA DAMAGE

3.1 HYPOTHESIS AND GENERAL RESEARCH OBJECTIVES

This study mainly focuses on the investigation of mechanisms underlying activation of p53 upon DNA damage in human cancer cells. p53 acts as a tumor suppressor by regulating diverse cellular processes including growth arrest, apoptosis, metabolism, anti-oxidation, DNA repair and genome integrity. The versatility of p53 functions is attributed to its central role in transcriptional activation or repression of hundreds of target genes. Some of these genes identified are functioning in cell metabolism and energy production, in which the co-enzyme nicotinamide adenine dinucleotide (NAD^+) and its derivatives are essential factors. The expansion of p53 functions into these physiological pathways leads to the possibility of regulation of cellular NAD^+ biosynthesis by p53. Establishing a link between p53 and NAD^+ biosynthesis will greatly facilitate our understanding of p53 activation because NAD^+-dependent histone deacetylases SIRTs are important regulators of p53. Therefore, I hypothesized that cellular NAD^+ biosynthesis can be regulated by p53 either directly or indirectly and this regulation may affect activities of cellular SIRT proteins and/or other NAD^+-dependent factors such as poly (ADP-ribose) polymerases (PARPs), which in turn regulate apoptotic function of p53. To test this hypothesis, p53 expression in human cells will be manipulated through RNAi and other methods. p53 will be activated by either DNA-damaging agents or other chemicals and assayed for its effects on the expression of cellular NAD^+ synthetases. On the other hand, activation of p53 will be examined in the

context of manipulating the expression of NAD^+ synthetases and SIRT proteins. In general, the inter-regulation between p53 and cellular NAD^+ synthetases will be investigated.

3.2 RESULTS

3.2.1 Common DNA-Damaging Agents Cause Distinct Responses In U2OS Cell

These experiments were designed and carried out for the goal of investigating the mechanisms underlying activation of p53's apoptotic function. A panel of human cancer cell lines and DNA-damaging agents were employed in this project. One dilemma in the study of p53 using cultured cell lines, most of which are either transformed or immortalized, is that the intactness of the p53 pathway is not guaranteed (http://p53.free.fr) [325]. Although almost half of human cancers contain a wild-type p53 gene, it is believed that these cells manage to inactivate or alter cellular pathways regulating or being regulated by p53. Commonly used DNA-damaging agents act through distinct mechanisms, and their effects largely depend on the dose being used [326]. Therefore, it is important to decide the experimental system to study p53-mediated DNA damage responses including apoptosis.

DNA-damaging agents are routinely used to provoke a p53 response in cultured cells. Exogenous genotoxic agents cause DNA damage either directly or indirectly. For example, Ionizing Radiation (IR) and UV-B/C light cause DNA double-stranded breaks and pyrimidine dimers resulting from cross-linking of adjacent cytosine or thymine bases, respectively. Agents that modify or intercalate with DNA also cause damages directly, resulting in oxidation, methylation and alkylation of DNA. Drugs clinically used for

cancer chemotherapy often target rapidly dividing cancer cells that perform extensive DNA replication and chromatin remodeling. 5-Fluorouracil (5-FU) is a pyrimidine analog that irreversibly inhibits thymidylate synthetase, shutting down the supply of thymidine for DNA replication. Camptothecin (CPT) and Etoposide (Eposin/VP-16) stabilize the complex of DNA with topoisomerase I and II, respectively, causing DNA strand breaks and apoptosis of the cell. Adriamycin, also known as Doxorubicin, not only interacts with DNA by intercalation but also inhibits the activity of topoisomerase II. Actinomycin D (Dactinomycin or Act.D) belongs to a class of polypeptide antibiotics and is primarily used as an investigative tool in cell biology to inhibit transcription. It does this by binding to DNA at the transcriptional initiation complex and in this way prevents elongation by RNA polymerase.

Several human cancer cell lines with wild-type p53, including HCT116, MCF7, A549, U87, A172 and U2OS, were examined for their responses toward various types of DNA-damaging agents including IR, UV, Camptothecin, Etoposide, 5-FU, Adriamycin and Actinomycin D. These cell lines demonstrated distinct cellular responses to the same type of DNA-damaging agent, suggesting that cellular DNA damage responses depend on cellular context (data not shown). The U2OS osteosarcoma cell line (ATCC® Number: HTB-96) was primarily used in my research because of its capacity to undergo both cell-cycle arrest, including G1/S and G2/M arrest, and apoptosis in response to distinct types of DNA damage agent. U2OS cells were challenged with aforementioned DNA-damaging agents with increasing doses and harvested after twenty-four hours for cellular DNA content analysis. Fixed cells were treated with RNase A to remove cellular RNA prior to P.I. (propidium idodide) staining. According to fluorescence intensity of their

DNA staining, cells can be sorted by fluorescence-activated cell sorting (FACS) into distinct cell-cycle phases: G1 phase (2N DNA), G2 phase (4N DNA), S phase (between 2N and 4N) and Sub-G1 population (less than 2N DNA). Fragmentation of nuclear chromatin is a characteristic of late-stage apoptosis. Consequently, apoptotic cells, and also necrotic cells, typically show DNA staining intensities lower than that of G1-phase cells (2N DNA). Thus, cells with sub-G1 DNA content are designated as "dead" cells.

U2OS cells clearly underwent cell-cycle arrest at the G2/M transition upon IR (Figure 6-A). Surprisingly, no cell death was observed at high dose of 20 Grays, even at later time-point (48 hours, data not shown). Etoposide, which presumably causes DNA double-stranded breaks (DSBs) by inhibiting topoisomerase II, also caused G2/M cell-cycle arrest without inducing cell death (Figure 6-B). UV radiation induced cell death at doses higher than 20 joules/m^2 (Figure 6-C). Camptothecin, a topoisomerase I inhibitor, induced cell death at concentrations higher than 0.5 μM (Figure 6-F). 5-FU exclusively caused cell-cycle arrest at G1/S (Figure 6-D). Interestingly, Adriamycin caused G2/M cell-cycle arrest at concentrations lower than 200 (ng/ml) but cells retained a normal pattern of cell-cycle distribution at concentrations higher than 400 (ng/ml), suggesting that additional cellular pathways were affected (Figure 6-E). These results provided information for subsequent experimental designs. The fact that some of these agents failed to induce a clear apoptotic response makes them unsuitable for the study of p53-induced apoptosis in U2OS cells.

3.2.2 Actinomycin D-Induced Cell Death In U2OS Cell Is p53-Dependent

Actinomycin D inhibits global transcription at relatively high concentrations and is a potent activator for p53. It binds to DNA duplexes and therefore also interferes

with DNA replication. The effective concentration of Act.D to suppress cellular transcription is at least 100 nM (data not shown). To avoid interfering with the induction of p53 downstream genes, working concentrations ranging from 1 nM to 80 nM were tested for apoptosis induction. U2OS cells became sensitive to Act.D at concentrations higher than 20 nM, which caused about 10% of cells to die at 24 hours (Figure 7-A and B). At the highest concentration tested (80 nM), Act.D killed about 60% of cells. In contrast, U2OS cells with p53 being stably knocked down by retrovirus-mediated RNA interference (RNAi) only showed about 30% cells death (80 nM Act.D) (Figure 7-A and B). Knock-down efficiency of p53 in U2OS cell was confirmed by western blotting (Figure 7-C). These data demonstrated that Act.D can induce a p53-dependent apoptotic response in U2OS cells. While 20 nM Act.D only induced about 10% of cells to die at 24 hours, extended incubation up to 48 hours killed about 30% of cells (Figure 12 and data not shown). To exclude potential effects of transcription inhibition, Act.D at 20 nM or lower was used in most experiments.

3.2.3 p53 Induces The Expression of Nmnat2 But Not Other NAD^+ Synthetases

To determine if p53 can regulate cellular NAD^+ synthesis, mRNA levels of key NAD^+ synthetases including Nampt, Nmnat1, Nmnat2 and Nmnat3 were compared in the presence or absence of p53. Methods that induce p53 expression without causing DNA damage were considered, because DNA lesions may activate p53 indirectly through upstream pathways such as ATM/ATR/DNA-PK that affect various cellular pathways, thereby complicating data analysis. An inducible p53 expression system provides a useful tool for the study of cellular events that are caused by p53 accumulation in the absence of DNA damage. I therefore used a lentivirus-based inducible expression vector (named

TPZ). The expression of genes of interest from this vector is tightly controlled and activated by the antibiotic Doxycycline or Tetracycline, a mechanism known as "Tet-On". Wild-type p53 open reading frame (ORF) was PCR amplified from IMR-90 human fibroblast cell line and cloned into TPZ vector. Constructs were verified by DNA sequencing. Lentiviruses were generated by 293T cell-based transfection of a packaging system consisting of TPZ vector containing p53 ORF, PAX and MD2.G vectors (Addgene) that provide necessary elements for assembly of infectious lentiviral particles. H1299 human non-small cell lung cancer cells (ATCC® Number: CRL-5803), which do not express endogenous p53 due to gene deletion, were infected with TPZ-p53 (wt) lentiviruses and used for the following study. H1299-p53 (wt) Tet-On cells were induced to express p53 by adding doxycycline (1 μg/ml) to cell culture medium. In the absence of doxycycline, there was no detectable p53 protein being expressed (Figure 8-A). Doxycycline efficiently induced p53 at 24, 48 and 72 hours, validating the H1299-p53 (wt) Tet-On cell line as a reliable system for controlled expression of p53 (Figure 8-A). First, semi-quantitative PCR was performed to examine changes in cellular mRNA levels for each NAD^+ synthetase, using cDNA synthesized from H1299-p53 (wt) Tet-On cells cultured with or without doxycycline (36 hours). Aliquots of PCR reactions for each gene were collected at increasing PCR cycles (intervals of two) starting from 20 cycles to ensure they were in linear range. Data clearly showed that induction of p53 elevated the mRNA levels of Nmnat2 but not that of Nmnat1, Nmnat3 and Nampt. In the meantime, p53 mRNA was only present in cells treated with Doxycycline (Figure 8-B). This result demonstrated that p53 could induce the expression of Nmnat2 gene in H1299 cells. Human Nmnat2 gene transcribes two alternative transcript variants (TV1: *NM_ 015039*

and TV2: *NM_170706*) that differ in their 5-end sequences. At this point, PCR primers used for detecting Nmnat2 transcripts were designed to target both transcript variants. This issue is addressed below. Further, real-time quantitative PCR (RT-qPCR) was conducted to confirm the finding of p53-induced expression of the Nmnat2 gene in H1299 cell, with samples collected at indicated time-points after p53 induction. Nmnat2 mRNA levels increased greater than 8-fold at 72 hours post p53 induction. In contrast, under the same condition, Nmnat1 and Nmnat3 mRNA levels only increased about 2-fold and 3-fold, respectively (Figure 8-C). These PCR results strongly supported the notion that p53 can activate the expression of the Nmnat2 gene, directly or indirectly.

3.2.4 DNA Damage Induces Nmnat2 In A p53-Dependent Manner

DNA damage activates p53's transcriptional activity, leading to induction of a large panel of p53 target genes such as $p21^{Waf1/Cip1}$. If p53 can induce the expression of Nmnat2 gene, it is logical to propose that p53-activating agents will also do this. To test this possibility, U2OS cells stably expressing either non-specific or p53-specific shRNA were used in parallel to compare Nmnat2 mRNA levels changes before and after Act.D treatment. As discussed above, Nmnat2 gene transcribes two mRNA species. Isoform-specific PCR primers were designed using the **Primer3** program to distinguish Nmnat2-TV1 from Nmnat2-TV2. To assure the specificity of PCR reactions, four forward primers (5-end specific) for each mRNA isoform and three reverse primers (targeting the common region) were picked, giving a total of twelve pairs for evaluation. Three pairs of PCR primers out of twelve for each isoform were confirmed to give a single PCR product and were therefore chosen to be used in real-time qPCR experiments (data not shown).

Both Nmnat2 isoforms were induced by Act.D (Figure 9-A). While Nmnat2-TV1 mRNA levels had increased approximate 6-fold at 72 hours, Nmnat2-TV2 mRNA levels had increased greater than 20-fold. At each time point, p53 knock-down cells showed significantly reduced increases in both Nmnat2-TV1 and Nmnat2-TV2 mRNA levels (Figure 9-A). To rule out the possibility that p53-dependent induction of Nmnat2 was exclusively caused by Act.D, two alternative drugs (Adriamycin and Nutlin-3) were used to activate endogenous p53 in U2OS cells. Nmnat2 isoforms were also induced by Adriamycin in a p53-dependent manner (Figure 9-B). Nutlin-3 is an antagonist of the MDM2 oncoprotein, which constantly targets p53 for protein degradation. Nutlin-3 binds to the p53-binding pocket of MDM2 and interrupts the p53-MDM2 interaction. Therefore, Nutlin-3 can stabilize p53 without causing DNA damage. As expected, Nmnat2 isoforms were induced by Nutlin-3 in a p53-dependent manner (Figure 9-B). Taken together, these data suggested that Nmnat2 can be induced by endogenous p53 upon DNA damage and thus might be a yet identified p53 target gene.

3.2.5 p53 Directly Binds To Two Putative Responsive Elements Within Human Nmnat2 Gene

Data thus far suggested that p53 is capable of inducing the Nmnat2 gene. However, whether p53 directly regulates the transcription of the Nmnat2 gene remains unknown. As a transcription factor, p53 regulates the expression of target genes by direct binding to gene promoter regions. Ei-Deiry *et al*. first defined the consensus p53-binding sequence in 1992 [39]. Since then, researchers have gained considerable knowledge of the structure and composition of p53 responsive elements (RE) in the human genome and have developed multiple strategies to discover and validate novel genes regulated by p53 [6, 27]. A p53-binding consensus sequence consists of two 10-base half sites and a spacer

as follows: RRRCWWGYYY...n... RRRCWWGYYY (R for purine, Y for pyrimidine, W is an A or T and the spacer is 0–13). General approaches to identify and validate functional p53 REs include chromatin immunoprecipitation (ChIP) and transactivation of REs in response to p53 activation. The purpose of ChIP is to examine potential interaction of p53 protein with candidate REs in response to p53-activating agents. Analysis of the human Nmnat2 gene (PubMed *gene ID: 23057*), which is located on chromosome1-1q25, reveals the existence of a 114-kilobase intronic region that separates the transcription initiation sites of TV1 and TV2 (Figure 10-A). A list of candidate p53 REs was generated with the P53MH algorithm, a program for p53 RE prediction, using the first 150 kilobases of the gene as input [327]. The top ten REs from the list were tested for p53-binding by ChIP. Specific PCR primers spanning each target region were first validated by regular PCR using human genomic DNA as template. Each set of primers generated a single PCR product (data not shown). Chromatin fragments (~500 base pairs) associated with p53 protein were amplified with specific primers targeting each candidate p53RE in the Nmnat2 gene. PCR results demonstrated that two putative p53-binding sites (designated as BS#1 and BS#2) were co-immunoprecipitated with p53 protein. The association of p53 protein with BS#1 and BS#2 was enhanced by Act.D (Figure 10-B). In contrast, a control antibody could not put down these chromatin fragments (Figure 10-B). Interestingly, BS#1 and BS#2 are both located in the intronic region of Nmnat2 gene (Figure 10-A). This is not rare because p53 REs in intronic regions have been found in other p53 target genes such as Tigar [27, 185, 327]. ChIP assay where Nutlin-3 was used to induce endogenous p53 resulted in the same observation, again suggesting that p53 directly binds to BS#1 and BS#2 in cells (data not

shown). Taken together, these ChIP data supported the conclusion that p53 protein directly binds to the human Nmnat2 gene promoter.

3.2.6 Identified p53-binding Sites In Nmnat2 Gene Are Functional For Driving The Expression Of Firefly Luciferase Reporter Gene

To determine whether BS#1 and BS#2 are functional p53 REs, these two sequences were placed upstream of the firefly luciferase reporter gene in the context of a mini CMV promoter. As discussed above, p53 RE contains two half sites of RRR**C**WW**G**YYY, in which the fourth "C" and seventh "G" are conserved nucleotides and essential for p53-binding. To further validate these two p53 REs, C/G to A/T mutant BS#1 and BS#2 were also generated and used as controls (Figure 11-A). Each reporter construct was co-transfected with a control renilla luciferase gene driven by the HSV-TK viral promoter, into either normal or p53 knock-down U2OS cells (Figure 11-B). Cells were treated with Act.D or Nutlin-3 to activate endogenous p53. Firefly luciferase activities were measured 48 hours post addition of the indicated drugs and normalized to that of Renilla luciferase, using the dual luciferase reporter assay kit (Promega). Results demonstrated that both wild-type BS#1 and BS#2 activated the expression of firefly luciferase reporter gene in response to Act.D or Nutlin-3 (Figure 11-C). Importantly, the functionality of these sequences was dependent on endogenous p53 activities because the induction of firefly luciferase was drastically blocked by p53 knock-down (Figure 11-C). As expected, both mutant BS#1 and BS#2 failed to activate any firefly luciferase activity upon activation of p53, further supporting the conclusion that BS#1 and BS#2 are authentic p53 responsive elements (Figure 11-C).

In summary, results thus far support the conclusion that NAD^+ synthetase Nmnat2 is a direct target gene of p53. This finding gave rise to one important question:

what is the role of Nmnat2 in the p53-mediated DNA damage response? Or, to be more specific, is Nmnat2 involved in p53-induced apoptosis? This question is addressed in the next section.

3.2.7 Knocking Down Both Nmnat2 Isoforms Protects U2OS Cell From DNA Damage-Induced Cell Death

DNA damage of many types activates p53 to induce apoptosis. Therefore, one question to be asked was whether Nmnat2 is involved in this process. To address this question, expression of Nmnat2 in U2OS cells was knocked down through retrovirus-mediated RNAi. Three pre-designed shRNA sequences (Openbiosysems) were chosen for constructing SMP retroviral shRNA expressing vectors. These shRNA sequences were designed to target common regions of Nmnat2 mRNA isoforms, thereby theoretically knocking down both. The shRNA sequence giving the best efficiency of knock-down (Openbiosystems *ID#100027*) was used throughout this project. RT-qPCR results showed that both Nmnat2-TV1 and Nmnat2-TV2 were knocked down in U2OS cells (Figure 12-A). As discussed in section 3.2.2, Act.D can induce p53-dependent apoptosis in U2OS cells. Accordingly, U2OS cells with non-specific, Nmnat2-specific or p53-specific shRNA were treated with Act.D with concentrations of 5nM or 20nM for 48 hours then measured for apoptosis induction. While 20nM Act.D killed greater than 30% of control cells, Nmnat2 knock-down and p53 knock-down cells were relatively tolerant, with about 8% and 12% cell death, respectively (Figure 12-B). This result suggested a crucial role of Nmnat2 in p53-induced apoptosis. Moreover, knocking down Nmnat2 protects U2OS cells from Camptothecin-induced apoptosis (Figure 12-C), showing that the protective effect of Nmnat2 is not limited to Act.D.

3.2.8 p53 Regulates Cellular NAD^+ Synthesis Upon DNA Damage

A role of p53 in regulating cellular metabolism has been discovered recently. Through regulating target genes such as *Tigar*, *SCO2* and *G6PDH*, p53 affects glycolysis and mitochondrial oxidative phosphorylation. NAD^+ is a key factor that is not only required for generation of cellular NADH/NADP/NADPH, which play critical roles in energy metabolism and anti-oxidation, but also for activities of other key factors such as SIRTs and PARPs. The finding that NAD^+ synthetase Nmnat2 is involved in p53-induced apoptosis upon DNA damage led to the hypothesis that p53 might regulate SIRT and PARP activities upon DNA damage. To evaluate this hypothesis, one important question to be answered is: Do p53 and Nmnat2 regulate cellular NAD^+ homeostasis upon DNA damage?

Cellular NAD^+ was measured using a NAD^+/NADH quantification kit (Biovision) according to the manufacturer's protocol. Act.D treatment caused an up-regulation of cellular NAD^+ levels in control U2OS cells at 72 hours, with about 45% increase compared to that of non-treated cell (Figure 13-A). Contrary to cells with control shRNA, p53 knock-down cells showed no increase in cellular NAD^+ levels before and after Act.D treatment (Figure 13-A). It should be pointed out that cellular NAD^+ levels were compared at a relatively late time (72 hours). It was rationalized that cellular NAD^+ synthesis after DNA damage might be co-related with apoptosis induction, which culminates after 72 hours in response to Act.D treatment. Theoretically, cellular NAD^+ levels could be affected by all Nmnat proteins. It is possible that Nmnat1 and Nmnat3 could compensate the inhibition of Nmnat2 by RNAi. To determine whether this actually happens after DNA damage, Nmnat1 and Nmnat3 were knocked down in U2OS cells and

knock-down efficiency for each gene was confirmed by RT-qPCR (Figure 13-B). U2OS cells with individual gene knock-down (control, p53, Nmnat2, Nmnat1 and Nmnat3) were treated with Act.D or non-treated. For each cell line, cellular NAD^+ were measured at indicated time point (36 and 72 hours) and compared to non-treated control. Data are presented as relative changes to non-damaged cells, such that they reflect the capacity of each cell line to synthesize cellular NAD^+ upon DNA damage. In cells with control shRNA, Act.D caused about 40~50% increase of cellular NAD^+ levels as measured at 36 hours and it did not increase further as measured at 72 hours (Figure 13-C). p53 knock-down cells showed a comparable increase of NAD^+ levels to cells with control shRNA at 36 hours. However, p53 knock-down cells failed to replenish their cellular NAD^+ at 72 hours, resulting in a decrease of about 40% compared to that at 36 hours (Figure 13-C). Intriguingly, Nmnat2 knock-down cells failed to keep their cellular NAD^+ pool at 36 hours with a 40% decrease compared to non-damaged cells, suggesting that Nmnat2 is involved in cellular NAD^+ synthesis after DNA damage (Figure 13-C). Interestingly, Nmnat2 knock-down cells managed to replenish their cellular NAD^+ partially at 72 hours, to a levels still lower than that of non-damaged cells (Figure 13-C). Nmnat3 knock-down cells failed to replenish their NAD^+ at 36 hours (~20% decrease), suggesting that Nmnat3 is also involved cellular NAD^+ synthesis after DNA damage (Figure 13-C). However, at 72 hours, NAD^+ levels in Nmnat3 knock-down cells had recovered to a levels higher than that of non-damaged cells. Under specified conditions, Nmnat1 knock-down cells behaved in the same way as control cells did, suggesting that Nmnat1 is not responsible for cellular NAD^+ synthesis after DNA damage. Comprehensive analysis of these data led to a simplified picture of cellular NAD^+ synthesis after DNA damage:

DNA damage activates p53-mediated transactivation of Nmnat2 (TV1 and TV2); cellular proteins such as SIRT and/or PARP consume NAD^+ that is replenished by Nmnat2, 3 and possibly other unknown factors. If this is the case, the p53-Nmnat2 pathway might regulate activities of SIRT and PARP proteins, which in turn affect p53 posttranslational modifications. This possibility is investigated in the next section.

3.2.9 Nmnat2 Is Involved In The Regulation Of p53 Acetylation After DNA Damage

As discussed in the last section, p53 is involved in the regulation of cellular NAD^+ levels upon DNA damage, presumably through transactivation of Nmnat2. Several important questions brought up by this finding were: **a)** Are SIRT proteins regulated by the p53-Nmnat2 pathway? **b)** Is there a feedback regulatory loop between p53 and Nmnat2? **c)** Are PARP proteins regulated by the p53-Nmnat2 pathway? These proposed regulatory mechanisms are illustrated in Figure 14-A. SIRT proteins are class III HDACs that depend on NAD^+ for their functions. SIRT1 can deacetylate many cellular proteins such as histones and p53. It was rationalized that if the p53-Nmnat2 axis affects SIRT1 activity, it should also affect deacetylation of its substrates including p53 and histones. To test this possibility, acetylation of p53-K382, histone2A-K5 and histone3-K9 were monitored before and after DNA damage in control and Nmnat2 knock-down cells. Act.D treatment induced acetylation of p53-K382 that increased over the time of incubation (Figure 14-B, lane 1-4). A reverse trend was observed in Nmnat2 knock-down cells where acetylation of p53-K382 reached its highest levels at 24 hours after DNA damage then decreased over time (Figure 14-B, lane 5-8). A similar observation was made for acetylation of histone2A-K5, which was inhibited in Nmnat2 knock-down cells at 72 hours (Figure 14-B, lane 5-8). Unexpectedly, no change was observed for acetylation of

histone3-K9, which is thought to be a prominent marker for transcriptional activation (Figure 14-B). Altered acetylation patterns of p53-K382 and histone2A-K5 in Nmnat2 knock-down cells suggested that Nmnat2 is involved in the regulation of cellular protein acetylation or deacetylation. Importantly, based on this result, it can be deduced that Nmnat2 is required for acetylation of p53-K382 after DNA damage (72 hours). Therefore, Nmnat2 probably inhibits SIRT1, which deacetylates p53 at K382.

Protein levels of SIRT1 in control cells decreased over time after DNA damage, displaying a trend opposite to that of acetylation of p53-K382 (Figure 14-B, lane 1-4). In contrast, in Nmnat2 knock-down cells, SIRT1 protein levels increased over time, a trend reverse to that of p53-K382 acetylation (Figure 14-B, lane 5-8). This observation is consistent with the notion that SIRT1 negatively regulates acetylation of p53-K382. Careful examination of the results revealed that the SIRT1 protein levels in Nmnat2 knock-down cells was lower than that in control cell under normal condition, indicating that Nmnat2 might positively regulate SIRT1 protein levels (Figure 14-B, lane 1 and 5).

In summary, experiments based on Nmnat2 knock-down cells produced the following information: 1) Nmnat2 is involved in the regulation of p53-K382 acetylation upon DNA damage; 2) Nmnat2 regulates p53-K382 acetylation both positively and negatively in a time-dependent manner; 3) Nmnat2 regulates SIRT1 abundance. As discussed in Chapter one, acetylation plays a critical role in the activation of p53 transcriptional activity. Therefore, by regulating acetylation of p53-K382, Nmnat2 possibly regulates the expression of p53 downstream genes upon DNA damage.

3.2.10 Nmnat2 Affects The Expression Of A Subset Of p53 Target Genes After DNA Damage

In the previous section, it was suggested that Nmnat2 regulates p53-K382 acetylation upon DNA damage, presumably by modulating SIRT1 activity. It was reasoned that Nmnat2 could regulate the expression of p53 downstream genes upon DNA damage. Answers to this question would also help to address the hypothesis that the p53-Nmnat2 pathway functions in a feedback manner. A sub-set of p53 target genes were therefore investigated for their expression after Act.D treatment, in the context of Nmnat2 knock-down. As a master transcription factor, p53 can transactivate or repress hundreds of genes that function in multiple cellular pathways. Among the large collection of p53-regulated genes, $p21^{Waf1/Cip1}$, encoded by the cyclin-dependent kinase inhibitor 1A *(CDKN1A)* gene, plays an important role in G1/S cell-cycle arrest and premature senescence. p53 constitutively transactivates the *MDM2* gene, whose product negatively regulates stability of p53 protein and its transcriptional activity. Tigar is involved in cell metabolism, where it diverts cytoplasmic glycolysis toward pentose phosphate pathway (PPP). PIG3, also known as tumor protein p53 inducible protein 3 (TP53I3), is a putative quinone oxidoreductase and thought to be involved in p53-mediated cell death. Growth arrest and DNA-damage-inducible protein GADD45 alpha (GADD45A) responds to environmental stresses by mediating activation of the p38/JNK pathway. The above-mentioned genes are well-known targets of p53 that function in distinct cellular pathways. Their expression levels in Nmnat2 knock-down cells were monitored. RT-qPCR was performed with cDNAs synthesized from U2OS cells (control verse Nmnat2 knock-down) treated with Act.D or non-treated. To be consistent with previous experiments, 24-hour and 72-hour time-points were chosen. Distinct expression patterns

were observed for these p53 target genes in Nmnat2 knock-down cells. The expression of $p21^{Waf1/Cip1}$ and p53 was not affected by Nmnat2 knock-down (Figure 15). However, the expression of MDM2, Tigar and Gadd45a after Act.D treatment was dramatically changed due to Nmnat2 knock-down (Figure 15). At 24 hours, knocking down Nmnat2 impaired the induction of MDM2, Tigar and Gadd45a, implying that Nmnat2 **promotes** the transcription of these genes. Intriguingly, at 72 hour, knocking down Nmnat2 enhanced the expression of MDM2, Tigar and Gadd45a, suggesting that Nmnat2 **inhibits** the transcription of these genes. In contrast, knocking down Nmnat2 had weaker effects over the expression of Pig3 gene, although slight enhancement at both time points was seen (Figure 15). The fact that the expression of p21 and p53 was not affected by Nmnat2 knock-down did not support the explanation of a global change in cellular transcription caused by Nmnat2 inhibition (Figure 15).

This result clearly suggested that Nmnat2 is involved in the regulation of p53 target gene expression upon DNA damage. However, opposite conclusions can be drawn based on time-points after DNA damage and target genes being examined. Observations made earlier demonstrated that acetylation of p53-K382 in Nmnat2 knock-down cells fluctuated in a way opposite to that in control cells upon DNA damage (Figure 14-B, lane 2-4 and 6-8). Linking the pattern of p53-K382 acetylation during the time course to the expression profiles of MDM2, Tigar and Gadd45a, several lines of mechanisms can be proposed: **a)** Nmnat2 proteins might inhibit acetylation of p53-K382 at early times after DNA damage (24 hours) but be required for enhanced acetylation of p53-K382 at late times after DNA damage (72 hours); **b)** altered acetylation of p53-K382 might be

relevant to dysregulated expression of p53 downstream genes; **c)** Nmnat2 proteins regulate SIRT1 in two ways (positively and negatively).

The notion that human Nmnat 2 gene transcribes two transcript variants that are translated into two protein isoforms led to one tempting hypothesis: Nmnat2 isoforms exert distinct functions. If this is true, understanding of the above discrepancies becomes easier. In the next section, efforts is put into addressing this issue.

3.2.11 Ectopic Expression Of Nmnat2 Isoform 2 (TV2) Suppresses The Expression Of A Subset Of p53 Downstream Genes

The role of Nmnat2 in DNA damage-induced cellular responses was investigated earlier, resulting in promising but somehow contradictory findings. Nmnat2 is clearly involved in regulating cellular processes including NAD^+ regeneration after DNA damage, SIRT1-mediated deacetylation of p53-K382 and expression of some p53 target genes. On the other hand, it seems that Nmnat2 has dual mechanisms that act in opposition to regulate these processes. A key to delineate this mystery might lie in the idea that Nmnat2 isoforms (named as Nmnat2-TV1 and Nmnat2-TV2 hereafter) are functionally different. Previously designed shRNA sequences against Nmnat2 in fact target both isoforms. The requirement of Nmnat2 for acetylation of p53-K382 at 72 hours post Act.D treatment strongly suggested that at least one isoform of Nmnat2 functions as a SIRT1 inhibitor (Figure 14-B). On the other hand, as a NAD^+ synthetase, Nmnat2 is thought to promote the activity of NAD^+-dependent SIRT proteins. It was necessary and interesting to determine if Nmnat2 isoforms exert distinct regulatory functions. First effort was taken to individually clone these isoforms.

Nmnat2 isoforms were cloned into Murine Stem Cell Virus (MSCV) vector derived from Murine Embryonic Stem Cell Virus (MESV) and LN retroviral vector

(Clontech). Using retroviral vector for expressing Nmnat2 proteins confers the advantage of stable expression and these constructs can also be used for transient transfection where needed. To prepare for future experiments, Nmnat1 and Nmnat3 were also cloned into the same expression system. All these genes were cloned from U2OS cells and confirmed by DNA sequencing. Successful cloning of all Nmnat genes from U2OS cells also assured that this cell line contains wild-type Nmnat genes. For each Nmnat protein, a Hemagglutinin (HA) tag was added to carboxyl terminal end for convenient detection. To confirm protein expression, MSCV constructs for each gene were transfected into U2OS cells and whole cell extracts were used for detection of each protein using an anti-HA antibody. All plasmids expressed desired proteins at predicted molecular weights (32, 34 and 28 kD for Nmnat1, Nmnat2-TV1 and Nmnat3, respectively) (Figure 16-A). Nmnat2-TV1 and Nmnat2-TV2 protein contain their unique amino terminal sequences. Nmnat2-TV1 protein consists of 307 amino acids with the first 28 amino acids being exclusive. Nmnat2-TV2 protein consists of 302 amino acids with the first 23 amino acids being exclusive (Figure 16-B). Western blotting confirmed that protein bands recognized by anti-HA antibody were also recognized by an anti-Nmnat2 (C-terminal) antibody (Figure 16-C). However, this anti-Nmnat2 antibody (Abcam) failed to visualize any endogenous Nmnat2 isoforms, probably due to instability of these proteins [328] (data not shown).

U2OS cells were infected with retroviruses carrying the indicated genes (empty, Nmnat2-TV1 or Nmnat2-TV2) and selected with puromycin for one week. Cells with empty, Nmnat2-TV1-HA or Nmnat2-TV2-HA are designated hereafter as U2OS-V, U2OS-TV1 and U2OS-TV2, respectively. These cell lines were treated with Act.D for 24 hours, 72 hours or were left un-treated. Real-time qPCR was then conducted with cDNA

synthesized from cells harvested at indicated time-points. The same set of genes was monitored as in sub-section 3.2.10. Strikingly, over-expression of Nmnat2-TV2 inhibited the expression of these p53 target genes at 72 hours (Figure 17-A). However, p53 mRNA levels was not affected by Nmnat2-TV2 over-expression, suggesting that Nmnat2-TV2 over-expression did so without causing global transcription repression (Figure 17-A). As shown earlier, knocking down both Nmnat2 isoforms enhanced the expression of MDM2, Tigar and Gadd45a but not that of $p21^{Waf1/Cip1}$ (Figure 15). Surprisingly, over-expression of Nmnat2-TV2 also inhibited the expression of $p21^{Waf1/Cip1}$ gene at 72 hours (Figure 17-A), indicating that Nmnat2-TV2 is a potent inhibitor for at least these p53 target genes. In contrast, over-expression of Nmnat2-TV1 slightly enhanced the expression of the same genes that were inhibited by Nmnat2-TV2 (Figure 17-A). Exogenous expression of Nmnat2-TV1 and Nmnat2-TV2 proteins in these cells was confirmed by western blotting (Figure 17-B). This is the first evidence supporting the hypothesis that Nmnat-2-TV1 and Nmnat2-TV2 exert distinct functions.

3.2.12 Nmnat2-TV1 And Nmnat2-TV2 Oppositely Regulate p53 Acetylation After DNA Damage

Knocking down both Nmnat2 isoforms resulted in dysregulated acetylation of p53-K382 upon DNA damage (Figure 14-B). This could be explained by the hypothesis that Nmnat2-TV1 and Nmnat2-TV2 have opposite effects on p53-K382 acetylation after DNA damage. With cells over-expressing individual Nmnat2 isoform, it is possible to investigate the effect of each isoform on p53 acetylation. First, gross acetylation of p53 protein was examined in U2OS cells over-expressing Nmnat2-TV1, Nmnat2-TV2 or empty vector before and after DNA damage. To this end, p53 protein was immunoprecipitated from whole cell extract prepared from cells collected at indicated

time-points post DNA damage, then resolved with SDS-PAGE and transferred to PVDF membrane for western blotting. Anti-acetyl-lysine antibody was used to probe gross acetylation of p53 protein. Intriguingly, over-expression of individual Nmnat2 isoform clearly resulted in divergent alterations of p53 acetylation after DNA damage. In U2OS-V cells, Act.D induced p53 acetylation that increased over time (Figure 18-A, lane 1-3). In U2OS-TV1 cells, DNA damage could not induce p53 acetylation over time, suggesting that Nmnat2-**TV1** functions as an **inhibitor** of p53 acetylation (Figure 18-A, lane 4-5). conversely, over-expressing Nmnat2-TV2 enhanced total p53 acetylation after DNA damage, suggesting that Nmnat2-**TV2** functions as an **activator** of p53 acetylation (Figure 18-A, lane 6-8). In the absence of DNA damage, interestingly, both Nmnat2-TV1 and Nmnat2-TV2 had no effect on p53 acetylation, indicating that these proteins are also regulated upon DNA damage. An alternative explanation could be that Nmnat2 isoforms reversely regulate cellular factors (e.g. SIRT deacetylases) that modify p53 acetylation upon DNA damage.

3.2.13 Ectopic Expression Of Nmnat2-TV2 Promotes DNA Damage-Induced Acetylation Of p53 On Lysine 382 And Lysine 120 In A Time-Dependent Manner

Regarding the complexity of p53 acetylation that occurs at multiple lysine residues of the protein, it should be kept in mind that gross acetylation of p53 does not necessarily reflect the real situation of specific acetylation events (e.g. p53-K382). Therefore, it was important to determine if Nmnat2 isoforms also reversely regulate p53 acetylation on specific lysine residues (e.g. K120, K320, K373 and K382). These lysine residues were mentioned here because of the availability of specific antibodies against them. U2OS-V, U2OS-TV1 and U2OS-TV2 cells were treated with Act.D and harvested

24 hours and 72 hours later. The same procedure of immunoprecipitation and western blotting was followed as before. In U2OS-V cells, acetylation of p53-K382 increased over time but acetylation of p53-K120 remained unchanged (Figure 18-B, lane 1, 4 and 7). In U2OS-TV2 cells, acetylation of p53-K382 was enhanced at 24 hours but decreased to a levels comparable to that of U2OS-V cells at 72 hours (Figure 18-B, lane 3, 6 and 9). Interestingly, acetylation of p53-K120 was greatly enhanced by Nmnat2-TV2 over-expression at 72 hours (Figure 18-B, lane 3, 6 and 9). In U2OS-TV1 cells, acetylation of p53-K382, although activated after DNA damage, was less than that in U2OS-V cells (Figure 18-B, lane 1, 2, 4, 5, 7 and 8). Acetylation of p53-K120 in U2OS-TV1 cells was comparable with that of U2OS-V cells. Unfortunately, anti-acetyl-p53 K320 and anti-acetyl-p53-K373 antibodies could not visualize any bands (not shown).

In summary, these data supported the hypothesis that Nmnat2 isoforms exert distinct regulatory functions over p53. Nmnat2-TV2 functions as a promoter for p53 acetylation after DNA damage, whereas Nmnat2-TV1 functions as an inhibitor for p53 acetylation. Importantly, Nmnat2-TV2 specifically stimulated acetylation of p53-K382 at 24 hours and p53-K120 at 72 hours after DNA damage. The notion that Nmnat2 proteins are NAD^+ synthetases regulating cellular NAD^+ synthesis upon DNA damage indicated that Nmnat2 isoforms probably regulate p53 acetylation through modulating NAD^+-dependent SIRT activities.

3.2.14 NAD^+-Dependent Histone Deacetylase SIRT1 And SIRT2 Deacetylate p53 On Lysine 382 And Lysine 120 Respectively After DNA Damage

Nmnat2 protein (TV1) has been found to be localized in cytoplasmic Golgi apparatus and associated with plasma membrane via palmitoylation [292, 329]. Previous

studies carried out by other groups were based on Nmnat2-TV1 [292, 328-335], which differs from Nmnat2-TV2 in its amino terminal end. Nmnat2-TV2 protein contains a cluster of five glutamic acids in its N-terminal sequences (Figure 16-B). Interestingly, glutamic acid is the target of cellular poly APD-ribosylation, implying that Nmnat2-TV2 might be subject to different regulations from Nmnat2-TV1. Cellular NAD^+-dependent SIRT and PARP proteins are found in distinct sub-cellular compartments. Sub-cellular localization of Nmnat, SIRT and PARP proteins is described in Figure 19-A. SIRT1 and SIRT2 are found in both the cytoplasm and the nucleus, therefore aimed as potential targets for Nmnat2 isoforms. Other SIRT proteins are found exclusively in the nucleus or mitochondria, with SIRT6, 7 in the nucleus and SIRT3, 4, and 5 in mitochondria [243].

To determine which SIRT proteins are responsible for acetylation of p53-K382 and p53-K120 upon DNA damage, specific SIRT1 and SIRT2 inhibitors were first tested. U2OS cells were treated with Act.D or non-treated in the presence or absence of each indicated SIRT inhibitor. Western blotting was performed to probe acetylation of p53-K382 and p53-K120 under these experimental conditions. In the absence of Act.D, SIRT1 inhibitor (Calbiochem) had no effect on p53-K382 acetylation (Figure 19-B, lane 1 and 3). Act.D activated acetylation of p53-K382 but not p53-K120 (Figure 19-B, lane 1and 2). However, combined treatment of Act.D and SIRT1 inhibitor greatly enhanced p53-K382 but not p53-K120 acetylation in U2OS cells (Figure 19-B, lane 2 and 4). This clearly demonstrated that p53-K382 deacetylation upon DNA damage is modulated by SIRT1. In other words, an increase of p53-K382 acetylation upon DNA damage might be due to the inhibition of SIRT1. It was clear that SIRT1 is not the deacetylase for p53-K120, as inhibition of SIRT1 could not enhance acetylation of this site. Inhibition of

SIRT2, in contrast, leads to enhanced acetylation of p53-K120 but not p53-K382 after DNA damage (Figure 19-C). This suggested that p53-K120 deacetylation was modulated by SIRT2 after DNA damage. Taken together, these results demonstrated that p53 deacetylation on K382 and K120 are regulated by SIRT1 and SIRT2 upon DNA damage, respectively.

Potential off-target effect of chemical inhibitor is always a concern in studying protein functions. Although SIRT1 and SIRT2 inhibitors specifically enhanced p53 acetylation on K382 and K120, respectively, there were still chances that other SIRTs or pathways were altered. To rule out this possibility, SIRT1 and SIRT2 were individually knocked down by retrovirus-mediated RNAi in U2OS cells. First, the specificity of gene knock-down was confirmed by detection of endogenous SIRT1 and SIRT2 protein levels in each knock-down cell line (Figure 19-D, top two panels). Acetylation of p53-K382 after DNA damage was enhanced by knocking down SIRT1 but not SIRT2 (Figure 19-D, third panel from top). Conversely, it was SIRT2 knock-down but not SIRT1 knock-down that promoted p53-K120 acetylation (Figure 19-D, fourth panel from top). It is worth noting that in the absence of DNA damage, p53-K120 acetylation was enhanced by both SIRT2 inhibitor and SIRT2 knock-down (lane 1, 3 and 5 in Figure 19-C, lane 1, 3 and 5 in Figure 19-D). This might suggest an important role of SIRT2 in preventing p53-induced apoptosis, as p53-K120 acetylation is critical for apoptotic function of p53 [96, 336-338].

Over-expression of Nmnat2-TV2 protein enhanced p53 acetylation on K382 and K120 after DNA damage (Figure 18-B), effects also observed from the inhibition of SIRT1 and SIRT2 (Figure 19-B, C and D). This strongly suggested that Nmnat2-TV2

plays an important role in inhibiting SIRT1 and SIRT2 activities after DNA damage, resulting in activation of p53 by enhanced acetylation. This also supports the hypothesis that the p53-Nmnat2 pathway functions in a feedback manner, by which p53-mediated transactivation of Nmnat2-TV2 promotes p53 acetylation. Nmnat2-TV1 may function as a promoter for SIRT activities (Figure 18-A), resulting in inhibition of p53 acetylation after DNA damage.

3.2.15 p53 Regulates Cellular Poly ADP-Ribose Homeostasis

In addition to SIRT proteins, another family of NAD^+-dependent proteins involved in cellular DNA damage responses is poly (ADP-ribose) polymerase (PARP). PARP-1 is the major enzyme of this family responsible for cellular poly ADP-ribosylation [277, 283]. PARP-1 is hyper-sensitive to DNA breaks and other types of DNA lesions [284]. It has been reported that upon activation by DNA breaks, the enzymatic activity of PARP-1 increases up to 500-fold [284]. This rapidly exhausts the stock of cellular NAD^+, leading to energy crisis and cell death if a cells fails to replenish its NAD^+ pool. The relationship between SIRT-mediated deacetylation and PARP-mediated poly ADP-ribosylation is not fully understood. The fact that both families of protein require NAD^+ indicates that inter-regulatory mechanisms might exist to coordinate these two types of PTMs that may occur on the same target protein (e.g. p53 and chromatin-associated factors). A possible role of p53 in regulating cellular poly ADP-ribosylation was also proposed in Section 3.2.9. Several preliminary experiments were carried out and results are presented in this sub-section. PARP proteins catalyze the formation of ADP-ribose polymer (PAR or pADPr), which usually has chain lengths ranging from 2 to 300 ADP-riboses and contains approximately 2% branching in the

chain. PARPs also catalyze the attachment of PAR to target proteins including p53 and PARP itself. The half-life of PAR is very short, which can be less than one minute, due to rapid degradation of the polymer by poly (ADP-ribose) glycohydrolyase (PARG). Cellular PAR was detected by a specific antibody (anti-pADPr (10H), Santa Cruz) that does not cross-react with ADP-ribose, 5'-AMP. U2OS (p53 wild-type) and Saos-2 human osteosarcoma (p53 null) cells were first compared for PAR levels. Using cell extract made with regular lysis buffer (1% SDS), PAR was almost undetectable (Figure 20-A, lane 1). However, adding ADP-HPD to lysis buffer greatly preserved PAR signals in cell extract (Figure 20-A, lane 1 and 3). ADP-HPD is an amino analog of ADP-ribose that acts as a potent and specific inhibitor of PARG [339, 340]. This suggests that disruption of cellular structures during lysis released PARG activities that rapidly degraded PAR signals in cell extracts even under stringent condition (1% SDS). Accordingly, ADP-HPD was included in lysis buffer in the following experiments. Interestingly, the PAR levels in Saos-2 cells was much higher in comparison with U2OS cells (Figure 20-A). This result implied that cellular p53 status might be co-related with PAR homeostasis. Next, two methods were employed to inhibit p53 in U2OS cells: ectopic expression of human papillomavirus (HPV) E6 oncoprotein and RNAi. HPV-E6 protein activates host E6-associated protein, which has ubiquitin ligase activity and acts to ubiquitinate p53, leading to its proteasomal degradation [341]. E6-mediated degradation of p53 resulted in elevation of cellular PAR levels in U2OS cells (Figure , 20-B). Similarly, knocking down p53 in U2OS cells caused an increase of cellular PAR levels, thereby ruling out the possibility of inactivation of PARG by HPV-E6 (Figure 20-C). In addition, exogenous expression of p53 in Saos-2 cells down-regulated cellular PAR levels (Figure 20-D).

Taken together, these results suggest that p53 negatively regulates cellular poly ADP-ribosylation.

Further experiments are needed to investigate more detailed mechanisms. However, regarding the newly discovered role of p53 in regulating NAD^+ metabolism, it is possible that p53 regulates cellular poly ADP ribosylation through the p53-Nmnat2 pathway. Therefore, these results are included in this chapter.

3.3 DISCUSSION

3.3.1 Nmnat2 Is A Direct Target Gene Of p53

In an effort to investigate the potential link between p53 and cellular NAD^+ synthesis, Nmnat2 was identified as a novel p53 target gene. To validate the finding, a series of experiments were designed and performed. First, with a inducible p53 expression system, mRNA levels of key NAD^+ synthetases including Nmnat1, Nmnat2, Nmnat3 and Nampt were monitored and compared before and after induction of p53 in H1299 human lung cancer cell line (p53 null). Induction of p53 clearly up-regulated the mRNA levels of Nmnat2 but had minimal effects on Nmnat1, Nmnat3 and Nampt (Figure 8-A and B). The role of p53 in regulating Nmnat2 expression was further supported by the observation that DNA-damaging agents Actinomycin D and Adriamycin both induced the expression of Nmnat2 in a p53-dependent manner (Figure 9-C). Furthermore, Nmnat2 was also activated by Nutlin-3, a stabilizer of endogenous p53 (Figure 9-C). Although initially PCR primers were designed to examine both Nmnat2 transcripts (TV1 and TV2), it was confirmed later that p53 is involved in the induction of both isoforms in response to DNA damage and Nutlin-3 treatment, with re-designed

isoform-specific PCR primers. Increases in mRNA levels are often due to enhanced transcription. However, other possible mechanisms were also considered. Like protein stability is regulated by ubiquitination-proteasomal pathway, mRNA turnover is regulated by a combination of *cis*-regulatory sequences on the RNA and *trans*-acting RNA-binding proteins [342]. Poly(A) tail shortening of messenger RNA could cause destabilization of mRNA cap-binding complex and exosome-mediated degradation. It has been reported that p53 induces Wig-1, which binds to AU-rich elements in 3-UTR region of p53 messenger and stabilize it [343, 344]. So it still remains open whether Nmnat2 mRNA is subject to p53-mediated stabilization. Experiments conducted later demonstrated that p53 directly binds to intronic region of the human Nmnat2 gene (Figure 10-B). Interestingly, p53-binding sites identified (BS#1 and BS#2) are located downstream of transcription initiation site of Nmnat2-TV1 but upstream of Nmnat2-TV2 (Figure 10-A). When placed upstream of a firefly luciferase reporter, BS#1 and BS#2 demonstrated different activities in response to p53 activation. It was noticed that BS#2 is a more potent responsive element for p53 than BS#1 (Figure 11-C). Under the same condition, BS#2 induced an approximate 75-fold increase of luciferase activity, whereas BS#1 only induced an approximate 20-fold increase (Figure 11-C). Also, it is interesting to point out that induction of Nmnat2-TV1 mRNA reached its highest levels (5 to 6-fold) at 72 hours after Act.D treatment, whereas Nmnat2-TV2 mRNA was induced greater than 20-fold (Figure 9-B). In contrast, Adriamycin and Nutlin-3 are better inducers for Nmnat2-TV1 over TV2 (Figure 9-C). Adriamycin induced the highest expression of Nmnat2-TV1 and Nmnat2-TV2, to approximately 7-fold and 3-fold at 24 hours, respectively (Figure 9-C). Nutlin-3 induced the expression of Nmnat2-TV1 and Nmnat2-TV2 approximate 9-fold and 5-fold

at 24 hours, respectively (Figure 9-C). Interestingly, both Adriamycin and Nutlin-3, under specified settings, induced cell-cycle arrest but not cell death (Figure 6 and data not shown). In contrast, Actinomycin D exclusively induced cell death in U2OS cells and was a better inducer for Nmnat2-TV2 over TV1 (Figure 7). Taken together, it is speculated that p53 BS#1 and BS#2 might correspond to individual Nmnat2 isoform. Based on their efficiency in activating firefly luciferase reporter and expression patterns of TV1 and TV2 in response to distinct drugs, it is proposed that BS#1 is the p53 RE for transactivation of TV1 and BS#2 is responsible for transactivation of TV2. If this is true, binding of p53 to BS#1 and BS#2 should be differently regulated upon DNA damage. These possibilities require further investigations. Based on current knowledge of p53 REs, the fourth cytosine and seventh guanine nucleotide in p53-binding half-site are essential for its functionality. It was predicted that mutations at these two residues should inactivate them. Indeed, both mutant BS#1 and mutant BS#2 failed to induce any firefly luciferase activities in response to p53 activation (Figure 11-C). Knocking down p53 drastically inhibited effects of BS#1 and BS#2 on the induction of firefly luciferase, clearly suggesting that functionality of both BS#1 and BS#2 is p53-dependent. In conclusion, Nmnat2 gene is a direct target gene of p53-mediated transcription.

3.3.2 The p53-Nmnat2 Pathway Functions In A Feedback Manner To Regulate p53

The scope of p53 functions keeps expanding due to the still growing list of its target genes [1, 71, 345]. The finding of NAD^+ synthetase Nmnat2 being a p53-regulated gene adds a new dimension to the complicated network of p53-mediated regulation. To understand the role of Nmnat2 in p53-mediated cellular DNA damage responses, Nmnat2 gene expression was inhibited with RNAi. Alignment of messenger RNAs of Nmnat2-

TV1 and Nmnat2-TV2 showed that they share most sequences except 5 ends, with about 400 and 200 bases exclusive to TV1 and TV2, respectively. Therefore, earlier studies were carried out with RNAi targeting the common regions of TV1 and TV2 mRNA, in either protein coding region or 3' untranslated region (3-UTR). Double knock-down (against both transcripts) of Nmnat2 produced several lines of evidence clearly suggesting roles of Nmnat2 in the regulation of p53 functions upon DNA damage. First, Nmnat2 knock-down U2OS cells were resistant to DNA damage-induced cell death (Figure 12-B and C). This observation established that Nmnat2 is indeed involved in p53-mediated DNA damage responses. Mechanisms underlying this phenotype could be complex, covering altered cellular NAD^+ synthesis and regulation of p53 activation.

Regarding the nature of Nmnat2 being a NAD^+ synthetase, it was logical to ask whether p53 regulates NAD^+ homeostasis. Recently, NAD^+-dependent histone deacetylase SIRT and poly (ADP-ribose) polymerase PARP drew large attentions due to their important regulatory roles in various cellular processes, especially DNA repair and maintenance of genomic integrity. DNA damage could stimulate cellular demand for NAD^+ to maintain and/or activate the activity of both SIRT and PARP proteins. Interestingly, p53 knock-down cells were capable of maintaining their NAD^+ pool at a relative early time-point (36 hours) after DNA damage. However, at a later time- point (72 hours), p53 knock-down cells could not replenish NAD^+ as control cells did, with approximate 45% decrease (Figure 13-A and C). It is possible that there was either accelerated cellular NAD^+ consumption or inhibited NAD^+ regeneration at later time-point after DNA damage. Knocking down Nmnat2 greatly impaired the capacity of cells to replenish cellular NAD^+ after DNA damage, suggesting that Nmnat2 was responsible

for DNA damage-induced NAD^+ synthesis (Figure 13-C). The fact that Nmnat3 was also involved in this process could be explained by the mitochondrial localization of this NAD^+ synthetase. It is argued that NAD^+ cannot freely diffuse across the mitochondrial outer membrane [247, 268, 270]. Therefore, SIRT and PARP proteins in mitochondria are likely regulated by local NAD^+ synthesis, catalyzed by Nmnat3. It is worth noting that these experiments measured the gross amount of cellular free NAD^+, and thus may not reflect local NAD^+ levels in each sub-cellular compartment. Strikingly, Nmnat1 knock-down cells were capable of maintaining their normal NAD^+ homeostasis, indicating that this nuclear NAD^+ synthetase is not involved in NAD^+ regeneration or consumption after DNA damage. If Nmnat2 is induced by p53 after DNA damage and plays a major role in regulating cellular NAD^+ pools, one might ask why p53 knock-down cells were still capable of replenishing cellular NAD^+ pools, with partially impaired capacity. One possible explanation could be: although inhibition of Nmnat2 in both p53 and Nmnat2 knock-down cells results in down-regulated NAD^+ regeneration after DNA damage, rates of NAD^+ consumption in these cells could be different. For example, p53 may affect cellular NAD^+ consumption by modulating glycolysis (through Tigar and SCO2) and cellular anti-oxidation (through GPX1). Nonetheless, the results suggested that Nmnat2 plays an important role in modulating cellular NAD^+ synthesis after DNA damage. By regulating Nmnat2, p53 might also affect cellular NAD^+ homeostasis, albeit underlying mechanisms are more complicated than expected.

A potential role of Nmnat2 in regulating cellular SIRT activities was proposed based on the finding that Nmnat2 regulates NAD^+ synthesis after DNA damage. To investigate whether SIRT activities are affected by Nmnat2, one simple way is to

examine acetylation status of protein substrates of SIRT in the context of Nmnat2 knock-down. SIRT1, a homolog to yeast Sir2, is the best studied SIRT and deacetylates p53-K382 both *in vitro* and *in vivo*. Therefore, acetylation of p53-K382 was used as readout for cellular SIRT1 activity. Acetylation of p53-K382 after DNA damage was altered in an unexpected manner in Nmnat2 knock-down U2OS cells. It was reasoned that knocking down the NAD^+ synthetase Nmnat2 would result in down-regulation of cellular SIRT1 activity, and consequently up-regulated p53-K382 acetylation. This indeed happened at 24 hours (Figure 14-B, lane 2 and 6). However, at 72 hours, p53-K382 acetylation was suppressed in Nmnat2 knock-down cells, indicating an up-regulation of cellular SIRT1 activity. It was also noticed that SIRT1 protein levels changed in a way inverse to that of p53-K382 acetylation after DNA damage. Interestingly, Nmnat2 knock-down down-regulated SIRT1 protein levels in the absence of DNA damage (Figure 14-B, lane 1 and 4 in SIRT1 panel). However, after DNA damage, SIRT1 protein levels was increased in Nmnat2 knock-down cells but inhibited in control cells, suggesting that Nmnat2 inhibits SIRT1 after DNA damage (Figure 14-B, lane 1-4 in SIRT1 panel). It was possible that SIRT1 is regulated by Nmnat2 proteins depending on cellular context. It has been reported that Nmnat1 in mice acts as a chaperon in a manner similar to heat-shock protein 70 (Hsp70) to protect against neurodegeneration [346, 347]. It was speculated that Nmnat2 might regulate SIRT1 protein stability in a similar way that Nmnat1 does [346]. Future investigations are needed to test this possibility. It was reported that SIRT1 can be regulated by p53, positively and negatively [348, 349]. Whether Nmnat2 is involved in these processes is open to test. Taken together the results clearly demonstrated that Nmnat2 can regulate acetylation of p53-K382 after DNA damage, possibly through

modulating cellular SIRT1 activity. More importantly, this is the first indication that Nmnat2 might be a SIRT1 inhibitor.

Based on the finding that Nmnat2 functions to regulate p53 acetylation, which plays a critical role the regulation of p53 functions [53, 96, 99, 100], the p53-Nmnat2 feedback regulatory loop was hypothesized. In the proposed model, p53 transactivates the Nmnat2 gene, and Nmnat2-mediated regulation of cellular SIRT1 in turn modifies p53 acetylation status and consequently its transcriptional activity toward target genes. Indeed, the expression of a sub-set of p53 target genes was altered in Nmnat2 knock-down U2OS cells. Nmnat2 was required for the expression of a subset of p53 target genes (Tigar, MDM2 and Gadd45a) at 24 hours. However, Nmnat2 inhibited the expression of the same genes at 72 hours (Figure 15). Why does one gene exert opposite regulatory functions? Considering that two isoforms of Nmnat2 are expressed from the human Nmnat2 gene, it was speculated that these two isoforms of Nmnat2 might exert distinct functions.

Nmnat2 isoforms were successfully cloned from U2OS cells and DNA sequencing confirmed that they are expressed in wild-type. Strikingly, ectopic expression of individual Nmnat2 isoforms affected p53 acetylation oppositely. Gross acetylation of p53 was inhibited by ectopic expression of Nmnat2-TV1 isoform after DNA damage, supporting the role of Nmnat2-TV1 in promoting cellular SIRT activities, hence inhibiting p53 acetylation (Figure 18-A). In contrast, ectopic expression of Nmnat2-TV2 enhanced gross acetylation of p53, supporting the role of Nmnat2-TV2 in inhibiting cellular SIRT activities hence promoting p53 acetylation (Figure 18-A). This result unequivocally supported the hypothesis that Nmnat2 isoforms function differently in

regulating p53 acetylation. Further, the effects of Nmnat2 isoforms on p53 acetylation at specific sites were examined. Actinomycin D induced acetylation of p53 on K120 and K382 but not on K320 or K373. Ectopic expression of Nmnat2-TV2 clearly enhanced acetylation of p53-K382 and p53-K120, in a time-dependent manner (Figure 18-B, lane 6 and 9). This suggested that Nmnat2-TV2 is an activator for p53 acetylation at K382 and K120 after DNA damage. The effect of ectopic expression of Nmnat2-TV1 on p53-K382 and K120 was not as significant as that of Nmnat2-TV2, despite inhibition of K382 acetylation seen at 72 hours (Figure 18-B, lane 6 and 7). It is possible that Nmnat2-TV1 targets p53 acetylation sites other than K382 and K120, as it obviously inhibited the gross acetylation of p53 after DNA damage (Figure 18-A, lane 4-6). Most importantly, the novel finding that Nmnat2-TV2 acts as an activator for p53 acetylation suggested that p53 might be able to facilitate its own activity by inducing Nmnat2-TV2 expression, forming a positive feedback regulation. On the other hand, through Nmnat2-TV1, p53 inhibits its own acetylation, forming a negative feedback regulation that probably acts to prevent over-activation of itself. The signaling intermediates in this model were proposed to be cellular SIRT proteins. Therefore, it was important to determine if SIRT proteins are involved in the regulation of p53 acetylation after DNA damage, specifically on K382 and K120.

To identify specific SIRT proteins targeted by each Nmnat2 isoform, it was taken into account that SIRT proteins are localized into distinct cellular compartments [243]. Regarding the cytoplasmic localization of Nmnat2, SIRT1 and SIRT2 were speculated to be the SIRTs regulated by Nmnat2 isoforms, because SIRT1 and SIRT2 undergo nucleocytoplasmic shuttling [350-352]. However, the roles of SIRT6 and SIRT7,

which are found exclusively in the nucleus, were also considered and will be investigated in future [253, 272]. Inhibition of endogenous SIRT1 and SIRT2 was accomplished by using specific inhibitors or RNAi. Both methods resulted in similar observations. Inhibition of SIRT1 significantly enhanced p53-K382 acetylation after DNA damage, similar to the effect of Nmnat2-TV2 over-expression at 24 hours. The fact that inhibition of SIRT1 had no effect on p53-K120 acetylation suggested that SIRT1 is not responsible for inhibiting acetylation of K120 after DNA damage. On the other hand, inhibition of SIRT2 stimulated p53 acetylation on K120 but not K382, suggesting that SIRT2 is responsible for inhibiting acetylation of K120 after DNA damage. Importantly, enhancement of p53-K120 acetylation was also caused by Nmnat2-TV2 over-expression, suggesting that Nmnat2-TV2 inhibits SIRT2 after DNA damage. Important information retrieved from these observations also included that p53 acetylation is subject to negative regulations by distinct SIRT proteins after DNA damage. It is logical to propose that a full activation of p53's apoptotic function requires removal of these inhibitory effects posed by cellular SIRT1, SIRT2 and possibly other SIRTs. One way to do so is to induce Nmnat2-TV2, a potential SIRT inhibitor. A simplified model is illustrated in Figure 21.

3.3.3 Activation Of p53 By Counteracting Inhibition

Activation of the tumor suppressor p53 is a topic under extensive study. There are still many gaps in our understanding toward how this protein is regulated during cellular stress responses [1, 36, 353]. One consensus is that protein posttranslational modifications are central to p53 activation [34, 36, 73]. Hot-spot mutations of the p53 gene in human cancers have been found within its DNA-binding domain, suggesting that transcriptional activity of p53 may mostly account for its tumor suppressive functions[17,

23]. Among the many functions of p53, induction of apoptosis is considered as the essential ability inherited from its ancestor and indispensible for tumor suppression [2, 21]. Therefore, p53 activity has to be tightly controlled in order to protect cells from unwanted cell death. Cells do this by at least two mechanisms. First, p53 protein is kept at low levels under normal conditions, where ubiquitin ligases such as MDM2 constantly target it for degradation. Second, p53 protein is kept in inactive state, which inhibits its DNA-binding affinity toward promoters of distinct target genes that mediate diverse cellular pathways. A simplified model of two levels of inhibition on p53 is illustrated in Figure 22.

A p53 response could be prompt. For example, upon DNA damage, p53 can be stabilized within a few minutes. DNA damage responsive protein kinases such as ATM/ATR/DNA-PK sense the presence of DNA lesions and phosphorylate p53 within amino terminal transactivation domain (TAD), leading to release of p53 from its negative regulators such as MDM2 and stabilization of the protein [354-356]. The intensity of signaling is mostly determined by the strength of stimuli. Constant and strong DNA damage signals fuel upstream pathways that eventually lead to activation of p53's apoptotic function and elimination of the cell. In the real world, DNA lesions could be generated either by environmental factors or intrinsically from cellular processes such as DNA replication and chromosome rearrangement. Under physiological conditions, cells manage to fix most lesions without inducing apoptosis. In other words, p53 might not accumulate to the extent as that induced by DNA-damaging agents in *in vitro* experiments. Therefore, p53 activity *in vivo* might rely on its activation through conformational changes, determined by a combination of PTMs such as acetylation.

Activation of p53 can be considered as counteraction against inhibition. For example, as discussed earlier in this section, p53 stabilization is an outcome of release of the protein from negative regulation of MDM2 and other factors such as ARF-BP1/Mule [357, 358]. Similarly, p53 is subject to de-acetylation catalyzed by HDACs, a large collection of proteins that constitutively remove acetyl moieties from a vast array of cellular proteins including p53 [359-364]. To become active to induce apoptosis, p53 protein has to be liberated from the multi-layers of inhibition. I speculated that in addition to DNA damage–induced protein stabilization (protein quantity), "PTMs switching" also plays an important role in the activation of p53 (protein quality). "PTMs switching" involves a series of modification/de-modification events. For example, p53 is regulated by a large array of protein kinases that relay signals from diverse pathways to p53 [365-367]. On the other hand, phosphatases such as serine/threonine phosphatase-1(PP-1) and PTEN can dephosphorylate p53 [368,369,370,371]. Ubiquitination of p53 plays an important role in the regulation of p53 protein stability and transcriptional activity, catalyzed by ubiquitin ligases such as MDM2, Pirh2 and ARF-BP1/Mule [357, 372-375]. Herpes virus-associated ubiquitin-specific protease (HAUSP), also known as ubiquitin specific peptidase 7 (USP7), de-ubiquitinates and stabilizes p53, leading to enhanced cell growth arrest and apoptosis [376-380]. In addition, p53 protein is subject to acetylation and deacetylation, catalyzed by a large group of lysine acetyltransferases (KATs or HATs) and HDACs (e.g. SIRTs) [381-384]. It is therefore appealing to propose that at any moment during a DNA damage response, p53 proteins are heterogeneous regarding PTM configuration of each molecule [385, 386]. In other words, some p53 sub-populations are modified or localized differently from others. To be simple, p53 proteins

can be artificially classified as pro-apoptotic or pro-survival in a cell. Shifting character from pro-survival to pro-apoptotic requires PTM changes of the protein. For example, acetylation of p53-K382 and other lysines within the C-terminus facilitates conformational changes of p53, resulting in exposure of its DNA-binding domain that was masked by the C-terminus [387-390]. Therefore, p53-K382 acetylation might be a pre-requisite for acetylation of lysines within the DNA-binding domain such as K120 and K164. However, it is equally possible that acetylation on some sites is inhibitory to others, although there is lack of evidence to date. As for p53 acetylation upon DNA damage, it was shown that acetylation of p53-K382 and p53-K120 is inhibited by SIRT1 and SIRT2, respectively, after DNA damage (Figure 19). This supports the notion that p53 is negatively regulated after DNA damage. Also, inhibition of SIRT2 in U2OS cells caused massive cell death, indicating that removal of negative regulation is necessary for full activation of p53's apoptotic function (data not shown). Work in this thesis provides evidence for how p53 is activated through inhibiting cellular SIRT activities. Significantly, p53 can do so by inducing the Nmnat2 gene that expresses two functionally distinct protein isoforms. In the presence of excess an amount of Nmnat2-TV1, a putative SIRT activator, acetylation of p53 was inhibited after DNA damage (Figure 18-A, lane 1-3 and 4-6). However, in the presence of excess an amount of Nmnat2-TV2, a potential SIRT inhibitor, acetylation of p53 was enhanced (Figure 18-A, lane 1-3 and 7-9). I predict that acetylation of p53 on other sites is regulated similarly. A thorough examination of p53 acetylation status after DNA damage by proteomics should identify more lysines that are inhibited by cellular SIRT proteins but required for activation of p53 apoptotic functions. The same prediction might be applied to other types of PTMs

such as methylation and ADP-ribosylation, which are also subject to extensive inhibitions posed by histone lysine-specific demethylases such as LSD1 and PARG, respectively [391, 392]. Interestingly, according to results presented in this thesis, p53 is also involved in the regulation of cellular poly ADP-ribose (PAR) homeostasis (Figure 20). Although significance and mechanisms are currently unknown, this finding may lead to new research directions related to p53 activation and other cellular pathways such as DNA repair.

3.4 FIGURE S AND LEGENDS

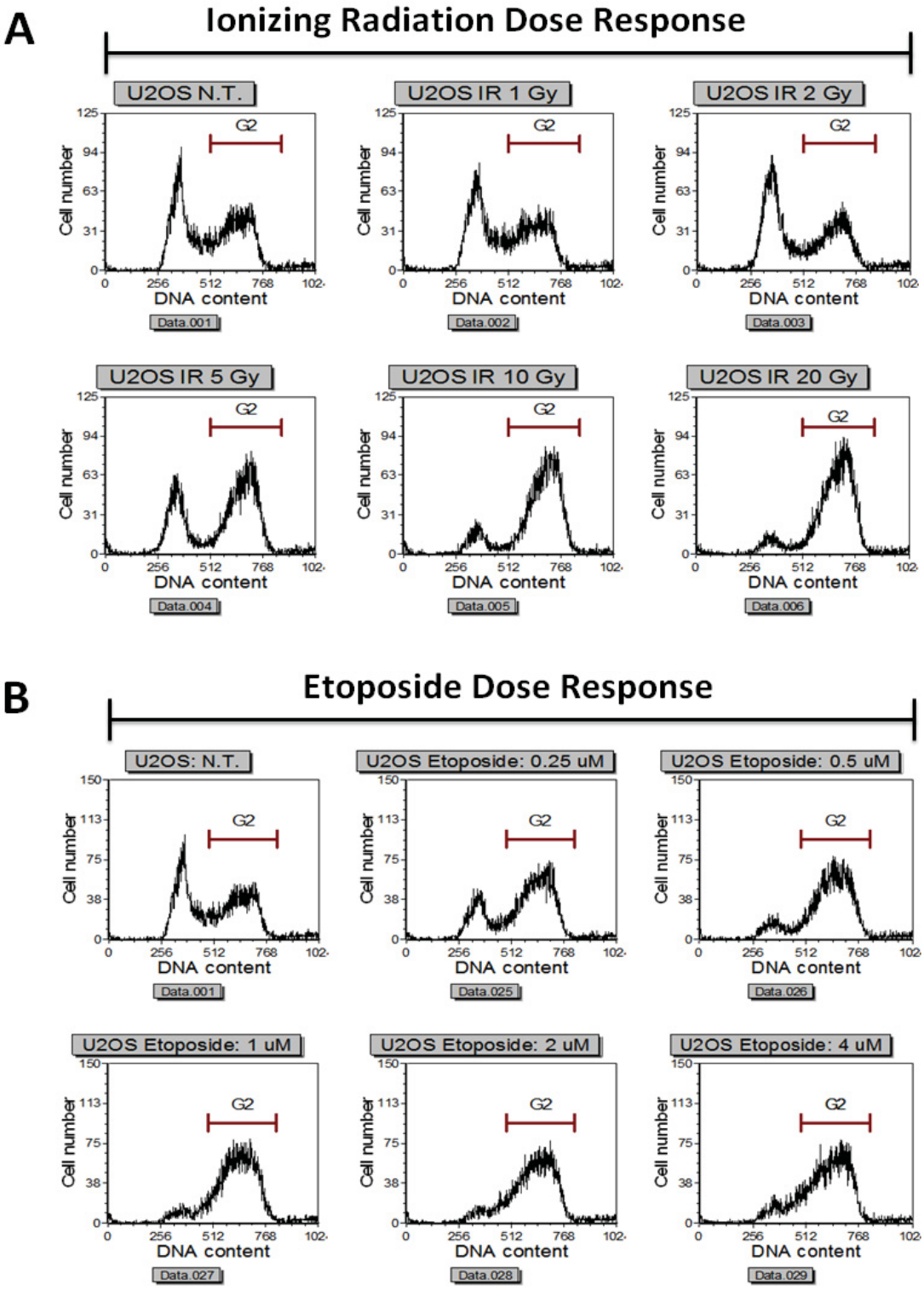


Figure 6. The type and dose of DNA damage has profound impacts on cell fate.

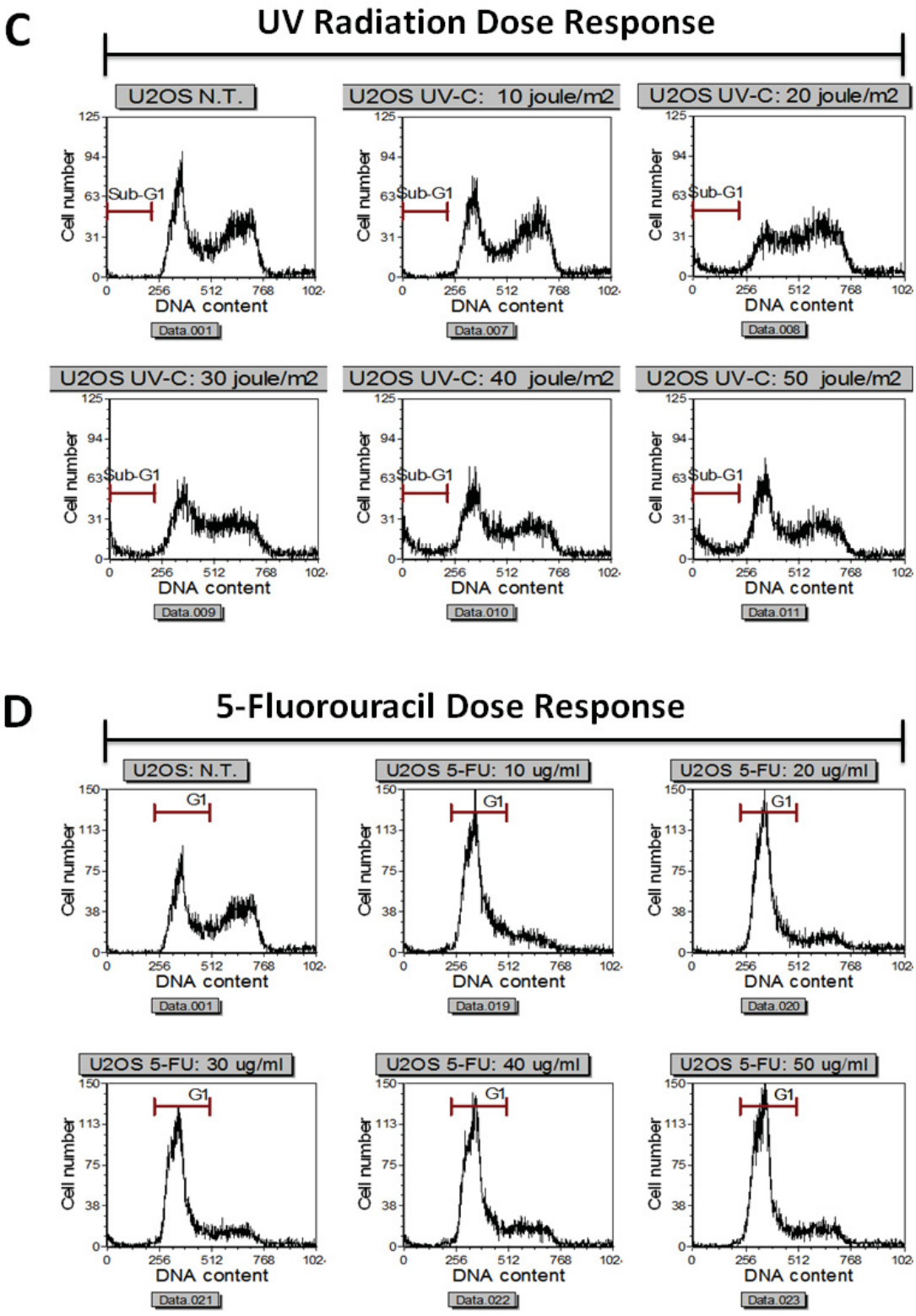


Figure 6. The type and dose of DNA damage has profound impacts on cell fate.

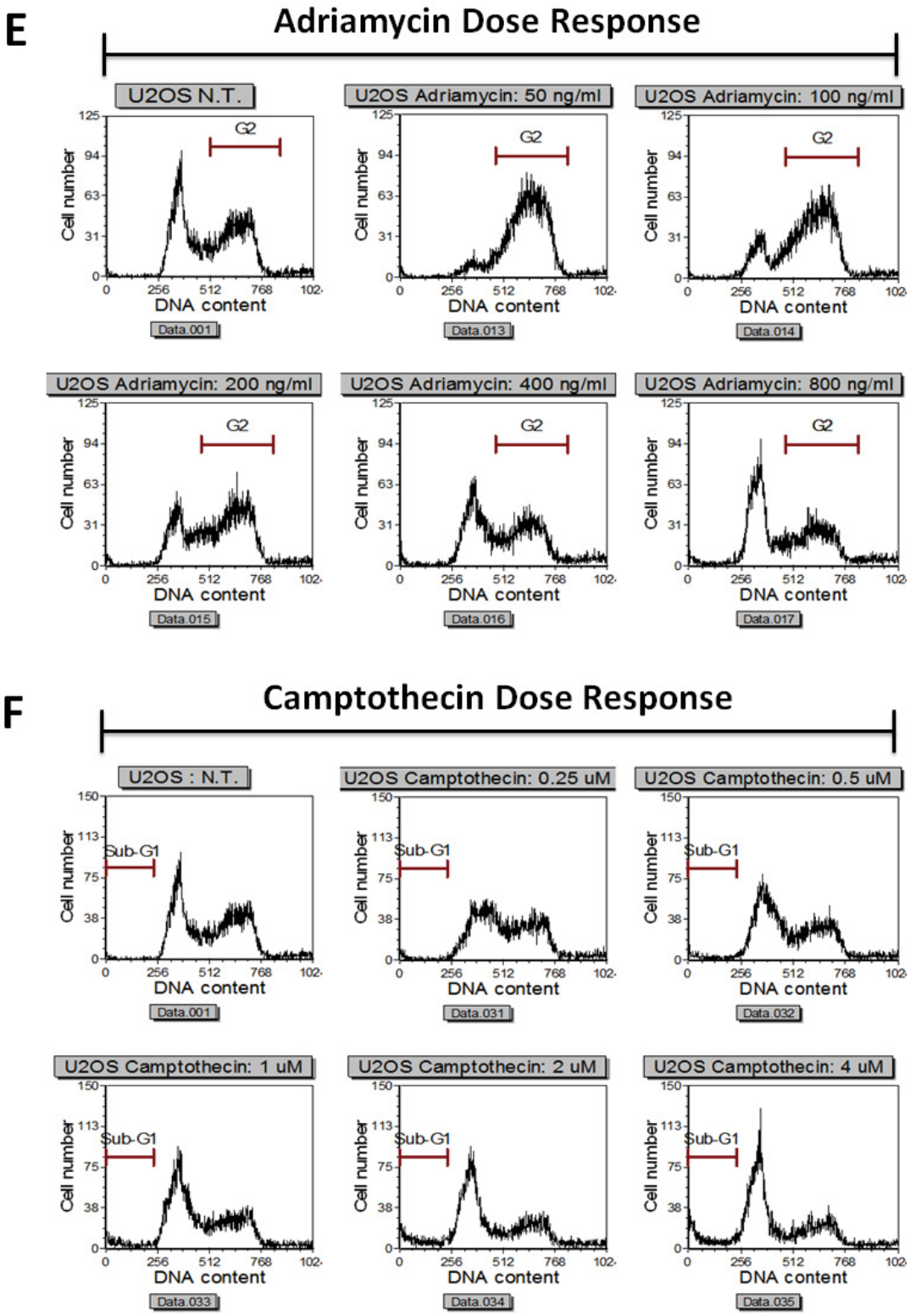


Figure 6. The type and dose of DNA damage has profound impacts on cell fate. U2OS

cells were treated with increasing doses of DNA-damaging agents or non-treated, then harvested 24 hours after treatment for cellular DNA content analysis. Cellular responses to each DNA-damaging agent are shown (red marker). Doses of each DNA-damaging agent used: **(A)** Ionizing radiation: zero, 1, 2, 5, 10 and 20 grays. **(B)** Etoposide concentrations: zero, 0.25, 0.5, 1, 2 and 4 μM. **(C)** UV-C (wavelength 254 nm): zero, 10, 20, 30, 40 and 50 joule/m^2. **(D)** 5-Fluorouracil: zero, 10, 20, 30, 40 and 50 μg/ml. **(E)** Adriamycin: zero, 50, 100, 200, 400 and 800 ng/ml. **(F)** Camptothecin: zero, 0.25, 0.5, 1, 2 and 4 μM. Harvested cells were fixed with 70% ethanol and then treated with RNase A (20 μg/ml) to degrade cellular RNA. Then, cells were stained with propidium iodide (50 μg/ml) and analyzed by FACS using FL-2A. Results are representative of three independent experiments.

A

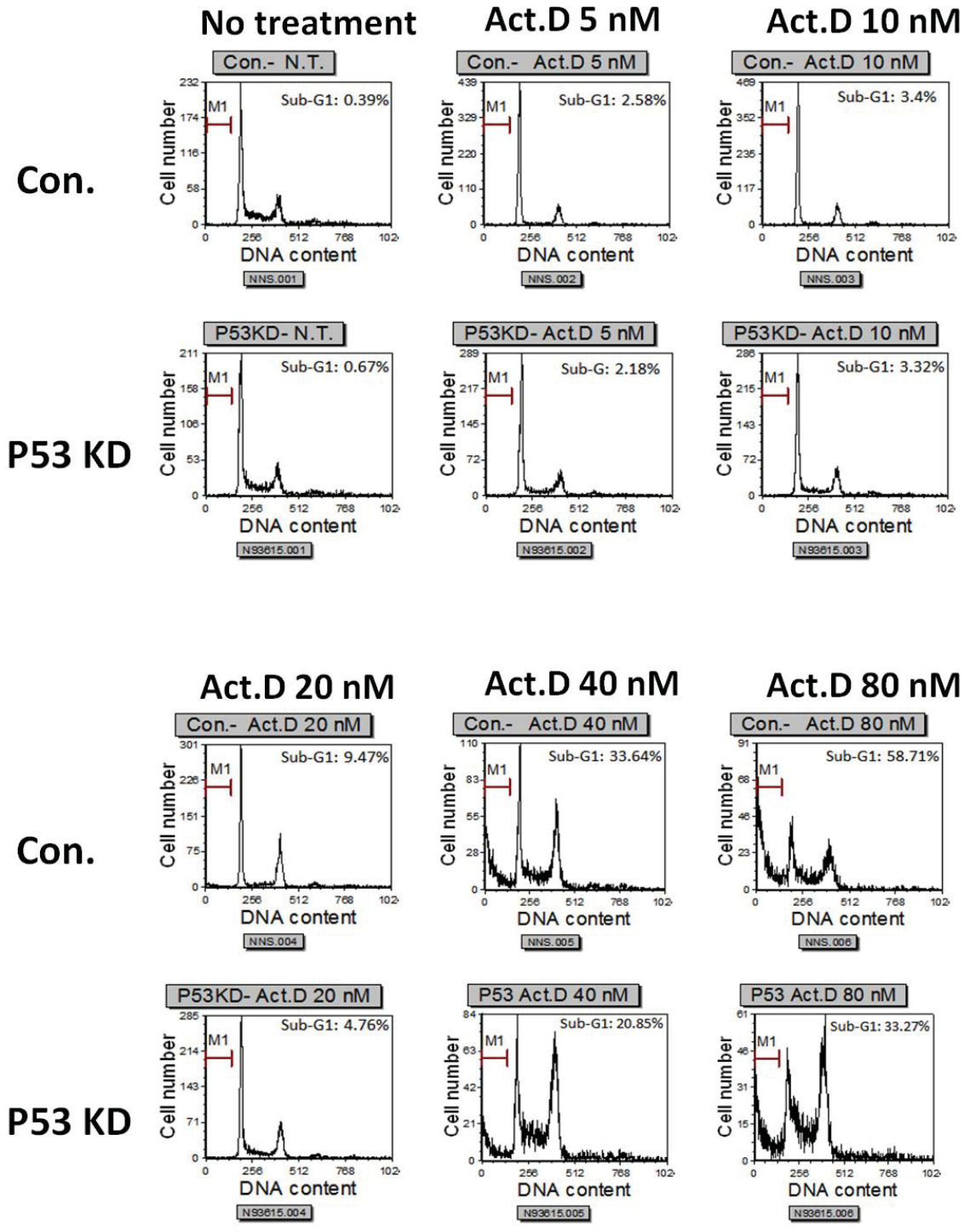
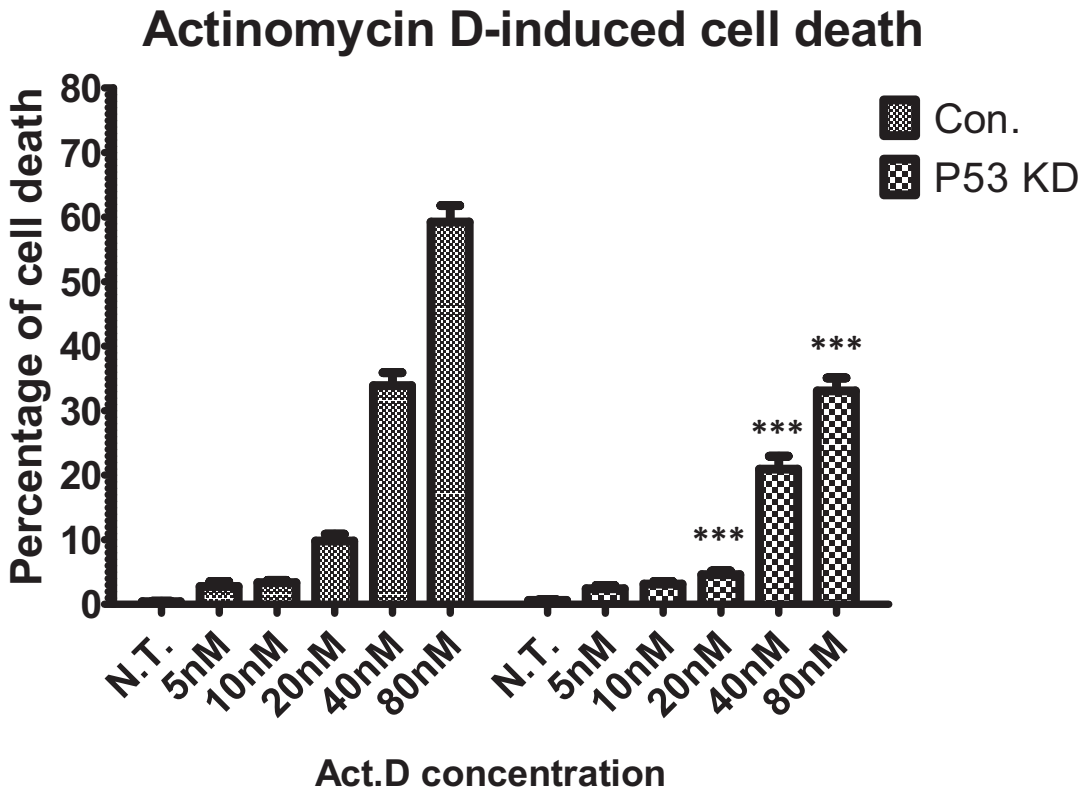


Figure 7. Actinomycin D induces p53-dependent cell death in U2OS cell.

B



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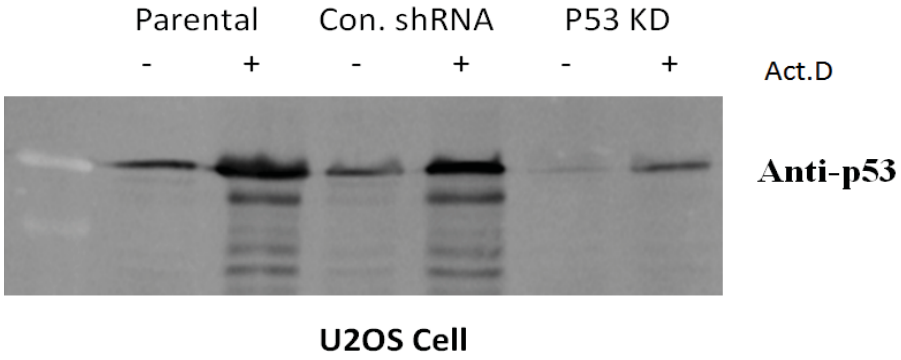


Figure 7. Actinomycin D induces p53-dependent cell death in U2OS cell. **(A)** U2OS cells with control shRNA (Con) or p53-specific shRNA (p53-KD) were damaged with

increasing doses of Actinomycin D (zero, 5, 10, 20, 40 and 80 nM) and harvested 24 hours later for cellular DNA content analysis by propidium iodide staining. Cells with sub-G1 DNA content were counted as dead cells (M1 marker). **(B)** Percentage of cell death induced by Actinomycin D with increasing concentrations in U2OS control (Con) and U2OS p53 knock-down (p53-KD) cells. **(C)** p53 protein levels in U2OS cell lines (parental, control shRNA, p53 knock-down) were examined by western blotting. Cells were either treated with 10 nM Actinomycin D or non-treated. Results in **(A)** are representative of three independent experiments. Columns in **(B)** represent mean values from three independent experiments with error bars showing SEM. Cell death in U20S p53 knock-down cells: *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.0001$ versus U2OS control cells at the same Act.D concentrations (20, 40 and 80 nM).

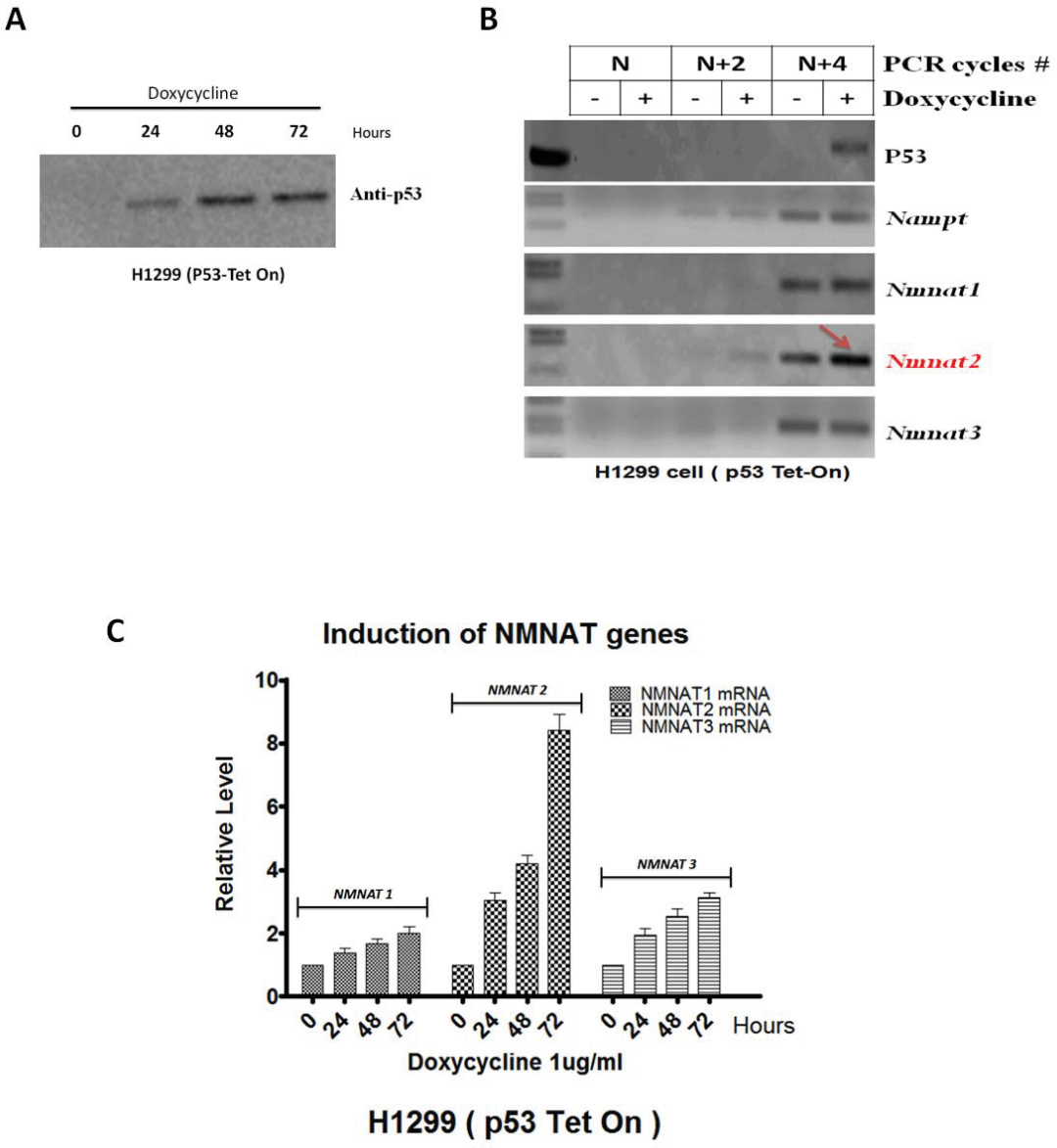


Figure 8. p53 induces the expression of NAD^+ synthetase Nmnat2 but not Nmnat1, Nmnat3 and Nampt. **(A)** Induction of p53 in H1299-p53-Tet On cells by doxycycline (1 μg/ml). Cells were harvested at zero, 24, 48 and 72 hours post treatment. Whole cell extracts were used to examine p53 protein expression by western blotting. **(B)** Induction of p53 in H1299-p53-Tet On cells elevated mRNA levels for Nmnat2 but not Nampt, Nmnat1 and Nmnat3. Cells were treated with doxycycline for 36 hours and harvested.

cDNAs were synthesized from these collected cells and used for semi-quantitative PCR. Primers for Nmnat2 mRNA were designed to amplify both transcript variants. **(C)** Real-time quantitative PCR was carried out to determine cellular mRNA levels for Nmnat1, Nmnat2 and Nmnat3 in response to p53 induction. H1299-p53-Tet On cells were grown in the presence of 1 μg/ml doxycycline and harvested at zero, 24, 28 and 72 hours. Relative mRNA levels were normalized to those from controls cells (no doxycycline). Figure s in **(A)** and **(B)** represent results from two independent experiments. RT-qPCR Data presented in **C** are mean values from three independent experiments, with error bars showing SEM.

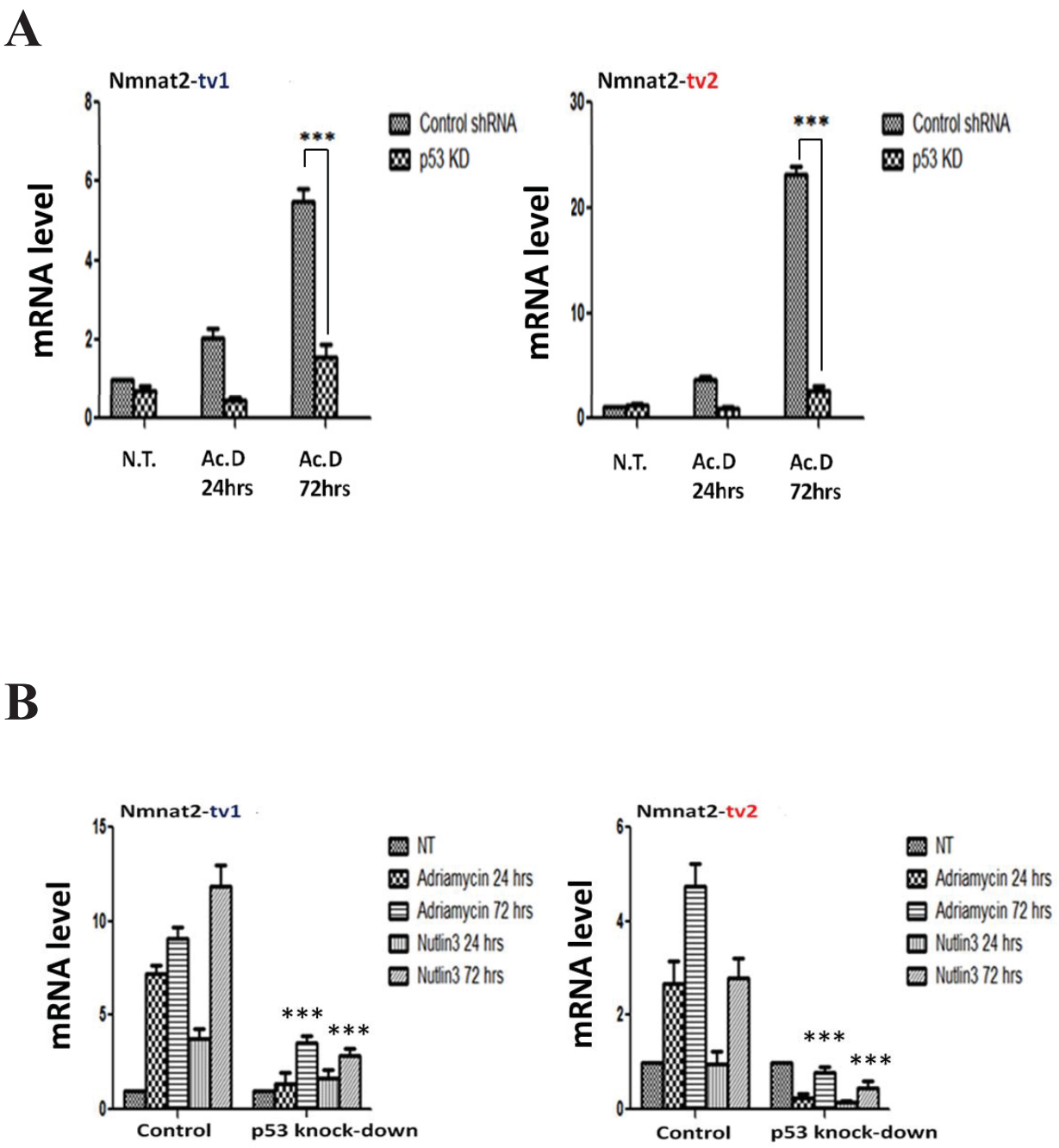


Figure 9. DNA damage induces p53-dependent expression of the Nmnat2 gene. **(A)** Act.D induced the expression of Nmnat2 in a p53-dependent manner. U2OS cells (control or p53 knock-down) were treated with Actinomycin D (20 nM) and harvested at

the indicated time-points. Nmnat2-TV1 (left) and Nmnat2-TV2 (right) messenger RNA levels were determined by RT-qPCR. **(B)** Adriamycin and Nutlin-3 induced the expression of Nmnat2 in a p53-dependent manner. Nmnat2-TV1 (left) and Nmnat2-TV2 (right) levels were determined by RT-qPCR. U2OS cells (control or p53 knock-down) were treated with another DNA damage reagent Adriamycin (200 ng/ml) or p53 activator Nutlin-3 (10 μM). Cells were harvested at indicated time-points. Real-time PCR experiments were repeated three times and presented as mean values with error bars showing SEM. mRNA fold changes in U2OS p53 knock-down cells were compared to that of U2OS control cells (Adriamycin 72 hours; Nutlin-3 72 hours): *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.0001$.

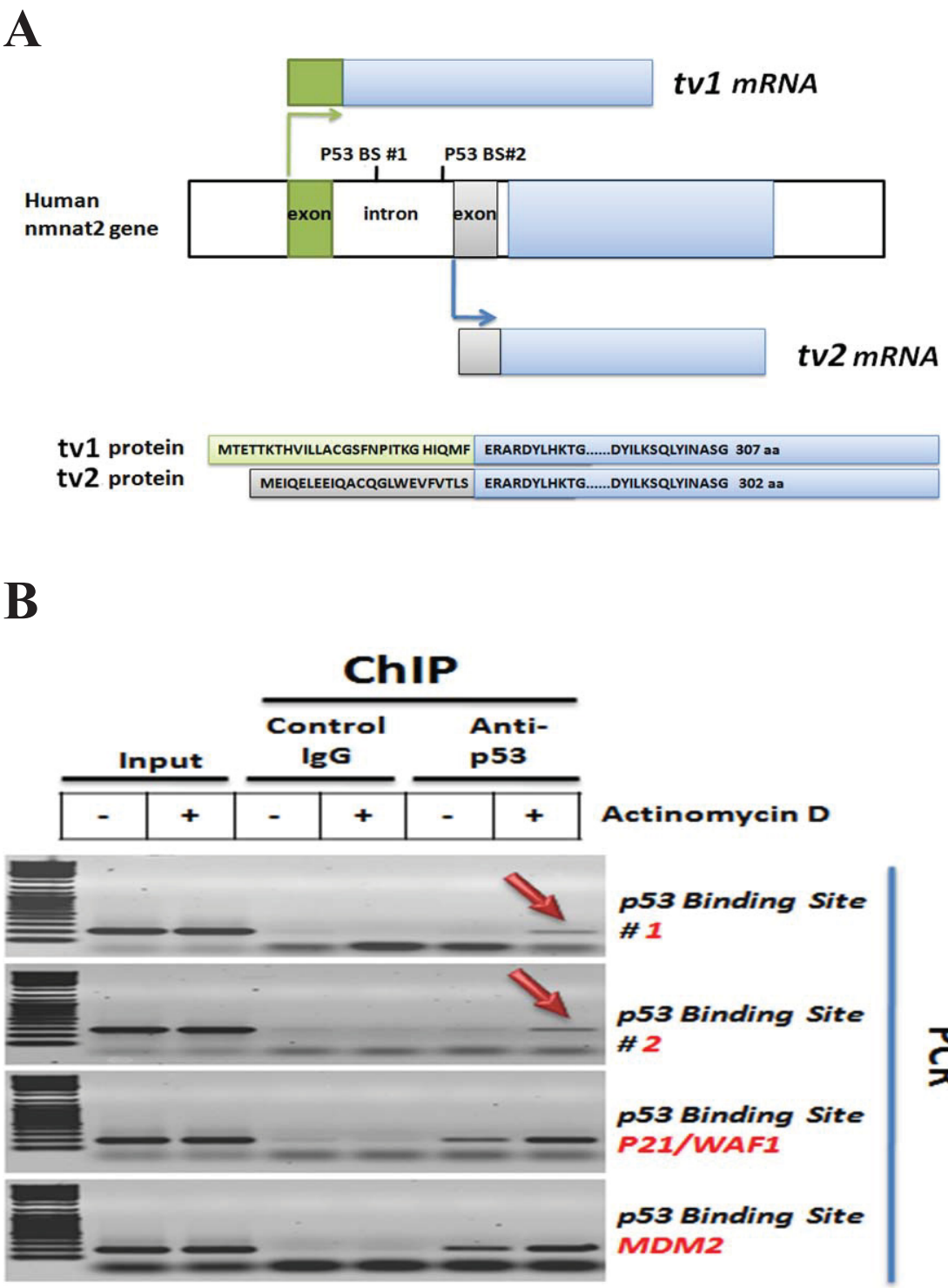


Figure 10. p53 directly binds to two putative responsive elements within the human Nmnat2 gene upon DNA damage. **(A)** Schematic of the human Nmnat2 gene (not to

scale) and the transcription start sites for each isoform. p53 responsive elements identified (p53BS#1 and p53BS#2) are located in the first intronic region. Transcript variant 1 and 2 differ in their first exon and are translated into two protein isoforms with distinct N-termini. **(B)** p53 BS #1 and p53 BS #2 were validated by chromatin immuno-precipitation (ChIP) to be bona fide p53 responsive elements. U2OS cells were damaged with Actinomycin D (20nM). Chromatin fragments that co-immunoprecipitated with p53 protein were amplified with PCR primers spanning individual p53RE. P53REs in the *p21*$^{WAF1/CIP1}$ and *mdm2* gene were used as positive controls. Mouse normal IgG was used as negative control for IP. Mouse monoclonal anti-p53 antibody (DO-1) was used for p53 pull-down. Results are representative of two independent experiments. Red arrows: PCR product for BS#1 and BS#2.

A

Wild type BS#1 : AGGTTTGGGCTGGAGAGTCAA-GAACAAGTCG TGGCTTGCCA-AGTGTGGACCACAT GGCGAT
Mutant BS#1: AGGTTTGGGCTGGAGAGTCAA-GAATAATTCG TGGTTTTCCA-AGTGTGGACCACAT GGCGAT

Wild type BS#2 : TGGGATCAAACTTATTACAG-TGGCATGTAT GGACTTGTGG-AAGGACAGATCATTCCAGCT
Mutant BS#2: TGGGATCAAACTTATTACAG-TGGAATATAT GGAATTATGG -AAGGACAGATCATTCCAGCT

B

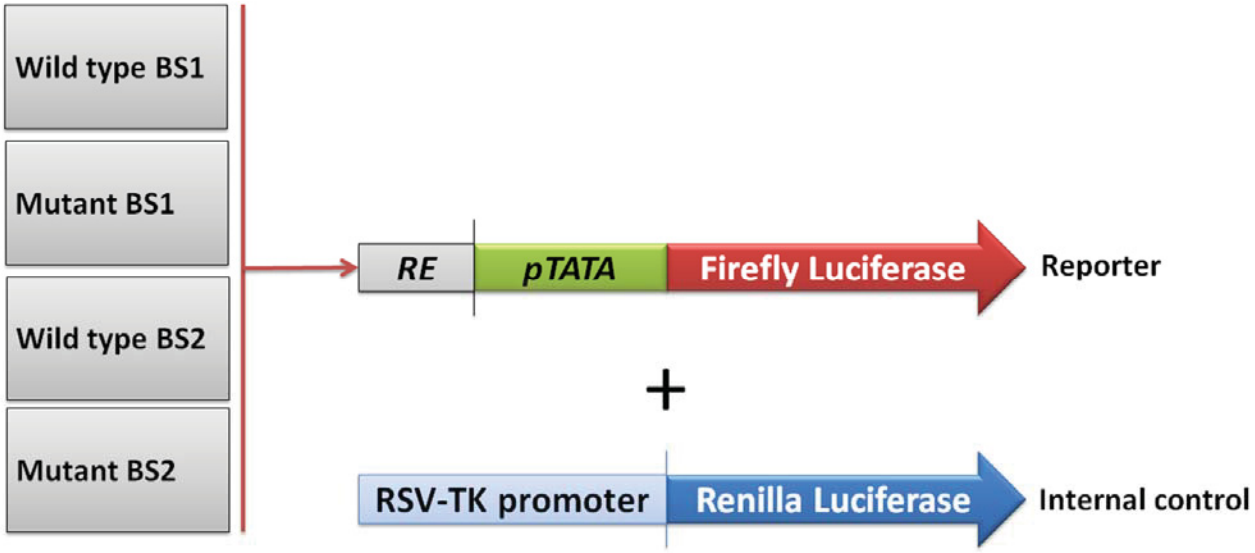


Figure 11. p53 activates firefly luciferase reporter gene via two wild-type responsive elements identified in human Nmnat2 gene.

C

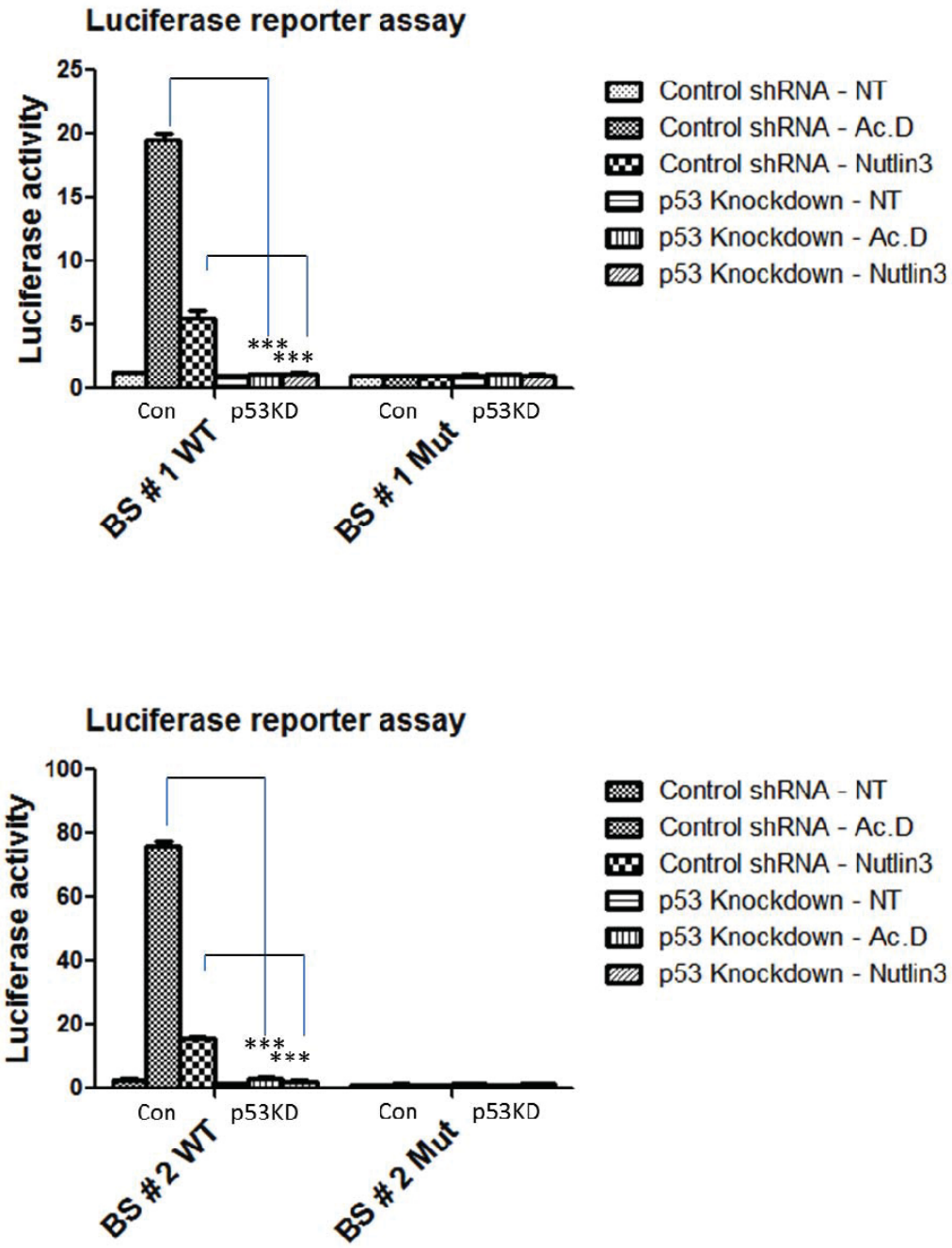
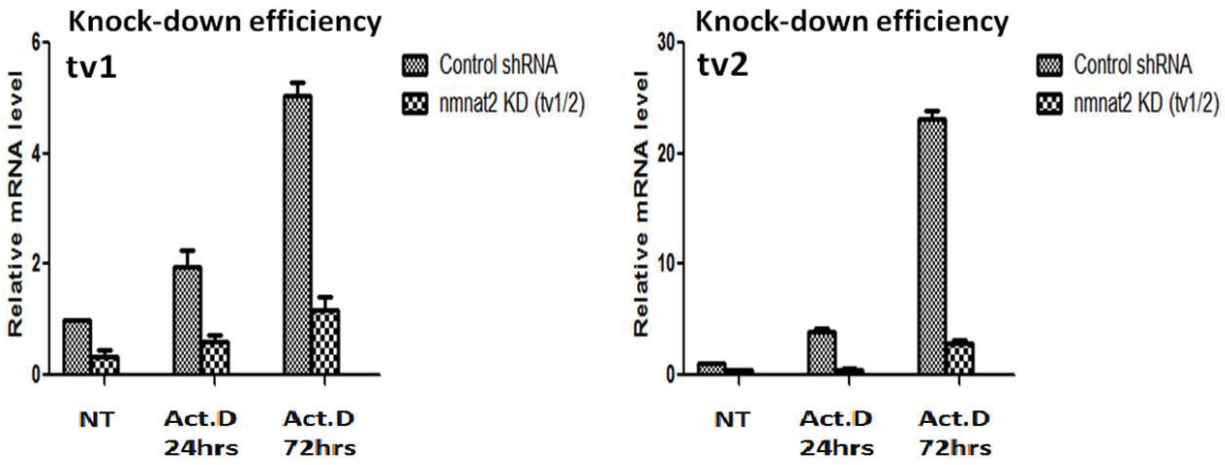


Figure 11. p53 activates firefly luciferase reporter gene via two wild-type responsive elements identified in human Nmnat2 gene. **(A)** Sequences of wild-type and mutant p53

BS#1 and p53BS#2. Nucleotides highlighted in red represent wild-type. Nucleotides highlighted in blue represent mutant. **(B)** The dual luciferase reporter system. **(C)** Wild-type p53BS#1 and p53BS #2 drive the expression of luciferase gene, whereas mutant p53BS#1 and p53BS #2 fail to induce luciferase activity. Luciferase reporter plasmids carrying individual p53BS (wild-type or mutant; #1 or #2) were transfected into U2OS cells (control or p53-KD). Cells were treated with Act.D (10 nM) or p53 activator Nutlin-3 (10 nM) and harvested 48 hours later for measurement of luciferase activities. Luciferase activities of each reporter were normalized to those from non-treated U2OS control cells. Data are presented as mean values with error bars showing SEM from three independent experiments. Firefly luciferase activities in U2OS p53 knock-down cells were compared to that of U2OS control cells with the same treatments (Act.D and Nutlin-3). *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.0001$.

A



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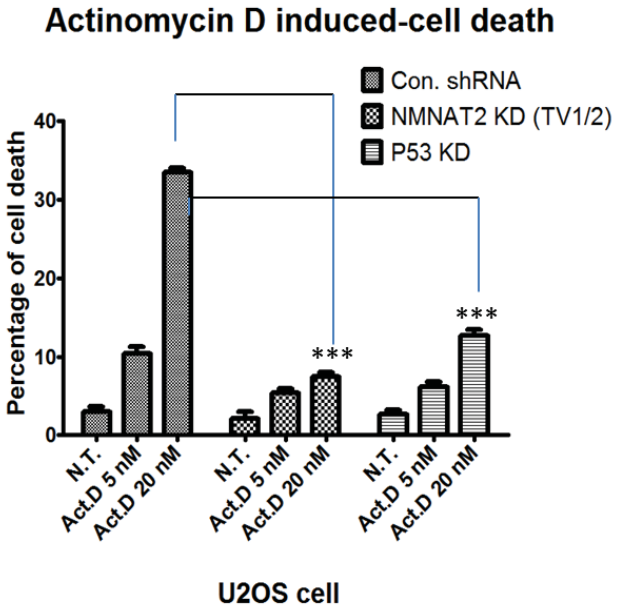


Figure 12. Knocking down Nmnat2 protects U2OS cell from DNA damage-induced cell death.

C

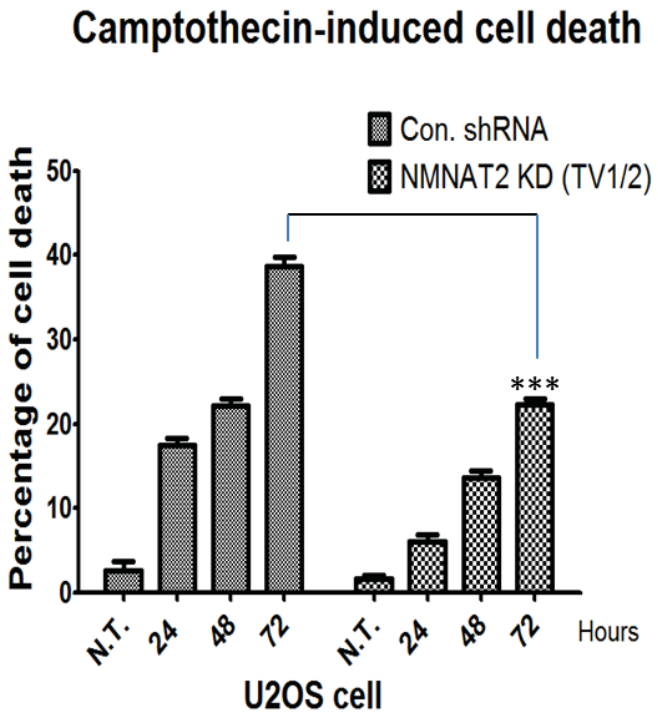


Figure 12. Knocking down Nmnat2 protects U2OS cell from DNA damage-induced cell death. **(A)** Nmnat2 gene expression was efficiently knocked down with retrovirus-mediated RNAi in U2OS cells. U2OS cells stably expressing control shRNA or Nmnat2-specific shRNA were treated with Act.D (20 nM) and collected at indicated time-points. Cellular mRNA levels of Nmnat2-TV1 (left) and Nmnat2-TV2 (right) were determined by RT-qPCR. **(B)** Knocking down Nmnat2 protects U2OS cells from Act.D-induced cell death. U2OS cells (control, Nmnat2-KD and p53-KD) were damaged with Act.D (5 nM or 20 nM) and harvested 48 hours later. The percentage of dead cells was determined by FACS analysis of cells with sub-G1 DNA content. **(C)** Knocking down Nmnat2 protects U2OS cells from Camptothecin-induced cell death. U2OS cells (control and Nmnat2-KD) were damaged with camptothecin (0.5 μM) and harvested after 24 hours. The

percentage of dead cells was indicated by sub-G1 DNA content. Data presented as mean values form three independent experiments with error bars showing SEM. Cell death in U2OS Nmnat2 knock-down and p53 knock-down cells was compared to that of U2OS control cells with the same treatment. *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.0001$.

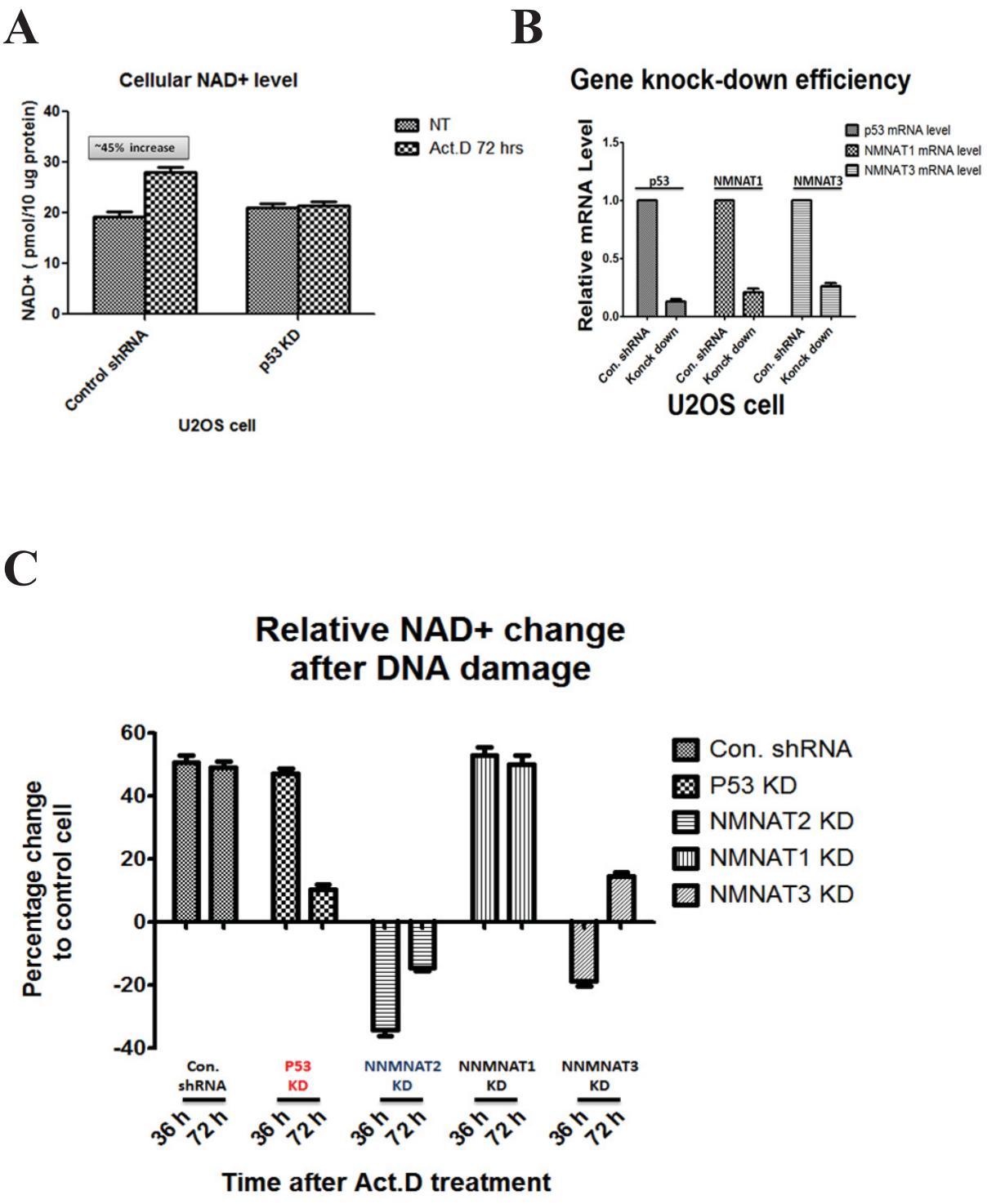


Figure 13. p53 modulates cellular NAD^+ levels upon DNA damage. **(A)** p53 is required for increases in cellular NAD^+ levels after Act.D treatment. U2OS cells (control or p53-

KD) were treated with Act.D (10 nM) and collected 72 hours later. Cellular NAD^+ was measured with NAD^+/NADH Quantitation Kit (biovision). Values were normalized to protein concentration (pmol/10 μg). **(B)** RNAi knock-down efficiency of p53, Nmnat1 and Nmnat3 in U2OS cells were determined with RT-qPCR and presented as relative amount to control cells. **(C)** Knocking down p53, Nmnat2 or Nmnat3 impaired the capacity to replenish NAD^+. U2OS cells (control, p53-KD, Nmnat1-KD, Nmant2-KD or Nmant3-KD) were damaged with Act.D (20 nM). Cells were harvested at 36-hour and 72 -hour time-points. Cellular NAD^+ measured by NAD^+/NADH Quantitation Kit (biovision). Data presented (except in **(B)**) are mean values from three independent experiments with error bars showing SEM.

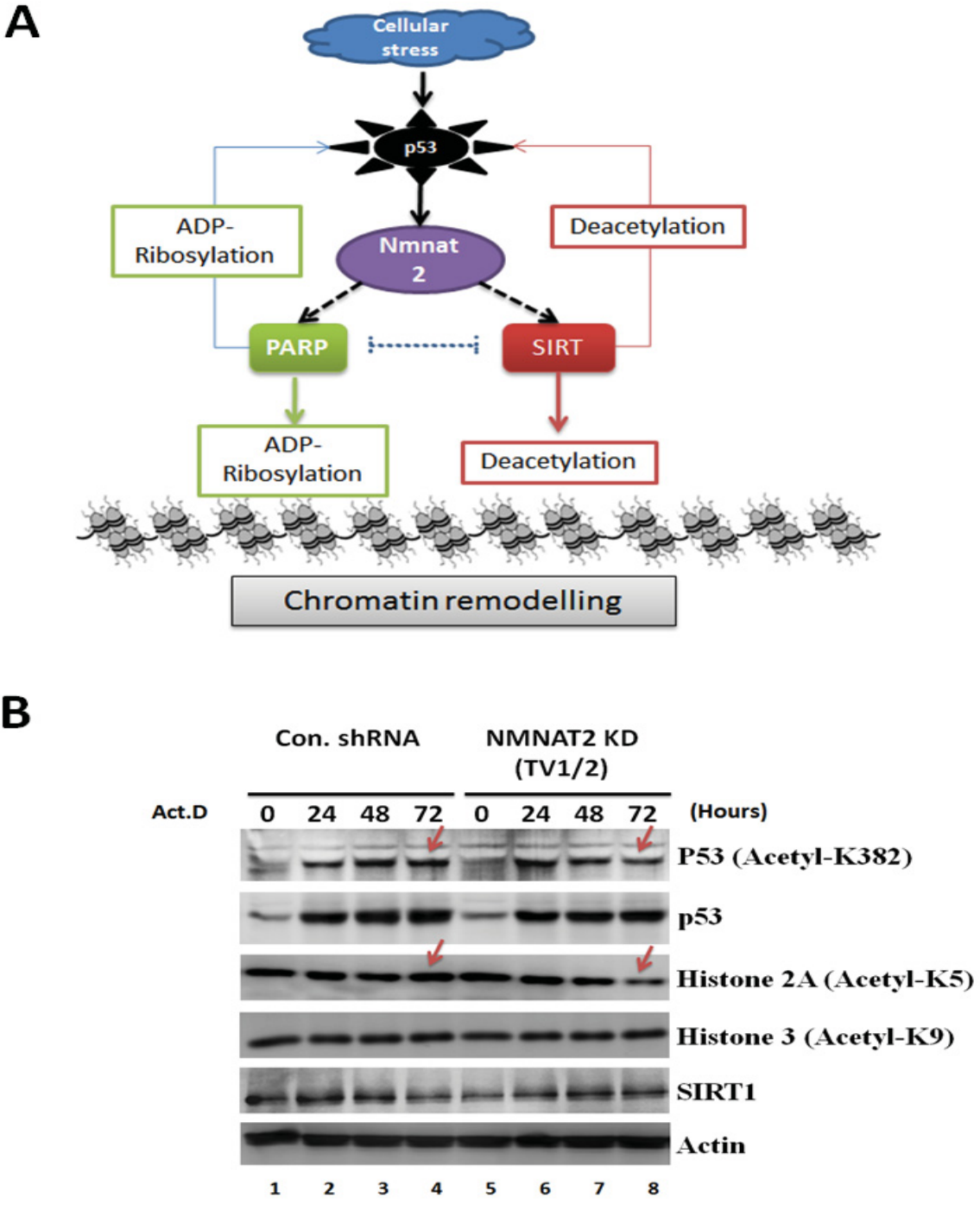


Figure 14. Knocking down Nmnat2 affects acetylation of p53 and histone upon DNA damage. **(A)** Proposed p53-Nmnat2 feedback regulatory loop. See text for details. **(B)**

Nmnat2 is involved in the regulation of p53-K382 and H2A-K5 acetylation. U2OS cells (control or Nmnat2-KD) were damaged with Act.D (20 nM) and harvested at indicated time-points. Ten micrograms cell extract was used in western blotting of proteins of interest. The results represent two independent experiments. Red arrows emphasize acetyl-p53-K382 and Acetyl-histone 2A-K5, respectively.

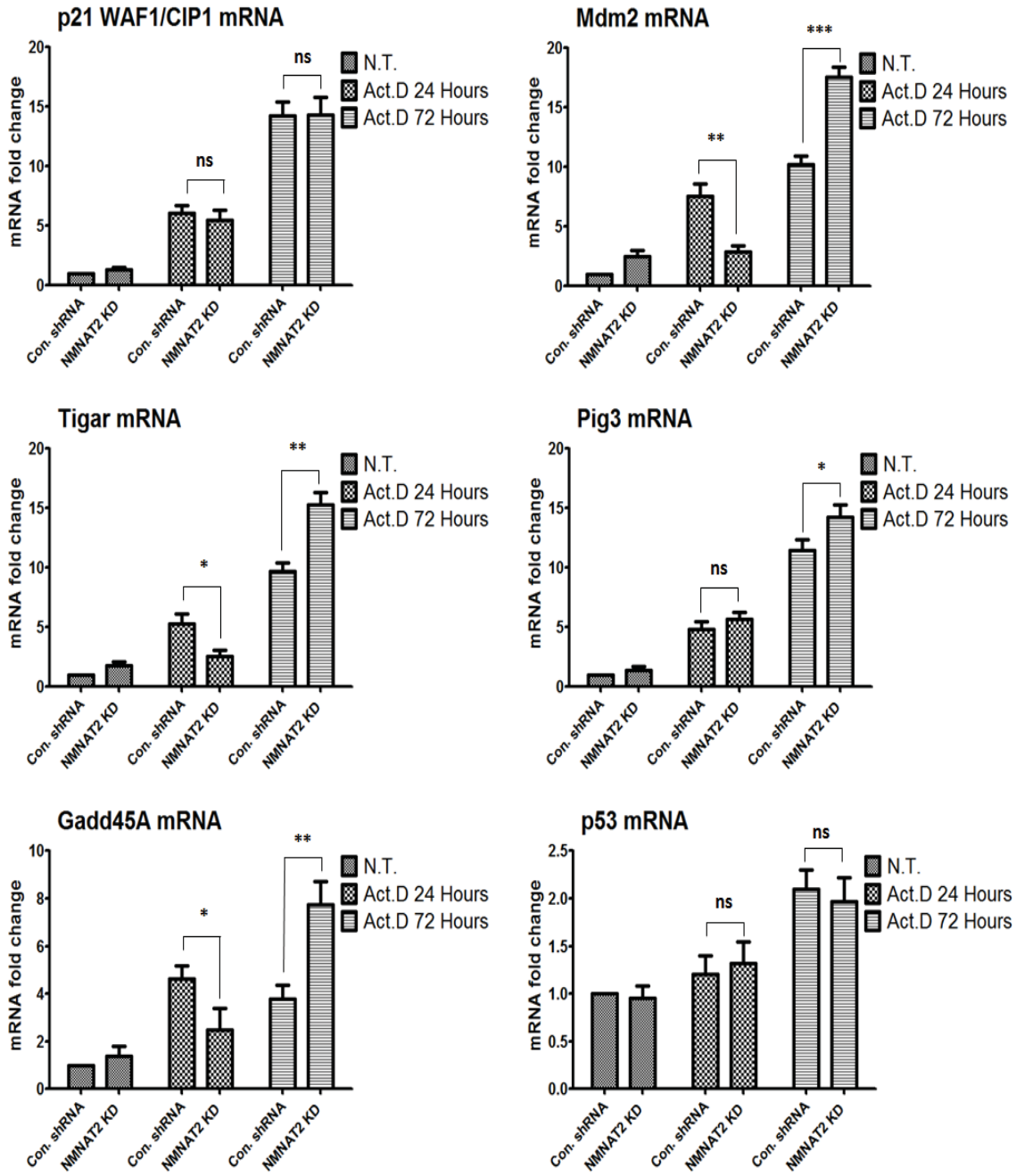


Figure 15. Knocking down Nmnat2 affects the expression of a subset of p53 target genes in a time-dependent manner in response to Actinomycin D treatment. U2OS cells (control and Nmnat2 knock-down) were treated with Act.D (20 nM) and harvested at the indicated time-points. Cellular mRNA levels of $p21^{waf1/cip1}$, mdm2, Tigar, pig3, Gadd45a

and p53 were determined by RT-qPCR and compared between control and Nmnat2 knock-down cells at each time-point. Real-time PCR was repeated three times and data is presented as mean values with error bars showing SEM. mRNA fold changes of each gene examined in U2OS control and Nmnat2 knock-down cells were compared at each indicated time-point. NS: not significant; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.0001$.

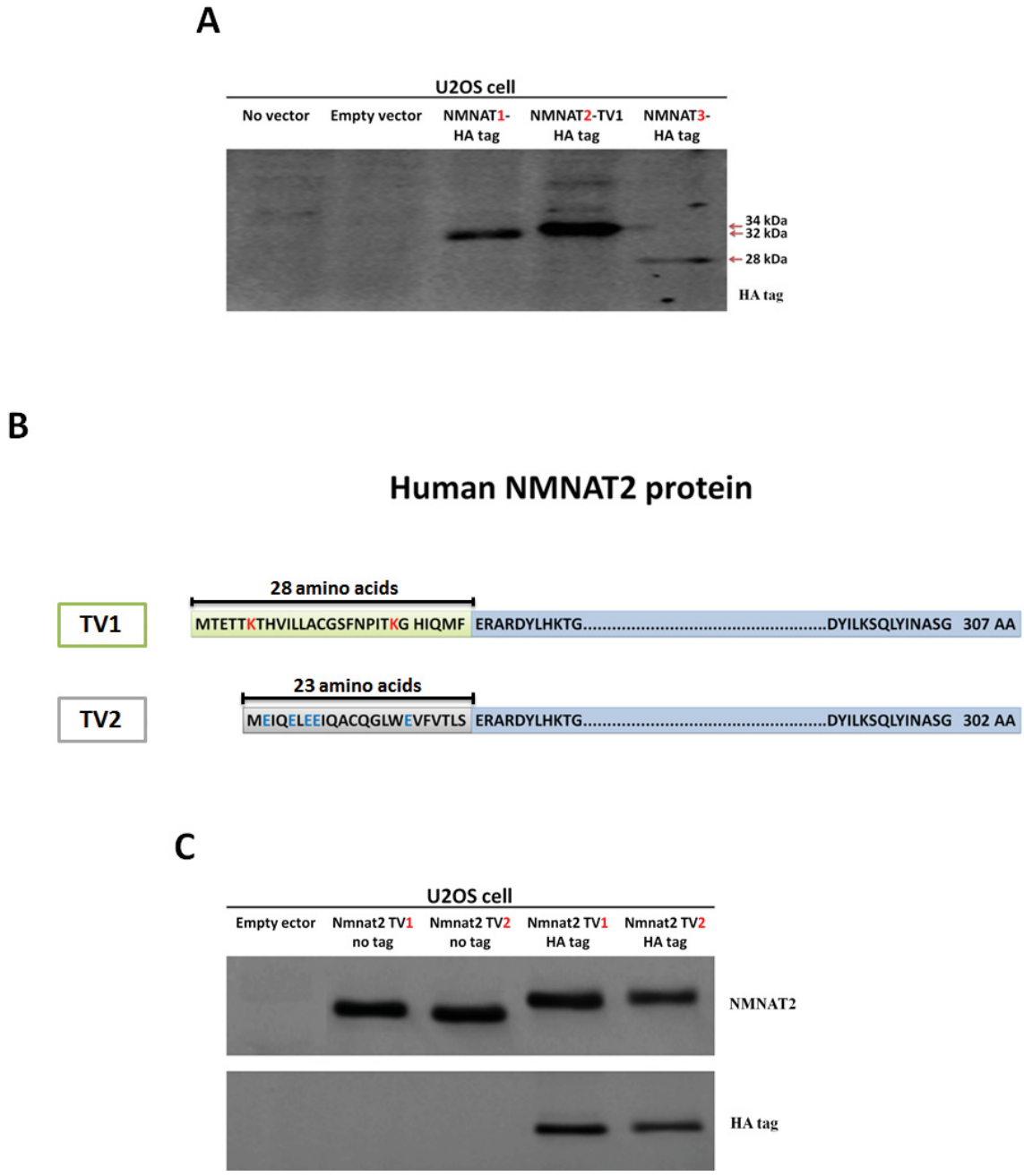


Figure 16. Cloning and expression of Nmnat2 isoforms. **(A)** Ectopic expression of Nmnat1-HA, Nmnat2-TV1-HA, and Nmnat3-HA in U2OS cells. MSCV vectors carrying HA-tagged Nmnat1, Nmnat2-TV1 and Nmnat3 were transfected into U2OS cells. The

expression of each Nmnat protein was confirmed by western blotting using an anti-HA antibody (HA probe, Santa Cruz). **(B)** Comparison of Nmnat2-TV1 and Nmnat2-TV2 protein N-terminal sequences. **(C)** Ectopic expression of Nmnat2-TV1 an Nmnat2-TV2 protein, with or without HA tags. Nmnat2 protein isoforms were detected with anti-HA and anti-Nmnat2 protein (C-terminal).

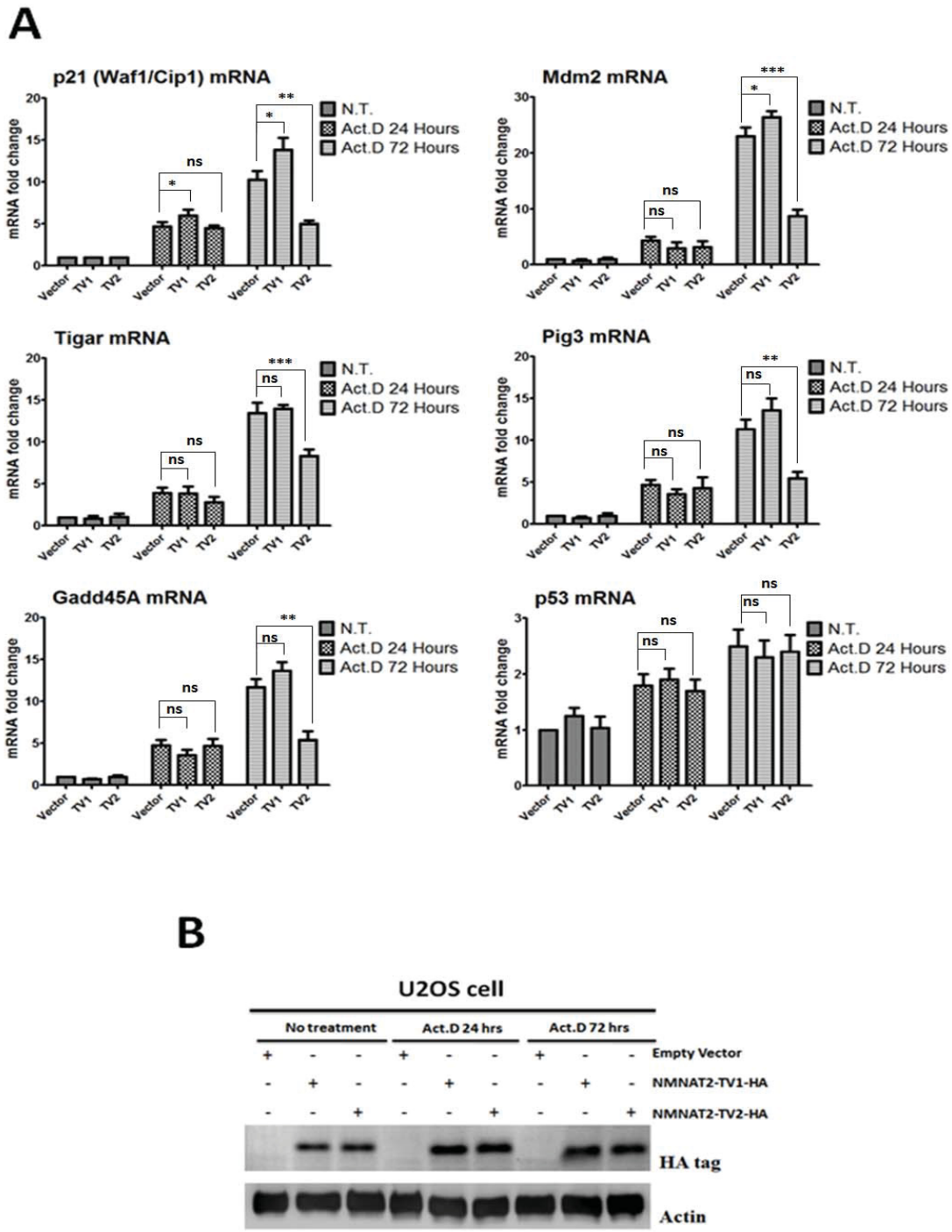


Figure 17. Over-expression of Nmnat2-TV2 suppresses the expression of a subset of p53 target genes after DNA damage. **(A)** Nmnat2-TV2 suppressed the expression of p21, mdm2, Tigar, pig3, and gadd45a at 72 hours after Act.D treatment. U2OS cells over-

expressing Nmnat-TV1-HA, Nmnat2-TV2-HA or empty vector were treated with Act.D (20 nM) and harvested at indicated time-points. Cellular mRNA levels of the same set of p53 target genes (Figure 15) were determined with RT-qPCR. Experiments were repeated three times and data are presented as mean values. mRNA fold changes of each gene examined in U2OS control, U2OS-TV1 and U2OS-TV2 cells were compared at each indicated time-point. NS: not significant; *, $P < 0.05$, **, $P < 0.01$, ***, $P < 0.0001$. **(B)** Expression of Nmnat2 proteins in U2OS cells was confirmed by western blotting.

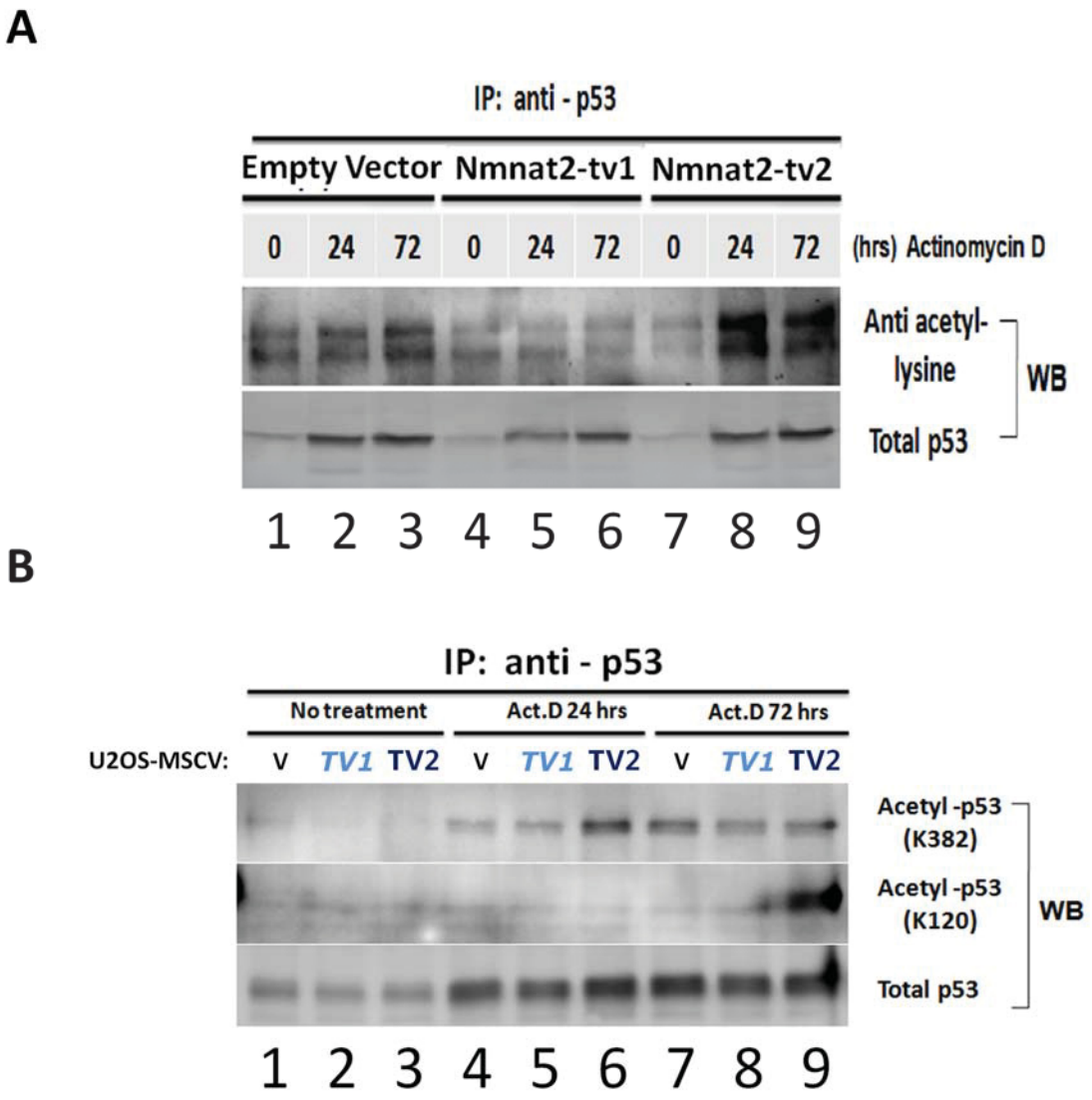


Figure 18. Nmnat2 isoforms regulate p53 acetylation oppositely upon DNA damage. **(A)** Over-expression of Nmnat2-TV1 and Nmnat2-TV2 had opposite effects on acetylation of p53 protein. U2OS cells (empty vector, Nmnat2-TV1 or Nmnat2-TV2) were treated with Act.D and harvested at the indicated time-points. Total p53 was immunoprecipitated and probed with anti-acetyl lysine antibody (Cell signaling). **(B)** Nmnat2-TV2 enhanced p53 acetylation at K382 and K120 in a time-dependent manner. The same procedure of IP-

western blotting was followed as described in **(A)**. Acetylated p53 at K382 or K120 was probed with antibodies specific for each site.

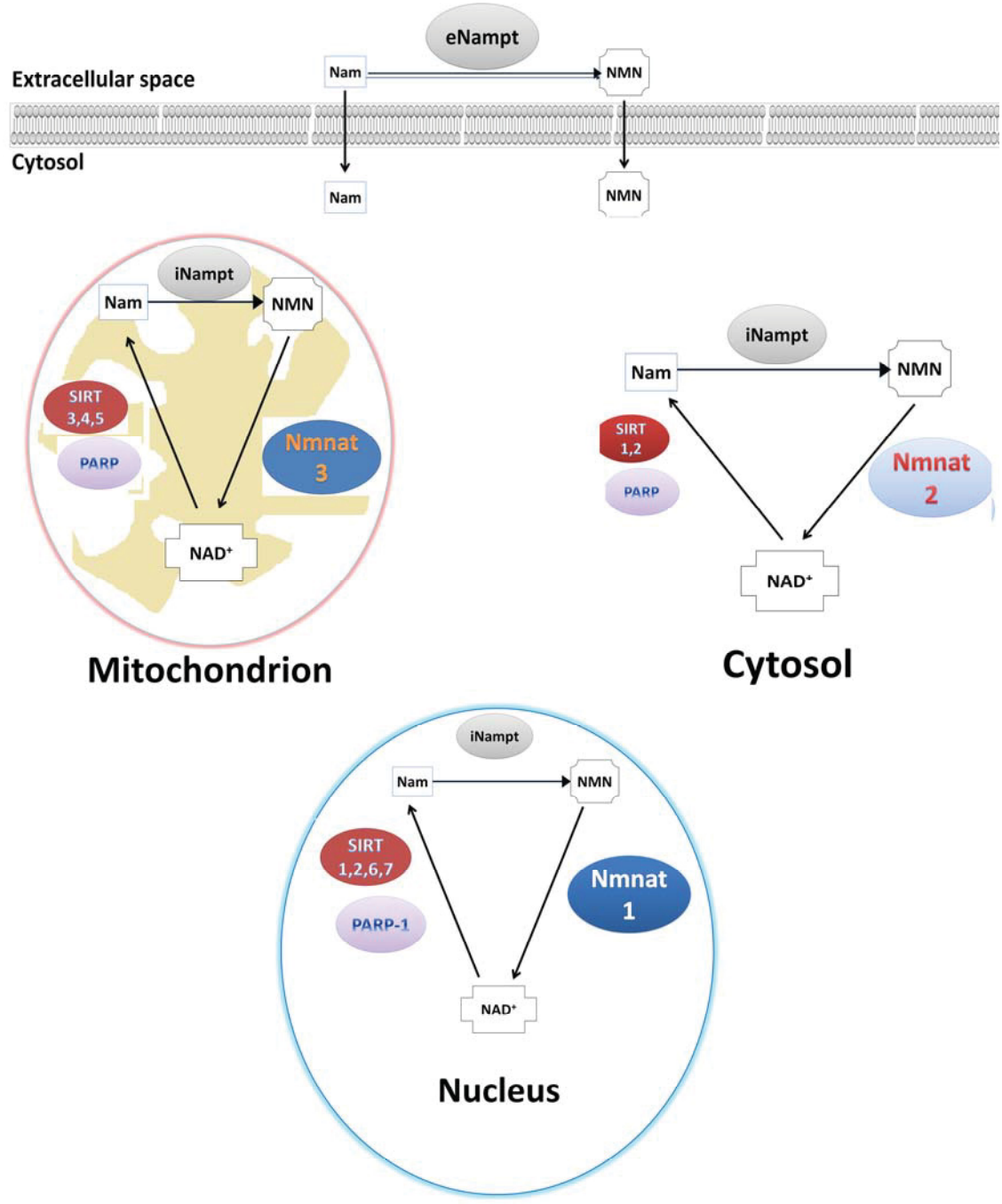


Figure 19. Histone deacetylase SIRT 1 and SIRT 2 target p53 protein on lysine 382 and lysine 120 respectively after DNA damage.

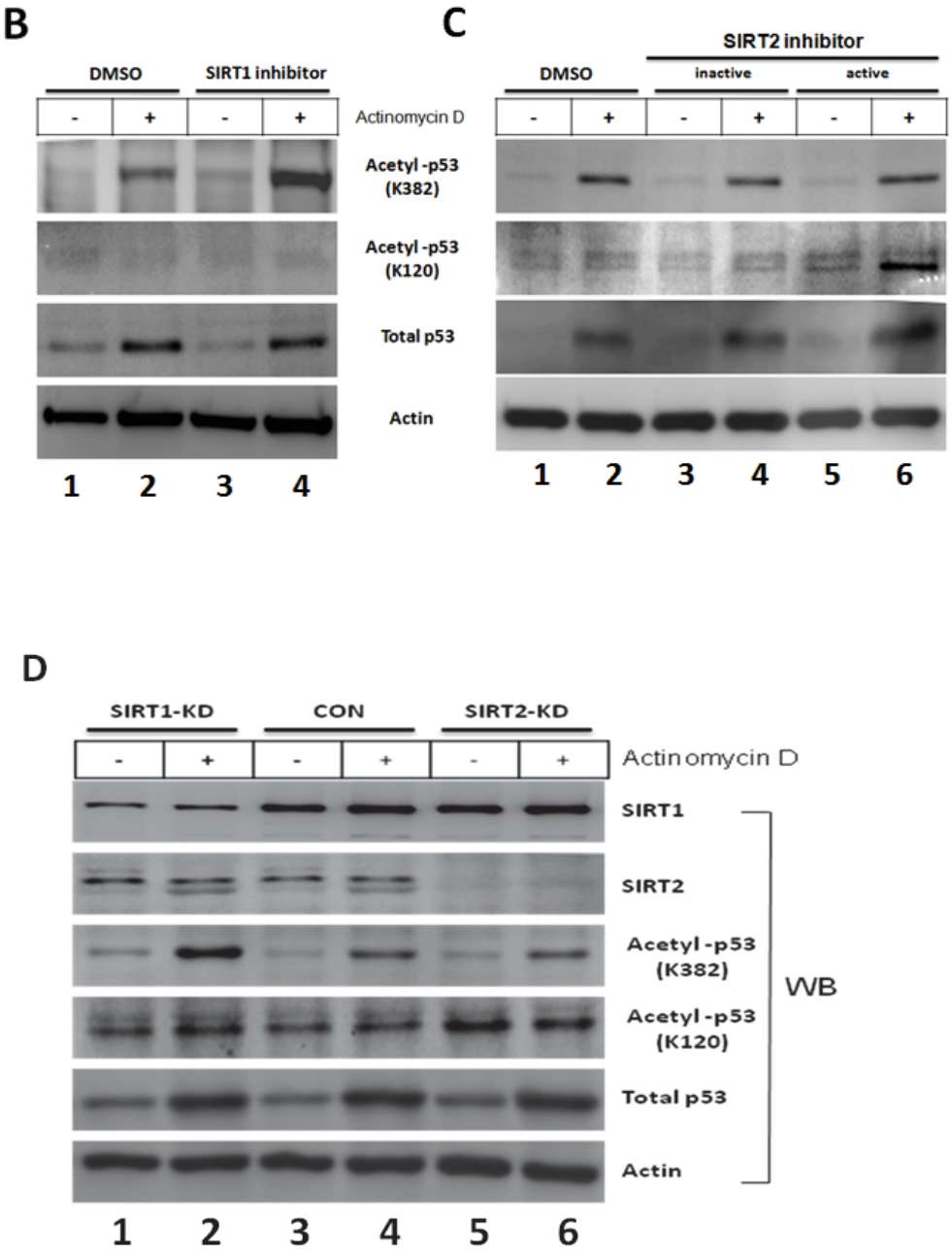


Figure 19. Histone deacetylase SIRT 1 and SIRT 2 target p53 protein on lysine 382 and lysine 120 respectively after DNA damage. **(A)** Schematic of sub-cellular localizations of Nmnats, SIRTs and PARPs. See text for details. **(B)** Inhibition of endogenous SIRT1 enhances p53 acetylation at K382 but not K120. U2OS cells were non-treated or treated

with Act.D (20 nM) and/or SIRT1 inhibitor III (100 μM) for 48 hours. Total and acetylated p53 (K382 and K120) were visualized by western blotting. **(C)** Inhibition of endogenous SIRT2 with inhibitor (AGK2, Calbiochem) enhanced acetylation of p53-K120 but not p53-K382. An inactive form of SIRT2 inhibitor (AGK7, Calbiochem) was used as control. U2OS cells were non-treated or treated with Act.D and/or SIRT2 inhibitors (10 μM) for 48 hours. Total and acetylated p53 (K382 and K120) were visualized by western blotting. **(D)** Knocking down SIRT1 or SIRT2 in U2OS cells has specific effects on p53 acetylation at K382 or K120, respectively. U2OS cells were infected with retroviruses carrying specific shRNA for SIRT1 or SIRT2 and selected with blasticidin (5 μg/ml) for 2 weeks. U2OS cells (control, SIRT1-KD or SIRT2-KD) were treated with Act.D or non-treated for 48 hours. Endogenous SIRT1, SIRT2, p53 and acetyl-p53 (K120 and K382) were visualized by western blotting. Results are representative of two independent experiments.

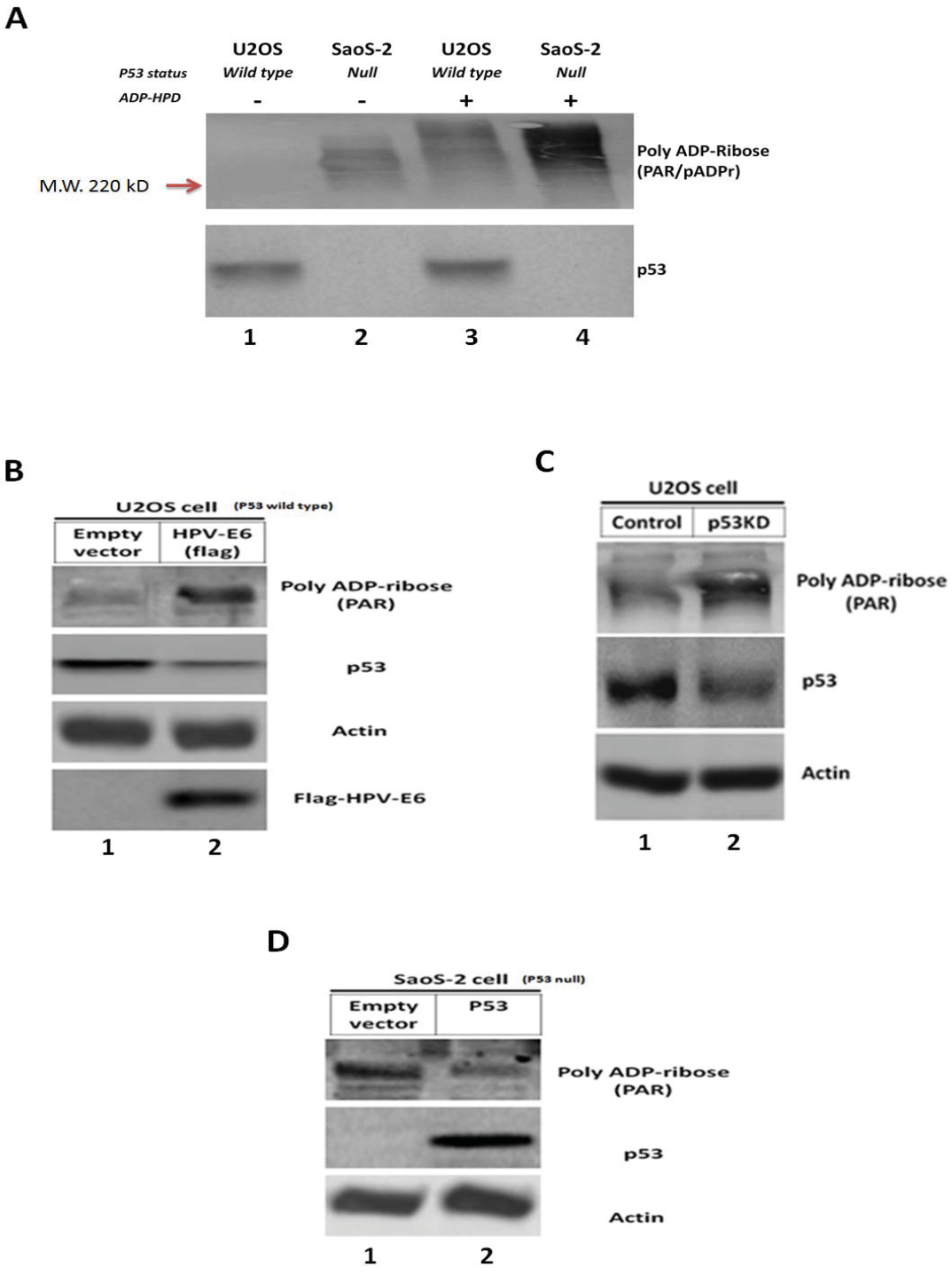


Figure 20. p53 regulates cellular poly ADP-ribosylation. **(A)** ADP-HPD inhibited poly ADP-ribose degradation in U2OS and Saos-2 cell extracts. ADP-HPD was included in

cell lysis buffer during preparation of whole cell extract. Cellular poly ADP-ribose was detected by anti-PAR antibody (10H, Alexis). **(B)** Human papilloma virus E6 protein caused degradation of p53 protein and enhanced cellular poly ADP-ribosylation. U2OS cells were transfected with HPV-E6 plasmid or empty vector, then grown in the presence of neomycin (1 mg/ml) for one week. Cellular PAR was examined with whole cell extract prepared from U2OS cells transfected with empty vector or HPV-E6. **(C)** Knocking down p53 in U2OS cells led to increased cellular PAR levels. U2OS cells (control and p53-KD) were compared for their endogenous PAR by western blotting. **(D)** Ectopic expression of p53 in Saos-2 cell (p53 null) decreased cellular PAR levels. MSCV-p53 or empty plasmids were transfected into Saos-2 cells. Cellular PAR was examined as above. Results are representatives of two independent experiments.

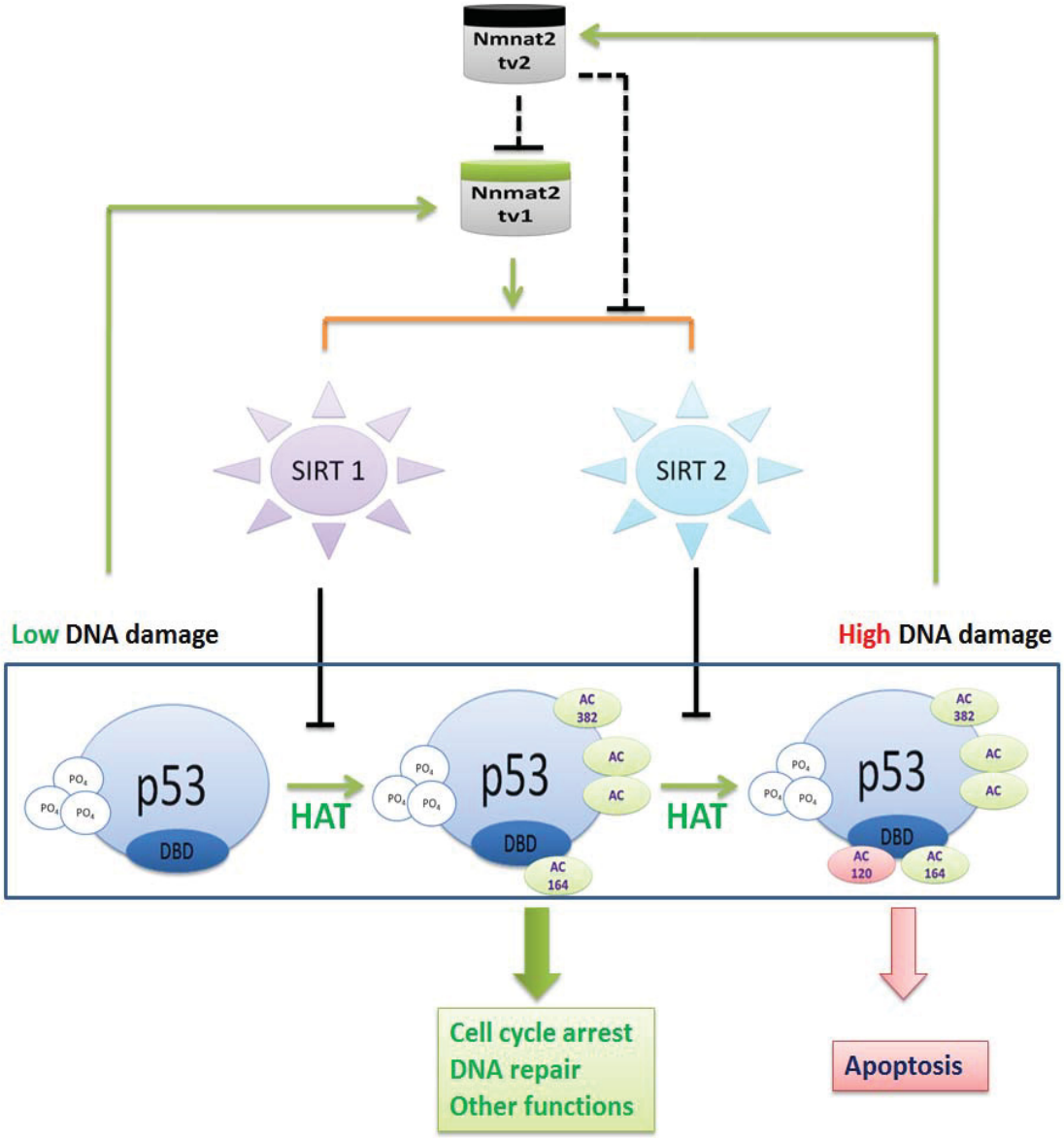


Figure 21. Schematic of proposed model: The p53-Nmnat2 feedback loop dictates cell fate upon DNA damage. Acetylation of p53 is negatively regulated by SIRT proteins during DNA damage responses, so that over-activation of p53 is controlled. Nmnat2-TV1

is induced by p53 to promote SIRT activities that prevent p53 from further activation and thus inhibit apoptosis. Nmnat2-TV2, also induced by p53 upon DNA damage, antagonizes TV1 and/or SIRT activities, thereby allowing hyper-acetylation of p53 and transcription of pro-apoptotic genes. The interaction between Nmnat2 TV1/2 might dictate p53 functions toward cell-cycle arrest or apoptosis. See text for more details.

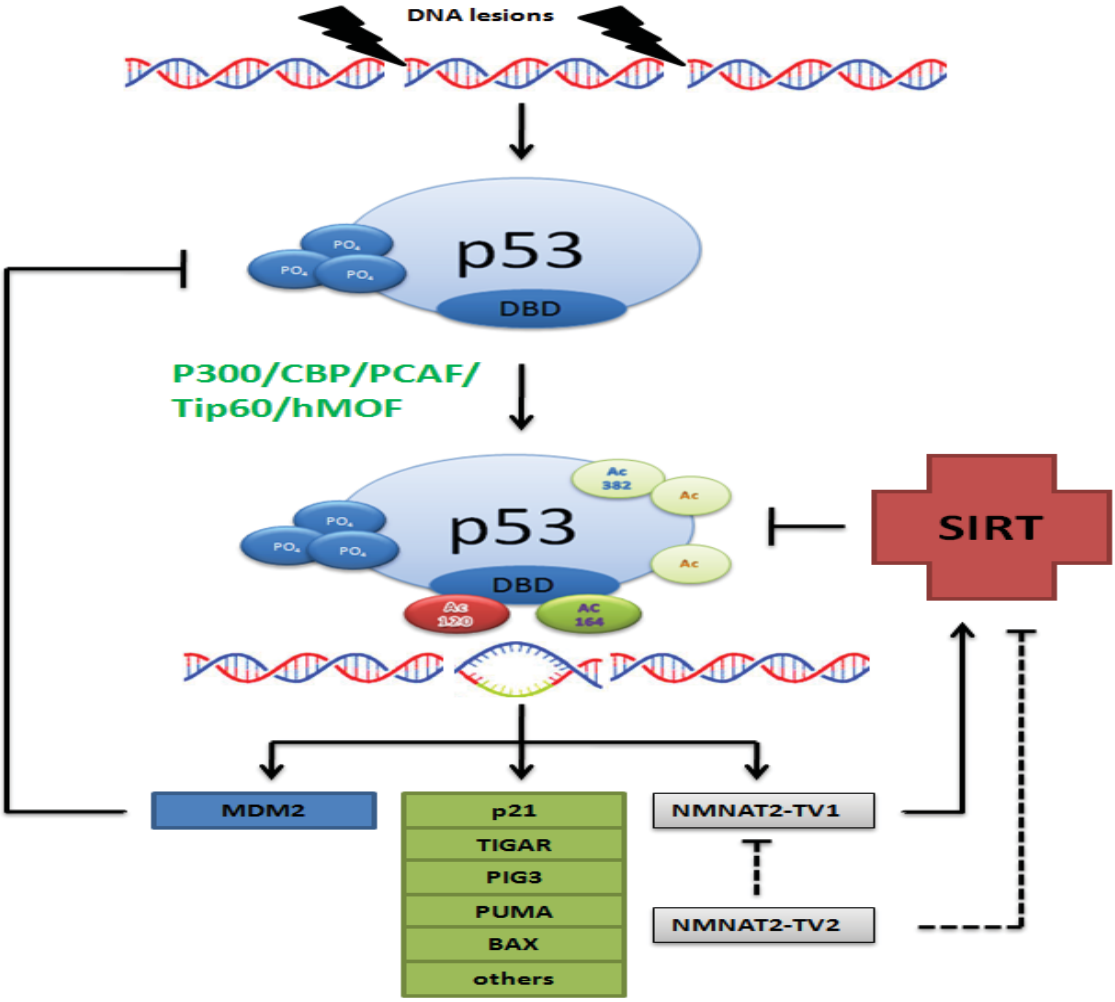


Figure 22. Appropriate p53 functions are controlled via multiple levels of inhibition. This simplified flowchart illustrates DNA damage-induced p53 activation. Negative feedback regulation by MDM2 keeps p53 protein at a low levels and also inhibits its acetylation by a group of histone acetyltransferases (HAT). Nmnat2 isoforms are induced by p53 to modulate its acetylation through SIRT deacetylases, in such way that appropriate p53 activation is fine-tuned. See text for more details.

CHAPTER 4

P53 PROTEIN SUB-POPULATIONS ARE REGULATED DIFFERENTLY IN HS68 HUMAN FIBROBLAST CELL UPON UV RADIATION

4.1 Hypothesis And General Objectives

The ability of p53 to induce cell death has been reported to act in two ways. Transactivation of pro-apoptotic genes, such as *bax*, *puma*, *p53AIP1*, *noxa* and others, plays an important role in p53-mediated apoptosis. p53 also can repress the expression of cellular anti-apoptotic genes such as *BIRC5/Survivin* [393-395]. Therefore, by regulating apoptosis-related genes transcriptionally, p53 indirectly induces cell death. On the other hand, there is evidence that p53 can trigger apoptosis in a manner independent of transcription. In general, p53 may translocate to mitochondrial outer membrane upon DNA damage, where it causes permeabilization of the membrane and consequent release of cytotoxic mitochondrial components including Cytochrome c, DIABLO, apoptosis-inducing factor (AIF) and endonuclease G. Previously, studies in Dr. Patrick Lee's laboratory revealed that the latent p53 population is sufficient to induce apoptosis in mouse embryonic fibroblasts (MEF) upon ionizing radiation (IR) [396, 397]. The DNA-dependent protein kinase (DNA-PK) was shown to phosphorylate latent p53 on serine15, which is critical for p53's apoptotic function in response to IR [396, 397]. Based on these findings, I continued to investigate mechanisms of activation of latent p53 in human cells, in response to UV radiation. It was hypothesized that certain sub-populations of latent p53 can be activated to trigger apoptosis in Hs68 human foreskin fibroblast cells. General goals of this project include: to determine if DNA-PK is involved in activation of

latent p53 upon UV radiation in Hs68 cells; to characterize latent p53; to investigate mechanisms of activation of latent p53 upon UV radiation.

4.2 RESULTS

4.2.1 DNA-PK Is Required For Serine 15 Phosphorylation Of Induced But Not Latent p53 Upon UV Radiation

The role of DNA-PK in activation of p53 upon DNA damage has been debated [398-400]. Ataxia telangiectasia mutated (ATM) and ataxia telangiectasia and Rad3-related protein (ATR), members of the phosphatidylinositol 3-kinase-related kinase (PI3K-related kinase) protein family to which DNA-PK also belongs, were thought to phosphorylate p53 on serine 15 in response to DNA breaks [401-406]. Previous experiments from the Lee group suggested that DNA-PK is required for phosphorylation of p53 on serine 15 and indispensible for latent p53-mediated apoptosis in response to IR [396, 398]. The fact that ATM/ATR/DNA-PK all phosphorylate p53 on serine 15 brings up one important question: What determines the specific kinase for phosphorylation of p53-Ser15 upon DNA damage? Regarding the role of DNA-PK in activating p53's apoptotic function, it is possible that the extent of DNA damage is a determinant factor. To address this question, DNA-PK catalytic subunit (DNA-PKcs) gene knock-out and wild-type mouse embryonic fibroblasts (MEF) were damaged with increasing doses of IR (5, 10 and 20 Grays) and harvested at five minutes and one hour post IR. Phosphorylation of p53-Ser15 was examined in these cells. Interestingly, although DNA-PK was not required for phosphorylation of p53-Ser 15 upon a low dose of IR (5 Gys), it was clearly required upon a high dose of IR (20 Gys) (Figure 23-A, lane 3, 7, 10 and 14). This result confirmed that DNA-PK is involved in the phosphorylation of p53-Ser15

upon IR, at high doses in mice. It was also reasoned that upon a low dose of IR, phosphorylation of p53-Ser15 might be regulated by other kinases, presumably ATM/ATR. The mechanisms underlying activation of specific upstream kinases in response to different IR doses remain elusive. This might reflect the cellular balance favoring cell death in response to high dose of IR, where DNA-PK dominates the activation of p53. Based on this and other observations made previously by Dr. Lee's group, the role of DNA-PK in activating the latent or pre-existing p53 in human cells in response to other types of DNA damage (e.g. UV radiation) was investigated.

The definition of latent p53 is somewhat obscure. Here, it is referred to as the p53 protein population under normal or non-damaged condition. p53 protein is unstable, constitutively undergoing proteasome-mediated degradation, which depends on the activity of ubiquitin ligases such as MDM2 [407]. Upon DNA damage, it is believed that phosphorylation of p53's N-terminal serine and threonine residues disrupts the interaction between p53 and MDM2, leading to accumulation of p53 [82, 401]. Thus, the so-called latent p53 is subject to rapid protein turnover. To study latent p53, one strategy is to block cellular protein translation, approached by treating cells with cycloheximide. Cycloheximide is an inhibitor of protein biosynthesis in eukaryotic organisms, acting to interfere with the step of translational elongation [408]. The strategy of experimental design is depicted in Figure 23-B. Human foreskin fibroblast Hs68 cells (passage number below thirty) were either UV irradiated (UV cross linker, Stratagene) with two doses of 50 joule/m^2 and 200 joule/m^2, or non-damaged. Immediately after UV radiation, cells were cultured in the presence or absence of cycloheximide and/or DNA-PK inhibitor (Calbiochem) (Figure 23-C). In parallel, Hs68 cells with p53 being stably knocked down

(Hs68-p53-KD) were treated the same. p53-Ser15 phosphorylation was examined in these cells after one hour. UV radiation induced phosphorylation of p53-Ser15 in Hs68 cells with control shRNA (Hs68-control) (Figure 23-C, top panel, lane 1, 5 and 9). In the presence of cycloheximide, p53-Ser15 phosphorylation was inhibited after both doses of UV, suggesting that this phosphorylation mainly occurred on newly translated p53 protein (Figure 23-C, top panel, lane 5, 6, 9 and 10). DNA-PK inhibitor also inhibited p53-Ser15 phosphorylation after both doses of UV ((Figure 23-C, top panel, lane 5, 7, 9 and 11). This indicated that DNA-PK is required for p53-Ser15 phosphorylation upon UV radiation. As discussed earlier, cycloheximide blocks new protein synthesis. Thus, phosphorylation of p53-Ser15 in the presence of cycloheximide was considered to occur on the latent population (Figure 23-C, top panel, lane 6 and 10). Unexpectedly, inhibition of DNA-PK did not block phosphorylation of the latent p53 population (Figure 23-C, top panel, lane 6, 8, 10 and 12). It has been reported that ATR kinase phosphorylates p53-Ser15 after UV [405]. However, in another experiment, LY294002 and Wortmannin (PI3-kinase inhibitors), although blocking phosphorylation of newly translated p53 (ser15) after UV, could not inhibit that of latent p53 (Figure 23-D, lane 4, 6, 8 and 10). These observations excluded the roles of DNA-PK, ATM/ATR and other PI3 kinases in phosphorylating the latent p53 on serine 15 after UV radiation. Furthermore, inhibition of DNA-PK and other PI3 kinases did not affect p53 stability after UV, indicating that phosphorylation of p53-Ser15 is not required for stabilization of p53 protein after UV damage (Figure 23-D, bottom panel, lane 3, 5, 7 and 9). It is possible that phosphorylation on other residues (e.g. serine 20) is relevant to p53 stabilization under this condition. Data presented thus far demonstrated that although DNA-PK is required

for serine 15 phosphorylation on latent p53 upon IR, it might only phosphorylate newly synthesized p53 upon UV radiation. However, it was interesting to see that in Hs68-p53-KD cells, phosphorylation of p53-Ser15 after UV radiation was not affected by cycloheximide and/or DNA-PK inhibitor (Figure 23-C, bottom panel, lane 5-8 and 9-12). This result clearly showed that p53 in Hs68-p53-KD cells might represent a p53 population that is not sensitive to cycloheximide treatment. More importantly, phosphorylation of p53-Ser15 in Hs68-p53-KD cells could not be blocked by DNA-PK inhibitor. Taken together, it was speculated that there probably is a stable p53 sub-population in Hs68 cells that is refractory to RNAi and protein degradation.

The Hs68-p53-KD cell line was established by retrovirus-mediated RNAi (pSUPER-Retro, Oligoengine), thus constantly expressing shRNA against p53. What appealed to me most was the possibility that this cell line could serve as an excellent investigative tool for the latent p53. Blocking protein synthesis with cycloheximide has its limitations, and is thus the last option to be taken for the study of p53 functions. Although cycloheximide was useful for the study of latent p53, its cytotoxic side effects make this drug unsuitable for time-course studies (e.g. over 12 hours).

4.2.2 Latent p53 That Is Resistant To RNAi Represents A Stable Protein Sub-Population In Hs68 Cell

Protein stability of p53 in Hs68-p53-KD cells was investigated by blocking new protein synthesis. As discussed earlier, p53 is constitutively degraded through proteasome under normal condition. Therefore, blocking new protein synthesis was expected to cause p53 loss over time. Hs68-control and Hs68-p53-KD cells were treated with cycloheximide and harvested at 0, 30, 60, 90, 120, 240 and 480 minutes after treatment. Total p53 protein levels were examined following the time course. Without new protein

synthesis, p53 protein levels in Hs68-control cells decreased over time (Figure 24-A, lane 1-7). In contrast, p53 protein levels in Hs68-p53-KD cells were relatively stable, although decrease was seen at early times after blocking new protein synthesis (Figure 24-A, lane 8-14). Interestingly, p53 protein levels were comparable between control and p53-KD cells after 240 minutes (Figure 24-A, lane 5-7 and 12-14). The existence of a "stable" sub-population of p53, which is different from the sub-population undergoing fast protein turnover, was proposed to explain this result. Without new proteins being translated, cellular pool of fast-turnover p53 was quickly depleted, within few hours. In contrast, the "stable" p53 sub-population was less sensitive to cycloheximide treatment. RNAi and cycloheximide inhibited the "fast-turnover" but not the "stable" sub-population. This could explain why p53 protein levels (i.e. stable sub-population) are comparable in both control and p53-KD cells 240 minutes after cycloheximide treatment (Figure 24-A, lane 5-7 and 12-14).

Accumulation of p53 after DNA damage is largely attributed to the disruption of p53-MDM2 interaction, accomplished by phosphorylation of both proteins by DNA damage-activated protein kinases such as ATM/ATR and Check-point kinase 1 and 2 (Chk1 and Chk2) [409-412]. In response to UV, p53 accumulated in Hs68-control cells (Figure 24-B, top panel, lane 1-7). Strikingly, in Hs68-p53-KD cells, UV radiation could not cause the accumulation of p53 at all (Figure 24-B, top panel, and lane 8-14). Interestingly, it seemed that kinetics of p53 accumulation in Hs68-control cells depended on the severity of DNA damage. Upon UV radiation of 50 joule/m^2, cells accumulated the highest levels of p53 protein at 6 hours. UV radiation of 250 joule/m^2 led to extended period of p53 accumulation up to 12 hours (Figure 24-B, top panel, lane 2-4 and 5-6). In

sharp contrast, p53 protein levels in Hs68-p53-KD cells remained constant during the time course after UV radiation (Figure 24-B, lane 8-14). This strongly suggested that latent p53 in Hs68-p53-KD cells was not subject to p53-MDM2 negative regulation, which dynamically maintains cellular p53 homeostasis.

To examine the stability of latent p53 after DNA damage, Hs68-control and Hs68-p53-KD cells were UV irradiated in the presence or absence of cycloheximide. As expected, in control cells, UV induced p53 accumulation (Figure 24-C, top panel, lane 1-5). Without new protein synthesis, cellular p53 levels decreased rapidly within 30 minutes (Figure 24-C, top panel, lane 1 and 6-9). Interestingly, UV radiation only slowed down the degradation of latent p53, which still proceeded in 4 hours (Figure 24-C, top panel, lane 6-9 and 10-13). In contrast, latent p53 in Hs68-p53-KD cells degraded with a slower rate (Figure 24-C, bottom panel, lane 1 and 6-9). Importantly, degradation of latent p53 protein in Hs68-p53-KD cells was blocked by UV radiation (Figure 24-C, bottom panel, lane 6-9 and 10-13). These results indicated that latent p53 in Hs68-p53-KD cells is regulated differently from that in Hs68-control cells or it represents a unique sub-population of p53. Accordingly, the latent p53 in Hs68 cells could be considered to consist of at least two sub-populations. One sub-population undergoes fast protein turnover, presumably regulated by ubiquitin ligases such as MDM2, and therefore can be rapidly stabilized by UV radiation. On the other hand, the "stable" sub-population undergoes slow protein turnover, independent of MDM2-mediated regulation, and therefore cannot be rapidly stabilized by UV radiation. It was possible that the failure of Hs68-p53-KD cells to accumulate p53 in response to UV was simply due to insufficient p53 mRNA abundance, resulting fromRNAi. However, this possibility obviously

conflicts with the observation that latent p53 in Hs68-p53-KD cells degraded much slower.

Based on what was proposed, at least two p53 sub-populations were activated by UV radiation in Hs68-control cells. However, in Hs68-p53-KD cells, only the "stable" p53 sub-population was activated. Therefore, Hs68-p53-KD cells could be employed as an substitutive research tool to cycloheximide treatment for the study of latent p53. This made it possible to investigate phosphorylation of the latent p53 in a relatively long time window. Hs68-control and Hs68-p53-KD cells were irradiated with two doses of UV (50 and 250 joule/m^2) and then monitored for phosphorylation of p53-Ser15 at time-points of 0, 12, 24 and 48 hours post UV. In Hs68-control cells, p53-Ser15 phosphorylation was induced by UV radiation. Interestingly, in response to low dose UV (50 joule/m^2), p53-Ser15 phosphorylation persisted for the first 24 hours then decreased (Figure 24-D, top panel, lane 1-4). In response to high dose UV (250 joule/m^2), p53-Ser15 phosphorylation remained at higher levels throughout the time course (Figure 24-D, top panel, lane 5-7). Comparison of corresponding total p53 levels at each time-point showed that p53 protein kept accumulating until 48 hours after high dose UV (250 joule/m2) but reached its peak at 24 hours after a lower dose of UV (50 joule/m2) (Figure 24-D, middle panel, lane 1-4 and 5-7). This suggested that the extent of p53 accumulation and phosphorylation of p53-Ser15 was determined by the severity of DNA lesions. It was possible that cellular DNA repair machinery could fix the majority of DNA lesions caused by low dose UV (50 joule/m^2) at 24 hours, thereby preventing further p53 activation. In contrast, in response to high dose UV (250 joule/m^2), which might exceed the capacity of cellular DNA repair; an extended activation of p53 was kept. This indicated that p53 protein is involved in

DNA repair of UV-induced lesions. p53 promotes cellular DNA repair in response to UV by transactivating genes such as *p48-DDB2* and *XPC* [163, 164]. P48-DDB2 is the key downstream gene responsible for the transport of XPC to sites of DNA damage in irradiated cells [165, 166]. Besides, p53 may function as a chromatin-accessibility factor in nucleotide excision repair (NER) and this function of p53 is independent of transcription [158]. Another p53-regulated DNA repair gene is *RRM2B,* whose product is ribonucleoside-diphosphate reductase subunit M2B, also known as P53R2 [413-416]. In Hs68-p53-KD cells, high dose UV did not trigger more phosphorylation of p53-Ser15 than low dose UV did (Figure 24-D, top panel, lane 9-11 and 12-14). As proposed, the p53 pool in Hs68-p53-KD cells is mainly composed of the "stable" sub-population. In contrast, the p53 pool in Hs68-control cells contains both "stable" and "fast-turnover" sub-populations. Therefore, it was reasonable to assume that DNA repair and probably other functions such as cycle arrest are mediated by the "fast-turnover" sub-population of p53, which is inducible and thus accumulates upon DNA damage. The lack of this p53 sub-population in Hs68-p53-KD cells might render these cells more sensitive to UV-induced apoptosis, possibly because of impaired DNA repair.

4.2.3 Knocking Down p53 Sensitizes Hs68 Cells To UV-Induced Apoptosis Due To Impaired Cellular DNA Repair

It was speculated that lack of the "fast-turnover" or inducible p53 sub-population in Hs68-p53-KD cells could lead to down-regulated cellular DNA repair thereby sensitizing cells to death because the "stable" sub-population of p53, as proposed, is capable of triggering apoptosis. It has been reported that p53 could cause mitochondrial outer membrane permeabilization (MOMP) independent of transcription, triggering cellular intrinsic apoptosis pathway. One key step in the intrinsic apoptosis pathway is

activation of pro-caspase 3, a member of the cysteine-aspartic acid protease (caspase) family [417, 418]. Release of Cyto. c from mitochondria into the cytoplasm facilitates the formation of apoptosome by pro-caspase 9, Cyto. c and Apaf-1. Pro-caspase 9 is then activated to cleave itself and other pro-caspases including pro-caspase 3 [419, 420]. Proteolytic processing of pro-caspase 3 produces two subunits, large and small, that dimerize to form the active enzyme . Activation of pro-caspase 3 is considered as a "no return" point, after which cells enter the execution phase of apoptosis. Therefore, if the "stable" sub-population of p53 is capable of triggering cell death, it most likely acts through MOMP-apoptosome-caspase 3 pathway[421].

To test this possibility, Hs68-control and Hs68-p53-KD cells were irradiated with two doses of UV (50 and 250 joule/m^2) and harvested at indicated time-points. Although caspase 3 cleavage could be triggered by UV radiation at 48 hours in Hs68-control cells, it occurred earlier (at 24 hours) and more extensively in Hs68-p53-KD cells (Figure 25-A, underlined lanes). This clearly showed Hs68-p53-KD cells were more sensitive to UV-induced apoptosis, through enhanced activation of pro-caspase 3.

Activation of caspase 3 will cause a series of proteolytic cleavage of cellular proteins including other pro-caspases and endonucleases such as DNA fragmentation factor (DFF), a heterodimeric protein of 40-kD (DFFB) and 45-kD (DFFA) subunits [422]. DFFA is the substrate for caspase-3 and triggers DNA fragmentation during apoptosis [422]. MOMP also releases endonuclease G, which cleaves chromatin DNA into nucleosomal fragments independently of caspases [422, 423]. To examine whether there is enhanced chromatin fragmentation or DNA breaks in Hs68-p53-KD cells after UV radiation, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end

labeling (TUNEL) was employed. TUNEL is a method for detection of DNA breaks by labeling the terminal end of nucleic acids, which can be identified by the enzyme of TdT. Accordingly, TUNEL also labels cells that contain severe DNA damage. Hs68-control and Hs68-p53-KD cells were grown on chamber slides and treated as indicated (Figure 25-B). Cells were stained with *In Situ* Cell Death Detection Kit-TMR red (TUNEL-based) from Roche Applied Science, according manufacturer's instruction. Cells stained red represent DNA nicks positive cells, resulting from DNA damage lesions or chromatin cleavage. Surprisingly, Hs68-p53-KD cells demonstrated strong staining of cellular DNA breaks, even in the absence of UV radiation (Figure 25-B. image A, B, C and D). This suggested an impaired cellular DNA repair in Hs68-p53-KD cells, where DNA lesions occurred even without UV radiation (Figure 25-B, image A and C). Non-damaged Hs68-control cells stained negative, confirmed that signals present in non-damaged Hs68-p53-KD cells were not likely artificially generated (Figure 25-B. image A and C). Hs68-control cells damaged with UV showed positive staining, indicating the presence of either DNA lesions or chromatin fragmentation. Hs68-p53-KD cells damaged with UV showed enhanced staining in comparison with both non-damaged Hs68-p53-KD and UV-damaged Hs68-control cells (Figure 25-B, image B, C and D). Enhanced staining of Hs68-p53-KD cells after UV could be due to accumulated irreparable DNA lesions and/or chromatin fragmentations resulting fromactivated endonucleases. Negative staining (without TdT) and positive staining (with TdT and pre-treated with DNase I) were included to control experimental procedures (Figure 25-B, image E-H). RNAi-mediated inhibition of the "fast-turnover" p53 sub-population might account for the accumulation of DNA lesions in Hs68-p53-KD cells. This also supports the role of p53 as

the “genome guardian”. However, the fact that those Hs68-p53-KD cells underwent enhanced apoptosis indicates that the “stable” sub-population of p53 is still capable of inducing apoptosis, or that the “fast-turnover” sub-population of p53 is anti-apoptotic. One important question to be asked, then, is how is this “stable” sub-population of p53 regulated?

4.2.4 UV Radiation Activates Serine 46 Phosphorylation Of The Stable Sub-Population Of p53 Protein

Phosphorylation of p53-Ser15 might not be crucial for activation of the “stable” p53, because UV radiation could not enhance it over time (Figure 24-D, top panel, lane 8-14). Therefore it was speculated that phosphorylation of other sites might be relevant. It has been reported that phosphorylation of serine 46 is critical for p53-mediated apoptosis [424-427]. Accordingly, phosphorylation of p53-Ser46 was examined in Hs68-control and Hs68-p53-KD cells after UV radiation. Another DNA-damaging agent Etoposide was included in this experiment because it potently stabilizes p53 without causing apoptosis at the concentration used (data not shown). Cells were damaged with either UV (250 joule/m^2) or Etoposide (50 μM) and harvested at 30 and 90 minutes post treatment. Although total p53 levels in Hs68-p53-KD cells were significantly lower than those of Hs68-control cells, phosphorylation of p53-Ser46 was comparable between these two cells at 90 minutes post UV radiation (Figure 26, lanes with red arrows). This result strongly suggested that phosphorylation of p53-Ser46 occurred on a specific sub-population of p53 protein, mostly likely the “stable” p53. Despite inducing high levels of p53, etoposide did not cause phosphorylation of p53-Ser46, suggesting phosphorylation of p53-Ser46 and accumulation of p53 protein are regulated separately. This result provided evidence for the first time that a sub-population of p53 can be specifically

activated upon UV radiation. As argued above, this sub-population of p53 might represent a “stable” form that is regulated differently from the “fast-turnover” p53.

4.2.5 The Latent p53 Sub-Population In p53 Knock-Down Hs68 Cells Is Localized In Peri-Nuclear Region

p53 constantly undergoes nucleocytoplasmic shuttling. This protein has two nuclear export sequences (NES) in N-terminal and C-terminal, and a nuclear localization sequence (NLS) in its C-terminal tetramerization domain [428-430]. Many hypotheses have been proposed for the mechanisms of p53 transport through the nuclear pore, with MDM2-mediated export and ubiquitination being two key elements [431]. Under normal conditions, p53 proteins are exported together with MDM2 into the cytoplasm, where both proteins are degraded [432, 433]. As proposed above, the “stable” sub-population of p53 protein might not be regulated by ubiquitination-proteasome degradation. Thus, sub-cellular localization of the “stable” p53 protein is an important question needing to be answered. To this end, sub-cellular localization of p53 protein in Hs68-control and Hs68-p53-KD cells was examined with immunofluorescence (IF) staining. Cells were grown on chamber slides to sub-confluence then fixed with paraformaldehyde. A polyclonal anti-p53 antibody (FL393, Santa Cruz) was used for immunofluorescence staining of p53 protein. Cell nucleus was visualized by 4', 6-diamidino-2-phenylindole (DAPI), which binds strongly to A-T rich regions in DNA. In Hs68-control cells, p53 was found primarily in the nucleus (Figure 27, image 1-3). However, it was surprising to see that, in Hs68-p53-KD cells, p53 was stained in the peri-nuclear region of the cell (Figure 27, image 4-6). In addition, interestingly, a sub-population of p53 showed punctate staining within the nucleus (Figure 27-A, image 6). It was originally speculated that peri-nuclear staining pattern might reflect mitochondrial localization of the “stable” p53 sub-

population. To test this possibility, Cyto. c, which is exclusively in mitochondria under normal conditions, was chosen as a marker for mitochondria in a co-localization experiment using confocal microscope (Zeiss LSM 510). Again, p53 protein in Hs68-control cells was stained primarily in the nucleus, with a small fraction in the cytoplasm. It seemed that cytoplasmic p53 is co-localized with mitochondria, as indicated by Cyto. c staining (Figure 27-B, image 1-3). In contrast, p53 staining in Hs68-p53-KD cells showed two patterns: dots in the nucleus and a crescent in the cytoplasm. When merged with Cyto. c staining, it seemed the cytoplasmic portion of p53 partially overlay with mitochondria (Figure 27-B, image 4-6). In an effort to confirm observed "mitochondrial localization" of the cytoplasmic p53, cells were examined under high magnification (100X). Interestingly, results showed that the cytoplasmic p53 fraction was in fact not co-localized with Cyto. c (Figure 27-C). While Cyto. c staining showed a pattern throughout the cytoplasm, p53 stained exclusively peri-nuclear. Close examination revealed that those p53, although in close vicinity to Cyto. c, were localized inside lumens of unknown structures, probably endoplasmic reticulum (ER) or Golgi apparatus (Figure 27-C, image 4-6). To confirm that Cyto. c represents mitochondria indeed, another protein, the mitochondrial heat-shock protein 70/GRP75 was examined for its co-localization with Cyto. c. Result clearly demonstrated a complete overlay of Cyto. c and GRP75, thus validating Cyto. c as a reliable mitochondrial marker. These results revealed that sub-cellular localization of p53 is heterogeneous. Although p53 was primarily stained in the nucleus in control cells, sub-populations could be localized into discrete areas such as peri-nuclear or nuclear organelles. Association of p53 with ribosomal RNA, nucleolus and PML body has been reported before [434-441]. Therefore it is likely that p53 stained

in Hs68-p53-KD cells was associated with cellular organelles. Further studies will be needed to identify the organelles in which the "stable" p53 sub-population resides.

4.3 Discussion

Results presented here demonstrate that composition of latent p53 protein pool is heterogeneous. p53 protein sub-populations are regulated differently, probably due to their discrete sub-cellular localizations. For example, p53 covalently associated with ribosomal RNA probably functions differently from the nuclear p53 sub-population and, correspondingly, they might be regulated differently. Previous studies in Dr. Lee's laboratory demonstrated that latent p53 in MEFs is sufficient for triggering apoptosis in response to IR, for which DNA-PK is required [396-398]. However, more detailed mechanisms have not been developed. For example, is transcriptional activity required for latent p53-mediated apoptosis? If not, what is the mechanism of action of the latent p53? Moreover, is DNA-PK involved in latent p53-mediated apoptosis in response to other types of DNA damage? The work described here was initiated to address these questions.

The Hs68 human foreskin fibroblast cell line was chosen because of its normal genetic background and non-transformed phenotype, which is close to that of MEFs. Cycloheximide was used to inhibit new protein synthesis, such that only the latent p53 would be available for the cell to respond to UV radiation. First, the potential role of DNA-PK in activating latent p53 in response to UV radiation was investigated. DNA-PK can phosphorylate p53 on serine 15, a key residue also targeted by many protein kinases from distinct cellular pathways [401, 405, 442-444]. Phosphorylation of p53-Ser15 is

largely dependent on cellular context. Experiments using DNA-PKcs gene knock-out MEFs demonstrated that p53-Ser15 phosphorylation was regulated by DNA-PK in response to high dose of IR (10, 20 Grays) (Figure 23-A). Interestingly, p53-Ser15 was phosphorylated equally well between wild-type and DNA-PK knock-out MEFs in response to low dose IR (5 grays), implying that DNA-PK is not always needed. In response to mild DNA damage, ATM/ATR might be charging the activation of p53, leading to cell growth arrest instead of apoptosis. Therefore, severity of DNA damage may decide which upstream pathway is activated and consequently, which p53 function is activated, to live or to die. Based on this notion, two doses of UV radiation (50 joule/m^2 and 250 joule/m^2) were used where permitted. UV-induced phosphorylation of p53-Ser15 was inhibited by both cycloheximide and DNA-PK inhibitor (Figure 23-C). Importantly, inhibiting DNA-PK had no effect on serine 15 phosphorylation of latent p53 because combined use of cycloheximide and DNA-PK inhibitor had the same effect as using cycloheximide alone. Furthermore, inhibiting other PI3 kinases including ATM/ATR had no effect on serine 15 phosphorylation of latent p53 (Figure 23-D). These experiments indicated thon serine 15 phosphorylation of latent p53 is regulated by other protein kinases in human fibroblasts upon UV radiation. However, the role of DNA-PK and other PI-3-like kinases in phosphorylating p53 at residues other than serine 15 could not be excluded. This experiment provided several lines of evidence. First, phosphorylation of p53-Ser15 occurred mostly on newly translated p53 proteins, as cycloheximide greatly inhibited this phosphorylation. Second, DNA-PK, and probably other PI-3 kinases as well (e.g. ATM /ATR) are required for serine 15 phosphorylation of induced p53 protein upon UV radiation. Third, intriguingly, p53-Ser15 phosphorylation

in Hs68-p53-KD cells could not be inhibited by cycloheximide and/or DNA-PK inhibitor (Figure 23-C). The last observation drew much interest because it implied that p53 protein in Hs68-p53-KD cells might represent a latent p53 sub-population.

According to classic views, latent p53 undergoes nucleocytoplasmic shuttling constitutively, mediated by oncoprotein MDM2, which exports p53 into the cytoplasm, where p53 is degraded in 26S proteasome [407]. Blocking new protein synthesis theoretically will lead to rapid depletion of cellular p53 protein pool. However, p53 protein in Hs68-p53-KD cells was less sensitive to cycloheximide treatment, compared with p53 in Hs68-control cells (Figure 24-A). It was reasoned if p53 proteins between these Hs68 cell lines (control *versus* p53-KD) were biochemically the same, p53 protein levels in Hs68-p53-KD cells should decrease with a faster rate after cycloheximide treatment, because RNAi down-regulated cellular p53 mRNA so new protein production. In fact, although Hs68-p53-KD cells produced much less p53 protein than Hs68-control cells did before inhibition of new protein synthesis, they maintained comparable p53 protein levels 4 hours later (Figure 24-A), implying p53 protein stability was regulated differently between these Hs68 cell lines. On the other hand, it was expected that UV damage should stabilize p53 in Hs68-p53-KD cells because MDM2-mediated p53 degradation would be inhibited after DNA damage. However, the result again was opposite to the prediction. Although Hs68-control cells accumulated significant amounts of p53 after UV radiation in a dose-dependent manner, Hs68-p53-KD cells failed to do so (Figure 24-B). This result indicated in another way that p53 protein stability in Hs68-p53-KD cells is regulated differently from Hs68-control cells. Furthermore, latent p53 in Hs68-control cells, although stabilized by UV, still underwent protein degradation within

4 hours (Figure 24-C, top panel). In contrast, latent p53 in Hs68-p53-KD cells, once stabilized by UV, survived to 4 hours without significant degradation (Figure 24-C, bottom panel). The possibility of UV-triggered mRNA stabilization or enhanced translation of p53 in Hs68-p53-KD cells was excluded because UV did not cause p53 protein accumulation in Hs68-p53-KD cells (Figure 24-B and C). Based on these results, it was proposed that latent p53 in Hs68-p53-KD cells represents a "stable" sub-population, which is not subject to MDM2-mediated degradation. In contrast, latent p53 in Hs68-control cells is composed of at least two sub-populations, described as "stable" and "fast-turnover" for their stability and regulation by MDM2-proteasome pathway (Figure 28). Based on this proposed model, UV radiation induced accumulation of the "fast-turnover" sub-population but not the "stable" sub-population. Accordingly, Hs68-p53-KD cells could be used as a model cell line which exclusively, or mainly, contains the "stable" protein sub-population of p53. This made it possible to study latent p53 in a long time window, a plan not accomplishable with cycloheximide.

First, p53-Ser15 phosphorylation after UV radiation was monitored following a time course up to 48 hours. It was noticed that the extent of p53 accumulation and phosphorylation of serine 15 was co-related to UV dose and time-point. High dose UV (250 joule/m^2) provoked a strong p53 response that lasted for at least 48 hours in Hs68-control cells. However, low dose UV (50 joule/m^2) provoked a p53 response that peaked at 24 hours then decreased over time (Figure 24-D, lane 1-7). Phosphorylation of p53-Ser15 in Hs68-p53-KD cells was not affected by UV dose, although total p53 protein levels was slightly induced by low but not high UV dose (Figure 24-D, lane 8-14). An indication from this result was: the stabilized "fast-turnover" p53 protein in Hs68-control

cells probably is responsible for cellular DNA repair. This could explain why p53 protein levels was kept at high levels in response to high UV dose but decreased after 24 hours in response to low UV dose. p53 is known as the "guardian of genome" for the prominent role of this protein in maintaining genome integrity, which is challenged every moment in the cell by intrinsically occurring DNA lesions [169]. Thus, loss of the "fast-turnover" sub-population of p53 in Hs68-p53-KD cells would make the cell incapable of repairing excess amounts of DNA lesions caused by UV radiation. On the other hand, the presence of "stable" p53 sub-population in Hs68-p53-KD cells might still be sufficient to trigger apoptosis upon UV radiation.

If the "stable" p53 sub-population indeed is pro-apoptotic, it likely acts through a mitochondrial intrinsic apoptosis pathway, which leads to activation of caspase 3 [418, 421]. Interestingly, cleavage of caspase 3 in Hs68-p53-KD cells was more efficient and robust than in Hs68-control cells in response to UV radiation (Figure 25-A). There are two possibilities to explain this observation. First, p53 accumulation in Hs68-control cells after UV damage suppressed cellular apoptosis, such that blocking p53 accumulation in Hs68-p53-KD cells led to enhanced apoptosis. Second, the "stable" sub-population of p53 was pro-apoptotic, such that blocking accumulation of "fast-turnover" p53 in Hs68-p53-KD cells released rather than inhibited the apoptotic function of the "stable" p53. As proposed above, induction of p53 after DNA damage might be relevant to cellular DNA repair [158, 163-166, 413-416]. Defects in cellular DNA repair could sensitize cells to DNA damage-induced apoptosis, a principle underlying cancer chemotherapy. Hs68 cells are not transformed and are genetically normal. It was speculated that knocking down p53 in this cell line might cause genetic instability, probably due to down-regulated

cellular DNA repair capacity. Indeed, Hs68-p53-KD cells harbored considerable amounts of DNA breaks even in the absence of UV radiation (Figure 25-B). In contrast, there were almost no detectable DNA breaks in Hs68-control cells under normal conditions. This result supported the notion that p53 is involved in maintaining genomic stability. After UV radiation, it seemed Hs68-p53-KD cells accumulated more DNA breaks, indicated by stronger staining intensity (TUNEL) (Figure 25-B). This could be caused by apoptosis-associated chromatin fragmentation or unrepaired DNA lesions. In contrast, Hs68-control cells contained much less DNA breaks after UV radiation. Taken together, it was concluded that impaired DNA repair capacity might sensitize Hs68-p53-KD cells to UV-induced apoptosis. According to the proposed model, the "fast-turnover" p53 sub-population, which was inhibited by RNAi, is required for p53's DNA repair function. However, how the "stable" p53 sub-population is activated by UV radiation remained an important question, in regard to its potential role in triggering apoptosis. It was reasoned that the "stable" p53 sub-population possibly is regulated differently from the "fast-turnover" sub-population. Therefore, p53 sub-populations might be phosphorylated by different upstream kinases. Phosphorylation of p53-Ser46 has been reported to be critical for p53's apoptotic function [424, 425, 445, 446]. It was predicted that the "stable" p53 sub-population might be phosphorylated on serine 46 for its apoptotic function. Indeed, p53-Ser46 in Hs68-p53-KD cells was phosphorylated equally well as in Hs68-control cells after UV radiation, although total p53 levels are significantly different (Figure 26). This strongly supported the hypothesis that p53 sub-populations are regulated differently, as the induced p53 (i.e. "fast-turnover" sub-population) in Hs68-control cells was not phosphorylated on serine 46 under the same condition (Figure 26). Several kinases were

proposed to be potential regulators of this phosphorylation, including DNA-PK, dual-specificity tyrosine-phosphorylation-regulated kinase 2 (DYRK2), p38MAPK and homeodomain-interacting protein kinase-2 (HIPK-2). Future studies will be needed to identify the specific kinases involved in activation of the "stable" p53 sub-population after UV radiation.

If the "stable" p53 is regulated differently from the "fast-turnover" one, they are likely localized to distinct sub-cellular compartments. Accordingly, localization of the "stable" p53 sub-population was also examined. Interestingly, p53 protein in Hs68-p53-KD cells was found in peri-nuclear region in the cytoplasm and punctate regions in the nucleus (Figure 27-A, B and C). Co-localization experiments with mitochondrial components such as Cyto. c and GRP75 revealed that latent p53 in Hs68-p53-KD cells was in fact localized in lumens of unknown structures, likely endoplasmic reticulum (ER), but not in mitochondria (Figure 27-C). p53 has been reported to be covalently associated with 5.8S ribosomal RNA in the cytoplasm [434, 439]. Therefore, it will be interesting to investigate whether the "stable" p53 sub-population in Hs68 cells is actually associated with ribosomes. Also, further studies are needed to examine other possible organelle, to which p53 might be associated, for example, ER and Golgi. The nuclear-dot staining of p53 also needs to be addressed. Nuclear structures such as nucleoli and PML bodies are interesting targets to test, as associations of p53 with these structures were reported previously [434-441]. The fact that p53 in Hs68-control cells was stained mainly in the nucleus also suggested that the majority of cellular p53 is "fast-turnover" sub-population which freely distributed throughout the nucleus, thus are predominately regulated by MDM2-mediated protein degradation. Although there are

still many questions remain to be answered, the results acquired so far suggest that cellular p53 proteins are localized to distinct sub-cellular compartments and regulated differently. The “stable” p53 sub-population, which is likely exists in association with cellular organelles, might play an important role in UV-induced apoptosis (Figure 28).

4.4 FIGURE S AND LEGENDS

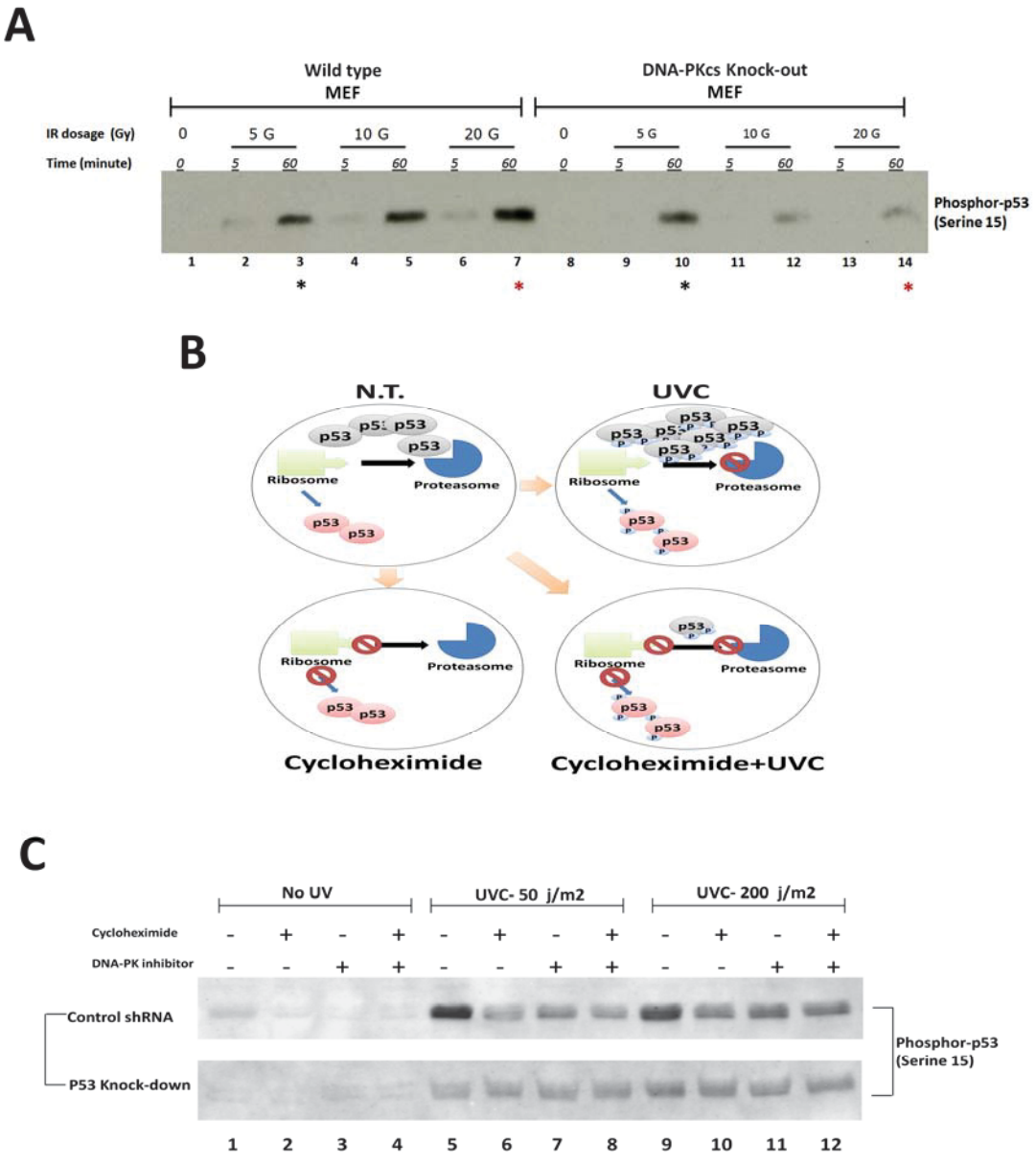


Figure 23. DNA-PK is required for phosphorylation of serine 15 on newly synthesized p53 but not latent p53 upon UV radiation.

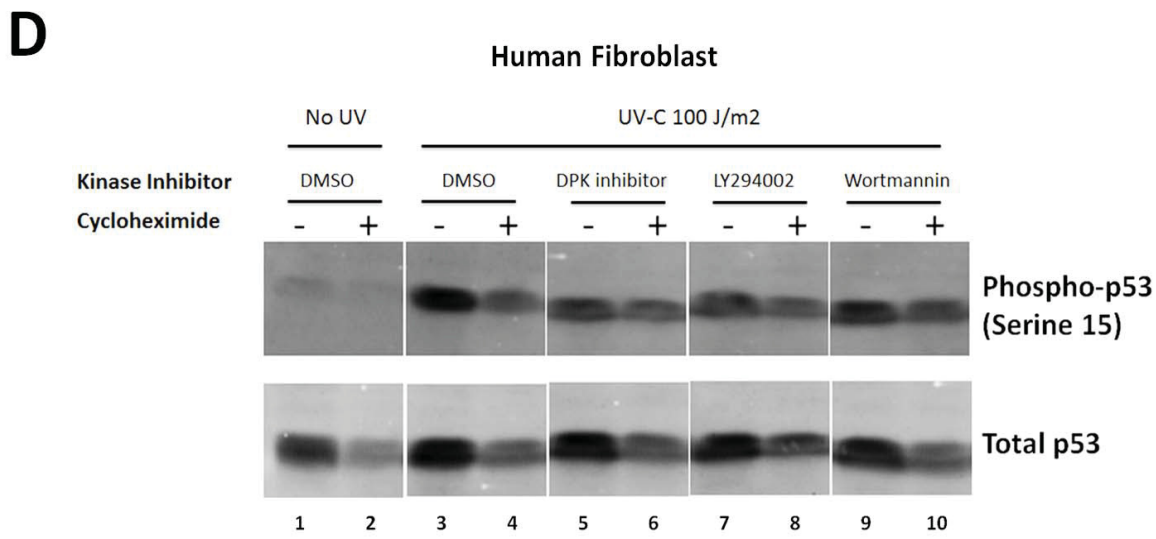


Figure 23. DNA-PK is required for phosphorylation of serine 15 on newly synthesized p53 but not latent p53 upon UV radiation. **(A)** Wild-type and DNA-PKcs -/- mouse embryonic fibroblasts (MEFs) were irradiated with increasing doses of IR at 5, 10 and 20 Gys, then harvested at indicated time-points. p53 serine 15 phosphorylation was examined by western blotting. **(B)** Schematic of experimental strategy of using the combination of cycloheximide and UV radiation to study latent p53. **(C)** Hs68 cells, (control and p53-KD) were treated with combinations of UV radiation (50 and 200 joule/m^2), cycloheximide (25 µg/ml) and DNA-PK inhibitor (5 µM) (Calbiochem). Cells were harvested one hour after treatment. p53 serine 15 phosphorylation was examined by western blotting. **(D)** Hs68 cells were non-damaged or UV irradiated (100 joule/m^2) in the presence of combinations of cycloheximide (25 µg/ml) and individual PI3 kinase inhibitors including DNA-PK inhibitor (5 µM), LY294002 (8 µM) and Wortmannin (5 µM). Cells were collected one hour after treatment and total p53 and phosphorylated p53

(serine 15) were examined by western blotting. Results are representative of two independent experiments.

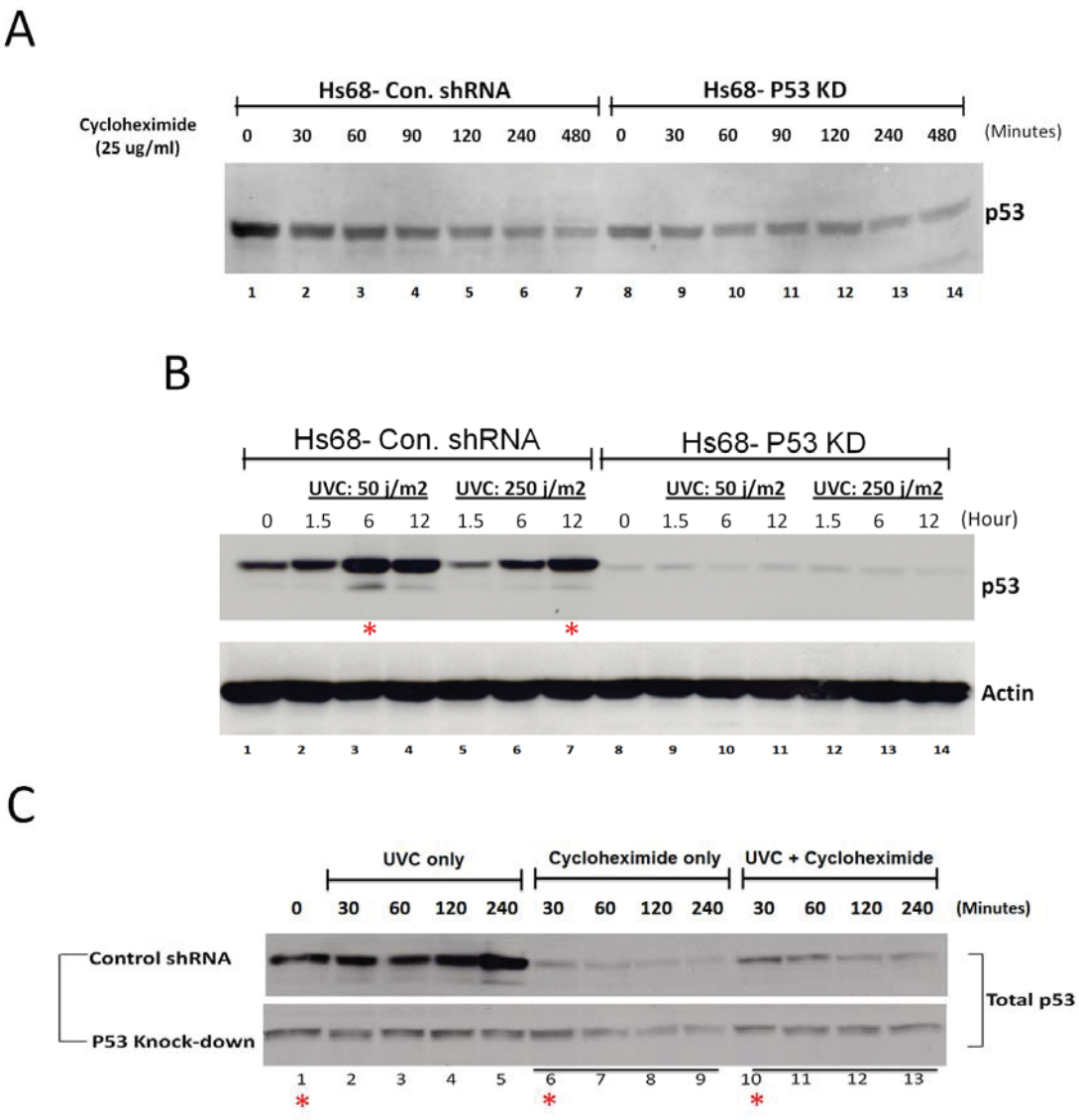


Figure 24. Latent p53 in HS68-p53-KD cells represents a stable protein sub-population.

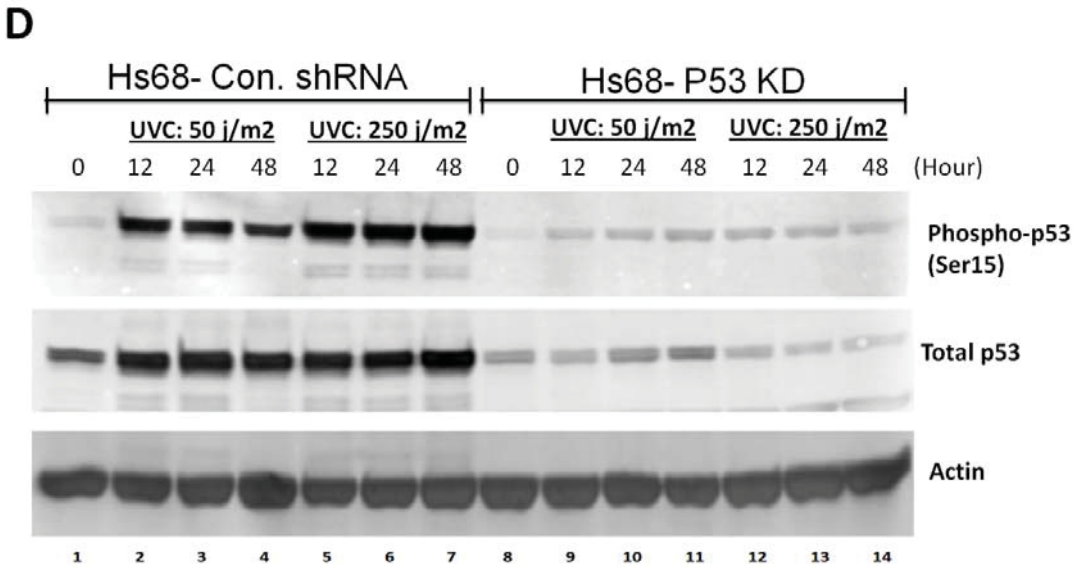


Figure 24. Latent p53 in HS68-p53-KD cells represents a stable protein sub-population. **(A)** Hs68-control and Hs68-p53-KD cells were grown in the presence of cycloheximide (25 μg/ml). Cells were collected at indicated time-points. Total p53 was examined by western blotting. **(B)** Hs68-control and Hs68-p53-KD cells were irradiated with two doses of UV (50 joule/m^2 or 250 joule/m^2). Cells were harvested at indicated time-points. Total p53 was examined by western blotting. **(C)** Hs68-control and Hs68-p53-KD cells were treated with cycloheximide or UV radiation alone, or in combination of both. After treatment, cells were collected at indicated time-points. Total p53 was examined by western blotting. Results are representative of two independent experiments. **(D)** Hs68-control and Hs68-p53-KD cells were UV irradiated (50 joule/m^2 or 250 joule/m^2) and harvested at 12, 24 and 48 hours post damage. Phosphorylated p53 (serine 15) and total p53 were examined by western blotting. Results are representative of two independent experiments.

A

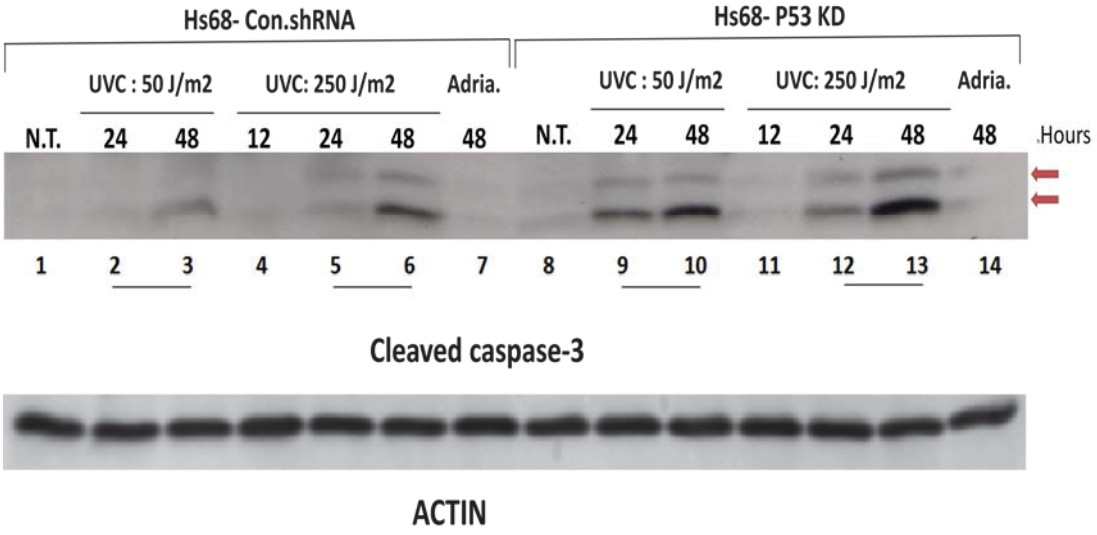


Figure 25. Knock-down of p53 sensitizes Hs68 human fibroblast cells to UV-induced cell death.

B

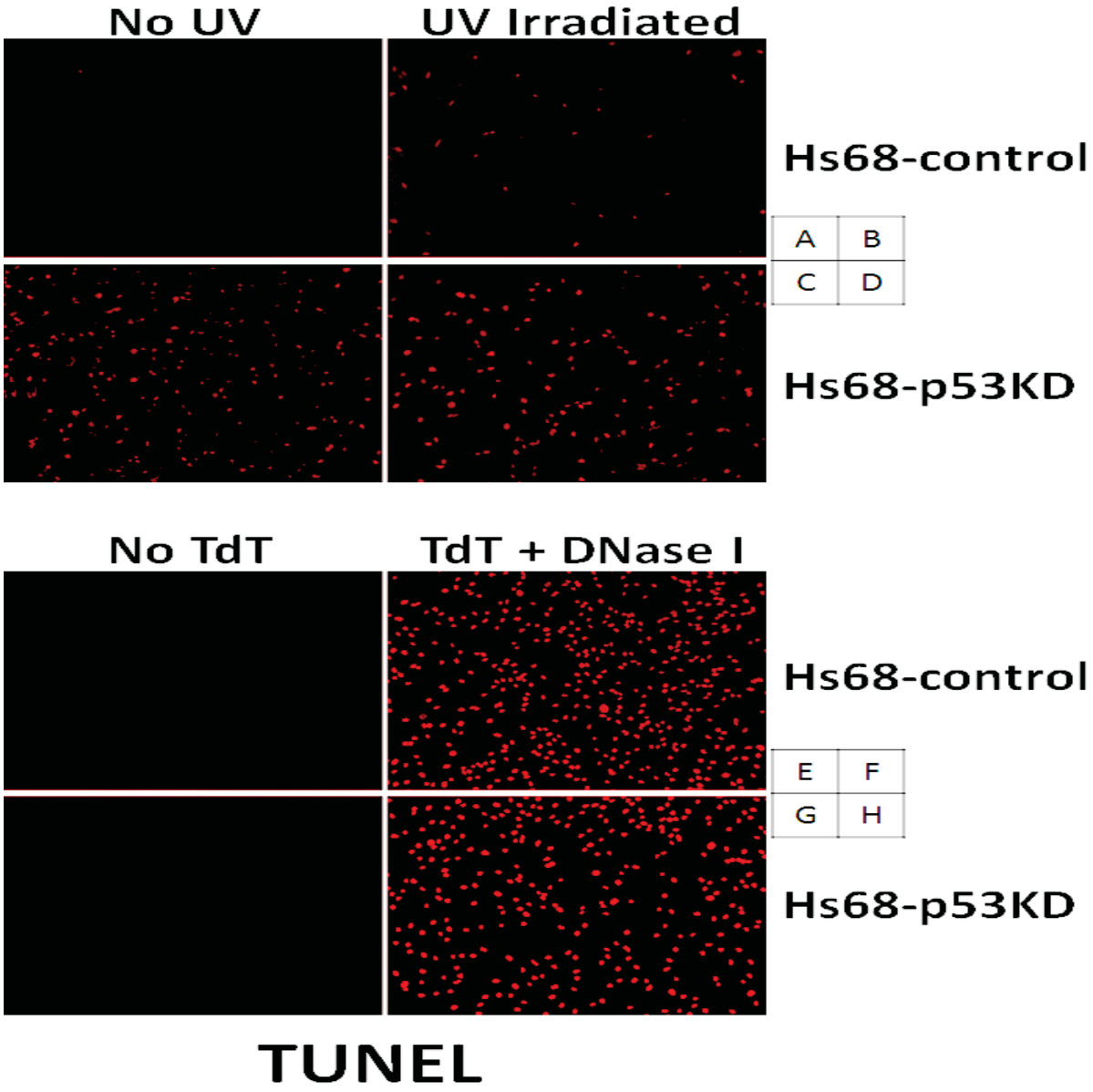


Figure 25. Knock-down of p53 sensitizes Hs68 human fibroblast cells to UV-induced cell death. **(A)** Hs68-control and Hs68-p53-KD cells were UV irradiated (50 joule/m^2 or 250 joule/m^2) or treated with Adriamycin (2 μg/ml). Cells were harvested at indicated time-points. Caspase 3 cleavage was examined by western blotting. Results are representative

of two independent experiments. **(B)** Knock-down of 53 leads to genomic instability due to impaired cellular DNA repair capacity. Detection of DNA breaks in Hs68 (control or p53-KD) was approached with TUNEL assay. Cells were either non-treated or UV-irradiated (100 joule/m^2). Red labelling represents the presence of DNA lesions within the cell. The intensity of signal co-relates with the amount of DNA breaks. Images E-H are control experiments showing negative staining without TdT and positive staining with TdT after DNase I treatment of fixed cells. Results are representative of three independent experiments.

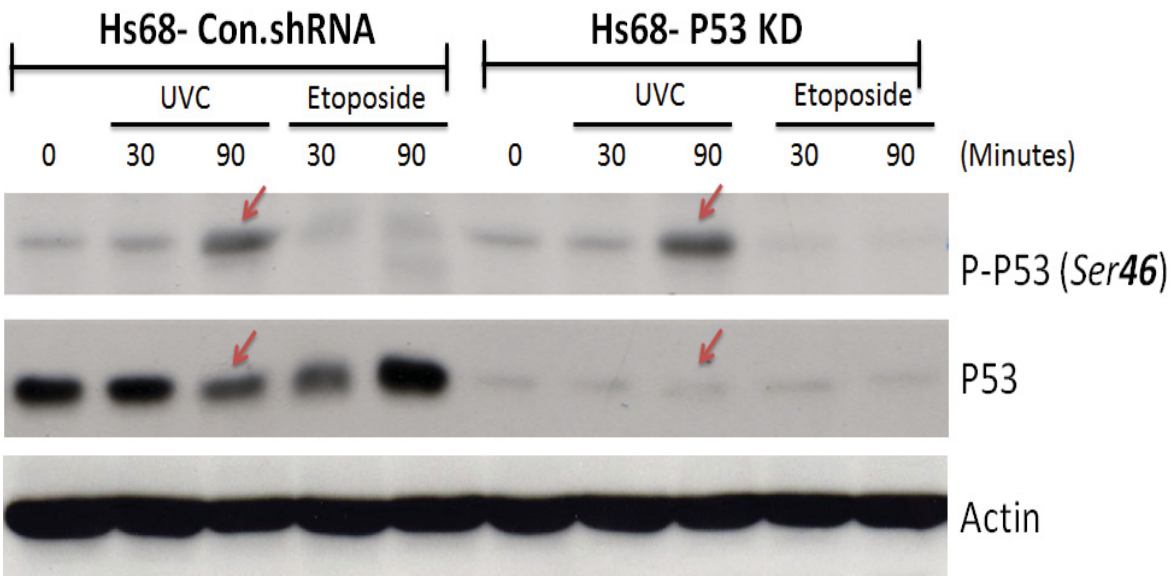


Figure 26. Phosphorylation of p53 serine 46 upon UV radiation cannot be inhibited by RNAi against p53 in Hs68 cells. Hs68 cells (control or p53-KD) were either UV irradiated (250 joule/m^2) or treated with etoposide (50 μM). Total p53 and phosphorylated p53 (ser 46) was examined at each time-point by western blotting. The results are representatives of two independent experiments.

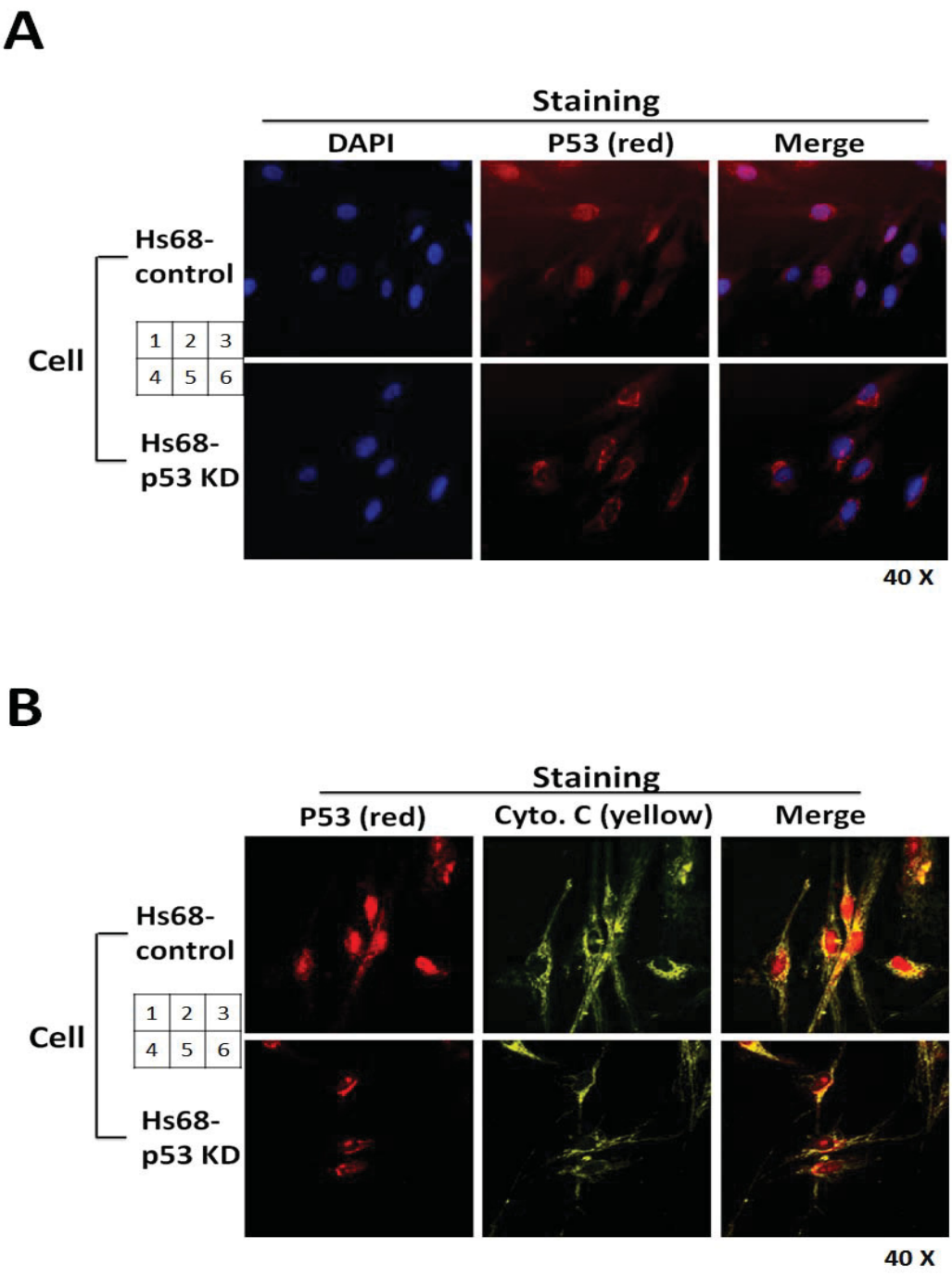
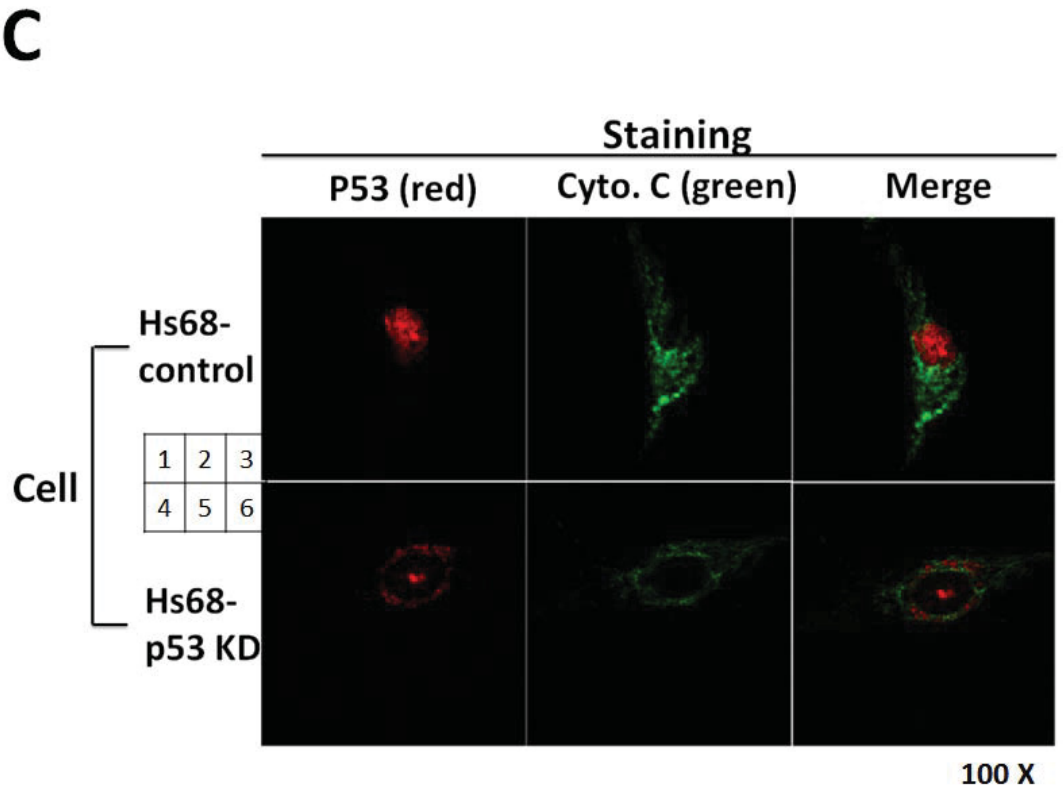


Figure 27. A sub-population of p53 is localized in peri-nuclear region.



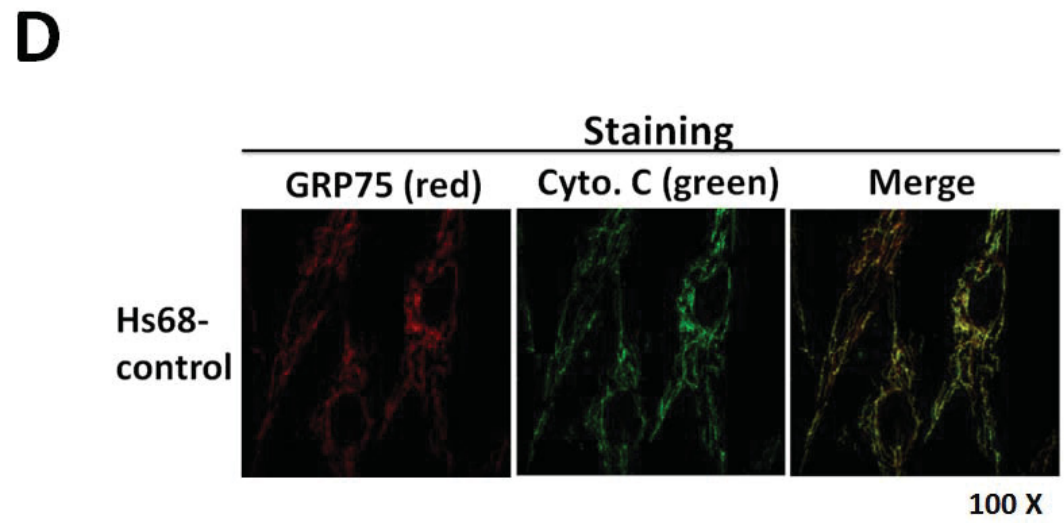


Figure 27. A sub-population of p53 is localized in peri-nuclear region. **(A)** Hs68 cells (control or p53-KD) were stained for endogenous p53 with a polyclonal anti-p53

antibody (FL-393, Santa Cruz). Cell nucleus was stained with DAPI. A Cy3-conjugated goat anti rabbit secondary antibody was use in this experiment. **(B)** Co-staining of p53 (red) and Cyto. c (yellow) in Hs68 cells (control or p53-KD). Cy3-conjugated goat anti-rabbit secondary antibody was used for p53. Cy5-conjugated goat anti-mouse secondary was used for detection Cyto. c. Images were acquired with 40X magnification on a confocal fluorescence microscope (Zeiss). Results are representative of three independent experiments. **(C)** Co-staining of p53 (red) and Cyto. c (green) in Hs68 cells (control or p53-KD). Cy3-conjugated goat anti-rabbit secondary antibody was used for p53. Cy5-conjugated goat anti-mouse secondary was used for detection Cyto. c. Images were acquired with 100X magnification on a confocal fluorescence microscope (Zeiss). **(D)** Co-staining of Grp75 (red) and Cyto. c (green) in Hs68 cells. Grp75 was detected with a rabbit polyclonal antibody. Cyto. c was detected with a mouse monoclonal antibody. Images were acquired with 100X magnification. Images are representative of three independent experiments.

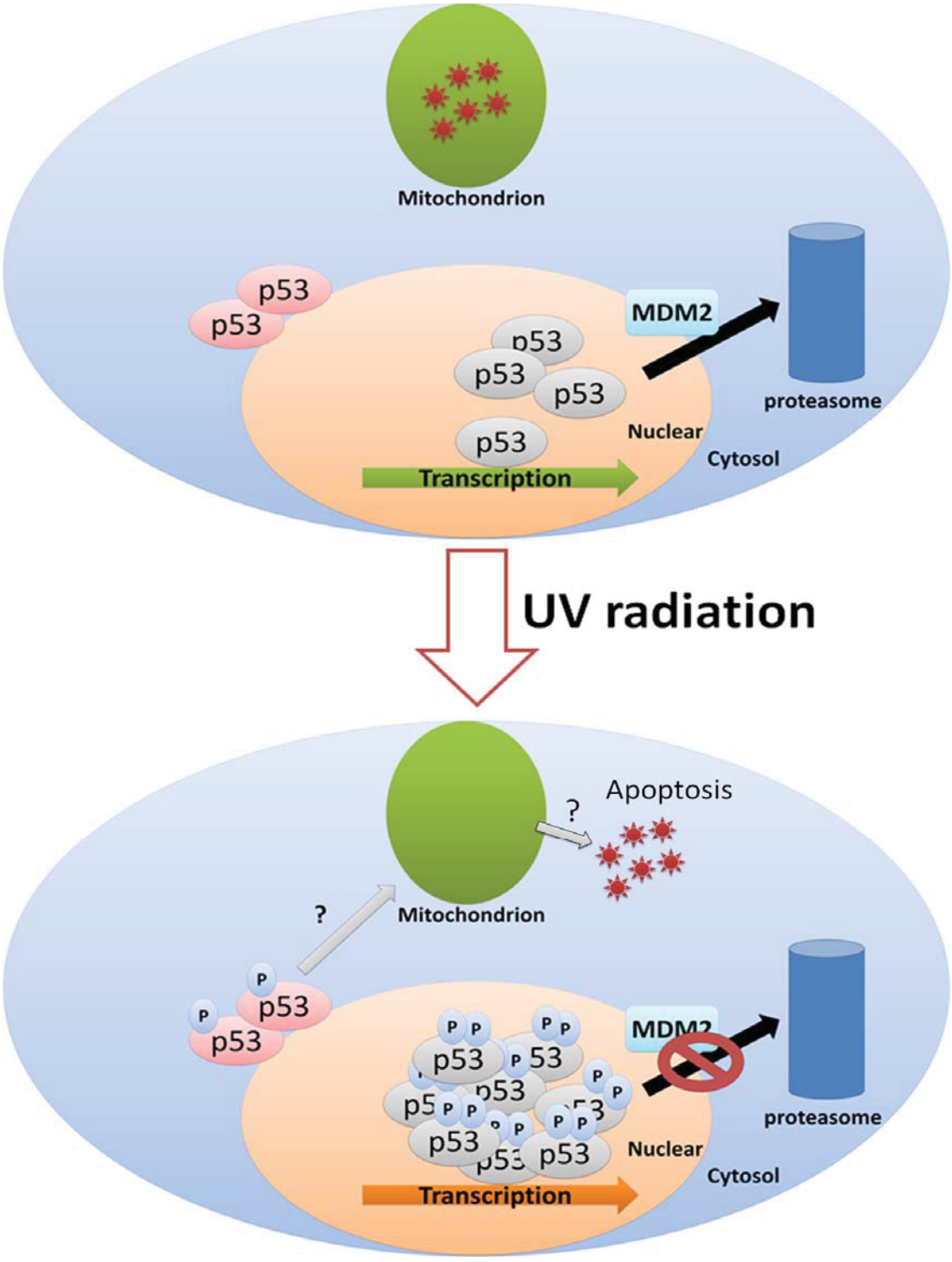


Figure 28. Proposed model: p53 protein sub-populations are regulated differently. Latent p53 proteins are localized into discrete sub-cellular regions. Nuclear p53 is targeted by

MDM2-mediated protein degradation. A stable p53 sub-population is probably associated with cellular structure or organelles. Upon UV radiation, nuclear p53 is stabilized by blocking MDM2 binding but stable p53 sub-population is activated by distinct mechanisms. See text for more details.

CHAPTER 5 CONCLUSION AND FUTURE DIRECTION

5.1 OVERVIEW: ACTIVATION OF P53'S APOPTOTIC FUNCTION

p53 is a tumor suppressor, as documented by numerous studies [1, 19, 72, 198, 215]. The prominent role of this gene in prevention of tumor formation has attracted extensive research efforts, resulting in an overwhelming collection of information. Contradictory conclusions were made based on experiments conducted with different models and methods. However, this also reflects that p53 has dual mechanisms in regulating cellular processes. It seems that p53 dictates cell fates into two directions: transient growth arrest and permanent elimination of the cell. Cellular stresses such as DNA damage, oncogene activation, hypoxia, nutrient depletion and telomere erosion can stimulate signaling pathways that often converge on the activation of p53. By regulating downstream genes with various functions, p53 directs cellular responses, to live or to die. For example, in response to mild DNA damage, p53 induces cell-cycle arrest allowing cells to repair DNA lesions. On the other hand, if DNA damage persists or cannot be repaired, p53 initiates apoptosis (Figure 3). Therefore, p53 can function as a protector or a killer. Mechanisms of the role-switching of p53 in regulating cellular stress responses are not fully understood. In my research, efforts were taken to address the question of how p53 is activated to induce apoptosis in response to DNA damage. Work in this thesis can be divided into two parts, which are covered under one topic of activation of p53's apoptotic function.

5.2 The Link Between p53 And Cellular NAD^+-Dependent Processes

Evidence presented here supports the conclusion that p53 can regulate its own acetylation by inducing the cytoplasmic NAD^+ synthetase gene Nmnat2, which expresses two protein isoforms with opposite regulatory roles toward cellular NAD^+-dependent histone deacetylases SIRT proteins, forming a p53-Nmnat2 feedback regulatory loop. Of the most importance is the discovery that Nmnat2-TV2 acts as an activator of DNA damage-induced acetylation of p53, whereas Nmnat2-TV1 acts as an inhibitor. In my proposed model, functions of p53 in cellular DNA damage responses are regulated by the balance of Nmnat2 isoforms in a feedback manner, through SIRT deacetylase-mediated protein deacetylation (Figure 21). Activation of p53's apoptotic function might depend on inhibition of cellular SIRT activities (e.g. SIRT1/2) by Nmnat2-TV2. On the other hand, p53's apoptotic function is inhibited by unrestrained cellular SIRT activities, which probably are activated by Nmnat2-TV1.

Establishment of the link between p53 and cellular NAD^+ synthesis pathway has profound impacts on research fields including neurodegeneration and cancer metabolism. The Nmnat2 gene was reported to be expressed at high levels in the brain [223, 328, 335, 447-449]. Studies carried out in the field of neuroscience suggested that NAD^+ synthetases including Nmnat2 mainly play protective roles, based on mouse model [328, 330, 347, 448-450]. However, these investigations were based on Nmnat2-TV1, as there is no mouse homologue of human Nmnat2-TV2 being found to date. Human and mouse Nmnat2-TV1 share a surprisingly high similarity (97%) in their protein sequences, indicating an essential role of this protein. It is possible that Nmnat2-TV2 is also

expressed in mice. Future studies are required to test this possibility. It will be interesting to investigate the role of the p53-Nmnat2 pathway in human neuro-degeneration.

The role of p53 in inhibiting cytoplasmic glycolysis and promoting mitochondrial functions has been reported [174, 198, 215, 313, 345, 451]. However, whether Nmnat2 proteins, especially Nmnat2-TV2, are involved in the regulation of cancer metabolism has not been investigated. Research in this direction may lead to discoveries of novel mechanisms of regulation in cancer metabolism and development of new strategies for cancer therapy.

If Nmnat2 isoforms function in opposition to regulate p53 functions, through SIRT proteins and possibly PARP as well, it is tempting to propose that cell fate might be dictated by the balance between these isoforms. Further studies are needed to address the mechanisms regulating this balance. In addition to being transcriptionally regulated by p53, it is possible that Nmnat2 proteins are subject to regulations by posttranslational modifications, which might regulate their protein stability, protein-protein interactions and NAD^+ synthetase activity. Nmnat2-TV2 protein contains five glutamic acids in its unique N-terminal sequence. It will be interesting to determine whether this protein is regulated by poly ADP-ribosylation. Nmnat2-TV1 protein contains two lysine residues within its unique N-terminal sequence. Protein acetylation prediction (web-based tools) suggests that these two lysines are excellent targets for acetylation. Therefore, Nmnat2 isoforms could be regulated by distinct cellular pathways (e.g. PARP and SIRT). Protein sequence alignment between Nmnat2 proteins and Nmnat1 protein revealed that N-terminal sequence of Nmnat2-TV1 but not Nmnat2-TV2 is homologous to that of Nmnat1. This implies that Nmnat2-TV1 might be functionally close to Nmnat1.

Biochemical characterization of Nmnat2 protein (based on TV1) suggested that this protein can form dimers [335]. It is wondered whether Nmnat2-TV2 dimerizes with Nmnat2-TV1 and, if it does, whether Nmnat2 heterodimers function differently from Nmnat2-TV1 homodimers in regulating cellular NAD^+ synthesis and SIRT activities. In other words, it is possible that Nmnat2-TV2 protein inhibits Nmnat2-TV1, for example, by changing its sub-cellular localization, protein stability or enzymatic activity. In addition to ectopic expression of Nmnat2 isoforms, it will be important to knock down each isoform individually in U2OS and other cell lines (e.g brain tissue origin). Isoform-specific gene knock-down of Nmnat2 has been undertaken and U2OS cell lines were established. Also, to identify other potential SIRT targets of Nmnat2 isoforms, knocking down and ectopic expression of SIRT3-7 will be beneficial.

5.3 p53 Protein Sub-Populations Are Regulated Differently

Cellular p53 protein undergoes fast-turnover, due to MDM2-mediated nuclear export and consequent cytoplasmic proteasome-dependent degradation [81, 355, 382, 452]. Protein stability is thought to play an important role in cellular stress-induced activation of p53. Upon DNA damage, upstream protein kinases such as ATM/ATR /DNA-PK are activated and initiate signaling cascades leading to stabilization and activation of p53 [86, 453]. However, p53 stabilization might not be necessary for its apoptotic function. Upon ionizing radiation, the latent p53 population in mouse embryonic fibroblasts (MEFs) can be activated by DNA-PK and induce apoptosis [396, 398]. Results presented here suggest that latent p53 in Hs68 human fibroblast cell line might consist of at least two sub-populations, referred as “fast-turnover” and “stable”.

The “fast-turnover” sub-population of p53 can be stabilized by UV irradiation, and is probably regulated by MDM2-mediated protein degradation. In contrast, the “stable” p53 is resistant to RNAi-mediated knock-down and could not be stabilized by UV radiation. It was proposed that the “fast-turnover” p53 is involved in cellular DNA repair, thus acting as a protector. Inhibition of “fast-turnover” p53 with RNAi resulted in genomic instability and sensitized Hs68 cells to UV-induced apoptosis. Furthermore, UV radiation caused phosphorylation of “stable” but not “fast-turnover” p53 sub-population on serine 46, a key residue for p53’s apoptotic function. Sub-cellular localization of the “stable” p53 was found to be in the peri-nuclear region and nuclear structures. Evidence presented here support the conclusion that p53 proteins are localized into distinct sub-cellular compartments and regulated differently. Activation of the “stable” p53, which is likely associated with cellular organelles or structures, could be one mechanism for p53 to induce apoptosis. The more detailed mechanisms of action are currently unknown. However, future efforts should focus on identification of specific kinases for activating the “stable” p53 sub-population, and whether this p53 sub-population directly translocates to the mitochondrial outer membrane after DNA damage, where it triggers the intrinsic apoptosis pathway. Also, the role of posttranslational modifications in activation of “stable” p53 sub-population requires clarification. It has been reported that acetylation of p53-K120 is critical for p53-induced transcription-independent apoptosis [100, 454, 455]. Hs68-p53-KD cell might serve as a useful investigative tool for these purposes. Strategies should be designed to test whether this levels of p53 regulation is impaired in cancer cells.

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