REGULATION OF THE P53-MDM2 FEEDBACK LOOP BY NUCLEOSTEMIN AND RIBOSOMAL PROTEINS

by

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TABLE OF ABBREVIATIONS

Act D	actinomycin D
ARF	alternative reading frame
ASPP	ankyrin repeat, SH3 domain, and proline-rich domain containing protein
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia RAD3-related
C-	carboxy-terminus
CDK	cyclin-dependent kinase
CHX	cyclohexamide
DBA	Diamond-Blackfan anemia
DC	dyskeratosis congenital
E2	ubiquitin carrier protein
E3	ubiquitin-protein isopeptide ligase
5-FU	5-fluorouracil
FdUMP	5-fluorodeoxyuridine monophosphate
FdUTP	5-fluorodeoxyuridine triphosphate
FUTP	5-fluorouridine triphosphate
GFP	green fluorescence protein
GST	glutathione-S-transferase
HR	homologus recombination
His	6× histidine tag
IB	immunoblot
IgG	immunoglobin type G
IMPDH	inosine monophosphate dehydrogenase
IP	immunoprecipitation
IR	ionizing radiation
MDM2	gene amplified on the mouse double minute chromosome
MMF	mycophenolate mofetil
MPA	mycophenolic acid
mRNA	message RNA
N-	amino-terminus
NE	nuclear extract
NER	nucleotide excision repair
NES	nuclear export signal
NLS	nuclear localization signal
NoLS	nucleolus localization signal
NS	nucleostemin
p300/CBP	two highly conserved homologous proteins encoded by separate genes: p300 and CREB-binding protein
PCAF	p300/CBP associated factor
PCR	polymerase chain reaction
PI	propidium iodide
Pol	Polymerase
PRD	proline-rich domain

rRNA	ribosomal RNA
RT	reverse transcription
SDS-PAGE	sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
siRNA	small interference RNA
TAD	transcriptional activation domain
TS	thymidylate synthase
Ub	ubiquitin
UBF	the HMG1 box containing upstream binding factor
UV	ultraviolet
WT	wild-type

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ABSTRACT

The MDM2-p53 feedback loop is tightly controlled in cells to prevent errant p53 activation and at the same time to enable rapid stress responses. Different stress signals regulate this feedback loop through different mechanisms. This dissertation seeks to investigate the regulation of the MDM2-p53 feedback loop by a novel nucleolar protein nucleostemin, (NS), and three ribosomal proteins L5, L11, and L23.

NS is essential for cell proliferation. Both downregulation and overexpression of the NS protein hinders cell proliferation, suggesting a physiological range of NS levels is important to maintain cellular homeostasis. However, the precise mechanism underlying this regulation is not yet known. Here I show that NS binds to the central acidic domain of MDM2 in the nucleoplasm and inhibits MDM2 E3 ubiquitin ligase activity, thus leading to the stabilization and activation of p53. Increases in NS levels in U2OS cells leads to higher expression of $p21^{cip1/waf1}$ and G1 cell cycle arrest. MPA-mediated GTP depletion induces the translocation of NS from the nucleolus to the nucleoplasm and enhances its interaction with MDM2 in early time points. This interaction may contribute to the early stage activation of p53 upon MPA treatment. Downregulation of NS by either siRNA-mediated depletion or mycophenolic acid (MPA) also activates p53 and induces G1 arrest. These effects require ribosomal proteins L5 and L11 as depletion of NS enhanced their interaction with MDM2. Knockdown of either gene abrogates both the NS-depletion-induced and MPA-induced p53 activation and cell cycle arrest. Thus, my study uncovers the mechanisms by which aberrant NS levels activate p53 and induce cell cycle arrest. This occurs through modulation of the MDM2-p53 pathway by NS itself and certain ribosomal proteins.

Previous studies have shown that three ribosomal proteins L5, L11 and L23 are important regulators of the MDM2-p53 feedback loop in response to nucleolar stress. Here we further investigate their role in response to nucleolar stress induced by MPA and 5-FU. I show that MPA treatment leads to nucleolar disruption and markedly increases the binding of L5 and L11 to MDM2 at later time points. Knockdown of either L11 or L5 by siRNA significantly decreased the MPA-induced stabilization and activation of p53. Similar to the cellular response to the MPA treatment, treatment with 5-FU increases the fraction of ribosome-free L5, L11, and L23 ribosomal proteins and their interaction with MDM2, leading to p53 activation and G1/S arrest. Conversely, individual knockdown of the expression of these ribosomal proteins by siRNA prevents the 5-FU-induced p53 activation and reverses the 5-FU-induced G1/S arrest.

Taken together, these results suggest an important role for the nucleolus as a cellular stress sensor. In response to nucleolar stress induced by MPA, 5-FU or knockdown of endogenous nucleolar protein NS, nucleolar proteins, including NS and certain ribosomal proteins are released from the nucleolus to the nucleoplasm, where they associate with MDM2 to inhibit its activity and stabilize p53.

CHAPTER ONE

INTRODUCTION

A. The p53 tumor suppressor.

The tumor suppressor protein p53 stands at the crossroads of cellular responses to a wide variety of cell stress signals. Diverse stresses including DNA damage, overexpressed oncogenes, hypoxia/anoxia, ribonucleotide depletion, ribosomal stress and loss of support/survival signals stabilize p53 at the protein level and its transcriptional activity is increased (Vousden and Lu, 2002). The activated p53 protein transcriptionally regulates many target genes that encode proteins inducing p53-dependent cell-cycle arrest, apoptosis, senescence, differentiation and DNA repair (Vousden and Lu, 2002). By inducing cell cycle arrest, p53 allows the cell to recover from these insults. If damage is sustained and irreparable, p53 triggers apoptosis to eliminate the damaged cell from the proliferating pool (Levine, 1997). Although not required for normal growth and development, mice with homozygous deletion of the p53 gene are highly tumor-prone and loss of p53 strongly enhances tumor development driven by other oncogenic alterations. The fact that *TP53* is mutated in about half of all cancers further demonstrates the crucial role of this tumor suppressor in protecting cells from malignant transformation. Recent studies have suggested that many tumors that retain wild-type p53 are defective in the ability to either induce or to respond to p53 (Vogelstein et al., 2000). Therefore, p53 is recognized as the principal guardian of the genome to prevent the initiation and progression of tumors.

As a sequence specific transcription factor, p53 has several well-defined domains (Figure 1.1). The central DNA-binding domain mediates sequence-specific DNA

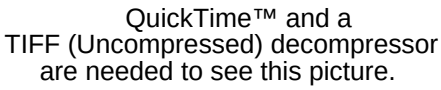

Figure 1.1. Human tumor suppressor protein p53. p53 is a transcriptional factor that contains several well-defined domains. The N-terminal transactivation domain and the proline-rich domain interact with proteins such as MDM2 or p300/CBP. The central sequence-specific DNA binding core harbors the vast majority of p53 mutations in human tumors. Residues R175, G245, R248, R249, R273, and R282 are the six hot spots for mutations. These residues directly contact with DNA. Lastly, the C-terminus contains the tetramerization domain and the basic regulatory domain, together that form a non-specific DNA interaction domain. Nuclear export of p53 is regulated by the nuclear export signals (NES) in the N- and C-termini. The three nuclear localization signals (NLSs) are located at C-terminus of p53. Evolutionarily conserved boxes are indicated as I–V.

binding. The majority of *TP53* mutations are located in this DNA binding domain, highlighting the importance of this region for p53 function (Ko and Prives, 1996). This domain has also been shown to interact with the ASPP (Ankyrin repeat, SH3 domain, and proline-rich domain containing protein) family of proteins, including ASPP1 and ASPP2,

and that interaction allows p53 to preferentially activate transcription from proapoptotic genes such as Bax and PIG3 (Bergamaschi et al., 2004). The N-terminal domain consists of two transcriptional activation domains (TA). The first forty-two residues of p53 constitute TA1, an essential domain for p53-mediated cell cycle arrest. This domain may also be required for some pro-apoptotic activities depending on the type of cellular stress the cell is exposed to (Scoumanne et al., 2005). The second activation domain TA2 (residues 43-92), also called the proline rich domain (PRD), interacts with components of the basal transcriptional machinery and transcription co-activators such as p300/CBP, which also have histone acetyl transferase activity (Fields and Jang, 1990; Raycroft et al., 1990). TA2 is thought to regulate the selective induction of apoptosis (Candau et al., 1997; Zhu et al., 1998). The carboxy-terminal tetramerization domain (TD) is responsible for oligomerization, which is required for the transcriptional activation of p53 target genes (Wang et al., 1993). The extreme C-terminal basic domain (BD) is an unstructured region of thirty residues. Following cellular insults, the p53 BD is a major site for post-translational modifications (Bode and Dong, 2004). It is involved in linear diffusion on the chromatin, an important mechanism by which transcription factors locate their target response elements in the genomic DNA (McKinney et al., 2004). Another important role of the p53-BD is in the recognition of a variety of DNA structures such as single–stranded DNA or damaged-DNA for DNA repair (Liu and Kulesz-Martin, 2001; Sengupta and Harris, 2005). However, the p53-BD can also play an inhibitory role for the expression of specific target genes, such as IGFBP3 and AQP3 (Harms and Chen, 2005). The C-terminal end of p53 also harbors nuclear localization and export signals, which are important for subcellular localization of p53.

There are over 4000 putative p53-binding sites existing in the human genome (Wang et al., 2001). Whether all of these are the *bona fide* target genes of p53 remains to be studied. Nonetheless, there are more than 150 genes that are proven to be regulated by p53 under physiologically relevant circumstances, many of which can be divided into groups that mediate a specific p53 function, such as inhibition of cell growth, DNA repair, activation of apoptosis or regulation of angiogenesis (Figure 1.2) (Vogelstein et al., 2000).

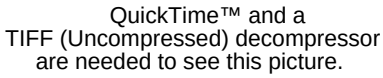

Figure 1.2. The p53 response. p53 mediates the response to various intra- and extracellular stresses. In general, these stresses induce p53 by stabilizing the p53 protein, so that accumulated p53 form tetramers and remain in the nucleus. Tetramerized p53 then acts as a transcription factor to induce or repress the expression of its down stream target genes. Depending on the cellular context, cell type and duration of the encountered stress, different subsets of the target genes are transciptionally activated. These

genes are responsible for different responses including (1) cell cycle arrest, (2) initiation of apoptosis, (3) accelerating DNA repair, (4) cell senescence, (5) inhibition of angiogenesis. In general, however, the effect of p53 activation is to inhibit cell growth, either through cell-cycle arrest or induction of apoptosis, thereby preventing tumor development. Mediators and downstream target genes and subsequent events are shown.

The best understood is p53-induced cell cycle arrest and apoptotic cell death (Bates and Vousden, 1999). The cellular response to p53 activation is variable and highly dependent on both the type of cell and the nature of the damaging agent and/or cellular stress. p53 mediated cell cycle arrest has been postulated to allow time for repair of DNA damage, preventing mutations from being passed onto daughter cells (Lane, 1992). The best characterized example of how p53 induces cell cycle arrest is in the case of $p21^{WAF1/CIP1}$ (el-Deiry et al., 1993; Harper et al., 1993). p21 binds and inhibits the activity of a number of cyclin dependent kinases (CDKs), resulting in cell cycle arrest mostly in G1 and sometimes in G2 (Bates et al., 1998; Bunz et al., 1998; Harper et al., 1995; Medema et al., 1998; Niculescu et al., 1998). Part of the mechanism by which p53 blocks cells at the G2 checkpoint involves inhibition of CDC2, the cyclin-dependent kinase required to enter mitosis. CDC2 is inhibited by three transcriptional targets of p53, GADD45, p21, and 14-3-3σ (Taylor and Stark, 2001). In response to DNA damage, the 14-3-3σ protein binds to phosphorylated CDC25 which is a tyrosine protein phosphatase for CDC2, and sequesters CDC25 in the cytoplasm where it can not activate CDC2; the GADD45 protein dissociates CDC2 from cyclin B, thus blocking G2-M phase transition (Jin et al., 2002a; Zhan et al., 1999).

When DNA damage is too severe for the cell to recover, p53 initiates programmed cell death to eliminate cells that may have acquired irreparable and

potentially oncogenic alterations. p53-induced apoptosis plays a critical role in p53-mediated tumor suppression. This effect is executed through a wide array of death effectors such as the BH3 only protein PUMA and NOXA (Nakano and Vousden, 2001; Oda et al., 2000; Yu et al., 2001). These proteins function by inducing the loss of inner mitochondrial membrane potential, leading to the release of cytochrome C and other apoptotic factors and subsequent activation of the caspase cascade that mediates apoptotic cell death (Danial and Korsmeyer, 2004; Jeffers et al., 2003; Villunger et al., 2003; Yu and Zhang, 2003). In addition to the transactivation of downstream target genes, p53 can repress gene expression (Ryan and Vousden, 1998; Yap et al., 2000). Some of the genes have anti-apoptotic function. The identification of the transcriptional repressor protein SIN3A and histone deacetylases as interacting proteins of p53 provides a molecular explanation for how p53 might repress gene expression through specific mediators (Figure 1.2) (Murphy et al., 1999). How p53 chooses its target genes for repression remains unclear. SIN3A binds to and stabilizes p53 through the proline-rich domain of p53, a region that is required for apoptotic function (Zilfou et al., 2001), again illustrating the close relationship between the transcriptional repression and apoptotic function of p53. Moreover, p53 can induce apoptosis by directly working in the mitochondria independently of its transcriptional activity. p53 binds to the pro-apoptotic proteins BAX and BAK, as well as the anti-apoptotic proteins such as Bcl-XL, leading to the release of cytochrome C (Chipuk et al., 2004; Leu et al., 2004; Mihara et al., 2003) and the initiation of the caspase cascade. Interestingly PUMA, a transcriptional target of p53, can activate this cytoplasmic function of p53 (Chipuk et al., 2005), suggesting that both transcriptional and non-transcriptional activities of p53 are important for the induction of cell death.

In addition, p53 plays an important role in the maintenance of genomic stability by mediating DNA repair (Avkin et al., 2006; Liu et al., 2005; Nowak et al., 2002). It has been shown that p53 is involved in various types of DNA repair, including nucleotide excision repair (NER), base excision repair (BER), nonhomologous end-joining (NHEJ) and homologous recombination (HR) (Adimoolam and Ford, 2003; Bertrand et al., 2004; Sengupta and Harris, 2005; Smith and Seo, 2002; Zurer et al., 2004). For example, p53-dependent transcriptional activity is important for regulation of NER by p53 (Adimoolam and Ford, 2003). p53 also binds to and modulates the activities of the NER-associated helicases XPB, XPD (Leveillard et al., 1996; Wang et al., 1995). It also regulates the expression of the DDB2 and XPC which are the global genomic repair (GGR)-specific damage recognition genes (Adimoolam and Ford, 2002; Hwang et al., 1999; Rubbi and Milner, 2003b), and serves as a chromatin accessibility factor for NER of DNA damage (Wang et al., 2003). p53 stimulated an in vitro reconstituted BER assay, potentially by binding APE-1 and regulating DNA polymerase β loading onto the apurinic/apyrimidinic (AP)-sites(Zhou et al., 2001). It has also been shown that p53 can physically bind to RAD51 and RAD54, major components of the HR machinery, and controls the level of HR (Linke et al., 2003; Sengupta et al., 2003). Hence, p53 regualtes DNA repair as well.

p53, through its cell-cycle-arrest and apoptotic activities, can have a strong inhibitory effect on cell growth. This makes it essential for cells to monitor p53 protein level and function during normal development. Multiple mechanisms exist to negatively control p53, including the regulation of protein activity, stability and subcellular localization through the action of numerous proteins that work directly or indirectly on

p53. One of the key negative regulators of p53 function is the murine double minute 2 (MDM2) oncoprotein.

B. The oncoprotein MDM2.

The MDM2 gene was originally discovered in a locus amplified on double minute chromosomes in a tumorigenic mouse cell line (Fakharzadeh et al., 1991). MDM2 overexpression enables primary human fibroblasts expressing E1A and activated Ras to form tumors in nude mice, thus MDM2 behaves as a *bona fide* oncogene (Seger et al., 2002). MDM2 plays an important role in the etiology of human cancer as it is amplified or overexpressed in a subset of human tumors expressing wild type p53 (Momand et al., 1998; Oliner et al., 1992). Amplification of the MDM2 gene in 7% of human tumors account for one mechanism of overexpression of MDM2 protein, and more recent data have shown that a naturally occurring polymorphism (SNP309) occurring within the MDM2 promoter leads to an increase in MDM2 mRNA and protein in human populations (Bond et al., 2004).

MDM2 is a nuclear phosphoprotein that possesses three conserved regions (Figure 1.3): The p53 binding domain, a central acidic region including a C4 zinc finger, and a C-terminal RING domain conferring E3 ligase activity. The N-terminal p53-interacting domain of MDM2 binds to the amino terminal transactivation domain of p53, thus interfering with the ability of p53 to interact with the transcription machinery (Chen et al., 1993; Lu and Levine, 1995). The central region of MDM2 is necessary for the interaction with the ribosomal protein L5, L23, L11**,** ARF and p300/CBP (CREB-binding protein). Recently, it has been shown that this region is essential for p53 degradation because an MDM2 mutant lacking the central acidic domain fail to ubiquitinate p53 and

degrade it (Kawai et al., 2003b; Meulmeester et al., 2003). The RING domain is common in E3 ligases and is required for MDM2 E3 ligase activity (Fang et al., 2000). Other

Figure 1.3. Human oncoprotein MDM2 and MDM2-interacting proteins. Functional domains of MDM2. The p53-binding domain binds to the amino terminal transactivation domain of p53. The central acidic domain is necessary for the interaction with the ribosomal protein L5, L23, ARF, YY1 and p300/CBP. This domain also contributes to p53 degradation. Downstream of acidic domain is the zinc finger domain of unknown function followed by another acidic domain. Lastly, the C-terminus contains the RING finger domain which is responsible for the E3 ubiquitin ligase activity of MDM2. A nuclear localization signal and a nuclear export signal shuttle MDM2 back and forth between the cytoplasm and the nucleus and provide another means by which p53 activity is tightly regulated. A nucleolar localization signal is located in the RING finger domain, although the biological significance of this regulation is unclear. The proteins that bind to different regions of MDM2 are also indicated.

motifs include a nuclear localization signal and a nuclear export signal. These signals shuttle MDM2 back and forth between the cytoplasm and the nucleus and provide yet

another means by which p53 acitivity is tightly regulated (Freedman and Levine, 1998; Roth et al., 1998). Within the RING domain, there is a nucleolar localization signal, which may be necessary to allow efficient nucleolar localization of both $p14^{ARF}$ and MDM2 (Lohrum et al., 2000). MDM2 prevents p53-dependent gene expression through diverse mechanisms (Figure 1.4). Biochemically, MDM2 functions as an E3 ubiquitin

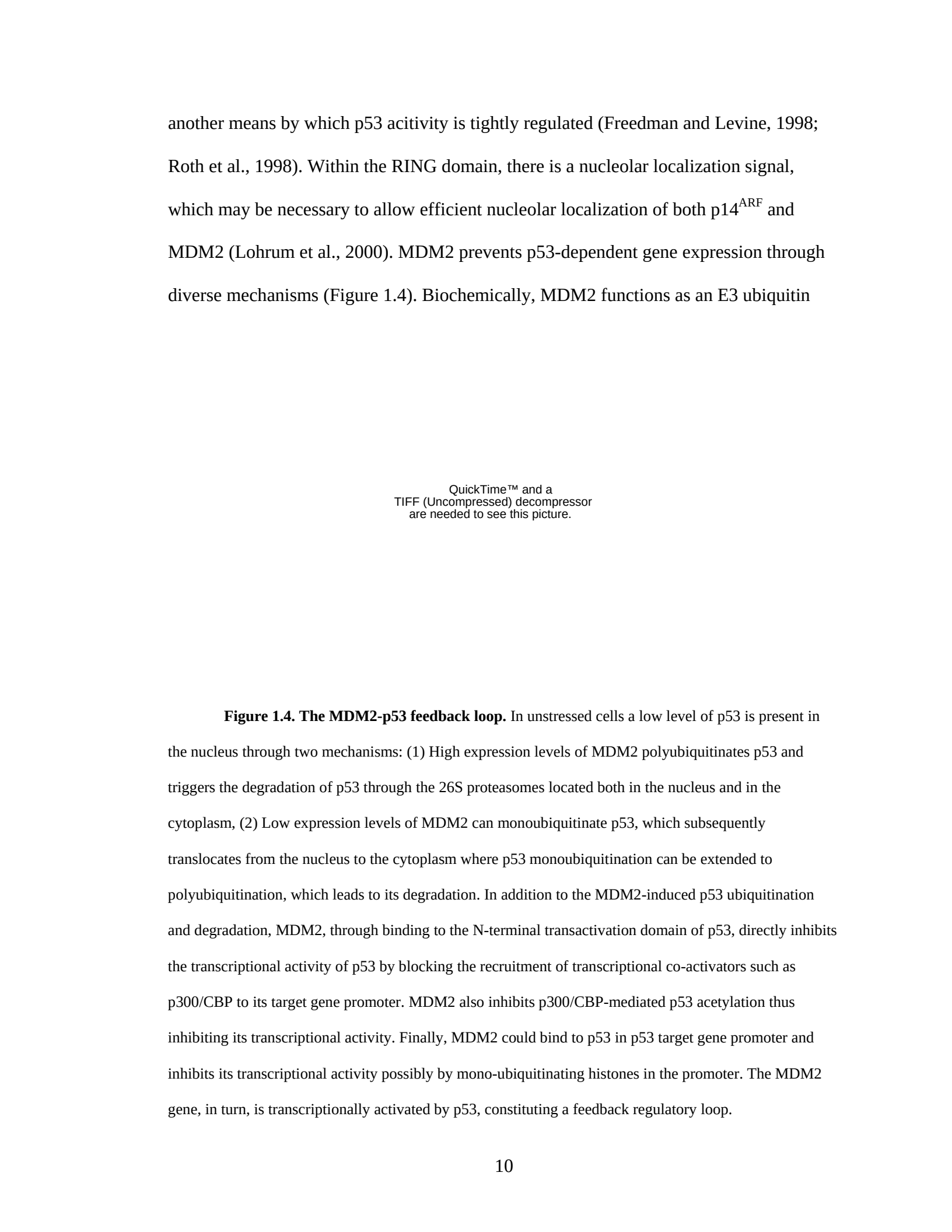

Figure 1.4. The MDM2-p53 feedback loop. In unstressed cells a low level of p53 is present in the nucleus through two mechanisms: (1) High expression levels of MDM2 polyubiquitinates p53 and triggers the degradation of p53 through the 26S proteasomes located both in the nucleus and in the cytoplasm, (2) Low expression levels of MDM2 can monoubiquitinate p53, which subsequently translocates from the nucleus to the cytoplasm where p53 monoubiquitination can be extended to polyubiquitination, which leads to its degradation. In addition to the MDM2-induced p53 ubiquitination and degradation, MDM2, through binding to the N-terminal transactivation domain of p53, directly inhibits the transcriptional activity of p53 by blocking the recruitment of transcriptional co-activators such as p300/CBP to its target gene promoter. MDM2 also inhibits p300/CBP-mediated p53 acetylation thus inhibiting its transcriptional activity. Finally, MDM2 could bind to p53 in p53 target gene promoter and inhibits its transcriptional activity possibly by mono-ubiquitinating histones in the promoter. The MDM2 gene, in turn, is transcriptionally activated by p53, constituting a feedback regulatory loop.

ligase.Along with E1 and E2 enzymes, MDM2 catalyzes the transfer of a chain of ubiquitin proteins onto several C-terminal lysine residues of p53, targeting p53 for rapid degradation by the 26S proteasome, which overcomes p53 tumor suppressor activity (Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). MDM2 can also ubiquitinate itself and induce its own degradation (Fang S, Jensen JP,2000; Honda R, Yasuda H, 2000). MDM2 was recently found to differentially catalyze monoubiquitination and polyubiquitination of p53 in a dosage-dependent manner (Li et al., 2003). As a consequence, low levels of MDM2 activity induce monoubiquitination and nuclear export of p53, whereas high levels promote polyubiquitination and nuclear degradation of p53. It seems likely that these distinct mechanisms are exploited under different physiological settings. For example, MDM2-mediated polyubiquitination and nuclear degradation of p53 may play a critical role in suppressing p53 function during the later stages of a DNA damage response or when MDM2 is malignantly overexpressed (Shirangi et al., 2002; Xirodimas et al., 2001b).On the other hand, MDM2-mediated monoubiquitination of p53 and subsequent cytoplasmic translocation may represent an important means of p53 regulation in the unstressed cell, where MDM2 is maintained at low level (Boyd et al., 2000; Freedman and Levine, 1999; Geyer et al., 2000; Stommel et al., 1999). Monoubiquitination may sabotage the transactivation potential of p53 by inducing its nuclear export, but this type of modifications is not sufficient for p53 degradation. It’s possible that movement of p53 into the cytoplasm may be important for transcription-independent functions of p53 such as interactions with mitochondrial proteins in the apoptosis response (Chipuk et al., 2004; Leu et al., 2004; Mihara et al., 2003). In addition to indirect mechanisms of transcriptional inhibition, MDM2 can

directly inhibit p53-dependent gene expression. It binds and occludes the p53 N-terminal transactivation domain, preventing the interaction of p53 with the basal transcription machinery (Chen et al., 1993; Momand et al., 1992; Oliner et al., 1993). Various stresses result in the acetylation of p53 by the histone acetyl transferases PCAF and p300/CBP, and acetylation has been suggested to be important for p53 stability and transcriptional activation even though this is controversial (Barlev et al., 2001; Espinosa and Emerson, 2001). However, this acetylation can be blocked by the association of p53 with MDM2 (Gu and Roeder, 1997; Ito et al., 2001; Jin et al., 2002b; Kobet et al., 2000; Sakaguchi et al., 1998). MDM2 was also reported to promote NEDD8 conjugation of p53. The C-terminal glycine residue of the ubiquitin-like protein NEDD8 can be covalently linked to lysine 370,372 and/or 373 of p53. This modification inhibits p53 transcriptional activity without affecting p53 protein stability (Xirodimas et al., 2004). The lysine residues modified by neddylation are three of the six lysines also targeted by ubiquitination. Whether neddylation augments ubiquitination is not yet clear. Finally, MDM2 induces monoubiquitination of histones surrounding the p53-response elements resulting in transcriptional repression (Minsky and Oren, 2004). To ensure that p53 is kept under tight control, MDM2 itself is a target gene of p53, thus forming a built-in auto-inhibitory feedback loop (Barak et al., 1993; Perry et al., 1993; Picksley and Lane, 1993; Wu et al., 1993). The importance of this regulatory loop is demonstrated in mice, where loss of *mdm2* expression leads to a very early embryonal lethality. This presumably occurs due to rampant p53-dependent apoptosis and consequently can be suppressed by the concurrent deletion of *p53* (Jones et al., 1995; Montes de Oca Luna et al., 1995).

While MDM2 is sufficient to target p53 for degradation, there is good evidence that it does not function alone. Several proteins have recently been shown to cooperate with MDM2 in the regulation of p53. The Yin Yang 1 (YY1) (Figure 1.3), a transcription factor that plays a key role in development, can increase the interaction between p53 and MDM2, so enhancing MDM2-dependent p53 polyubiquitination and degradation (Gronroos et al., 2004; Sui et al., 2004). Another protein gankyrin, a seven ankyrin-repeat protein, was originally identified as an oncoprotein commonly overexpressed in hepatocellular carcinomas and independently identified as a protein associated with the 19S regulatory complex of the 26S proteasome (Dawson et al., 2002; Higashitsuji et al., 2000; Hori et al., 1998). Gankyrin binds to MDM2 and facilitates binding of MDM2 to p53, and enhances the ability of MDM2 to mono- and poly-ubiquitinate p53. Gankyrin also recruits the MDM2/p53 complex to the 26S proteasome and accelerates the degradation of p53 in an MDM2-dependent manner (Higashitsuji et al., 2005). p300/CBP has also been shown to cooperate with MDM2 to promote poly-ubiquitination of p53 (Grossman et al., 2003). p300/CBP protein binding to MDM2 is necessary for efficient poly-ubiquitination of p53 to occur since in this report authors suggested that MDM2 only can promote the mono-ubiquitination of p53 under most conditions (Grossman et al., 2003). HAUSP (Herpes-virus-associated ubiquitin-specific protease), a ubiquitin hydrolase, can de-ubiquitinate p53 directly (Li et al., 2002), but its principal function in the p53 pathway seems to be in the de-ubiquitination and stabilization of MDM2, resulting in enhanced degradation of p53. This modification is influenced by yet another protein, Daxx (death domain-associated protein), which forms a complex with MDM2

and HAUSP, preventing the auto-ubiquitination of MDM2 and thus promoting p53 degradation (Horn and Vousden, 2007).

Although MDM2 has been extensively characterized as a regulator of p53, there is considerable evidence that MDM2 has p53-independent functions in cell cycle control, differentiation, cell fate determination, DNA repair and basal transcription (Ganguli and Wasylyk, 2003). For example, MDM2 directly interacts with the RB tumor suppressor protein and inhibits the ability of RB to inhibit E2F1 function. This leads to perturbation of RB-mediated G1 cell cycle arrest (Xiao et al., 1995). There is evidence that wild-type MDM2 can efficiently ubiquitinate and degrade RB (Uchida et al., 2005). MDM2 has been shown to interact with E2F1/DP1 and stimulate E2F1/DP1-dependent transactivation of E2F responsive promoters and subsequent stimulation of DNA synthesis (Martin et al., 1995). Additionally, MDM2 blocks the apoptotic activity of E2F1 (Loughran and La Thangue, 2000). Finally, MDM2 can promote p21 proteasomal turnover independently of ubiquitination (Jin et al., 2003). These experiments indicate that MDM2 promotes cell proliferation by regulating other important components of the cell cycle in addition to regulating p53. The NUMB protein has been identified as an MDM2 interacting protein that is also degraded by MDM2 (Juven-Gershon et al., 1998; Yogosawa et al., 2003). *Drosophila* NUMB antagonizes Notch signaling and is involved in neural cell differentiation and cell fate determination. The implications of this interaction between these two proteins await further experiments. MDM2 interacts with the general transcription factor TFIID and activates the promoter of cyclin A, a gene that is important for S-phase entry (Burley and Roeder, 1996; Leveillard and Wasylyk, 1997). MDM2 has also been shown to bind to a number of other factors involved in RNA

biosynthesis and cell surface receptor turnover such as L5/5S ribonucleoprotein particle and androgen receptor (Ganguli and Wasylyk, 2003).

C. Regulation of the MDM2-p53 regulatory feedback loop

Since the MDM2-p53 feedback loop is critical for cells to maintain a low level of p53, it must be tightly regulated in cells. A growing number of stress signals that can lead to p53 activation have been identified, including DNA damage, oncogene activation, ribosomal stress and so on. Depending on the stress signal, there are several different and independent pathways through which MDM2 function can be inhibited and p53 can be activated (Figure 1.5).

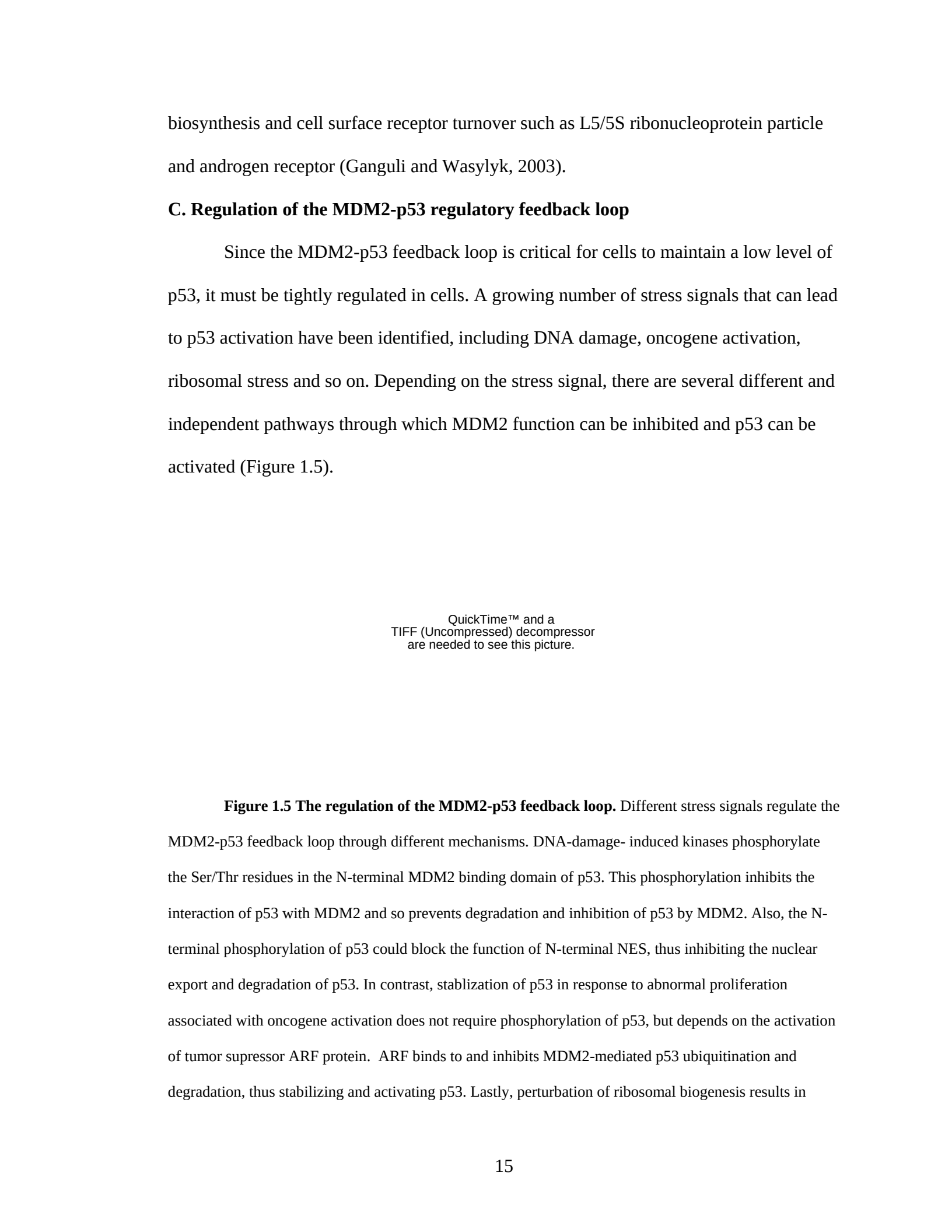

Figure 1.5 The regulation of the MDM2-p53 feedback loop. Different stress signals regulate the MDM2-p53 feedback loop through different mechanisms. DNA-damage- induced kinases phosphorylate the Ser/Thr residues in the N-terminal MDM2 binding domain of p53. This phosphorylation inhibits the interaction of p53 with MDM2 and so prevents degradation and inhibition of p53 by MDM2. Also, the N-terminal phosphorylation of p53 could block the function of N-terminal NES, thus inhibiting the nuclear export and degradation of p53. In contrast, stablization of p53 in response to abnormal proliferation associated with oncogene activation does not require phosphorylation of p53, but depends on the activation of tumor supressor ARF protein. ARF binds to and inhibits MDM2-mediated p53 ubiquitination and degradation, thus stabilizing and activating p53. Lastly, perturbation of ribosomal biogenesis results in

nucleolar stress which may release ribosomal proteins into the nucleoplasm where they bind to MDM2 and inhibit the E3 ubiquitin ligase activity of MDM2, thus leading to increased p53 activity.

DNA damage

DNA damage activates and stabilizes p53 primarily through post-translational modifications. DNA damage activates damage-sensor kinases, including Ataxia telangiectasia mutated kinase (ATM) and ataxia telangiectasia RAD3-related kinase (ATR), which phosphorylate p53 on serine 15, while their downstream kinases checkpoint kinase-2 (CHK2) and checkpoint kinase-1 (CHK1), respectively, phosphorylate p53 on serine 20 (Bartek and Lukas, 2003). Phosphorylation of serine 15 also triggers the phosphorylation of threonine18. These residues constitute part of a helical domain that interacts with a hydrophobic pocket in the MDM2 N-terminus (Kussie et al., 1996). The phosphorylation of threonine 81 in the proline-rich domain (PRD) enables the binding of the prolyl isomerase PIN1 to induce cis-trans prolyl isomerization of proline 82 within the PRD. Together, these modifications and associated conformational changes disrupt MDM2-p53 binding and promote acetylation and phosphorylation of the p53 C-terminus (Toledo and Wahl, 2006). DNA damage-induced changes in the phosphorylation of MDM2 are also thought to contribute to blocking the degradation of p53. The mechanism of this effect does not appear to involve changes in association between p53 and MDM2 (Meek and Knippschild, 2003). Phosphorylation of MDM2 at serine 395 by ATM, and at the adjacent tyrosine 394 by the c-Abl protein kinase (also a downstream effector of ATM), inhibit, independently of each other, the ability of MDM2 to mediate the degradation of p53 and, in the case of serine 395 phosphorylation, blocks MDM2-mediated nuclear export of p53 (Meek and Knippschild,

2003). *In vitro* and cell transfection studies have suggested this p53 regulation model that emphasizes the importance of phosphorylation to produce structural changes in p53. This enables competition between MDM2 and p300 for binding to the N-terminal p53 transactivation domain and inducing competing modifications in the p53 C-terminal regulatory domain. However, *in vivo* models have suggested that these modifications play only very subtle, modulatory roles in regulating p53 function (Toledo and Wahl, 2006). The interaction of p53 with proteins that control p53 function, either directly or through the regulation of p53 stability appears to be key in turning on and off the p53 response.

Oncogene-mediated p53 activation

One of the key mediators of oncogene-induced activation of p53 is $p14^{ARF}$ ($p14^{ARF}$ in humans, $p19^{ARF}$ in mouse, hereafter referred to as ARF). ARF, the alternate reading frame protein, is a small basic protein (pI>12) encoded by the INK4a locus that also encodes the cyclin-dependent kinase inhibitor p16INK4a (Lowe and Sherr, 2003). ARF has been shown to have a variety of functions. One of its key roles is to bind and inhibit the activity of MDM2 in response to activation of viral and cellular oncoproteins such as Ras and c-Myc (Honda and Yasuda, 1999; Kamijo et al., 1998; Zhang et al., 1998). ARF prevents MDM2 from targeting p53 through two possible mechanisms: 1) separating the two proteins from different cellular compartments and 2) inhibiting MDM2-mediated p53 ubiquitination. ARF interacts with MDM2 and blocks MDM2-mediated p53 degradation through direct inhibition of MDM2 E3 activity (Honda and Yasuda, 1999; Midgley et al., 2000). It was found that experimentally overexpressed ARF resides in the nucleolus and could induce the nucleolar localization of MDM2. This could result in the inhibition of p53 degradation due to separation of MDM2 from p53

(Weber et al., 1999). However, the essential role of nucleolar retention is challenged by the finding that mutated ARF does not induce growth arrest despite its ability to retain MDM2 in the nucleolus (Korgaonkar et al., 2002). Moreover, various truncated human ARFs that contain the 1-29 region did not necessarily accumulate in the nucleolus, but nevertheless stabilized MDM2 and p53 (Llanos et al., 2001). In addition, in certain cell lines, induction of ARF leads to stabilization of p53 and cell cycle arrest without the relocalization of MDM2 to the nucleolus (Lin and Lowe, 2001; Llanos et al., 2001). Therefore, relocalization to the nucleolus is not essential for the inhibition of MDM2 by ARF in all cells, although it may contribute to the suppression of MDM2 under certain circumstances. Interestingly, ARF can also prevent the inhibition of p53 acetylation by MDM2, illustrating another role for ARF in the activation of p53, independent of regulation of MDM2's E3 activity (Ito et al., 2001).

MDMX

MDMX (also known as MDM4), another key regulator of p53, is a p53-binding protein that shares extensive homology with MDM2, including a RING finger at its C-terminus (Shvarts et al., 1996) and an N-terminal region that binds to p53. In contrast with MDM2, MDMX lacks a critical cystine in its RING domain, which precludes it from acting as a ubiquitin ligase. Unlike its namesake, the MDMX gene is not transcriptionally activated by p53 in stressed cells (Stad et al., 2001), and its promoter apparently lacks p53 responsive elements (Marine and Jochemsen, 2005). As in the case of MDM2, MDMX deficiency causes early embryonic lethality rescued by p53 loss (Marine and Jochemsen, 2005; Migliorini et al., 2002; Parant et al., 2001), suggesting that MDMX is critical for the MDM2-p53 feedback loop regulation. Another important

implication of these *in vivo* studies is that MDM2 and MDMX are non-redundant p53 inhibitors, as each regulator is normally unable to compensate for the loss of the other. Consistent with the *in vivo* studies, overexpression of MDMX has been found in several tumor types (Danovi et al., 2004). MDMX can bind to the p53 transactivation domain, and experimental evidence indicates that it inhibits p53 transactivation by limiting access to essential transcriptional co-activators, and, presumably, to the basal transcription machinery (Marine and Jochemsen, 2005). Although this function of MDMX is independent of MDM2 (Francoz et al., 2006; Xiong et al., 2006), there are some interesting levels of cross talk between these two proteins. MDMX binds to MDM2 through their RING domains and enhaces the ability of MDM2 to ubiquitinate and degrade p53 (Gu et al., 2002; Linares et al., 2003). Moreover, siRNA against MDMX results in increased p53 protein abundance and activity (Gu et al., 2002; Kawai et al., 2003a). Interestingly, DNA damage and ARF overexpression lead to the ubiquitination and degradation of MDMX by MDM2 (de Graaf et al., 2003; Kawai et al., 2003a; Pan and Chen, 2003), adding another layer of complexity to the intricate balance between MDM2 and p53. Together, these *in vivo* and *in vitro* observations suggest that MDM2 and MDMX have different roles in inhibiting p53. However, the functional significance of MDMX in tumor development and maintenance remains to be further investigated. Recent evidence from mouse models suggest that MDM2 mainly controls p53 stability, whereas MDMX functions as an important p53 transcriptional antagonist (Toledo and Wahl, 2006).

Ribosomal/Nucleolar stress

In eukaryotes, the nucleolus is a nuclear sub-compartment whose key function is

the synthesis and processing of ribosomal RNAs (rRNAs) and their assembly into ribosomal subunits (r-subunits) (Raska et al., 2006b). Three classical regions of the nucleolus have been defined by their ultrastructural appearance and by their roles in ribosome synthesis. These regions are 1) the fibrillar centers (FC), which are the sites of the repeated rRNA genes; 2) the dense fibrillar component (DFC), which surrounds the fibrillar centers and into which the nascent rRNA extends and some of its processing occurs; and 3) the granular component (GC), where ribosome is assembled (Thiry and Lafontaine, 2005). rRNA synthesis requires four basal transcription factors: the promoter selectivity factor SL1; transcription initiation factor IA (TIF-IA/Rrn3); upstream binding factor (UBF) and RNA polymerase I. The product of Pol I transcription is a long precursor rRNA (pre-rRNA) that contains the sequence for the mature ribosomal RNAs (18S, 5.8S and 28S rRNA), two external transcribed spacers (ETS) and two internal transcribed spacers (ITS). This primary transcript is subsequently chemically modified at numerous sites and subjected to a series of endo- and exonucleolytic-processing steps to produce mature rRNAs. The fourth ribosomal RNA, 5S rRNA, is independently transcribed as a precursor by RNA polymerase III in the nucleoplasm and then transported to the nucleolus (Raska et al., 2006a). The mature rRNAs associate with 79 ribosomal proteins to form the 40S (small) and 60S (large) ribosomal subunits. After their assembly, the large and small subunits are transported to the cytoplasm to initiate protein synthesis (Nazar, 2004).

Ribosomal biogenesis is tightly coordinated with cell growth and proliferation. The synthesis of rRNA is linked to cell cycle progression. rRNA transcription is maximal in S and G2 phases, repressed in mitosis and increased in G1. The coordination between

cell cycle and ribosome production ensures that translation of mRNAs occurs at appropriate levels and during a specific window of the cell cycle, thus leading to accurate control of cell growth and proliferation. Uncoupling protein synthesis from cell growth and proliferation can result in tumorigenesis. Indeed, several gene products involved in ribosomal biogenesis and protein translation are associated with tumor susceptibility. Mutations in the gene that encode small ribosomal protein S19 have been identified in Diamond-Blackfan anemia (DBA), a condition characterized by anemia and an increased susceptibility to hematopoietic malignancies (Choesmel et al., 2007; Da Costa et al., 2003; Draptchinskaia et al., 1999). Mutation or knockdown of S19 leads to defects in pre-rRNA processing and 40S ribosome subunit maturation (Choesmel et al., 2007; Da Costa et al., 2003; Flygare et al., 2007). Germ line mutation of *DKC1*, the gene altered in Dyskeratosis congenital (DC), has a direct effect on ribosome assembly and has been associated with increased risk for cancer (Ruggero et al., 2003; Ruggero and Pandolfi, 2003). *DKC1* encodes a putative pseudouridine synthase, dyskerin, which mediates post-transcriptional modification of rRNA through the site-specific conversion of uridine to pseudouridine (Lafontaine and Tollervey, 1998). Hypomorphic *DKC1* mutant mice recapitulate the clinical features of DC, and more than 50% of the mice develop tumors during their lifespan. The cells from the mutant mice harbors impaired rRNA processing activity (Ruggero et al., 2003). These results indicate that *DKC1* is an important tumor suppressor gene and suggest that defects in ribosome modification may relate to the tumor susceptibility in DC patients. A recent genetic screen study in zebrafish shows that 11 of the 12 lines with elevated cancer incidence were heterozygous for mutations in different ribosomal genes, suggesting that some ribosomal proteins may act as haploid-

insufficient tumor suppressors (Amsterdam et al., 2004). Together, these results suggest that ribosomal proteins have signaling functions in regulating cell proliferation and also raise a number of questions: 1) how is ribosome biogenesis coupled to cell cycle progression? and 2) is there a cell cycle checkpoint for ribosome biogenesis?

It has been demonstrated that a variety of physiological or pathological stimuli, such as serum deprivation, high cell culture density, glucocorticoid treatment of lymphoid cells, heat shock, and cell differentiation can rapidly down-regulate the rate of rRNA transcription and result in growth arrest (Grummt, 1999; Reeder, 1999). Growth inhibitory factors repress RNA polymerase I transcription through pRB-UBF interactions (Ciarmatori et al., 2001; Hannan et al., 2000). Knockdown of nucleolar protein p120, which is necessary for 60S ribosome subunit formation also induces G1 arrest in human lymphocytes (Fonagy et al., 1992). A conditional deletion of S6 ribosomal protein in the liver of adult mice leads to failure of liver proliferation following partial hepatectomy (Volarevic et al., 2000). These findings suggest the existence of a cell cycle checkpoint downstream of the aberrant ribosome biogenesis. The existence of a p53-dependent signaling mechanism between ribosomal biogenesis and cell cycle control was established when a nucleolar protein involved in rRNA processing and assembly known as BOP1 was identified (Pestov et al., 2001). Expression of a dominant negative version of BOP1 causes defects in rRNA processing that induces a cell cycle arrest. This phenotype was alleviated by deletion of p53. Therefore, p53 can functionally link ribosome biogenesis to cell cycle progression. The intimate link between Pol I activity, nucleolar integrity and p53-mediated damage control has also been demonstrated by genetic inactivation of TIF-IA, an essential Pol I transcription factor that mediates growth

factor- and nutrient-dependent control of Pol I transcription (Schnapp et al., 1990). In mouse embryonic fibroblasts, Cre-mediated depletion of TIF-IA leads to disruption of nucleoli, cell cycle arrest, upregulation of p53, and induction of apoptosis (Yuan et al., 2005). Strikingly, RNA interference (RNAi)-induced depletion of p53 can overcome proliferation arrest and apoptosis induced by TIF-1A deletion, reinforcing the central role of p53 in surveillance of ribosome biogenesis. A recent genetic model offers further insight into the basis of how ribosomal biogenesis feedback can regulate cell cycle progression through a p53 checkpoint. The S6 ribosomal protein heterozygous deletion in the T-cell lineage leads to a reduction in the number of T lymphocytes in the peripheral lymphoid organs. In response to stimulation through the T-cell receptor, the S6 haploinsufficient blast T cells failed to proliferate due to a cell cycle block, while deletion of p53 rescued the proliferative defect and restored near normal numbers of T lymphocytes in the spleen. Therefore, the correlation between aberrant ribosomal biogenesis and p53 activation supports the notion that the nucleolus can signal to p53 and regulate cell cycle progression. It is worth noting that Rubbi and Milner (Rubbi and Milner, 2003a) recently proposed that the nucleolus is a major cellular stress sensor and transmits signals to p53 activation. They demonstrate using focused micropore irradiation, that p53 levels are not stabilized by high level of nuclear irradiation unless the nucleolus is disrupted. By directly destabilizing the nucleolus in the absence of DNA damage, they demonstrated that p53 levels are stabilized. From these data, they proposed a model in which the integrity of the nucleolus regulates p53 activity irrespective of DNA damage.

Again, these observations raise a question of how p53 might “sense” the

ribosomal stress or nucleolar stress? Release of ribosomal proteins and other nucleolar components after disruption of ribosome biogenesis or the nucleolus may play major roles in signaling to p53. Our lab and others have shown that ribosomal proteins such as L5, L11 and L23 are major MDM2 binding partners and can inhibit the E3 ubiquitin ligase activity of MDM2, thus leading to stabilization and activation of p53 (Dai and Lu, 2004; Dai et al., 2004; Lohrum et al., 2003; Zhang et al., 2003). Indeed, treating cells with siRNA for L11 or L5 abrogates the induction of p53 in response to ribosomal stress, such as treatment with the RNA pol I inhibitor Actinomycin D (Bhat et al., 2004; Dai and Lu, 2004; Dai et al., 2004). In addition to L proteins, other nucleolar factors such as B23 and nucleolin have been implicated in signaling to p53. B23, also known as nucleophosmin, interacts directly with MDM2 and inhibits MDM2-mediated p53 ubiquitination and degradation in resonse to UV, thus inhibiting the MDM2-p53 feedback loop (Kurki et al., 2004). Nucleolin, another abundant nucleolar protein, which plays a critical role in rRNA processing (Ginisty et al., 1999), has also been shown to directly bind to MDM2 and inhibit both p53 ubiquitination and MDM2 auto-ubiquitination (Saxena et al., 2006). It is unclear why multiple proteins involved in ribosome biogenesis interact with MDM2. Perhaps they represent parallel or collaborating pathways that ensure an effective p53 response, similar to the ever-expanding signaling network between ATM and p53.

D. Nucleostemin

Nucleostemin (hereafter referred to as NS) was initially identified as a novel nucleolar protein preferentially expressed in the nucleoli of neural stem cells, embryonic stem cells, and several cancer cell lines (Tsai and McKay, 2002). Though NS is also

enriched in mesenchymal, stromal stem cells, *c-kit*$^{+}$ bone marrow cells, adult testes and several types of human cancers (Baddoo et al., 2003; Kafienah et al., 2006; Liu et al., 2004), it is not exclusively expressed in stem/progenitor cells. NS mRNA expression was, for instance, found in proliferating diploid fibroblasts or T lymphocytes (Fan et al., 2006) and NS protein is readily detectable in early passage mouse embryonic fibroblasts (MEFs) but not at later passages when cells become senescent (Beekman et al., 2006; Zhu et al., 2006). NS expression therefore appears closely associated with cellular proliferation, regardless of the origin or the differentiation status of the cell. There are several observations supporting an active role for NS in the control of cellular proliferation. Upon differentiation of neuronal stem cells, NS expression decreases before cell cycle withdrawal, suggesting that NS down-regulation leads to cell cycle exit rather than occurring as a consequence of the cell proliferation block (Tsai and McKay, 2002). Moreover, when bone marrow stem cells are stimulated with fibroblast growth factor 2 to proliferate, NS expression increases in a dose-dependent manner, and knockdown of NS abolishes the proliferative effect of fibroblast growth factor 2 (Kafienah et al., 2006). Finally, NS knockdown in an osteosarcoma cell line (U2OS) increased the number of noncycling cells (Tsai and McKay, 2002). NS interacts with telomeric-repeat binding factor 1 (TRF1) and enhances the degradation of the TRF1. TRF1 is known to negatively regulate telomere length through blocking the access of telomerase to the telomeres (van Steensel and de Lange, 1997; Zhu et al., 2006). Consistent with its high expression in ES cells, targeted deletion of NS leads to early embryonic lethality in homozygous NS-null embryos (Beekman et al., 2006; Zhu et al., 2006). Heterozygous NS-null mice have premature senescence of embryonic fibroblast cells (Zhu et al., 2006). These data suggest

the important roles of NS in establishing early embryogenesis and delaying cellular senescence of MEFs.

NS protein contains several domains: the N-terminal basic (B) domain, a coiled-coil domain followed by a GTP-binding domain, the intermediate domain, and an acidic domain in the carboxyl end (Figure 1.6).

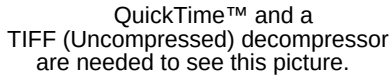

Figure 1.6. Human nucleolar protein Nucleostemin. Nucleostemin is named because of its nucleolar localization and preferential expression in stem cells. Nucleostemin protein contains several domains. The N-terminal basic domain interacts with p53. A coiled-coil domain with unknown function follows the basic domain. The central GTP-binding domain is unique since it contains circularly permuted GTP-binding motifs. Both the B-domain and the wild-type GTP-binding domain are required for the nucleolar targeting of nucleostemin. Lastly, the C-terminus contains the intermediate domain and acidic domain. I-domain acts as the gating mechanism that prevent the non-GTP-bound nucleostemin from moving into the nucleolus. The four nuclear localization signals (NLSs) are located at N-terminus of nucleostemin.

NS and its homologues, guanine nucleotide binding protein-like 3 (GNL3L) and Ngp-1 (hereafter referred to as Ngp1), belong to a subfamily of GTPases featured by their

nucleolar distribution and a unique domain of circularly permuted GTP-binding motifs, where the G4 motif is located N-terminally to the G1, G2, and G3 motifs (Daigle et al., 2002; Leipe et al., 2002). To date, only one gene in this family in multicellular organisms has been experimentally shown to contain intrinsic GTPase activity, namely the human Lsg1 protein (Reynaud et al., 2005). NS is predominantly distributed in the nucleolus, but it dynamically shuttles between the nucleolus and the nucleoplasm in a GTP-driven fashion. It enters the nucleolus in a GTP-bound form, mediated by its basic (B) domain and GTP-binding motif G4 (Tsai and McKay, 2005). In the nucleolus, NS interacts with the ribosomal L1-domain-containing 1 (RSL1DL), another nucleolar protein (Meng et al., 2006) which provides the nucleolar binding site for NS. When NS is in the GTP-unbound state, it does not interact with RSL1DL and its nucleolar targeting ability is suppressed by the intermediate (I)-domain (ID). This I-domain-mediated nucleoplasmic-retaining mechanism does not depend on the B- or the G-domain, but is disabled by the GTP-bound G-domain. Without the I-domain, the G-domain is localized in the nucleolus regardless of its GTP-binding state. It remains unclear why most nucleolar components including NS shuttle between the nucleolus and the nucleoplasm, but this movement may allow cells to respond to a variety of environmental stimuli in a fast and dynamic fashion (Carmo-Fonseca et al., 2000).

Phylogenetically, NS is most closely related to GNL3L in vertebrates. They share the same yeast orthologue: Grn1p in *Schizosaccharomyces pombe* and Nug1p in *Saccharomyces cerevisiae*. Grn1p is involved in the processing of 35S pre-ribosomal RNA (rRNA), the nuclear export of Rpl25, and the maintenance of cell growth (Du et al., 2006). Mutation of *Nug1* inhibits the export of 60S subunit from the nucleolus (Du et al.,

2006; Kallstrom et al., 2003). Although the yeast orthologue of NS and GNL3L displays general activities in growth and ribosome biogenesis, rodent NS and GNL3L are distinctly expressed in different tissues. Also, only human GNL3L, but not NS, can rescue the *Grn1*-deficient growth phenotype in fission yeasts (Du et al., 2006). Furthermore, the expression of NS does not correlate completely with the sites of nascent rRNAs and 28S RNA-containing ribosomes (Politz et al., 2005). These results suggest that NS and GNL3L have evolved specific properties in vertebrates, become functionally diverged from each other and from Grn1p, and NS is not simply a rRNA-processing protein. Alternatively, NS may be associated with other nucleolar functions.

Though the molecular basis for the requirement of NS for cell cycle progression remains unclear, NS interaction with the tumor suppressor p53 provides some clue. Mutation analysis indicates that overexpression of a mutated NS, which lacks the GTP-binding domain, prevents cells from entering mitosis and causes apoptosis in a p53-dependent manner (Tsai and McKay, 2002).The p53-binding of NS, which is mediated through the N-terminal basic domain of NS, is required for the induction of cell death by the overexpression of this NS mutant. This finding suggests a role for NS in the regulation of the p53 pathway. The fact that NS shuttles between nucleolus and nucleoplasm in a GTP-regulated manner also allows NS the opportunity to interact with p53 (Tsai and McKay, 2005). Interestingly, an NS gain-of-function phenotype can only be achieved by an optimal level of overexpression and that a high-level overexpression of NS may inversely affect cell proliferation (Zhu et al., 2006). Supporting this idea, stable U2OS cell lines, with a NS transgene expressed at different levels, demonstrated that low-overexpression lines had a clear growth advantage over the wild-type cells, whereas the moderate- and

high-overexpression lines displayed a similar or slowed growth rate compared to the control (Zhu et al., 2006). Based on these results, it is proposed that cell proliferation and senescence are controlled by a threshold level of NS. However, the mechanisms underlying the regulation remain completely unknown. Therefore, this thesis research project addresses the question of what is the mechanism underlying the cell cycle regulation by optimal level of NS. The approach I have taken is to manipulate the protein level of NS by overexpression or siRNA knockdown of NS and to detect the effect of different levels of NS on the p53-MDM2 feedback regulatory loop. Our results fit with the model proposed above, that is, ectopic overexpression or depletion of NS activates p53 and induces G1 cell cycle arrest through inhibition of MDM2. Overexpression of NS stabilizes p53 and induces cell cycle arrest by binding to and inhibiting MDM2-mediated p53 suppression. MPA-mediated GTP depletion induces the translocation of NS from the nucleolus to the nucleoplasm and enhances its interaction with MDM2 at early time points. This interaction may contribute to the early stage activation of p53 upon MPA treatment. Depletion of NS by either siRNA or mycophenolic acid (MPA) treatment may trigger nucleolar stress that in turn activate p53, via enhancement of the binding of the ribosomal proteins L5 and L11 to MDM2. Consequently, p53 is relieved from the MDM2-mediated suppression.

In this thesis, I also further investigate the role of certain ribosomal proteins in response to nucleolar stress induced by MPA and 5-FU. I show that MPA treatment leads to nucleolar disruption and markedly increases the binding of L5 and L11 to MDM2 at later time points. Knockdown of either L11 or L5 by siRNA significantly decreases the MPA-induced stabilization and activation of p53. Similar to the cellular response to the

MPA treatment, 5-FU treatment increases the fraction of ribosome-free L5, L11, and L23 ribosomal proteins and their interaction with MDM2. Consequently, MDM2 can no longer efficiently target p53 for ubiquitination and degradation, leading to p53 stabilization and activation.

Thus my data supports a role for NS in cell cycle regulation by demonstrating that overexpression of NS or nucleoplasmic translocation of NS upon MPA treatment at early time points may affect p53 stabilization and activation through inhibition of its negative regulator MDM2. My data also further supports a role for certain ribosomal proteins in activation of the p53 checkpoint by demonstrating that in response to nucleolar stress induced by MPA, 5-FU or reduction of NS by siRNA or MPA, there is an increased binding of ribosomal proteins to MDM2, leading to MDM2 inhibition and p53 activation.

CHAPTER TWO

Aberrant expression of nucleostemin activates p53 and induces cell cycle arrest via inhibition of MDM2

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SUMMARY

The nucleolar protein nucleostemin (NS) is essential for cell proliferation and early embryogenesis. Either depletion or overexpression of NS reduces cell proliferation. However, the mechanisms underlying this regulation are still unclear. Here we show that NS regulates p53 activity through inhibition of MDM2. NS binds to the central acidic domain of MDM2 and inhibits MDM2-mediated p53 ubiquitination and degradation. Consequently, ectopic overexpression of NS activates p53 and induces G1 cell cycle arrest. Interestingly, reduction of NS by siRNA-mediated depletion also activates p53 and induces G1 arrest. These effects require ribosomal proteins L5 and L11 as depletion of NS enhances their interaction with MDM2 and knockdown of either of them abrogates the NS-depletion-induced p53 activation and cell cycle arrest. These results suggest a p53-dependent cell cycle checkpoint that monitors the changes of the cellular levels of NS via MDM2 inhibition mechanisms.

INTRODUCTION

The tumor suppressor protein p53 responds to diverse stresses to regulate many target genes whose protein products induce cell-cycle arrest, apoptosis, senescence and DNA repair (Oren, 2003; Vogelstein et al., 2000). In unstressed cells, p53 is controlled at a low level by its inhibitor MDM2, an E3 ubiquitin ligase that ubiquitinates and targets p53 for proteasome-mediated degradation (Fang et al., 2000; Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997), through a feedback mechanism (Picksley and Lane, 1993; Wu et al., 1993). In response to stresses p53 is stabilized and activated largely through inhibition of MDM2. These include DNA damage, oncogenic, and nucleolar

stresses (Lindstrom et al., 2007). DNA damage induces phosphorylation of both p53 and MDM2, unties the p53-MDM2 feedback loop, and consequently activates p53 (Banin et al., 1998; Canman et al., 1998; Maya et al., 2001; Schon et al., 2002; Siliciano et al., 1997). Oncogenic stress induces expression of the tumor suppressor protein ARF, which prevents MDM2 from targeting p53 and activates p53 (Palmero et al., 1998; Sherr and Weber, 2000; Zindy et al., 1998). Recently, the nucleolar stress (also called ribosomal stress) has been proposed because many external or internal insults funnel into the disruption of the nucleolus, leading to p53 activation (Rubbi and Milner, 2003a). This stress can be initiated by perturbation of ribosomal biogenesis by factors that inhibit rRNA synthesis, processing, and ribosome assembly, such as the treatment with actinomycin D (Act D) or 5-fluorouracil (5-FU) (Ashcroft et al., 2000; Gilkes et al., 2006; Sun et al., 2007), serum starvation (Bhat et al., 2004), expression of dominant negative mutant Bop1 (Pestov et al., 2001), or genetic disruption of ribosomal protein S6 and TIF-IA (Panic et al., 2006; Yuan et al., 2005).

Our lab and others have shown that a number of ribosomal proteins, including L5, L11, L23 and S7, bind to MDM2 and inhibit MDM2-mediated p53 suppression (Chen D, 2007; Dai and Lu, 2004; Dai et al., 2006b; Dai et al., 2004; Jin et al., 2004; Lohrum et al., 2003; Zhang et al., 2003). These ribosomal proteins might play a crucial role in p53 activation in response to the nucleolar stress. In addition to ARF and ribosomal proteins, other nucleolar proteins also target the p53-MDM2 pathway. For example, nucleophosmin (also called B23) binds to MDM2 and acts as a negative regulator of the p53-MDM2 interaction in response to UV damage or viral stress (Kurki et al., 2004). The nucleolar phosphoprotein nucleolin binds to MDM2, resulting in the inhibition of p53

ubiquitination and MDM2 auto-ubiqutination and activation of p53 (Saxena et al., 2006). Taken together, these findings point to a critical role for the nucleolar proteins in transmitting the nucleolar stress signals to p53-mediated cell growth inhibition.

The nucleostemin (NS), a recently identified nucleolar protein, regulates cell cycle progression as well. It was initially identified as a putative nucleolar GTP-binding protein that is expressed in neural stem cells, embryonic stem cells, hematopoietic primitive cells, and tumor cell lines (Tsai and McKay, 2002). The expression of NS decreases drastically prior to cell cycle exit upon differentiation of the stem cells, indicating that it is essential for cell proliferation (Tsai and McKay, 2002). Consistently, NS is essential for early embryogenesis as a complete loss of function cause embryonic lethality at as early as E3.5 (Beekman et al., 2006; Zhu et al., 2006). Interestingly, both depletion and overexpression of NS in cultured neural stem cells, cancer cells, and mouse embryonic fibroblast cells disrupts cell-cycle progression, suggesting that an optimal level of NS is essential for maintaining the proliferation of those cells (Beekman et al., 2006; Tsai and McKay, 2002; Zhu et al., 2006). However, the molecular mechanisms underlying the role of NS in regulating cell proliferation remain unclear. It has been shown that NS directly bind to p53 through its N-terminal basic domain, which is required for NS overexpression-induced cell death, suggesting that NS may regulate the p53 pathway (Tsai and McKay, 2002). However, how NS regulates the p53 and whether it regulates MDM2 activity are still unknown. Also, how depletion of NS results in cell cycle arrest is also unclear.

In light of the fact that many nucleolar proteins target MDM2, we reasoned that NS may also target MDM2 for p53 response. Indeed, we find that both overexpression and

reduction of NS activate p53 through inhibition of MDM2. Overexpression of NS stabilizes p53 and induces cell cycle arrest by binding to and inhibiting MDM2-mediated p53 suppression. Depletion of NS by siRNA triggers p53 activation via enhancing the binding of the ribosomal proteins L5 and L11 to MDM2. Consequently, p53 is relieved from the MDM2-mediated suppression. These results reveal a novel and critical role for the NS in regulation of the p53-MDM2 feedback circuit.

MATERIALS AND METHODS

Cell lines, plasmids, and antibodies. Human H1299, U2OS, and SJSA cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 5% CO2 humidified atmosphere as previously described (Dai et al., 2004). Expression plasmid encoding Flag-tagged NS (Flag-NS) was constructed by inserting full-length NS cDNA into pcDNA3-2Flag vector. The cDNA was amplified by PCR from a NS cDNA clone (Clontech) using primers 5'-CGCGGATCCATGAAAAGGCCTAAGTTAAAG-3' (P1) and 5'-CGCTCTAGATTACACATAATCTGTACTGAAG-3' (P2). The GFP-NS plasmid was cloned by inserting full-length NS cDNA using PCR into pEGFP-C1 (Clontech) vector. The primers used were P1 and 5'-CCCAAGCTTGGGTTACACATA ATCTGTACTGAAG-3' (P3). Deletion mutants of the NS were also generated by PCR and cloned into pcDNA3-2Flag vector. The HA-MDM2 expression vector has been described (Dai and Lu, 2004). The V5-tagged deletion mutants of MDM2 were constructed by inserting PCR products into the pcDNA3-V5 vector. Anti-L5 (Dai and Lu, 2004), anti-L11 (Sun et al, 2007), anti-HA, and anti-MDM2 (2A10 and 4B11) (Dai and

Lu, 2004; Dai et al., 2004) antibodies have been described. Anti-p21 (NeoMarkers), anti-p53 (DO-1, Santa Cruz), anti-MDM2 (SMP14, Santa Cruz), anti-NS (Chemicon), anti-Flag (sigma), and anti-V5 (Invitrogen) were purchased. Rabbit polyclonal anti-NS antibodies were generated using purified His-tagged full-length NS protein expressed in *Escherichia coli* as an antigen.

Cotransfection, immunoblot and co-immunoprecipitation analyses. Cells were transfected with plasmids as indicated in figure legends using TransIT®-LT1 reagents following the manufacturer's protocol (Mirus Bio Corporation). Cells were harvested at 48 hours posttransfection and lysed in lysis buffer consisting of 50 mM Tris/HCl (pH 8.0), 0.5% Nonidet P-40 (NP-40), 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.25 μg/ml pepstatin A, and 1 mM leupeptin. Equal amounts of cleared cell lysates were used for immunoblot analysis as described previously (Dai et al., 2004). Co-IP assays were conducted as described previously (Dai et al., 2004). Bound proteins were detected by immunoblot using antibodies as indicated in figure legends.

In Vivo Ubiquitination Assays. H1299 cells were transfected with plasmids as indicated in figure legend. The cells were treated with 20 μM of MG132 for 6 hours prior to harvest and harvested at 36 hours posttransfection. *In vivo* ubiquitination assays were conducted as described previously (Dai et al., 2004). Eluted proteins were analyzed by immunoblot with anti-p53 (DO-1) antibodies.

RNA Interference (RNAi). RNAi-mediated knockdown of endogenous L5, L11, and NS was performed essentially as described (Dai et al., 2004). The target sequences for L5, L11, and the control scrambled II RNA were described (Dai and Lu, 2004; Dai et

al., 2004). The target sequences for NS were 5'-AAGCTGTACTGCCAAGAAC-3' (NS siRNA-1, used for all experiments except where indicated) and 5'-AAGAACTAAAACA GCAGCAGA-3' (NS siRNA-2) (Tsai and McKay, 2002). All the siRNA duplexes with a 3' dTdT overhang were synthesized by Dharmacon (Lafayette, CO). These siRNA duplexes (0.2 μM) were introduced into cells using SilentFect (BioRad) following the manufacturer's protocol. Cells were harvested 48 to 72 hours after transfection for immunoblot, IF staining, RT-real-time PCR, and cell cycle analyses.

Reverse Transcription (RT) and Real-time PCR Analyses. Total RNA was isolated from cells using Qiagen RNeasy Mini Kits (Qiagen, Valencia, CA). Reverse transcriptions were performed as described (Dai and Lu, 2004). Quantitative real time PCR was performed on an ABI 7300 real time PCR system (Applied Biosystems) using SYBR Green Mix (Applied Biosystems) as described previously (Sun et al., 2007). All reactions were carried out in triplicate. Relative gene expression was calculated using the ΔCτ method following the manufacturer's instruction. The primers for *p21*, *mdm2*, and *GAPDH* were described (Sun et al., 2007).

Cell Cycle Analyses. U2OS Cells were transfected with plasmids or siRNAs as described in figure legends. Cells transfected with GFP or GFP-NS were treated with 200 ng of nocodazole/ml for 16 hours before harvesting. Cells were fixed and stained in 500 μl of propidium iodide (PI, Sigma) stain buffer (50 μg/ml PI, 30 μg/ml polyethylene glycol 8000, 200μg/ml RNase A, 0.1% Triton X-100, 0.38 M NaCl, pH 7.2) at 37°C for 30 minutes. The cells were then analyzed for DNA content using a Becton Dickinson FACScan flow cytometer. Data was analyzed using the CellQuest and Modfit software programs.

RESULTS

NS interacts with MDM2 in Cells. It has been shown that overexpression of NS resulted in inhibition of cell proliferation (Tsai and McKay, 2002; Zhu et al., 2006). This effect may be, at least partially, attributed to its ability to bind to p53 (Tsai and McKay, 2002). To further gain insight into the mechanisms underlying this inhibition, we sought to test whether NS also interacts with MDM2 because MDM2 is a master regulator of p53 (Fang et al., 2000; Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997). To do so, we cotransfected H1299 cells with Flag-NS and HA-MDM2 followed by co-immunoprecipitation (co-IP)-immunoblot (IB) assays. As shown in Fig. 2.1A, MDM2 was indeed specifically co-immunoprecipitated with NS by the anti-Flag antibody in cells co-transfected with both HA-MDM2 and Flag-NS (lane 3). Conversely, NS was specifically co-immunoprecipitated with MDM2 in cells by the anti-HA antibody when co-transfected with HA-MDM2 (lane 6). To determine whether endogenous NS and MDM2 also interact with each other, cell lysates from SJSA cells were used because these cells express high level of MDM2 (Dai and Lu, 2004). As shown in Fig. 2.1B, endogenous NS was specifically co-immunoprecipitated with MDM2 with a monoclonal anti-MDM2 antibody and endogenous MDM2 was co-immunoprecipitated with NS with a polyclonal anti-NS antibody. These results suggest that NS interacts with MDM2 in cells.

NS constantly shuttles between the nucleolus and the nucleoplasm in a GTP-regulated manner (Tsai and McKay, 2005). To test which compartment NS interacts with MDM2, H1299 cells were co-transfected with GFP-NS and HA-MDM2 followed by immunofluorescence (IF) staining using anti-MDM2 antibodies. As shown in Fig. 2.1C,

ectopic MDM2 was primarily expressed in the nucleoplasm while GFP-NS was predominantly expressed in the nucleolus but a portion of GFP-NS was also present in the nucleoplasm. We did not observe a significant relocalization of MDM2 into the nucleolus by GFP-NS (data not shown). These results suggest that NS most possibly interacts with MDM2 in the nucleoplasm.

The coiled-coil and GTP-binding domains of NS bind to the central acidic domain of MDM2. To further characterize the MDM2-NS interaction, we mapped the binding domains of both proteins using transfection, followed by co-IP analysis. We first generated a set of Flag-tagged deletion mutants of NS. These deletion mutants and wild-type (wt) Flag-NS were co-transfected with HA-MDM2 followed by co-IP assays as shown in Fig. 2. 2A. HA-MDM2 was co-immunoprecipitated with a mutant deleted with the N-terminal basic domain (NS^{47-549}, lane 3) and a mutant deleted with both N-terminal basic domain and the C-terminal domains, including the acidic domain (NS^{47-270}, lane 5), although to a less extent as compared to wt NS (lane 2). By contrast, a N-terminally deleted mutant ($NS^{268-549}$) did not bind to MDM2 (lane 4). These results suggest that MDM2 binds to the central coiled-coil and GTP binding domains of NS (Fig. 2.2B). To map the NS binding domain in MDM2, we generated a panel of V5-tagged MDM2 deletion mutants. Similar assays were performed using anti-V5 antibodies. As shown in Fig. 2.2C, a mutant deleted with the N-terminal 210 residues ($MDM2^{210-491}$) was co-immunoprecipitated with Flag-NS (lane 3). Further deletion of a portion of acidic domain ($MDM2^{236-491}$) markedly reduced the binding of MDM2 to NS (lane 4) while further deletion of entire acidic domain ($MDM2^{284-491}$) abolished the MDM2-NS binding (lane 5). Also, the N-terminal fragment containing p53-binding domain ($MDM2^{1-150}$) did not

bind to NS. These results suggest that the central acidic domain of MDM2 is essential for its binding to NS (Fig. 2.2D). It has been shown that p53 binds to NS at the N-terminal basic domain (Tsai and McKay, 2005). Thus, p53 and MDM2 bind to NS at different domains. Indeed, we observed that NS co-immunoprecipitated with both p53 and MDM2 simultaneously in cells when all the proteins are expressed (data not shown), suggesting that NS could form a tertiary complex with MDM2 and p53 in cells.

Overexpression of NS inhibits MDM2-mediated p53 ubiquitination and degradation. The central acidic domain of MDM2 is critical for its function to mediate p53 degradation (Kawai et al., 2003b; Meulmeester et al., 2003). In fact, many negative regulators of MDM2, including ARF (Midgley et al., 2000; Stott et al., 1998), L5 (Elenbaas et al., 1996), and L23 (Dai et al., 2004; Jin et al., 2004), target this domain and inhibit the MDM2-p53 circuit. Therefore, we next wanted to determine if NS binding regulates MDM2-mediated p53 ubiquitination and degradation. We introduced exogenous proteins into H1299 cells as shown in Fig. 2.3A. As expected (Fang et al., 2000; Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997), overexpression of MDM2 remarkably reduced p53 levels (lane 3 of Fig. 2.3A). In contrast, further expression of NS partially rescued MDM2-mediated p53 degradation (lane 4 of Fig. 2.3A). Interestingly, like other MDM2 regulators (Dai et al., 2006b; Lohrum et al., 2003; Xirodimas et al., 2001a), overexpression of NS also stabilized MDM2 independently of p53 (lane 3 of Fig. 2.3B and lane 9 of Fig. 2.1A). These results suggest that excess expression of NS blocks MDM2-mediated p53 degradation. To determine if this stabilization of p53 is due to inhibition of MDM2-mediated p53 ubiquitination, H1299 cells were introduced with exogenous MDM2, p53 and NS and treated with the

proteasome inhibitor MG132 for 6 hours for *in vivo* ubiquitination assays. As shown in Fig. 2.3C and by others (Fang et al., 2000; Haupt et al., 1997; Honda et al., 1997), MDM2 ubiquitinated p53 (lane 3). By contrast, expression of NS (lane 4) inhibited MDM2-mediated p53 ubiquitination. These results indicate that NS can stabilize p53 by alleviating MDM2-mediated p53 ubiquitination and degradation.

Overexpression of NS activates endogenous p53 and induces G1 cell cycle arrest. Next, we examined the effect of NS on endogenous p53 by introducing Flag-NS into human osteosarcoma U2OS cells that contain wild-type p53. Interestingly, overexpression of Flag-NS induced p53 in a dose-dependent fashion (Fig. 2.4A). Correspondingly, the levels of the p53 targets, $p21^{cip1}$ (p21 thereafter) and MDM2, were also induced (Fig. 2.4A). Consistently, overexpression of NS prolonged half-life of endogenous p53 in U2OS cells (data not shown). These results, together with the results above, suggest that overexpression of NS induces the level of p53 by blocking MDM2-mediated p53 degradation. To test whether the induced p53 and p21 by NS mediates cell cycle arrest, we transfected U2OS cells with GFP-NS. Cells were treated with the mitotic inhibitor nocodazole before FACS analysis to block cells in G2/M (Dai et al., 2004). Therefore, cells found in G1 phase are previously arrested and do not reach G2/M phase. GFP-positive cells were then gated for cell cycle analysis. As shown in a representative result in Fig. 2.4B and summarized in Fig. 2.4C, a significant portion of GFP-NS expressing cells were arrested in the G1 phase as compared to GFP expressing cells. Therefore, ectopic overexpression of NS induced G1 cell cycle arrest.

Knockdown of NS by siRNA-mediated ablation also activates p53 and induces G1 cell cycle arrest. To further define the physiological relevance of NS in the regulation

of the MDM2-p53 pathway, we performed siRNA-mediated knockdown experiments. Surprisingly, knockdown of endogenous NS also induces p53 levels as well as the levels of p53 targets, p21 and MDM2 (Fig. 2.5A). p53's activation by knockdown of NS was confirmed by two siRNAs against two different sequences in the *NS* gene, thus this effect was less likely an off-target effect of siRNA transfection. Consistently, knockdown of NS induced the mRNA expression of *p21* and *MDM2*, as determined by RT-real-time PCR assays (Fig. 2.5B). Furthermore, knockdown of NS by two individual siRNAs drastically induced cell cycle arrest in G1 phase as indicated by the significant increase of the G1 phase cells and decrease of S phase cells in NS siRNA transfected cells compared to scrambled RNA transfected cells (Fig. 2.5C and 2.5D). These results suggest that depletion of NS activates p53 and induces G1 arrest.

Knockdown of NS by siRNA does not disrupt the nucleolus but enhances the interaction of MDM2 with ribosomal proteins L5 and L11. NS is an essential nucleolar protein that constantly shuttles between the nucleolus and the nucleoplasm (Tsai and McKay, 2005) and is expressed in most proliferating cells (Tsai and McKay, 2002). Treatment of cells with either actinomycin D or MPA relocalizes NS from the nucleolus to the nucleoplasm (Tsai and McKay, 2005). Thus, it is possible that NS is an integrated component of the nucleolus and that depletion of NS could trigger a nucleolar stress that in turn activates p53. Because nucleolar stress usually lead to the disruption of the nucleolus (Rubbi and Milner, 2003a), we first determined the nucleolus integrity upon NS knockdown by IF staining. To our surprise, knockdown of NS did not significantly alter the nucleolar structure as determined by the nucleolar marker B23 (Fig. 2.6A). This is consistent with a previous observation showing that NS deficiency does not cause

nucleolar disruption in embryonic blastocysts (Beekman et al., 2006). However, whether nucleolar disruption is absolutely required for the stress-induced p53 activation remains to be determined. It has been suggested that genetic inactivation of ribosomal protein S6 induces p53-dependent checkpoint while not disrupting the nucleolus (Panic et al., 2007; Panic et al., 2006). We therefore determined if the MDM2-binding ribosomal proteins could still participate in the p53 activation induced by NS depletion as these ribosomal proteins inhibit MDM2-mediated p53 suppression in response to the nucleolar stress (Dai et al., 2006a; Dai and Lu, 2004; Dai et al., 2004; Jin et al., 2004; Lohrum et al., 2003; Zhang et al., 2003). We investigated the ribosomal proteins L5 and L11, but not L23, in the following studies, since knockdown L23 itself also induces and activates p53 (Dai et al., 2004; Jin et al., 2004). To test this idea, we first examined whether NS knockdown enhances the interaction between MDM2 and these L proteins. U2OS cells were transfected with scrambled or NS siRNA followed by co-IP assays with anti-MDM2 antibodies. As shown in Fig. 2.6B, NS knockdown indeed enhanced the interaction between MDM2 and L5 or L11. These results suggest that knockdown of NS may trigger a nucleolar stress that enhances the interactions between MDM2 and the two L proteins.

Knockdown of L5 or L11 by siRNA inhibits NS knockdown-induced p53 activation and G1 arrest. The enhanced interaction of MDM2 with L5 and L11 suggests that these ribosomal proteins may play a role in NS knockdown-induced p53 activation. Therefore, we examined if knocking down their levels by siRNAs would influence NS knockdown-induced p53 level and activity in U2OS cells. As shown in Fig. 2.7, reduction of either L5 (Fig. 2.7A) or L11 (Fig. 2.7B) levels by siRNA markedly inhibited the NS knockdown-induced levels of p53 as compared to that in scrambled RNA

transfected cells. Consistently, NS knockdown induction of p21 and MDM2 protein levels was drastically impaired by siRNA against L5 or L11, but not a scrambled sequence (Fig. 2.7A and 2.7B). This impairment was also true to the mRNA level of *p21* and *MDM2*, as measured by real-time RT-PCR assays (Fig. 2.7C). Consistently, ablation of either L5 or L11 by siRNA significantly reduced the NS knockdown-induced G1 arrest (Fig, 2.8A and 2.8B). These results reveal that L5 and L11 are required for NS knockdown-mediated induction of p53 activation and G1 arrest.

DISCUSSION

It has been shown that the optimal levels of NS are essential for normal cell homeostasis. Either aberrant overexpression or depletion of NS inhibits cell proliferation (Tsai and McKay, 2002; Zhu et al., 2006). In this study, we have further illustrated the mechanisms underlying the role of NS in cell cycle regulation. We show that both aberrant high and low levels of NS induce G1 arrest through activation of p53 as a result of inhibition of MDM2. Upon ectopic overexpression the excess NS molecules directly bind to MDM2 and inhibit MDM2-mediated p53 ubiquitination and degradation. When the level of NS is significantly reduced by siRNA-mediated depletion, the nucleolar stress occurs, leading to the association of ribosomal proteins L5 and L11 with MDM2 and subsequent inhibition of MDM2 function. Consequently, p53 is activated in both cases (Fig. 2.9). Therefore, p53 acts as a key cell cycle checkpoint mediator that senses the aberrant cellular levels of NS via distinct mechanisms that funnel into the inhibition of MDM2.

Our mapping studies show that NS binds to the central acidic domain of MDM2. This region has been shown to be critical for mediating p53 degradation (Kawai et al., 2003b; Meulmeester et al., 2003). Indeed, many MDM2 regulatory proteins, such as L5, L23, and ARF, bind to this region and regulate MDM2-mediated p53 turnover (Dai et al., 2004; Elenbaas et al., 1996; Jin et al., 2004; Midgley et al., 2000; Stott et al., 1998). Similar to the effect of these proteins on MDM2 function, NS binding to MDM2 also results in inhibition of E3 ligase activity of MDM2 towards p53, leading to stabilization and activation of p53. Since MDM2 is a cytoplasm-nuclear shuttle protein and predominantly localized in the nucleoplasm (Roth et al., 1998; Tao and Levine, 1999) and NS is a nucleoplasm-nucleolar shuttle protein and predominantly localized in the nucleolus (Tsai and McKay, 2002; Tsai and McKay, 2005), one question would be how these proteins interact in cells. It is possible that under normal growth conditions, the steady-state nucleoplasmic NS is extremely low and would not significantly interact with MDM2. That is why we can only detect a weak binding for the endogenous proteins in normally cultured cells. However, when NS is overexpressed such as ectopic expression, a significant portion of the protein is accumulated in the nucleoplasm and thus able to interact with MDM2. The dynamic nature of both proteins in nuclear shuttling would allow their interaction to respond to cell growth and stress signals. It is conceivable that in response to certain cellular stress, NS may be redistributed from the nucleolus to the nucleoplasm where it can target MDM2. Indeed, we observed that the NS-MDM2 interaction was significantly enhanced at the early stage of MPA treatment, which depletes intracellular GTP levels and leads to the nucleoplasmic localization of NS (see Chapter 3).

It has been shown that p53 can bind to NS at the N-terminal basic domain (Tsai and McKay, 2002) and our mapping study show that MDM2 interacts with the coiled-coil and GTP-binding domains of NS, suggesting that NS could bind to p53 and MDM2 simultaneously. Indeed, we observed the tertiary complex of NS association with both p53 and MDM2 (data not shown). Thus, MDM2 does not compete with p53 for NS binding. This is consistent with the regulation of the MDM2-p53 pathway by other nucleolar proteins such as ARF and ribosomal proteins, which do not disrupt the p53-MDM2 binding. Instead, they associate with both p53 and MDM2 and inhibit MDM2-mediated p53 ubiquitination and degradation (Dai and Lu, 2004; Dai et al., 2006b; Dai et al., 2004; Honda and Yasuda, 1999; Jin et al., 2004; Midgley et al., 2000; Zhang et al., 2003).

Similar to overexpression of NS, depletion of NS by siRNA-mediated ablation also induces p53. Further analysis reveals that ribosomal proteins L5 and L11 are required for the NS-depletion induced p53 levels, activation, and cell cycle arrest, suggesting that depletion of NS triggers a nucleolar stress that activates p53. Unlike other nucleolar stresses such as those induced by treatment of 5-FU, actinomycin D, or the genetic knockdown of TIA-IA (Lohrum et al., 2003; Sun et al., 2007; Yuan et al., 2005), but like the case of S6 knockdown (Panic et al., 2007), knockdown of NS by siRNA did not significantly result in the disruption of the nucleolus, which is consistent with the observation that NS deficiency does not cause nucleolar disruption in embryonic blastocysts (Beekman et al., 2006). This raises a question whether the nucleolar disruption is required for the nucleolar stress. Because NS is localized into the distinct region of the nucleolus that is not actively involved in the ribosomal biogenesis (Politz et

al., 2005), how the NS depletion causes the nucleolar stress remains to be determined. Nevertheless, our results demonstrate that the ribosomal proteins L5 and L11 are required for NS depletion induced p53 activation. Consistently, NS depletion by siRNA treatment enhances the interaction of MDM2 with L5 and L11. One possibility is that NS may regulate nuclear export of the ribosomal proteins and depletion of NS would lead to the accumulation of free L5 and L11 in the nucleoplasm where they interact with MDM2. This is an interesting future topic worth testing. Interestingly, heterozygous deletion of NS in mice did not trigger significant p53 activation (Zhu et al., 2006). This result may suggest that the loss of one copy of the *NS* gene would not reduce NS to a level that is low enough to trigger a nuceloalr stress. Also, p53 knockout did not rescue the lethality by NS knockout in mice (Beekman et al., 2006), suggesting that NS may have p53-independent function especial for early embryogenesis. Further characterizing this p53-independent function would provide insights into the role of NS in cell proliferation.

In summary, our results suggest that maintaining a balanced level of NS is essential for normal cell proliferation. Aberrant high levels of NS, such as the case of ectopic overexpression, or abnormally low levels of NS, such as those induced by siRNA-mediated depletion or cell differentiation, activate p53 and induce G1 arrest. However, the mechanisms underlying these p53 activation pathways appear to be different. At high levels, NS interacts with and directly inhibits the E3 ligase activity of MDM2 whereas at low levels nucleolar stress occurs, leading to the enhanced interaction of MDM2 with L5 and L11 and inhibition of MDM2 function. Thus, our study uncovers the cellular and molecular mechanisms by which aberrant NS levels activate p53 and

induce cell cycle arrest: modulation of the MDM2-p53 pathway by NS itself and ribosomal proteins.

ACKNOWLEDGEMENTS

We thank Jayme Gallegos for proofreading this manuscript and other members of the laboratory for active discussion. This work is supported by grants to H.L from NIH/NCI (CA095441, CA93614 and CA079721).

FIGURE LEGENDS

Fig. 2.1. NS interacts with MDM2 in cells. (**A**). Ectopically expressed NS interacts with ectopically expressed MDM2 in cells. H1299 cells were transfected with Flag-NS and HA-MDM2 individually or together. Cell lysates were immunoprecipitated with anti-Flag (lanes 1 to 3) or anti-HA (lanes 4 to 6) antibodies followed by IB with anti-Flag or anti-HA antibodies. * indicates non-specific anti-HA antibody-reacting bands. (**B**). Endogenous NS interacts with endogenous MDM2 in cells. Cell lysates from SJSA cells were immunoprecipitated with monoclonal anti-MDM2 (4B11) antibodies (lane 3) or mouse IgG (lane 2), polyclonal anti-NS antibodies (lane 6) or rabbit IgG (lane 5) followed by anti-MDM2 and anti-NS antibodies. (**C**). NS co-localizes with MDM2 in the nucleoplasm. H1299 cells were co-transfected with GFP-NS and HA-MDM2. Forty-eight hours posttransfection the cells were immunostained with anti-MDM2 antibody followed by staining with goat-anti-mouse secondary antibody (red) and DAPI. GFP-NS was shown in green.

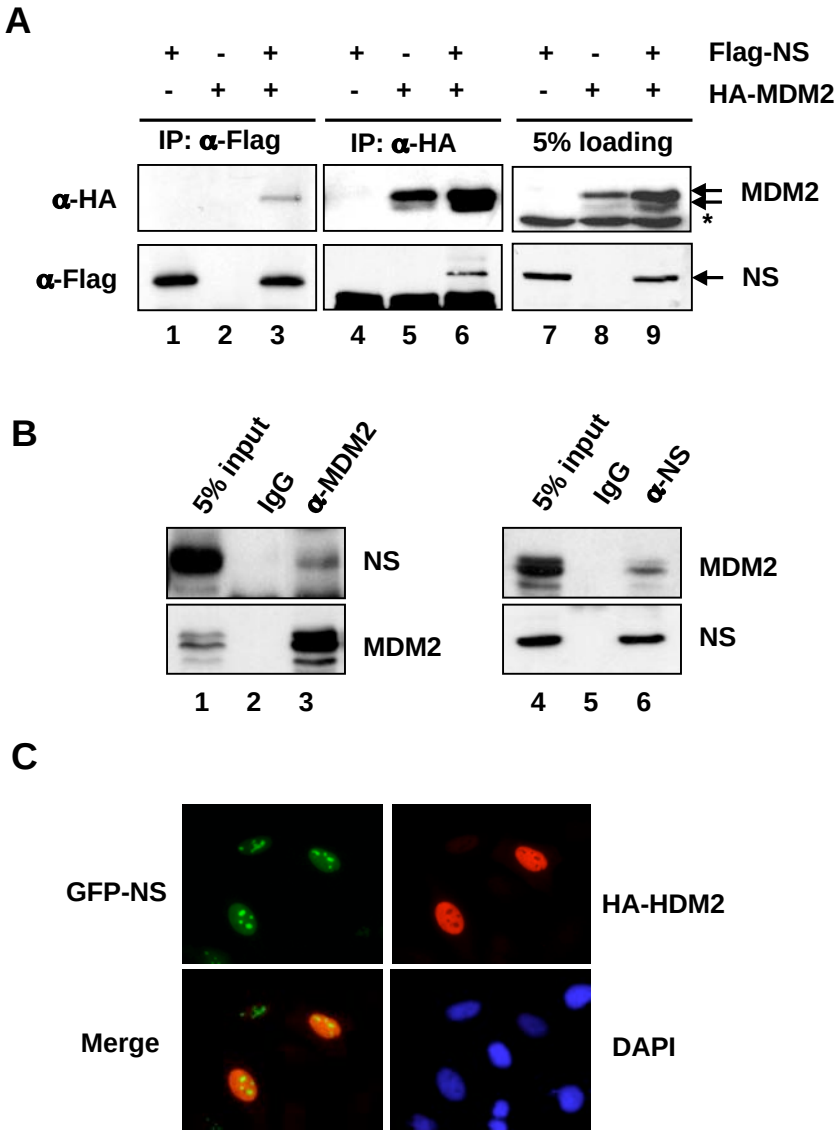

A
+ - + + - + + - + Flag-NS
- + + - + + - + + HA-MDM2
IP: α-Flag
IP: α-HA
5% loading
α-HA
α-Flag
MDM2
NS
*
1 2 3 4 5 6 7 8 9
B
5% input
IgG
α-MDM2
5% input
IgG
α-NS
NS
MDM2
MDM2
NS
1 2 3 4 5 6
C
GFP-NS
HA-HDM2
Merge
DAPI

Fig. 2.2. The coiled-coil and GTP binding domains of NS bind to the central acidic domain of MDM2. (**A**). MDM2 binds to the coiled-coil and GTP binding domains of NS. H1299 cells were transfected with Flag-tagged full length NS (wt) or its deletion mutants together with HA-MDM2 plasmid. Cell lysates were immunoprecipitated with the anti-Flag antibody followed by IB using antibodies as indicated. The lysates were also directly loaded onto a SDS gel for IB using the anti-HA antibody (bottom panel). (**B**). Schematic diagram of the NS with the indication of the MDM2-binding domain (black bar). BD, basic domain; AD, acidic domain; CC, coiled-coil domain; G4 and G1, putative GTP binding motifs. (**C**). NS binds to the central acidic domain of MDM2. H1299 cells were transfected with plasmids encoding V5-tagged MDM2 fragments together with Flag-NS plasmid as indicated. Cell lysates were immunoprecipitated with an anti-V5 antibody followed by IB using antibodies as indicated. The lysates were also directly loaded onto a SDS gel for IB using anti-Flag antibody (bottom panel). (**D**). Schematic diagram of the MDM2 protein with indication of the NS binding acidic domain (AD) (black bar). ZF represents zinc finger domain while RF represents ring finger domain.

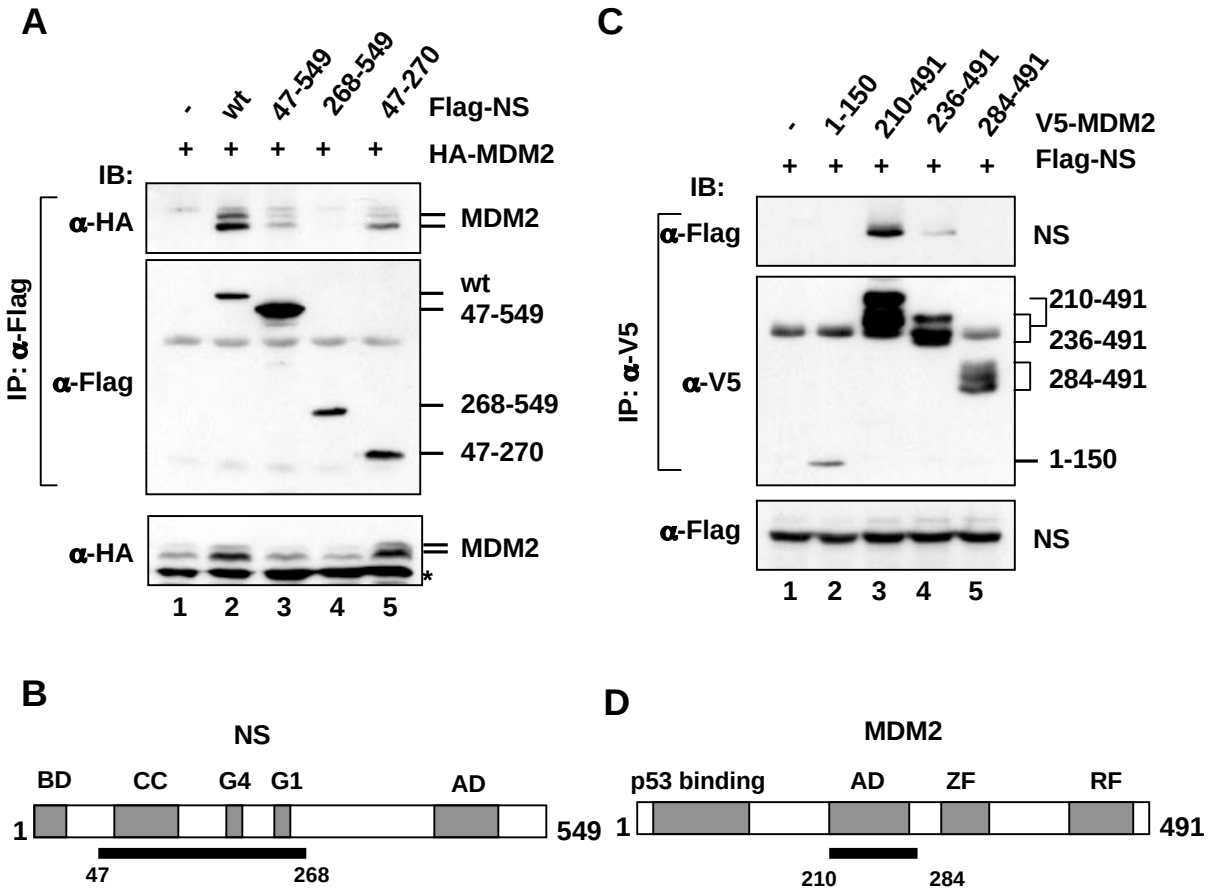

A
- wt 47-549 268-549 47-270
Flag-NS
+ + + + +
HA-MDM2
IB:
α-HA
MDM2
IP: α-Flag
α-Flag
wt
47-549
268-549
47-270
α-HA
MDM2
*
1 2 3 4 5
C
- 1-150 210-491 236-491 284-491
V5-MDM2
+ + + + +
Flag-NS
IB:
α-Flag
NS
IP: α-V5
α-V5
210-491
236-491
284-491
1-150
α-Flag
NS
1 2 3 4 5
B
NS
BD CC G4 G1 AD
1
549
47
268
D
MDM2
p53 binding AD ZF RF
1
491
210
284

Fig. 2.3. NS inhibits MDM2-mediated p53 ubiquitination and degradation. (**A**). NS inhibits MDM2-mediated p53 degradation. H1299 cells were transfected with plasmids as indicated. Cell lysates were subjected to IB using antibodies as indicated on the left. (**B**). NS stabilizes MDM2 independently of p53. H1299 cells were transfected with HA-MDM2 in the presence or absence of Flag-NS as indicated followed by IB using antibodies as indicated. (**C**). NS inhibits MDM2-mediated p53 ubiquitination. H1299 cells were transfected with plasmids as indicated. The transfected cells were treated with MG132 (20 μM) for 6 hrs before harvesting. Ubiquitinated p53 species were detected by immunoblot with the anti-p53 (DO-1) antibody (upper panel). Ubiquitinated p53 (p53-(Ub)n) is indicated. The expression of total p53, MDM2, and NS is shown in lower panels. * indicates non-specific anti-HA antibody-reacting bands.

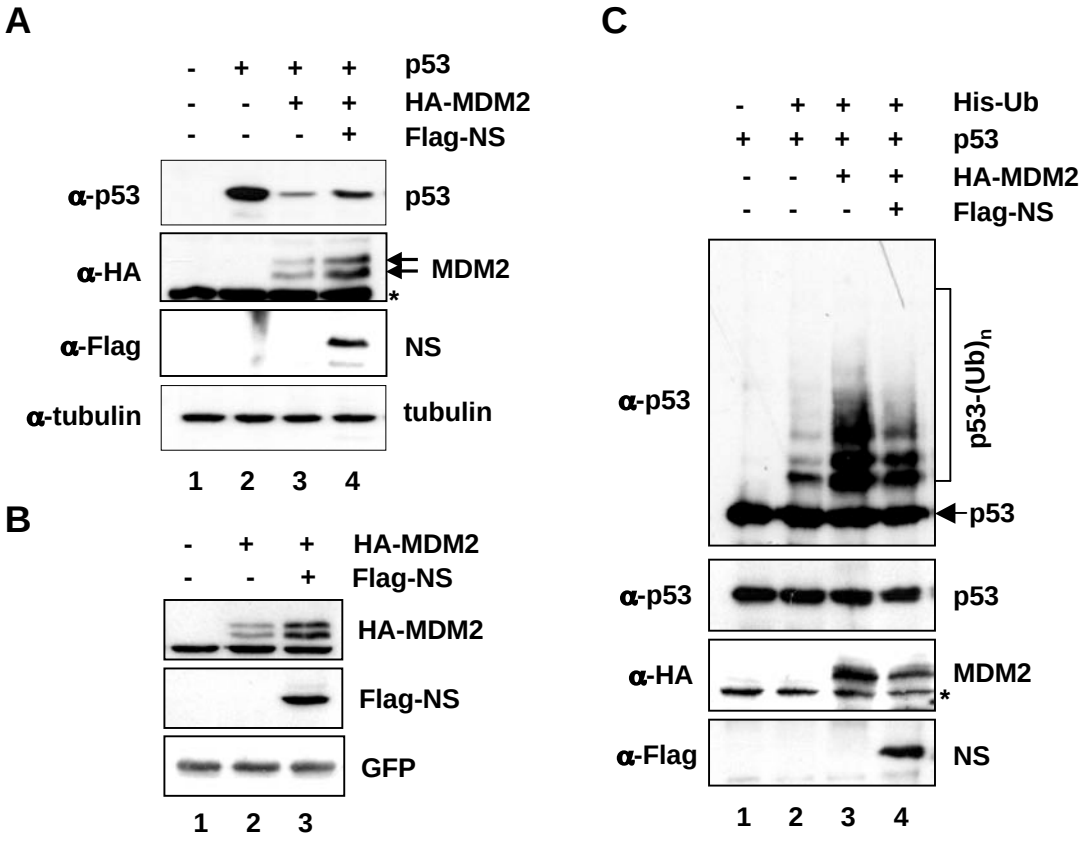
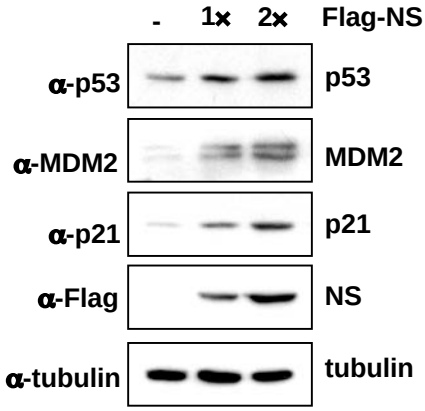
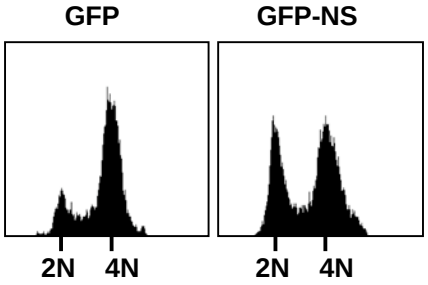

A
- + + + p53
- - + + HA-MDM2
- - - + Flag-NS
α-p53
p53
α-HA
MDM2
*
α-Flag
NS
α-tubulin
tubulin
1 2 3 4
B
- + + HA-MDM2
- - + Flag-NS
HA-MDM2
Flag-NS
GFP
1 2 3
C
- + + + His-Ub
+ + + + p53
- - + + HA-MDM2
- - - + Flag-NS
α-p53
p53-(Ub)n
p53
α-p53
p53
α-HA
MDM2
*
α-Flag
NS
1 2 3 4

Fig. 2.4. Overexpression of NS activates p53 and induces G1 arrest. (**A**). Overexpression of NS induces p53 levels. U2OS cells were transfected with increasing amounts of Flag-NS. Cell lysates were assayed by IB for the expression of p53, MDM2, p21, and Flag-NS. (**B**), (**C**). Overexpression of NS induces G1 arrest. U2OS cells were transfected with GFP or GFP-NS and treated with 200 ng/ml of nocodazole as described in Materials and Methods. GFP-expressing cells were then gated for cell cycle analysis. The histograms of PI staining from one representative experiment are shown in (B). G1 and G2/M phase cells are indicated as 2N and 4N, respectively. The mean percentage of cells arrested in G1 phase is shown in (C).

A


-
1×
2×
Flag-NS
α-p53
p53
α-MDM2
MDM2
α-p21
p21
α-Flag
NS
α-tubulin
tubulin

B


GFP
GFP-NS
2N
4N
2N
4N

C

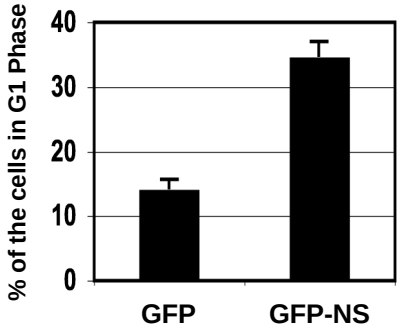

% of the cells in G1 Phase
40
30
20
10
0
GFP
GFP-NS

Fig. 2.5. Knockdown of endogenous NS activates p53 and induces G1 cell cycle arrest. (**A**). Knockdown of NS induces p53 levels. U2OS cells were transfected with scrambled or one of the two NS siRNA against different sequences. Cell lysates were assayed for the expression of p53, MDM2, p21, and NS as indicated using IB assays. (**B**). Knockdown of endogenous NS increases the mRNA expression of p53 target *mdm2* and *p21*. Total RNAs were extracted from U2OS cells transfected with siRNAs as in (A) and subjected to reverse transcription reactions followed by real-time PCR assays. Relative expression of *p21* and *mdm2* genes were normalized against the expression of *GAPDH*. (**C**) (**D**). Knockdown of endogenous NS induces G1 arrest. U2OS cells were transfected with siRNA as mentioned in (A). 72 hours posttransfection, the cells were harvested and stained with PI for cell cycle analysis. The histograms of PI staining from one representative experiment are shown in (C). The mean percentages of cells in G1 or S phase are shown in (D). Panels **B** and **D** were done by Mu-shui Dai.

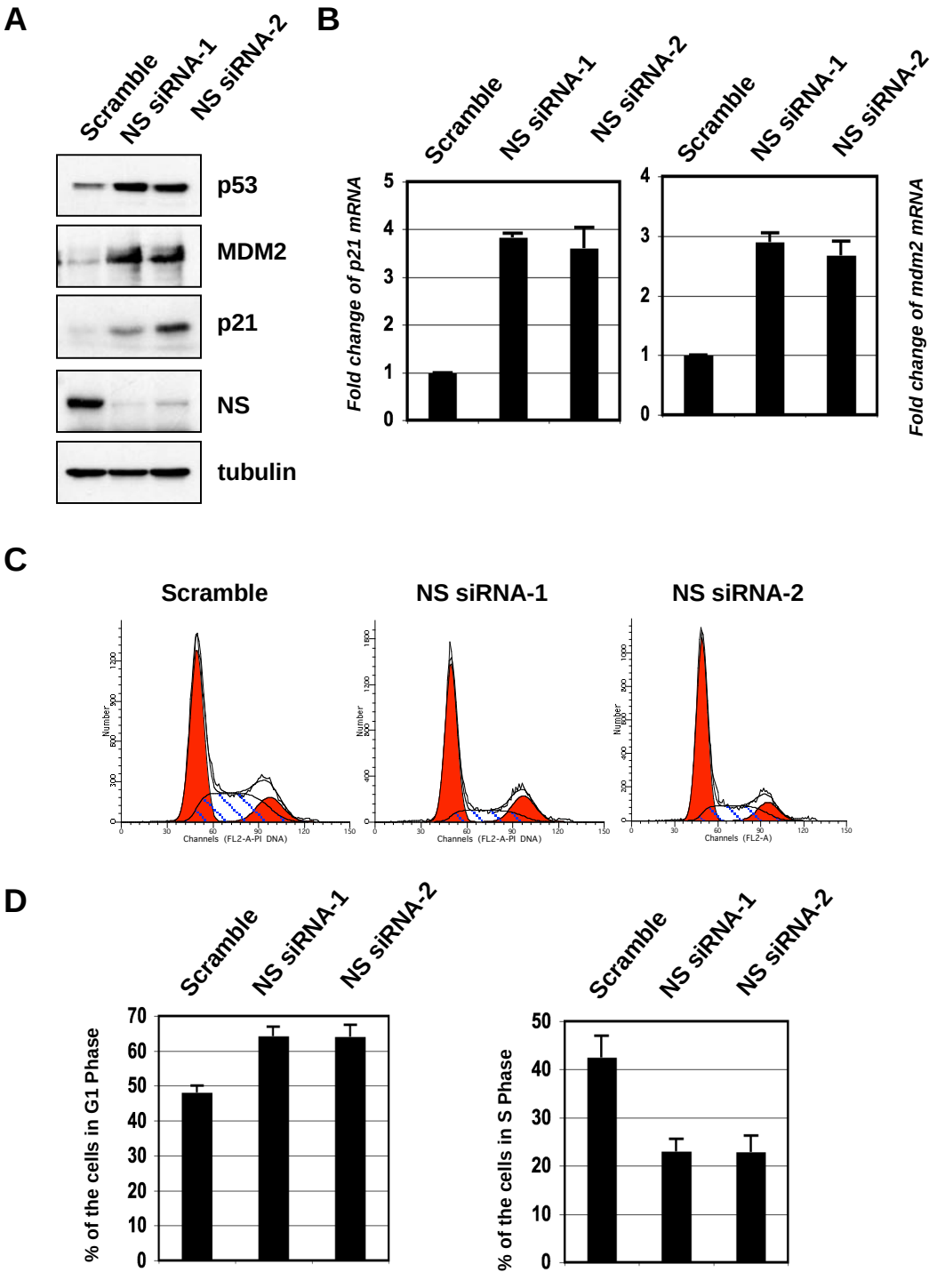
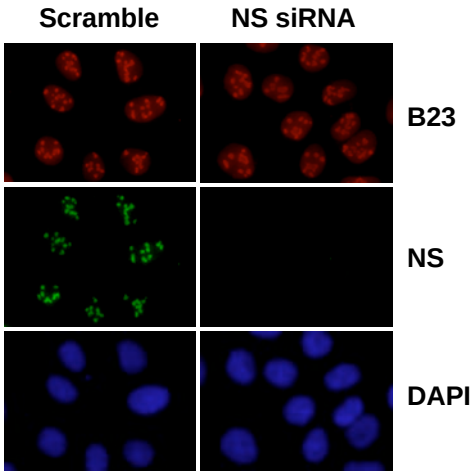

A
Scramble
NS siRNA-1
NS siRNA-2
p53
MDM2
p21
NS
tubulin
B
Scramble
NS siRNA-1
NS siRNA-2
Fold change of p21 mRNA
Scramble
NS siRNA-1
NS siRNA-2
Fold change of mdm2 mRNA
C
Scramble
NS siRNA-1
NS siRNA-2
Number
Channels (FL2-A-PI DNA)
Channels (FL2-A)
D
Scramble
NS siRNA-1
NS siRNA-2
% of the cells in G1 Phase
% of the cells in S Phase

Fig. 2.6. Knockdown of NS does not lead to the nucleolar disruption but enhances the interaction of MDM2 with ribosomal proteins L5 and L11. (**A**). Knockdown of endogenous NS does not disrupt the nucleolus. U2OS cells were transfected with scrambled or NS siRNA as indicated. The cells were then stained with anti-B23 (red) and anti-NS (green) antibodies as well as DAPI for DNA. (**B**). Knockdown of endogenous NS enhances the interaction of MDM2 with L5 and L11. U2OS cells were transfected with scrambled or NS siRNA. Cell lysates were subjected to co-IP using anti-MDM2 (4B11) antibodies followed by IB to detect the level of L11, L5, and MDM2.

A


Scramble
NS siRNA
B23
NS
DAPI

B

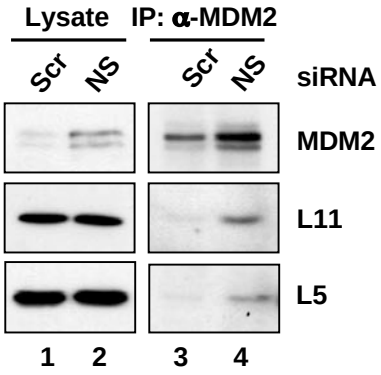

Lysate
IP: α-MDM2
Scr
NS
Scr
NS
siRNA
MDM2
L11
L5
1
2
3
4

Fig. 2.7. NS knockdown-induced activation of p53 requires ribosomal proteins L5 and L11. (**A**). Knockdown of L5 abolished the induction of p53 by knockdown of NS. U2OS cells were transfected with scrambled, NS siRNA, or L5 siRNA as indicated. Cell lysates were subjected to IB to detect the expression of p53, MDM2, p21, L5, or NS as indicated. (**B**). Knockdown of L11 abolished the induction of p53 by knockdown of NS. U2OS cells were transfected with scrambled, NS siRNA, or L11 siRNA as indicated. Cell lysates were subjected to IB to detect the expression of p53, MDM2, p21, L11, or NS as indicated. (**C**). Knockdown of L5 or L11 abolished the induced expression of *p21* and *mdm2* mRNA by knockdown of NS. U2OS cells were transfected with scrambled, NS siRNA, L5 siRNA, or L11 siRNA as indicated. Total RNAs were extracted and subjected to RT reactions followed by real-time PCR assays. Relative expression of *p21* and *mdm2* genes was normalized against the expression of *GAPDH*. Panel **C** was done by Mu-shui Dai.

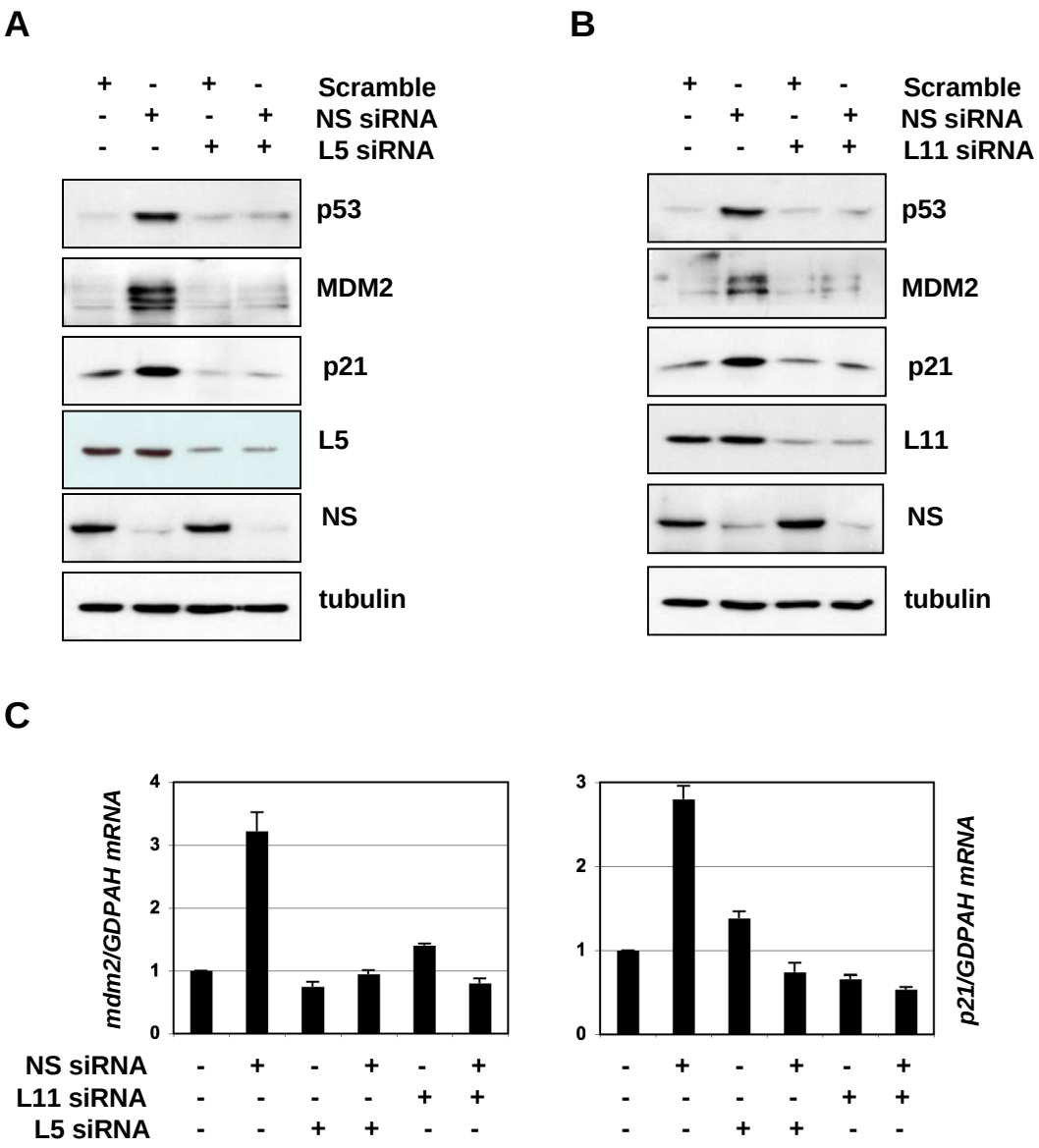
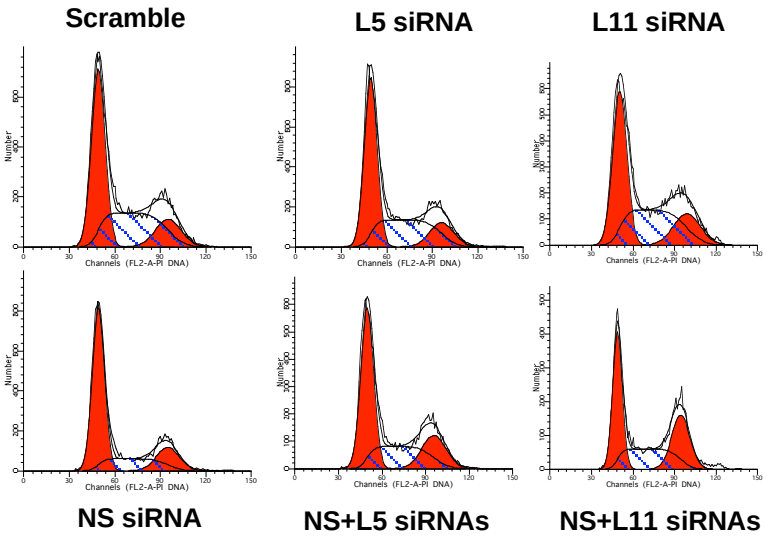

A
+ - + - Scramble
- + - + NS siRNA
- - + + L5 siRNA
p53
MDM2
p21
L5
NS
tubulin
B
+ - + - Scramble
- + - + NS siRNA
- - + + L11 siRNA
p53
MDM2
p21
L11
NS
tubulin
C
mdm2/GDPAH mRNA
0
1
2
3
4
NS siRNA - + - + - +
L11 siRNA - - - - + +
L5 siRNA - - + + - -
p21/GDPAH mRNA
0
1
2
3
- + - + - +
- - - - + +
- - + + - -

Fig. 2.8. NS knockdown-induced G1 arrest requires the ribosomal proteins L5 and L11. U2OS cells were transfected with scrambled, NS, L5, or L11 siRNA as indicated. 72 hours posttransfection, the transfected cells were harvested and stained with PI for cell cycle analysis. The histograms of PI staining from one representative experiment are shown in (A). The mean percentages of cells in G1 or S phase are shown in (B).

A


Scramble
L5 siRNA
L11 siRNA
NS siRNA
NS+L5 siRNAs
NS+L11 siRNAs

B

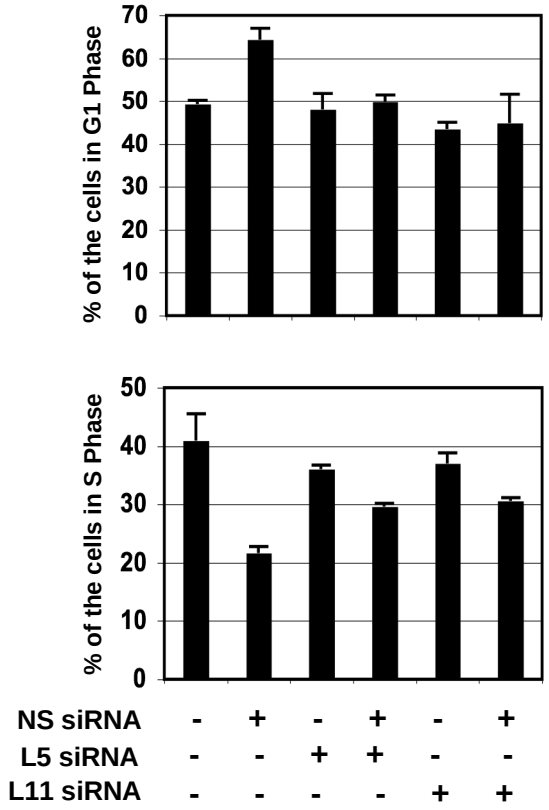
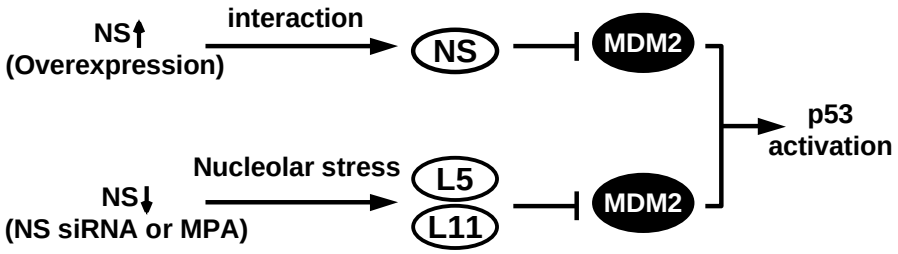

% of the cells in G1 Phase
% of the cells in S Phase
NS siRNA - + - + - +
L5 siRNA - - + + - -
L11 siRNA - - - - + +

Fig. 2.9. Schematic model illustrates that the potential mechanisms underlying the p53 activation by aberrant high levels of NS upon ectopic overexpression or low levels of NS by siRNA knockdown (See text for further discussion).


NS↑
(Overexpression)
interaction
NS
MDM2
p53
activation
Nucleolar stress
NS↓
(NS siRNA or MPA)
L5
L11
MDM2

CHAPTER THREE

Mycophenolic acid-mediated p53 activation involves Nucleostemin and ribosomal proteins L5 and L11

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Running Title: MPA activation of p53 requires L5 and L11

Key Words: NS, MDM2, p53, ribosomal proteins, L5, L11, MPA, and cell cycle

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This chapter has been combined with chapter two and submitted to MCB. The paper is currently in revision.

SUMMARY

Mycophenolate mofetil (MMF), a prodrug of mycophenolic acid (MPA), is widely used as an immunosuppressive agent. MPA selectively inhibits inosine monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme for the de novo synthesis of guanine nucleotides, leading to depletion of guanine nucleotides (GTP) pool. Its chemotherapeutic effects have been attributed to its ability to induce cell cycle arrest and apoptosis. MPA treatment has also been shown to induce and activate p53. However, the mechanism underlying the p53 activation pathway is still unclear. Here, we show that MPA treatment results in translocation of NS from the nucleolus to the nucleoplasm and significant reduction of NS protein levels. Interestingly, MPA treatment enhances the interaction of MDM2 with NS at early time points and with L5 and L11 at later time points. Knockdown of endogenous L5 or L11 markedly impairs the induction of p53 and G1 cell cycle arrest induced by MPA. These results suggest that MPA may trigger a nucleolar stress-induced p53 activation response that requires the inhibition of MDM2 imposed by NS and ribosomal protein L5 and L11.

INTRODUCTION

Inosine monophosphate dehydrogenase (IMPDH) is an essential, rate-limiting enzyme for the de novo synthesis of guanine nucleotides. It catalyzes the nicotinamide adenine dinucleotide (NAD)-dependent oxidation of inosine-5’-monophosphate (IMP) to xanthosine-5’-monophosphate (XMP), which is the committed step in de novo guanine nucleotide biosynthesis (Jackson et al., 1975).This reaction is particularly important to B and T lymphocytes, which are singularly dependent on the de novo pathway, rather than

the salvage pathway, for purine biosynthesis (Allison et al., 1977). There are two separate, but very closely related, IMPDH isoenzymes, termed type I and type II, that share 84% amino acid identity (Natsumeda et al., 1990). Expression of IMPDH, particularly the type II enzyme, is significantly up-regulated in many tumor cells, including leukemia cells (Carr et al., 1993; Gharehbaghi et al., 1994; Jackson et al., 1975; Nagai et al., 1991; Nagai et al., 1992), thus IMPDH is a target for cancer as well as immunosuppressive chemotherapy. Inhibitors of IMPDH such as mycophenolate mofetil (MMF, Cellcept), a prodrug of mycophenolic acid (MPA), have been used in organ and stem cell transplantation and in autoimmune diseases as highly effective immunosuppressive agents (Allison and Eugui, 2005).

MPA, the active metabolite of MMF, is a selective inhibitor of IMPDH (Allison and Eugui, 2005). It can effectively induces cell-cycle arrest in late G1 phase in lymphocytes (Heinschink et al., 2000; Laliberte et al., 1998; Quemeneur et al., 2003), and results in differentiation (Inai et al., 2000; Inai et al., 1998; Messina et al., 2005) or apoptosis (Gu et al., 2003; Li et al., 1998; Messina et al., 2004b; Takebe et al., 2006) in cultured cell lines depending on cell type. It has been shown that MPA treatment inhibits the induction of cyclin D3, a major component of cyclin-dependent kinase (CDK), and degradation of $p27^{kip1}$, a CDK inhibitor, resulting in the G1 cell cycle arrest (Laliberte et al., 1998). MPA causes apoptosis in interleukin-3 (IL-3)-dependent murine hematopoietic cell lines through inhibiting both the Ras-MAPK and mTOR pathways (Gu et al., 2003). Also, the induction of apoptosis in multiple myeloma cell lines occurs through both caspase-dependent (Takebe et al., 2006) and caspase-independent (Ishitsuka et al., 2005) mechanisms. However, these signaling pathways are only the potential downstream

targets, the upstream mechanisms that sense the depletion of guanine nucleotide and trigger the cell cycle arrest or apoptosis are still not very clear. Interestingly, it has been reported that certain specific inhibitors of ribonucleotide biosynthesis, including MPA, cause a reversible p53-dependent G1 arrest in the absence of detectable DNA damage, and it has been proposed that p53 can serve as a sensor of ribonucleotide pool perturbation (Linke et al., 1996). p53 has also been shown to mediate the cell cycle arrest and apoptosis in response to guanine nucleotide depletion in human neuroblastoma cell lines (Messina et al., 2004a; Messina et al., 2004b). However, how p53 senses this nucleotide depletion-induced stress remain elusive. In this study, we show that MPA treatment results in disruption of the nucleolus as evident by the massive translocation of nucleophosmin (also called B23), a nucleolar marker, as well as NS from the nucleolus to the nucleoplasma followed by significant reduction of NS protein levels. Interestingly, MPA treatment enhances the interaction of MDM2 with NS at early time points and with L5 and L11 at later time points. Knockdown endogenous L5 or L11 markedly impairs the induction of p53 and G1 cell cycle arrest induced by MPA. These results suggest that MPA may trigger a nucleolar stress-induced p53 activation response that requires the inhibition of MDM2 imposed by NS and ribosomal protein L5 and L11.

MATERIALS AND METHODS

Cell lines, plasmids, and antibodies. Human U2OS cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 5% CO2 humidified atmosphere as previously described (Dai et al., 2004). The cells were treated

with MPA (Sigma) or vehicle methanol where indicated. Anti-L5 (Dai and Lu, 2004), anti-L11 (Sun et al, 2007), and anti-MDM2 (2A10 and 4B11) (Dai and Lu, 2004; Dai et al., 2004) antibodies have been described. Anti-p21 (NeoMarkers), anti-p53 (DO-1, Santa Cruz), anti-MDM2 (SMP14, Santa Cruz), and anti-NS (Chemicon) were purchased. Rabbit polyclonal anti-NS antibodies were generated using purified His-tagged full-length NS protein expressed in *Escherichia coli* as an antigen.

Immunoblot and co-immunoprecipitation analyses. Cells were lysed in lysis buffer consisting of 50 mM Tris/HCl (pH 8.0), 0.5% Nonidet P-40 (NP-40), 1 mM EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.25 μg/ml pepstatin A, and 1 mM leupeptin. Equal amounts of cleared cell lysates were used for immunoblot analysis as described previously (Dai et al., 2004). Co-IP assays were conducted as described previously (Dai et al., 2004). Bound proteins were detected by immunoblot using antibodies as indicated in figure legends.

RNA Interference (RNAi). RNAi-mediated knockdown of endogenous L5 and L11, was performed essentially as described (Dai et al., 2004). The target sequences for L5, L11, and the control scrambled II RNA were described (Dai and Lu, 2004; Dai et al., 2004). All the siRNA duplexes with a 3' dTdT overhang were synthesized by Dharmacon (Lafayette, CO). These siRNA duplexes (0.2 μM) were introduced into cells using SilentFect (BioRad) following the manufacturer's protocol. Cells were harvested 48 to 72 hours after transfection for immunoblot, IF staining, RT-real-time PCR, and cell cycle analyses.

Reverse Transcription (RT) and Real-time PCR Analyses. Total RNA was isolated from cells using Qiagen RNeasy Mini Kits (Qiagen, Valencia, CA). Reverse

transcriptions were performed as described (Dai and Lu, 2004). Quantitative real-time PCR was performed on an ABI 7300 real-time PCR system (Applied Biosystems) using SYBR Green Mix (Applied Biosystems) as described previously (Sun et al., 2007). All reactions were carried out in triplicate. Relative gene expression was calculated using the ΔCτ method following the manufacturer's instruction. The primers for *p21, mdm2,* and *GAPDH* were described (Sun et al., 2007).

Cell Cycle Analyses. U2OS Cells were transfected with scrambled, L5, or L11 siRNA as indicated in figure legends. Cells were fixed and stained in 500 µl of propidium iodide (PI, Sigma) stain buffer (50 µg/ml PI, 30 µg/ml polyethylene glycol 8000, 200µg/ml RNase A, 0.1% Triton X-100, 0.38 M NaCl, pH 7.2) at 37°C for 30 minutes. The cells were then analyzed for DNA content using a Becton Dickinson FACScan flow cytometer. Data was analyzed using the CellQuest and Modfit software programs.

RESULTS

GTP depletion by MPA reduces NS levels, activates p53, and induces G1 cell cycle arrest. It has been shown that GTP binding favors the retention of the NS into the nucleolus and lowering the intracellular GTP level by MPA treatment redistributed NS from the nucleolus to the nucleoplasm (Tsai and McKay, 2005). MPA inhibits the rate-limiting enzyme IMPDH of *de novo* guanine nucleotide biosynthesis, leading to depletion of intracellular GTPs (Allison, 2005), and has been shown to activate p53 and induce p53-dependent G1 arrest as well (Linke et al., 1996; Messina et al., 2005). Thus, we hypothesized that MPA treatment might activate the p53 pathway by regulating NS.

To test whether MPA activation of p53 involves NS and whether it activates p53 in U2OS cells, we treated the cells with different doses of MPA. Cells were harvested at 12 hours after the treatment for immunoblot analysis. As shown in Fig. 3.1A, MPA induced the levels of p53, MDM2 and p21 in a dose-dependent fashion in U2OS cells at concentrations as low as 0.5 μM. Surprisingly, MPA treatment resulted in drastic reduction of NS levels in a dose-dependent manner (second panel of Fig. 3.1A). Because 10 μM of MPA is a clinically relevant dose (Weigel et al., 2001) and also led to a peak induction of p53 and drastic reduction of NS, we decided to use this dose for the following experiments. In order to determine the kinetics of MPA-induced p53 activation and NS reduction, we also performed time-dependent response of cells to treatment with 10 μM of MPA. As shown in Fig. 3.1B, the induction of p53 as well as p21 and MDM2 was observed at as early as 4 hours post-treatment and reached a platform from 8 to12 hours. Also, significant reduction of NS was observed at 12 hours after treatment. Thus, we chose 12 hours as a time point for the following experiments. The induced p53 by MPA treatment was transcriptionally active, since the p53 target *p21* and *mdm2* mRNA levels were significantly induced, as determined by real-time PCR assays (Fig. 3.1C). Also, MPA treatment induced G1 arrest and resulted in a loss of the G2/M phase peak in U2OS cells (Fig. 3.4). Altogether, these results suggest that GTP depletion by MPA treatment reduces intracellular NS level and activates p53.

GTP depletion mediated by MPA treatment induces redistribution of NS into the nucleoplasm and enhances the interaction of MDM2 with NS and ribosomal protein L5 and L11. We have shown that depletion of intracellular deoxynucleotide pool by 5-fluorouracil (5-FU) resulted in a nucleolar stress-p53 activation response (Sun et al.,

2007). To determine whether p53 activation mediated by MPA-induced GTP depletion also involves a nucleolar stress, we examined the cellular localization of the endogenous NS in response to MPA treatment. As shown in Fig. 3.2A, MPA treatment led to redistribution of NS into the nucleoplasm, consistent with the previous study (Tsai and McKay, 2005). However, consistent with our IB results (Fig. 3.1A and 3.1B), MPA treatment also resulted in significant reduction of the overall level of NS in a time-dependent manner. In contrast to the case of NS knockdown, B23 was largely redistributed into the nucleoplasm, suggesting that MPA treatment affects the integrity of the nucleolus structure and redistributes NS into the nucleoplasm while also reducing its level. Therefore, MAP may also trigger a nucleolar stress.

Since NS most likely interacts with MDM2 in the nucleoplasm, redistribution of NS in the nucleoplasm by MPA treatment would allow NS to interact with MDM2. Thus, we examined whether MPA treatment enhances the binding of ribosomal proteins and NS to MDM2. As shown in Fig. 3.2B, the interaction between NS and MDM2 was enhanced at the early time points (4 and 8 hours) but returned to an undetectable level at a later time point (12 hours). These results suggest that NS binding to MDM2 may contribute to p53 activation at early stage of MPA treatment. The decrease of the interaction in later stage may simply reflect the decrease of NS level upon MPA treatment. As noted above, MPA treatment disrupts the nucleolus integrity and activates p53, suggesting that GTP depletion by MPA induces a nucleolar stress. Because our lab and others have shown that several ribosomal proteins including L5, L11, and L23 participate in such nucleolar stress-induced p53, we also tested whether L5 and L11 play a role in MPA-induced p53 activation. Interestingly, MPA treatment drastically enhanced the interaction of MDM2

with the ribosomal proteins L5 and L11 at later time points (Fig. 3.2B). These results indicate that depletion of GTP by MPA treatment also results in a nucleolar stress-p53 response in cells.

Reduction of endogenous L5 or L11 by siRNA alleviates MPA-induced p53 activation and cell cycle arrest. To validate the requirement of L5 and L11 for MPA-induced p53 activation, we performed siRNA-mediated ablation experiments. Indeed, reduction of either L5 (Fig. 3.3A) or L11 (Fig. 3.3B) levels by siRNA markedly inhibited the MPA-induced level of p53 as compared to that in scrambled RNA transfected cells. Consistently, knocking down either L5 or L11 abrogated MPA-induced p21 and MDM2 protein levels (Fig. 3.3A and 3.3B) as well as their mRNA levels as measured by real-time RT-PCR assays (Fig. 3.3C and 3.3D). Consistently, knocking down either L5 or L11 significantly reduced the MPA-induced G1 cell cycle arrest and rescued the G2/M phase peak (Fig, 3.4A and 3.4B). These results demonstrate that L5 and L11 are required for MPA-mediated induction of p53 activation and G1 arrest.

DISCUSSION

Previous studies has shown that MPA treatment leads to p53-dependent cell cycle arrest and/or apoptosis (Linke et al., 1996; Messina et al., 2004a; Messina et al., 2004b). Consistent with these studies, a very recent study shows that MPA inhibits the 45S precursor rRNA synthesis, causes the concomitant translocation of nucleolar proteins including nucleolin, nucleophosmin and NS from the nucleolus to the nucleoplasm. This efflux correlates temporally with the induction of p53 in cell lines with wild-type p53 (Huang et al., 2007). These studies, although unclear about the molecular mechanism,

demonstrate that the status of intracellular ribonucleotide synthesis is constantly monitored at least partially by p53-dependent checkpoint.

This study demonstrates that NS and ribosomal proteins play crucial roles in GTP depletion-induced p53 activation. This effect is probably mediated by a sequential increase in nucleoplasmic MDM2-NS and MDM2-Ls (L5 and L11) protein complexes. By binding to MDM2, NS and those two ribosomal proteins inhibit the E3 ligase function of MDM2, and consequently stabilize and activate p53 (Dai and Lu, 2004; Lohrum et al., 2003; Zhang et al., 2003) (also see Chapter 2). NS dynamically shuttles between the nucleolus and the nucleoplasm and GTP-binding domain functions as a molecular switch (Tsai and McKay, 2005), allowing NS quickly redistribute from the nucleolus to the nucleoplasm, where it can target MDM2 in response to MPA treatment. Indeed MPA treatment markedly increased the interaction of NS with MDM2. However, this interaction was transient, taking place at 4-8 hours after MPA treatment. These results suggest that NS may play a role in early p53 stabilization when the interaction of MDM2 with L5 and L11 is not significantly induced. However, it is difficult to determine whether MPA-induced early p53 activation requires NS using siRNA-mediated ablation because knockdown of NS itself also induces p53. Interestingly, our data shows that MPA causes a significant reduction of NS 12 hours post-treatment. How MPA leads to decrease of NS is currently unclear. Because MPA induces the level of MDM2 (Fig. 3.1A and 3.1B) and MDM2 binds to NS (Fig. 2.1), one possibility is that MDM2 may ubiquitinate NS and target it for proteasome-mediated degradation. However, we did not observe significant increase of ubiquitination of NS upon overexpression of MDM2 and MPA treatment also reduced the level of NS in *p53*$^{-/-}$*mdm2*$^{-/-}$ MEF cells (data not shown).

Thus, MPA-mediated reduction of NS is through an unknown mechanism(s) independently of MDM2.

Interestingly, similar to the cellular response to the treatment with 5-FU (Gilkes et al., 2006; Sun et al., 2007) or low doses of actinomycin D (Dai et al., 2004), treatment with MPA also led to nucleolar disruption and markedly increased the binding of L5 and L11 to MDM2 (Fig.3.2B), suggesting that depletion of cellular GTP pool causes a nucleolar stress as well. Furthermore, knockdown of either L11 or L5 by siRNA significantly decreased the MPA-induced stabilization and activation of p53. Together, these results suggest that ribosomal proteins L5 and L11 are also required for MPA-induced-p53 activation. Thus, our results also provide additional evidences showing that ribosomal proteins L5 and L11 may be required for common nucleolar-stress-p53 activation pathway under growth inhibitory conditions.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

Fig. 3.1. MPA treatment decreases NS, stabilizes and activates p53. (**A**). Dose-response of p53 induction and activation by MPA. U2OS cells were treated with different doses of MPA as indicated for 12 hrs. Cell lysates were assayed for expression of p53, p21, MDM2, and NS by IB analysis. (**B**). Time-dependent effect of MPA on p53 induction and activation. U2OS cells were treated with 10 μmol/L of MPA for different time courses as indicated. Cell lysates were assayed for expression of p53, p21, MDM2, and NS by IB analysis. (**C**). MPA treatment induces the expression of *p21* and *mdm2* mRNA levels. U2OS cells were treated with methanol or 10 μmol/L of MPA for 12 hours. Total RNAs were prepared from the cells and retrotranscribed. Real-time PCR analysis was then conducted to determine the relative expression of the *p21* and *mdm2* mRNA as normalized against *GAPDH* mRNA. Panel **C** was done by Mu-shui Dai.

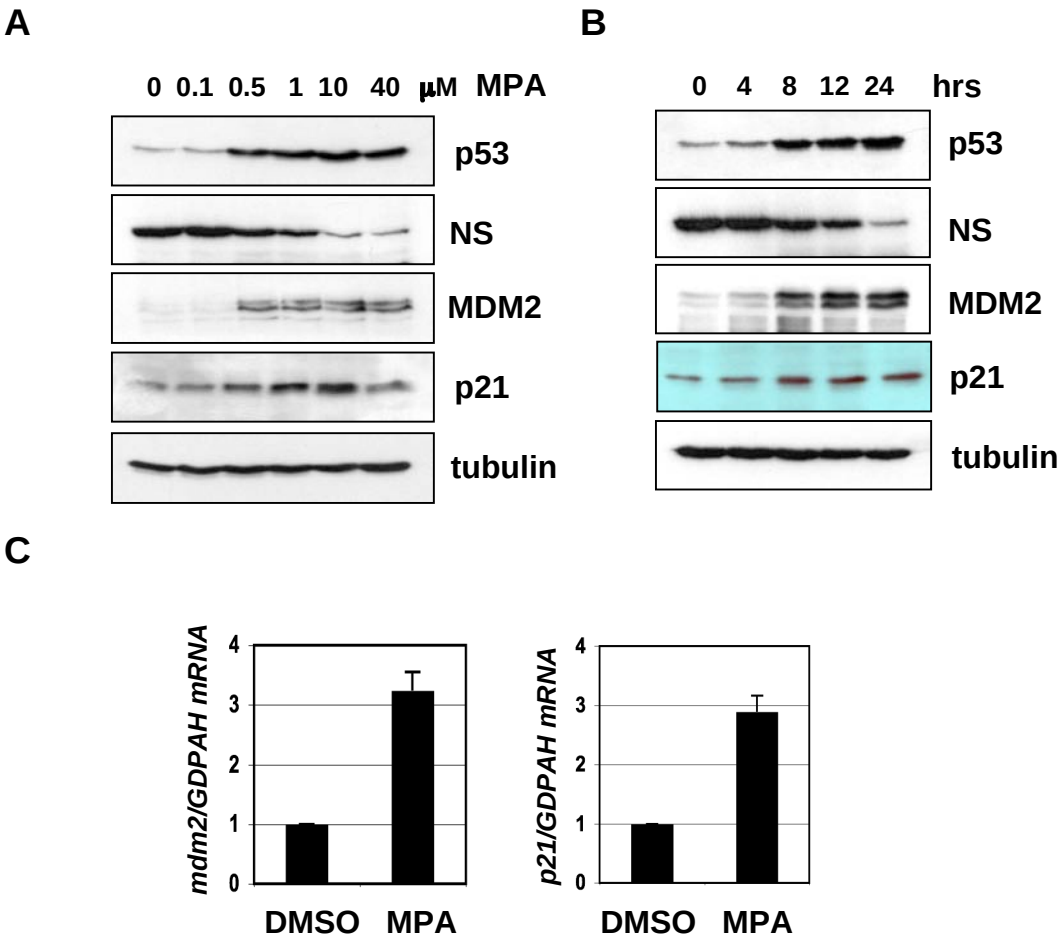
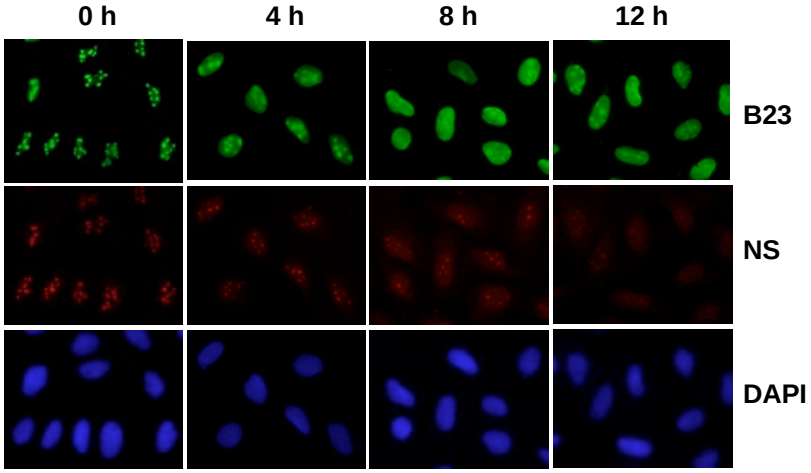

A
0 0.1 0.5 1 10 40 μM MPA
p53
NS
MDM2
p21
tubulin
B
0 4 8 12 24 hrs
p53
NS
MDM2
p21
tubulin
C
mdm2/GDPAH mRNA
0
1
2
3
4
DMSO
MPA
p21/GDPAH mRNA
0
1
2
3
4
DMSO
MPA

Fig. 3.2. MPA treatment induces redistribution of NS into the nucleoplasm and enhances the interaction of MDM2 with NS at early time points and with L5 and L11 at late time points. (**A**). MPA treatment induces redistribution of NS into the nucleoplasm. U2OS cells were treated with methanol or 10 μmol/L of MPA for different time points (h). The cells were immunostained with anti-B23 (green) and anti-NS (red) as well as DAPI for DNA. (**B**). MPA treatment enhances the interaction of MDM2 with NS at early time points and with L5 and L11 at later time points. U2OS cells were treated with 10 μmol/L of MPA for different time courses as indicated. Cell lysates were immunoprecipitated with anti-MDM2 (4B11 and SMP14) antibodies followed by immunoblot using anti-NS, anti-L5, anti-L11, or MDM2 (2A10) antibodies (lanes 5 to 8). The lysates were also directly loaded onto a SDS gel for immunoblot analysis with above antibodies (lanes 1 to 4).

A


0 h
4 h
8 h
12 h
B23
NS
DAPI

B

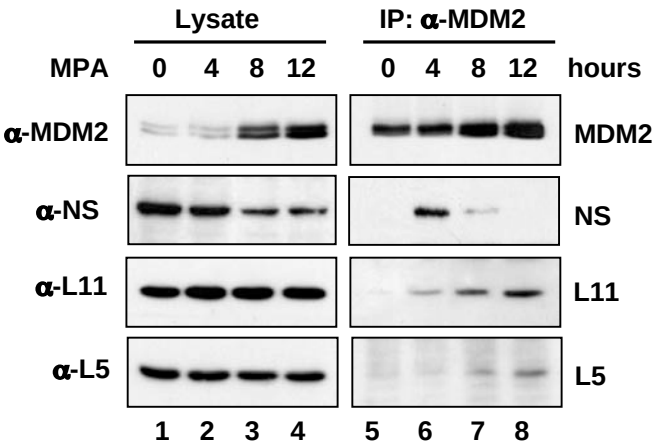

Lysate
IP: α-MDM2
MPA
0 4 8 12
0 4 8 12
hours
α-MDM2
MDM2
α-NS
NS
α-L11
L11
α-L5
L5
1 2 3 4
5 6 7 8

Fig. 3.3. MPA-induced p53 activation requires the ribosomal proteins L5 and L11. (**A**). Ablation of endogenous L5 by siRNA inhibits MPA-induced p53. U2OS cells were transfected with scrambled or L5 siRNA for 48 hours as indicated. Twelve hours before harvesting, the cells were treated with methanol (lanes 1 and 3) or 10 μmol/L of MPA (lanes 2 and 4). Cell lysates were assayed for expression of p53, p21, and MDM2 by IB with specific antibodies. (**B**). Ablation of endogenous L11 by siRNA inhibits MPA-induced p53. U2OS cells were transfected with scrambled or L11 siRNA for 48 hours as indicated. Twelve hours before harvesting, the cells were treated with methanol (lanes 1 and 2) or 10 μmol/L of MPA (lanes 3 and 4). Cell lysates were assayed for expression of p53, p21, and MDM2 by IB with specific antibodies. (**C**) (**D**). Ablation of endogenous L5 or L11 by siRNA inhibits the levels of *p21* and *mdm2* mRNA induced by MPA. Total RNAs was prepared from cells transfected with scrambled, L5, or L11 siRNA followed by treatment with methanol or MPA as above (A) or (B) and retrotranscribed. Real-time PCR analysis was then conducted to determine the expression of the *mdm2* (C) and *p21* (D) mRNA levels. The expression of *GAPDH* mRNA was used as control. Panels **C** and **D** were done by Mu-shui Dai.

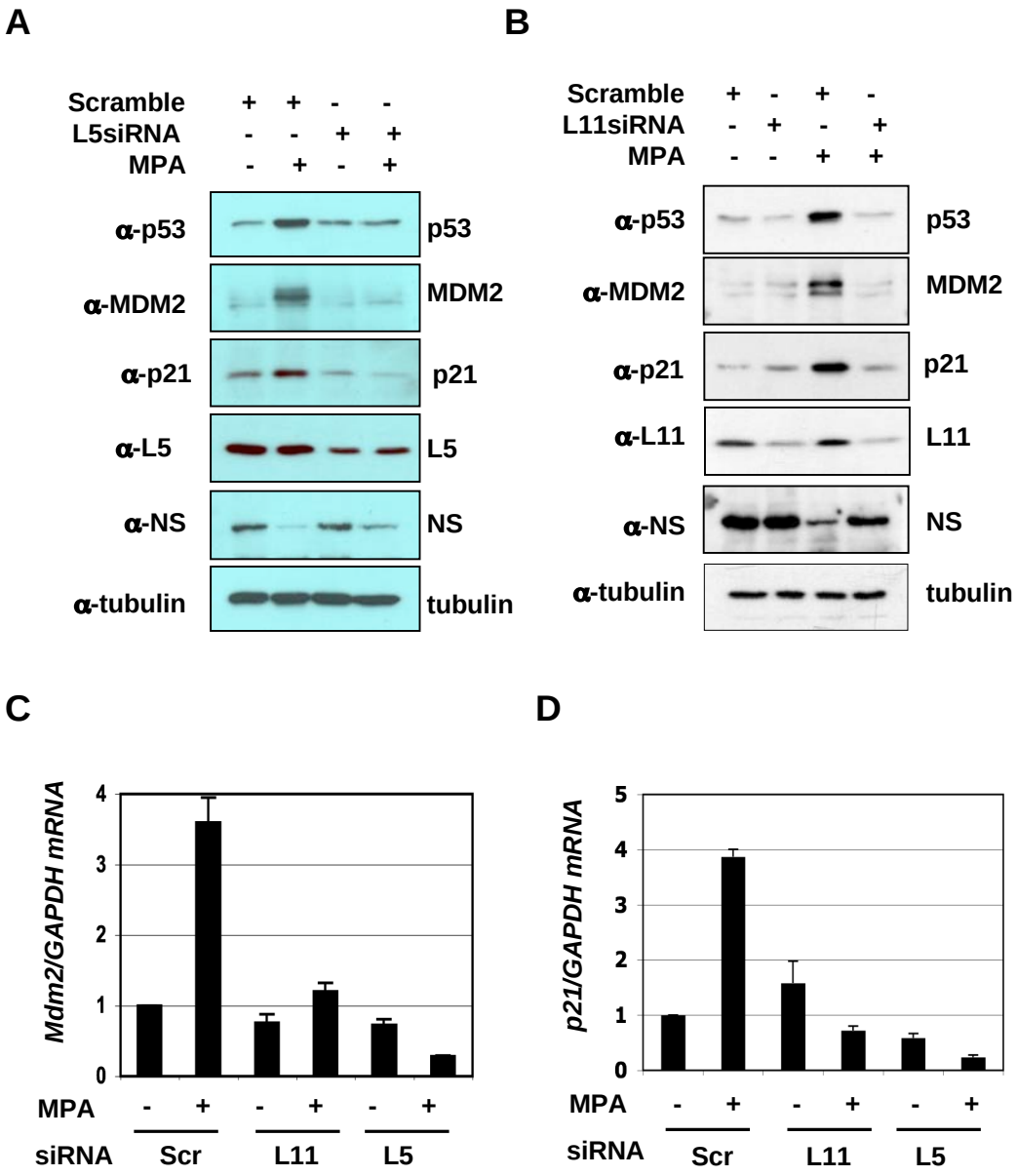
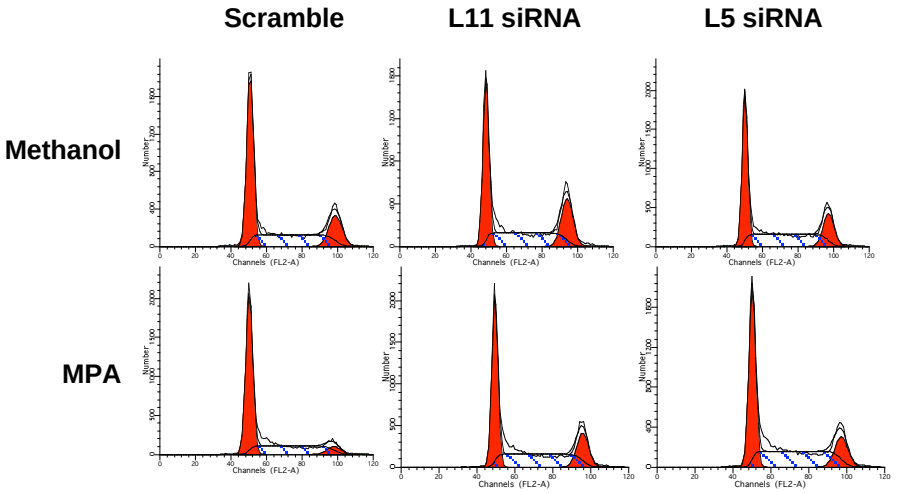
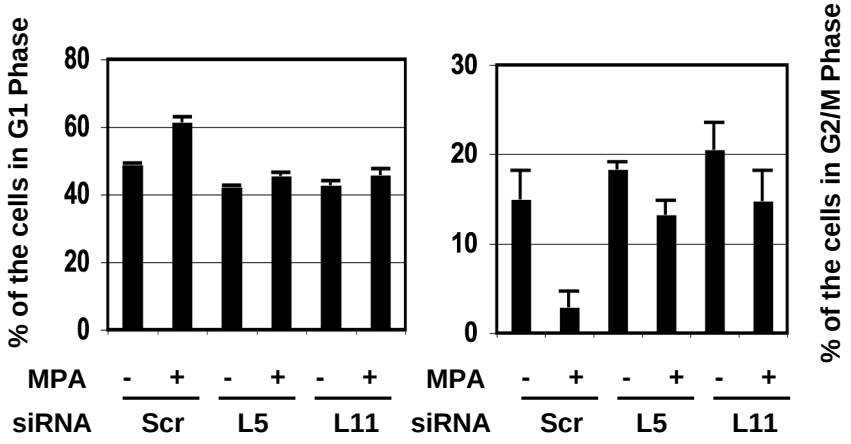

A
Scramble + + - -
L5siRNA - - + +
MPA - + - +
α-p53
p53
α-MDM2
MDM2
α-p21
p21
α-L5
L5
α-NS
NS
α-tubulin
tubulin
B
Scramble + - + -
L11siRNA - + - +
MPA - - + +
α-p53
p53
α-MDM2
MDM2
α-p21
p21
α-L11
L11
α-NS
NS
α-tubulin
tubulin
C
Mdm2/GAPDH mRNA
0
1
2
3
4
MPA - + - + - +
siRNA Scr L11 L5
D
p21/GAPDH mRNA
0
1
2
3
4
5
MPA - + - + - +
siRNA Scr L11 L5

Fig. 3.4. MPA treatment induces G1 cell cycle arrest that requires the ribosomal proteins L5 and L11. U2OS cells were transfected with scrambled, L5, or L11 siRNA followed by treatment with methanol or 10 μmol/L of MPA for 12 hours before harvesting as indicated. The cells were then stained with PI followed by flow cytometry analysis for cell cycle profile. The histograms of PI staining from one representative experiment are shown in (A). The mean percentages of cells in G1 or G2/M phase are shown in (B).

A


Scramble
L11 siRNA
L5 siRNA
Methanol
MPA
Number
Channels (FL2-A)

B


% of the cells in G1 Phase
% of the cells in G2/M Phase
80
60
40
20
0
30
20
10
0
MPA - + - + - +
siRNA Scr L5 L11
MPA - + - + - +
siRNA Scr L5 L11

CHAPTER FOUR

5-Fluorouracil activation of p53 involves an MDM2-ribosomal protein interaction

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Running Title: 5-FU enhances MDM2-ribosomal protein interaction

Key Words: 5-FU, MDM2, p53, ribosomal proteins, L5, L11, L23 and cell cycle

[1] The abbreviations used are: 5-FU, 5-Fluorouracil; FdUMP, 5-Fluorodeoxyuridine monophosphate; FdUTP, 5-Fluorodeoxyuridine triphosphate; FUTP, 5-Fluorouridine triphosphate; E3, ubiquitin-protein isopeptide ligase; rRNA, ribosomal RNA; siRNA, small interference RNA; TS, thymidylate synthase.

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SUMMARY

5-Fluorouracil (5-FU) is a widely used chemotherapeutic drug for the treatment of a variety of solid tumors. The anti-tumor activity of 5-FU has been attributed in part to its ability to induce p53-dependent cell growth arrest and apoptosis. However, the molecular mechanisms underlying p53 activation by 5-FU remain largely obscure. Here we report that 5-FU treatment leads to p53 stabilization and activation by blocking MDM2 feedback inhibition through ribosomal proteins. 5-FU treatment increased the fraction of ribosome-free L5, L11, and L23 ribosomal proteins and their interaction with MDM2, leading to p53 activation and G1/S arrest. Conversely, individual knockdown of these ribosomal proteins by siRNA prevented the 5-FU-induced p53 activation and reversed the 5-FU-induced G1/S arrest. These results demonstrate that 5-FU treatment triggers a ribosomal stress response so that ribosomal proteins L5, L11, and L23 are released from ribosomes to activate p53 by ablating the MDM2-p53 feedback circuit.

INTRODUCTION

The antimetabolite agent 5-fluorouracil (5-FU) is widely used in the treatment of many types of cancers, including colorectal carcinomas, breast cancers, and cancers of the areodigestive tract (Longley et al., 2003). It has been suggested that 5-FU achieves its therapeutic efficacy by two mechanisms. First, 5-FU is converted to 5-Fluoro-dUMP (FdUMP) and 5-fluoro-dUTP (FdUTP) in cells. FdUMP binds to the nucleotide-binding site of thymidylate synthase (TS), an enzyme that catalyzes the reaction from dUMP to dTMP, and inhibits its enzymatic activity, resulting in depletion or imbalance of intracellular deoxynucleotide pool (Houghton et al., 1995). Consequently, 5-FU

suppresses DNA synthesis and repair and results in DNA damage (Longley et al., 2003). Second, 5-FU can also be converted to 5-fluoro-UTP (FUTP), which incorporates into RNA molecules, particularly rRNA, and leads to inhibition of rRNA processing (Ghoshal and Jacob, 1994; Kanamaru et al., 1986; Wilkinson and Pitot, 1973). As a result, 5-FU induces cell cycle arrest and/or apoptosis.

The effect of 5-FU on cell growth arrest and apoptosis has been attributed to the ability of this drug to induce the level and activity of the tumor suppressor p53 (Bunz et al., 1999; Kaeser et al., 2004). Consistent with this statement is the fact that mutations or deletions of p53 result in the resistance of cells to 5-FU (Ahnen et al., 1998; Bunz et al., 1999; Liang et al., 2002; Longley et al., 2002; Lowe et al., 1994; Lowe et al., 1993; O'Connor et al., 1997). In unstressed cells, p53 level and activity are controlled by its physiological regulator MDM2, an E3 ubiquitin ligase that ubiquitinates and targets p53 for proteasome-mediated degradation (Fang et al., 2000; Haupt et al., 1997; Honda et al., 1997; Kubbutat et al., 1997), through a feedback mechanism (Picksley and Lane, 1993; Wu et al., 1993). This regulation ensures a proper low level of p53 in cells. In response to stress, cellular signaling pathways are activated to untie the MDM2-p53 feedback loop, consequently activating p53 (Oren, 2003; Vogelstein et al., 2000). For example, in response to DNA damage signals, phosphorylation of both MDM2 and p53 prevents the MDM2-p53 interaction and relieves p53 from MDM2 inhibition (Bhat et al., 2004; Dai and Lu, 2004; Dai et al., 2006b; Dai et al., 2004; Jin et al., 2004; Lohrum et al., 2003; Zhang et al., 2003). Thus, 5-FU might induce p53 by triggering a DNA damage-phosphorylation pathway. However, mutation of the N-terminal and DNA damage responsive phosphorylation sites does not prevent the induction of p53 by 5-FU (Kaeser

et al., 2004). Also, a study showed that the cytotoxicity of 5-FU was not due to the inhibition of TS (Longley et al., 2002). Instead this cytotoxicity was due to the incorporation of 5-FU into RNAs, as uridine, which inhibits the incorporation of 5-FU into RNAs, but not thymidine, which prevents the inhibition of TS, relieves the toxicity of 5-FU to cells (Bunz et al., 1999; Geoffroy et al., 1994; Pritchard et al., 1997). Hence, it was later believed that 5-FU might activate p53 through an RNA processing-related mechanism (Longley et al., 2003). However, it remains completely unclear what this mechanism is.

Ribosomal RNA (rRNA) processing is a critical step for ribosomal biogenesis, which converts rRNA precursors into mature rRNAs (Ruggero and Pandolfi, 2003). Emerging evidence suggests that perturbation of ribosomal biogenesis by the inhibition of rRNA processing, synthesis, and ribosome assembly causes ribosomal stress, leading to p53 activation (Ashcroft et al., 2000; Pestov et al., 2001). Examples for such stress include treatment of cells with a low dose of actinomycin D (Ashcroft et al., 2000), loss-of-function mutations of the rRNA processing factor Bop1 (Pestov et al., 2001), or serum starvation (Bhat et al., 2004). This type of stress has been demonstrated using a number of reagents that disrupt the structure of the nucleolus (Rubbi and Milner, 2003a). Recently, our lab and others have shown that several ribosomal proteins, including L5, L11, and L23, target the MDM2-p53 feedback loop in response to such nucleolar or ribosomal stress (Bhat et al., 2004; Dai and Lu, 2004; Dai et al., 2006b; Dai et al., 2004; Jin et al., 2004; Lohrum et al., 2003; Zhang et al., 2003). These ribosomal proteins directly bind to MDM2 and inhibit MDM2-mediated ubiquitylation of p53, thus stabilizing and activating p53 (Bhat et al., 2004; Dai and Lu, 2004; Dai et al., 2006b; Dai

et al., 2004; Jin et al., 2004; Lohrum et al., 2003; Zhang et al., 2003). Since the 5-FU active metabolite FUTP could incorporate into rRNA and inhibit rRNA processing (Ghoshal and Jacob, 1994; Kanamaru et al., 1986; Wilkinson and Pitot, 1973), we hypothesized that 5-FU treatment might also trigger nucleolar/ribosomal stress and consequently turn on the ribosomal proteins-MDM2 pathway to activate p53.

This study is aimed to test this hypothesis. In our study, we found that indeed 5-FU treatment stabilized p53 by enhancing the interaction of MDM2 with L5, L11, and L23. Also, individually knocking down these L proteins markedly reduced the 5-FU-dependent induction of p53 and its target genes MDM2 and p21, and attenuated the ability of 5-FU to induce G1/S phase arrest. Furthermore, 5-FU treatment induced the release of more ribosome-free ribosomal proteins. Therefore, these results reveal the ribosomal proteins L5, L11, and L23 as critical players in 5-FU-mediated p53 activation.

MATERIALS AND METHODS

Cell Culture and 5-FU Treatment. Human osteosarcoma U2OS and human p53-null lung small cell carcinoma H1299 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 5% CO2 humidified atmosphere as previously described (Dai et al., 2004). The cells were treated with different doses of 5-FU (Sigma) and harvested at the indicated time courses for immunoblot and co-immunoprecipitation (co-IP) assays.

Immunoblot and Co-immunoprecipitation Analyses. Cells were lysed in lysis buffer consisting of 50 mM Tris/HCl (pH 8.0), 0.5% Nonidet P-40 (NP-40), 1 mM

EDTA, 150 mM NaCl, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM dithiothreitol (DTT), 0.25 μg/ml pepstatin A, and 1 mM leupeptin. Equal amounts of clear cell lysate were used for immunoblot analysis, as described previously (Dai et al., 2004). Co-IP assays were conducted as described previously (Dai et al., 2004). Beads were washed twice with lysis buffer, once with SNNTE buffer (50 mM Tris/HCl (pH 7.4), 5 mM EDTA, 1% NP-40, 500 mM NaCl, and 5% sucrose) and once with RIPA buffer (50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% (w/v) sodium deoxycholate). Bound proteins were detected by immunoblot using antibodies as indicated in the figure legends. Anti-L5 (Dai and Lu, 2004), anti-L23 (Dai et al., 2004), and anti-MDM2 (2A10 and 4B11) (Dai and Lu, 2004; Dai et al., 2004) antibodies have been described. Anti-p21 (NeoMarkers), anti-p53 (DO-1, Santa Cruz), and anti-nucleophosmin (B23) (Zymed) were purchased. Rabbit polyclonal anti-L11 was generated using purified His-tagged full length L11 protein (Dai and Lu, 2004) expressed in *E. Coli* as an antigen.

Cell Transfection and In Vivo Ubiquitination Assays. H1299 cells were transfected with plasmids, as indicated in each figure legend using TransFectin Reagent following the manufacturer's protocol (Bio-Rad). Plasmids encoding p53 and HA-tagged MDM2 were described (Dai et al., 2004). Twenty-four hours post-transfection, the cells were treated with or without 5-FU for 12 hrs. The cells were harvested at 36 hrs posttransfection and assayed for protein expression by immunoblot analysis. For *in vivo* ubiquitination assays, the transfected cells were also treated with 20 μM of MG132 for 6 hrs prior to harvest. *In vivo* ubiquitination assays were conducted as described previously

(Dai et al., 2004). Eluted proteins were analyzed by immunoblot with anti-p53 (DO-1) antibodies.

RNA Interference (RNAi). RNAi-mediated knockdown of endogenous L5, L11, and L23 was performed as described previously (Dai et al., 2004). The 21-nucleotide siRNA duplexes with a 3' dTdT overhang were synthesized by Dharmacon (Lafayette, CO). The target sequence for L11 was 5'-AAGGTGCGGGAGTATGAGTTA-3' (Bernardi et al., 2004). The target sequence for L5 and L23 as well as the control scrambled II RNA were described previously (Dai and Lu, 2004; Dai et al., 2004). These siRNA duplexes (0.2 μM) were introduced into cells using siLentFect (Bio-Rad), following the manufacturer's protocol. The transfected cells were treated with or without 10 μg/ml of 5-FU for 12 hrs before harvest. Cells were harvested 48 hrs after transfection for immunoblot, real-time RT-PCR, and cell cycle analyses.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) and Real-time PCR Analyses. Total RNA was isolated from cells using Qiagen RNeasy Mini Kits (Qiagen, Valencia, CA) and treated with DNase I (Invitrogen). Reverse transcriptions were performed as described (Dai and Lu, 2004). Quantitative real time PCR was performed on an ABI 7300 real time PCR system (Applied Biosystems) using SYBR Green Mix (Applied Biosystems). All reactions were carried out in triplicate. Relative gene expression was calculated using the $\Delta C\tau$ method following the manufacturer's instructions. The following primers were used: p21: 5'-CTGGACTGTTTTCTCTCGGCTC-3' and 5'-TGTATATTCAGCATTGTGGGAGGA-3'; MDM2: 5'-ATGAATCCCCCCCTTCCAT-3' and 5'-CAGGAAGCCAATTCTCACGAA-3'; GAPDH: 5'-GATTCCACCCATGGCAAATTC-3' and 5'-AGCATCGCCCCACTTGATT-3'.

Cell Cycle Analysis. Cells were transfected with siRNAs as described above and treated with 10 μg/ml of 5-FU for 12 hrs. Cells were fixed with a solution of 95% ethanol and 5% acetic acid and stained in 500 μl propidium iodide (PI, Sigma) stain buffer (50 μg/ml PI, 30 μg/ml polyethylene glycol 8000, 200μg/ml RNase A, 0.1% Triton X-100, 0.38 M NaCl, pH 7.2) at 37°C for 30 minutes. The cells were then analyzed for DNA content using a Becton Dickinson FACScan flow cytometer. Data was analyzed using the CellQuest and Modfit software programs.

Immunofluorescence Staining. Cells treated with 5-FU or mock DMSO were fixed and stained with monoclonal anti-B23 antibody followed by staining with Alexa Fluor 546 (red) goat anti-mouse antibody (Molecular Probes, OR) as well as 4', 6'-diamidino-2-phenylindole (DAPI) for DNA staining. Stained cells were analyzed under a Zeiss Axiovert 25 fluorescent microscope.

Polysome Profile Analysis. Postmitochondrial supernatant (PMS) extractions, sucrose gradient sedimentation of polysomes, and analysis of the polysomes/mRNPs distribution of proteins were carried out as previously described (Dai et al., 2004). Briefly, cells were incubated with 100 μg/ml of cycloheximide (CHX) for 15 minutes. The cells were homogenized in polysome lysis buffer containing 30 mM Tris-HCl (PH 7.4), 10 mM $MgCl_2$, 100 mM KCl, 0.3% NP-40, 50 μg/ml CHX, 30 U/ml RNasin inhibitor, 1 mM DTT, 1mM PMSF, 0.25 μg/ml pepstatin A. After incubation on ice for 5 minutes, the lysates were centrifuged at 12,000 g at 4°C for 8 minutes. Supernatants were subjected to sedimentation centrifugation in a 15%-47% sucrose gradient solution containing 30 mM Tris-HCl (PH 7.4), 10 mM $MgCl_2$, 100 mM KCl in a Beckman SW41 rotor at 37,000 rpm at 4°C for 2 hrs. Fourteen fractions were collected from each tube and

30 μl of each fraction was assayed for protein levels by immunoblot. RNAs were extracted from the fractions by phenol/chloroform extraction and ethanol precipitation.

RESULTS

5-FU Treatment Stabilizes p53 in Cells. It has been shown that 5-FU treatment induces p53 level and activity (Bunz et al., 1999; Kaeser et al., 2004). To determine the minimal dose that is necessary for activating p53, we conducted 5-FU dose-dependent p53 induction assays using human osteosarcoma U2OS cells. Cells were harvested at 12 hrs after 5-FU treatment for immunoblot analysis. As shown in Fig. 4.1A, 5-FU induced the levels of p53, MDM2 and p21 in a dose-dependent fashion, with lowest activation dose for p53 being 0.1 μg/ml. Since 10 μg/ml of 5-FU led to peak induction, we decided to use this dose for the following experiments. In order to determine the kinetics of 5-FU-induced p53 activation, we also examined the time-dependent response after treatment with 10 μg/ml of 5-FU. As shown in Fig. 4.1B, the p53 induction was observed at 4 hrs post-treatment and reached at a plateau from 8 to12 hrs. Thus, we chose 12 hrs as a time point for the following experiments.

To test whether the induction of p53 by 5-FU is due to the stabilization of p53, we performed half-life assays. U2OS cells were treated with or without 10 μg/ml of 5-FU for 12 hrs. The cells were then incubated with 50 μg/ml of CHX and harvested at different time points for immunoblot analysis. As shown in Figs. 4.1C and 4.1D, 5-FU treatment significantly prolonged the half-life of p53 from less than half hour in DMSO treated cells to 2 hrs in 5-FU treated cells. These results demonstrate that 5-FU stabilizes p53.

One important regulator of p53 stability is the E3 ubiquitin ligase MDM2, which works as a p53 feedback inhibitor (Brooks and Gu, 2006; Dai et al., 2006b; Picksley and Lane, 1993; Wu et al., 1993). To determine whether 5-FU-induced p53 stabilization is due to inhibition of MDM2-mediated p53 ubiquitination and degradation, we transfected p53-null H1299 cells with p53 and/or MDM2 plasmids followed by 5-FU treatment. As shown in Fig. 4.1E, MDM2 drastically reduced the level of p53 (compare lane 3 to lane 2). This reduction of p53 was impaired in the presence of 5-FU treatment (compare lane 4 to lane 3). Furthermore, 5-FU significantly reduced MDM2-mediated p53 ubiquitination, particularly the polyubiquitination of p53 (Fig. 4.1F, compare lane 5 to lane 4). These results suggest that 5-FU treatment stabilizes p53 by inhibiting MDM2-mediated ubiquitination and degradation of p53.

5-FU Treatment Enhances the Interaction of MDM2 with Ribosomal Proteins L5, L11, and L23. Previous studies by others and our lab showed that three ribosomal proteins including L5, L11, and L23 directly bound to MDM2 and inhibited MDM2-mediated p53 ubiquitination and degradation, leading to p53 stabilization and activation, in response to ribosomal stress (Dai et al., 2006a; Dai and Lu, 2004; Dai et al., 2004; Jin et al., 2004; Lohrum et al., 2003; Zhang et al., 2003). Because 5-FU metabolites could incorporate into RNAs and inhibit rRNA processing (Ghoshal and Jacob, 1994; Kanamaru et al., 1986; Wilkinson and Pitot, 1973) and meddling with rRNA processing causes ribosomal stress and often leads to the disruption of the nucleolus (Ashcroft et al., 2000; Pestov et al., 2001; Rubbi and Milner, 2003a), it is likely that 5-FU might also activate p53 by employing these ribosomal proteins to block the MDM2-p53 feedback loop. To test this idea, we first tested whether 5-FU treatment enhances the interaction

between MDM2 and these L proteins by treating U2OS cells with 5-FU followed by immunoprecipitation (IP) with anti-MDM2 antibodies and immunoblot analysis. As shown in Fig. 4.2A, 5-FU treatment indeed enhanced the interaction between MDM2 and L5, L11, or L23 when anti-MDM2 antibodies were used for co-IP. Consistently, the enhancement of the MDM2-L5 interaction by 5-FU was also evident when a reciprocal IP was conducted with anti-L5 antibodies (Fig. 4.2B). Of note, this reciprocal IP could not be done with anti-L11 antibodies, as all the L11-antibodies tested were not suitable for co-IP with endogenous MDM2 (data not shown). When anti-L23 antibodies were used for similar co-IP assays, we found that 5-FU also markedly induced the interaction between MDM2 and L23 (Fig. 4.2C). The enhanced co-IP of MDM2 by the anti-L23 antibody was specific for L23 since no MDM2 was detected when the control pre-immune serum was used (Fig. 4.2D). Although the overall levels of L5, L11, and L23 were not altered by the treatment of 5-FU (left panels of Fig. 4.2A), MDM2 levels were significantly induced (Fig. 4.1B and the left top panels of Fig. 4.2A and 4.2C). To exclude the possibility that the enhanced interaction between MDM2 and the L proteins might be due to the increased levels of MDM2, we treated the cells with MG132, a proteasome inhibitor, to normalize the levels of MDM2 in cell, treated with or without 5-FU, and then performed similar co-IP assays. We found that the enhanced interaction between MDM2 and each of the L proteins by 5-FU treatment was still noted when MG132 was used, as shown in the representative results for L23 (Fig. 4.2E) and L11 (Fig. 4.2F). Taken together, these results demonstrate that 5-FU treatment can elevate the interactions between MDM2 and the three L proteins.

5-FU Treatment Results in An Increase in the Free Form of Ribosomal Proteins

***Proteins*.** To illustrate how 5-FU may enhance the MDM2-L protein interactions, we first tested whether 5-FU treatment could alter the nucleolar structure by using the B23 protein as a nucleolar marker (Rubbi and Milner, 2003a). As shown in Fig. 4.3A, 5-FU treatment resulted in diffusion of B23 into the nucleoplasm, suggesting that 5-FU might have disrupted the normal nucleolus structure and caused nucleolar stress (Rubbi and Milner, 2003a). Since none of our polyclonal antibodies against these L proteins is suitable for immunofluorescence staining (data not shown), we were unable to assess the distribution of these L proteins in response to 5-FU treatment using this method. To overcome this obstacle, we next conducted polysome profile assays as described previously (Bhat et al., 2004; Dai et al., 2004), which allowed us to examine the distribution of ribosome-associated and ribosome-free forms of these proteins. Cytoplasmic extracts prepared from 5-FU or mock DMSO treated U2OS cells were subjected to linear sucrose gradient sedimentation centrifugation. Fourteen fractions were collected and subjected to WB assays for detection of MDM2, L5, L11 and L23. Consistent with our previous study (Dai et al., 2004), MDM2 was not co-eluted with either the polysomes (fractions 1-7) or the monosomes (fractions 8-10), both of which contain L11, L5, and L23 (Fig. 4.3B). Instead, MDM2 stayed near the top of the gradient where the ribosome-free fractions of L11, L5 and L23 were also detected (Fig. 4.3B). The profile of polysome, monosome and free mRNPs (small ribonuclear protein) was verified by determining the distribution of rRNAs (data not shown). Interestingly, treatment of 5-FU resulted in a marked increase of the ribosome-free L11, L5 and L23, as compared to the cells treated with DMSO (Fig. 4.3B). Estimation of the ratio of free form to ribosome-

associated L proteins revealed an approximately 2~8-fold increase of the free ribosomal proteins in 5-FU treated cells (Fig. 4.3C). Together with results in Fig. 2, these results demonstrate that 5-FU treatment causes ribosomal stress and subsequently induces the release of ribosome-free L proteins that interact with MDM2.

Knockdown of L5, L11 or L23 by siRNA Inhibits 5-FU-induced p53 Activation. The enhancement of the ribosomal protein-MDM2 interactions has been shown to suppress MDM2 inhibitory activity toward p53 (Dai and Lu, 2004; Dai et al., 2004; Jin et al., 2004; Lohrum et al., 2003; Zhang et al., 2003). Thus, we predicted that this effect would also happen in response to 5-FU treatment. To demonstrate that L11, L5 and L23 indeed play roles in 5-FU-induced p53 activation, we examined if knocking down their expression by small interference RNAs (siRNAs) would influence the 5-FU-induced p53 level and activity in U2OS cells. As shown in Fig. 4.4, reduction of L5 (Fig. 4.4A) or L11 (Fig. 4.4B) levels by siRNA markedly inhibited the 5-FU-induced levels of p53, as compared to that in scrambled RNA duplex transfected cells. Consistently, 5-FU induction of p21 and MDM2 protein levels was also drastically impaired by L5 and L11, but not scrambled, siRNA, respectively (Fig. 4.4A and 4.4B). This trend was also true to the mRNA level of p21 and MDM2, as measured by real-time RT-PCR assays (Fig. 4.4D and 4.4E). These results demonstrate that L5 and L11 are required for 5-FU-mediated induction of p53 level and activity.

Consistent with our previous observation (Dai et al., 2004), L23 siRNA induced p53 (Fig. 4.4C) as well as the protein (Fig. 4.4C) and mRNA (Fig. 4.4D and 4.4E) levels of MDM2 and p21, Interestingly, when L23 siRNA transfected cells were treated with 5-FU, the induction of p53, p21 and MDM2 levels were not further enhanced in

comparison with the scrambled RNA transfected to 5-FU treated cells (Fig. 4.4C-4.4E). Instead, the levels of *mdm2* mRNA (Fig. 4.4E) and the levels of p21 and MDM2 proteins (Fig, 4.4C) were reduced by L23 siRNA in the presence of 5-FU, as compared to the cells treated with 5-FU alone. This lack of synergy between L23 siRNA and 5-FU treatment indicates that L23 also plays a role in 5-FU-induced p53 activation. As shown in previous reports (Bhat et al., 2004; Dai and Lu, 2004), siRNAs against these ribosomal proteins did not negate protein synthesis within a few days after transiently depleting these proteins. Thus, our results demonstrate that L5, L11 and L23 are crucial for 5-FU-triggered p53 activation.

Reduction of Endogenous L5, L11 or L23 by siRNA Alleviates 5-FU-induced Cell Cycle Arrest. It has been shown that 5-FU treatment results in G1/S phase cell cycle arrest (Boyer et al., 2004; De Angelis et al., 2006; Hernandez-Vargas et al., 2006; Li et al., 2004; Maybaum et al., 1980; Pizzorno et al., 1995). To determine whether the above L proteins are also required for this 5-FU effect, we conducted cell cycle analysis after introducing L5, L11, L23 or scramble siRNAs into U2OS cells. As shown in Figs. 4.5A and 4.5B, 5-FU treatment resulted in the accumulation of cells at later G1 or early S phase and the concurrent loss of G2 cells, consistent with previously reported results (Boyer et al., 2004; De Angelis et al., 2006; Hernandez-Vargas et al., 2006; Kaeser et al., 2004; Li et al., 2004; Maybaum et al., 1980; Pizzorno et al., 1995). Consistent with the above results in Fig. 4, knockdown of either endogenous L5 or L11 by siRNA alleviated this 5-FU effect on cell cycle progression. Without 5-FU treatment, L5 or L11 siRNA alone would not significantly affect the cell cycle profile (Figs. 4.5A and 4.5B). Consistent with our previous observation (Dai et al., 2004) and the result in Fig. 4.4,

knockdown of L23 by siRNA induced G1 cell cycle arrest (Figs. 4.5A and 4.5B). Again, L23 siRNA did not synergize the effect of 5-FU on the cell cycle, suggesting that L23 was also required for 5-FU-induced G1/S arrest. These results demonstrate that L5, L11, and L23 are each required for the cell cycle arrest induced by 5-FU.

DISCUSSION

It has been shown that 5-FU activates p53 to induce cell cycle arrest and/or apoptosis (Bunz et al., 1999; Kaeser et al., 2004). However, the mechanism underlying this 5-FU-p53 signaling pathway has remained unknown (Longley et al., 2003). Here we provide the first evidence demonstrating that ribosomal proteins L5, L11, and L23 play crucial roles in mediating 5-FU-induced p53 activation. We showed that 5-FU treatment enhanced the interaction between MDM2 and L5, L11, and L23 in cells (Fig. 4.2). These enhanced interactions are not due to the induced expression of MDM2 by 5-FU treatment, as the enhanced interactions were still noted when MDM2 levels were normalized by MG132 (Fig. 4.2E and 4.2F). Further supporting the role of these L proteins in 5-FU-induced p53 activation is the evidence showing that knockdown of either of the three L proteins reduced the 5-FU-induced levels of p53 and its targets, p21 and MDM2 (Fig. 4.4), and consequently alleviated 5-FU-induced G1/S phase arrest (Fig. 4.5). Taken together, our results as described above link L5, L11 and L23 with the 5-FU-p53 signaling pathway.

The ribosomal proteins L5, L11, and L23 have been shown to bind to MDM2 and inhibit MDM2 suppression of p53 activity in response to nucleolar/ribosomal biogenesis stress (Bhat et al., 2004; Dai and Lu, 2004; Dai et al., 2006b; Dai et al., 2004; Jin et al.,

2004; Lohrum et al., 2003; Zhang et al., 2003). This stress is induced by perturbation of ribosomal biogenesis through inhibition of rRNA synthesis, processing and assembly (Ashcroft et al., 2000; Pestov et al., 2001; Rubbi and Milner, 2003a). In response to this type of stress it is believed that the nucleolus is disrupted and ribosomal proteins are consequently released from the nucleolus to interact with MDM2 in either the nucleoplasmic or the cytoplasmic compartments (Bhat et al., 2004; Dai et al., 2004). Because the incorporation of the 5-FU metabolite FUTP into RNAs, particularly rRNA, has been suggested to represent a major mechanism underlying the cytotoxicity of 5-FU to cells due to interference with rRNA processing (Ghoshal and Jacob, 1994; Kanamaru et al., 1986; Wilkinson and Pitot, 1973), we predicted that 5-FU would cause ribosomal stress similar to that by actinomycin D (Ashcroft et al., 2000; Bhat et al., 2004; Dai and Lu, 2004; Dai et al., 2004; Lohrum et al., 2003). Indeed, 5-FU treatment resulted in the inhibition of MDM2-mediated p53 ubiquitination and degradation (Fig. 4.1E and 4.1F), the alteration of the nucleolar structure (Fig. 4.3A), and the increase of ribosome-free forms of L5, L11 and L23 (Fig. 4.3B-4.3C). Together with the results of Figs. 4.2, 4.4 and 4.5, these results suggest that 5-FU activates p53 by causing ribosomal stress and releasing these L proteins to block the MDM2-p53 negative feedback regulation (Fig. 4.5C).

The finding that the above L proteins play critical roles in 5-FU-induced p53 activation has several implications. First, it provides another line of evidence showing the importance of these L proteins in regulating the p53 pathway, in addition to their responses to a low dose of actinomycin D (Bhat et al., 2004; Dai and Lu, 2004; Dai et al., 2004; Lohrum et al., 2003), further supporting the model that the nucleolus plays a

central role in p53 response to cellular stress (Rubbi and Milner, 2003a). Also, our results using siRNAs specifically against the L proteins demonstrate a key role for these L proteins in the cell cycle checkpoint control. By doing so, these ribosomal proteins may coordinate ribosomal biogenesis with the cell cycle progression, thus maintaining the integrity of ribosomal biogenesis and genomes in response to ribosomal stress. Finally, these L proteins may have implications in the development of cellular resistance to 5-FU. Emerging evidence suggests that alterations of the ribosomal biogenesis pathway might contribute to tumorigenesis (Ruggero and Pandolfi, 2003). Thus it is possible that the expression of these L proteins may be altered by mutation or deletion in certain tumors, leading to impotence of 5-FU treatment in tumors. Because these L proteins are small proteins, further characterization of their interactions with MDM2 would be informative for designing small molecules or peptides that could restore the sensitivity of cancer cells to 5-FU treatment. These are interesting topics for future investigation.

ACKNOWLEDGEMENTS

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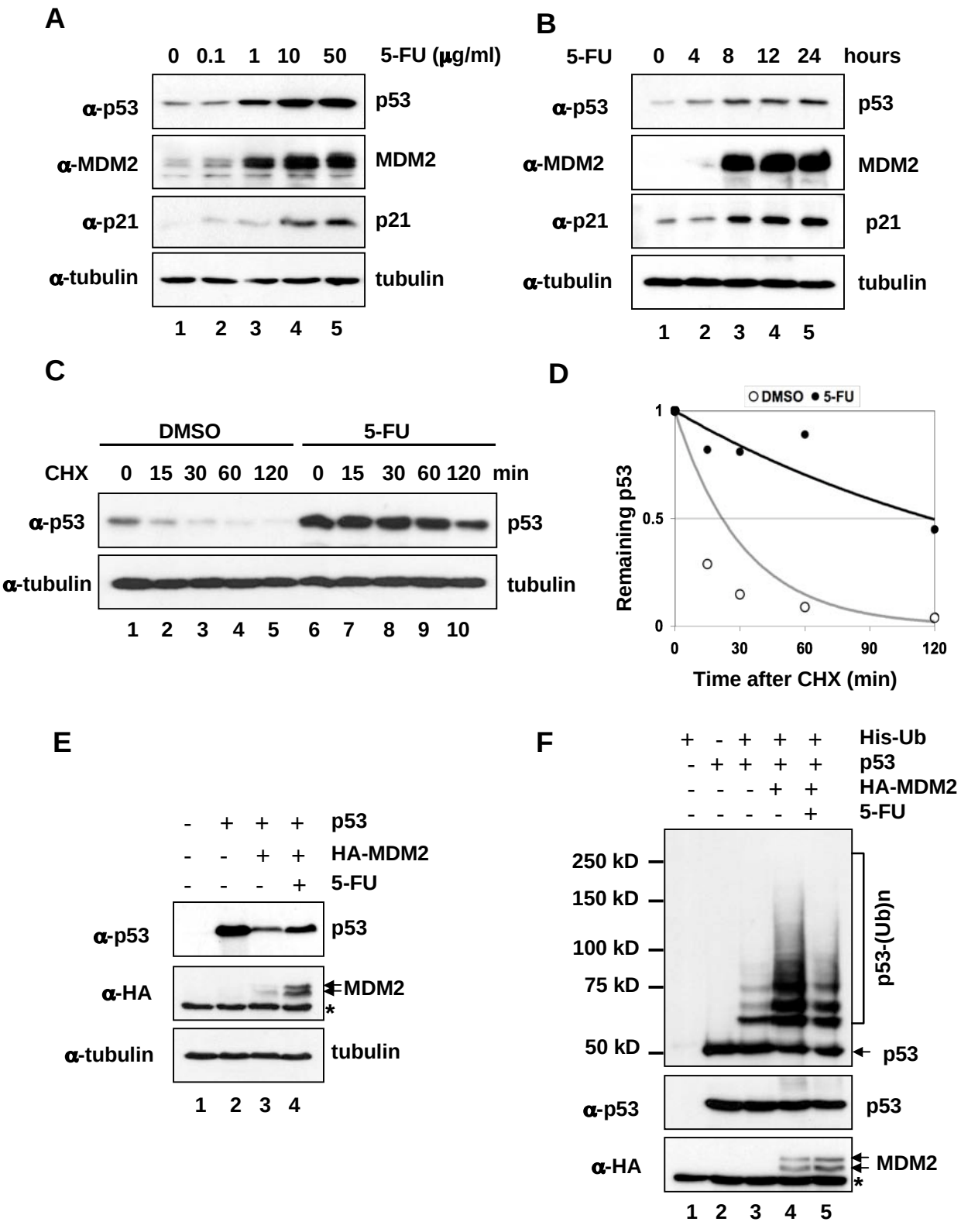
FIGURE LEGENDS

Fig. 4.1. 5-FU treatment stabilizes p53 by inhibiting MDM2-mediated p53 ubiquitination and degradation. (**A**). Dose-response of p53 induction and activation by 5-FU. U2OS cells were treated with different doses of 5-FU as indicated for 12 hrs. Clear cell lysates were assayed for expression of p53, p21, and MDM2 by immunoblot using antibodies as indicated. (**B**). Time-dependent effect of 5-FU on p53 induction and activation. U2OS cells were treated with 10 μg/ml of 5-FU for different time courses as indicated. Clear cell lysates were assayed for expression of p53, p21, and MDM2 by immunoblot using antibodies as indicated. (**C**), (**D**). 5-FU treatment stabilizes p53. U2OS cells were treated with 10 μg/ml of 5-FU for 12 hrs and then 50 μg/ml of CHX was added into the media. The cells were harvested at different time points as indicated and assayed for levels of p53 and tubulin by immunoblot. The bands were quantified and normalized with loading controls determined by tubulin expression and plotted in (D). (**E**). 5-FU treatment inhibits MDM2-mediated p53 degradation. H1299 cells were transfected with p53 (0.2 μg) and HA-MDM2 (2 μg) plasmids as indicated. 24 hrs posttransfection, the cells were treated with or without 10 μg/ml of 5-FU for 12 hrs. Clear cell lysates were assayed for expression of p53 and MDM2 by immunoblot using antibodies as indicated. * indicates non-specific anti-HA antibody-reacting bands. (**F**). 5-FU treatment inhibits MDM2-mediated p53 ubiquitination. H1299 cells were transfected with combinations of p53 (0.2 μg), HA-MDM2 (2 μg), and His-ubiquitin (His-Ub) (1 μg) plasmids as indicated. Twenty-four hours posttransfection, the cells were treated with 10 μg/ml of 5-FU for 12 hrs (lane 5). All transfected cells were treated with MG132 (20 μM) for 6 hrs before harvesting. Ubiquitinated proteins were detected by immunoblot with the anti-p53

(DO-1) antibody (upper panel). Ubiquitinated p53 (p53-(Ub)n) is indicated. The expression of total p53 and MDM2 is shown in lower panels. * indicates non-specific anti-HA antibody-reacting bands. Panel **F** was done by Mu-shui Dai.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α-p53
p53
α-MDM2
MDM2
α-p21
p21
α-tubulin
tubulin
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B
5-FU 0 4 8 12 24 hours
α-p53
p53
α-MDM2
MDM2
α-p21
p21
α-tubulin
tubulin
1 2 3 4 5
C
DMSO
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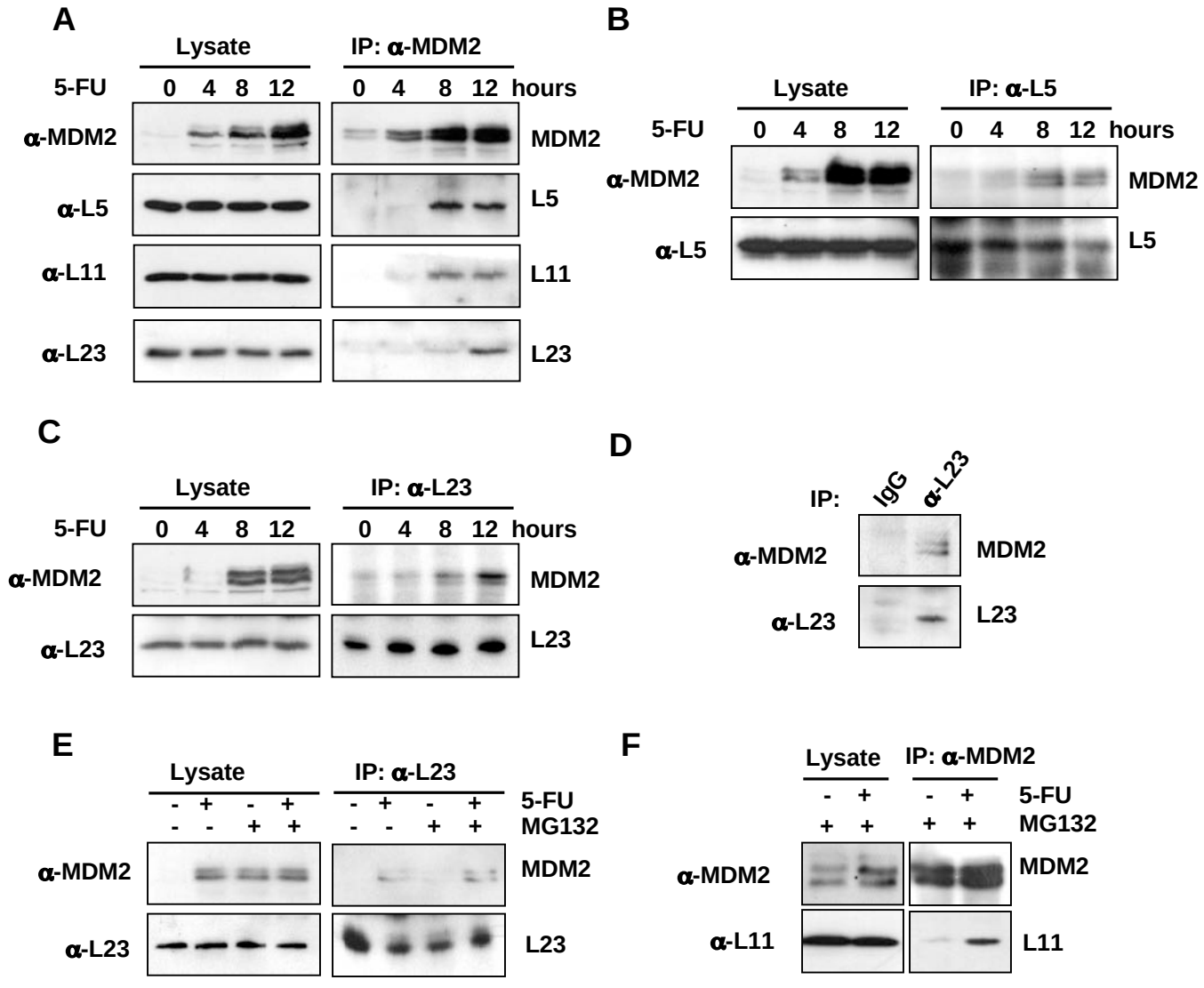
α-p53
p53
α-tubulin
tubulin
1 2 3 4 5 6 7 8 9 10
D
DMSO 5-FU
Remaining p53
1
0.5
0
0 30 60 90 120
Time after CHX (min)
E
- + + + p53
- - + + HA-MDM2
- - - + 5-FU
α-p53
p53
α-HA
MDM2
*
α-tubulin
tubulin
1 2 3 4
F
+ - + + + His-Ub
- + + + + p53
- - - + + HA-MDM2
- - - - + 5-FU
250 kD
150 kD
100 kD
75 kD
50 kD
p53-(Ub)n
p53
α-p53
p53
α-HA
MDM2
*
1 2 3 4 5

Fig. 4.2. 5-FU treatment enhances the interactions of MDM2 with L5, L11, and L23. (**A**). 5-FU treatment enhances the interaction between MDM2 and L5, L11, or L23. U2OS cells were treated with 10 μg/ml of 5-FU for different time courses as indicated. The clear cell lysates were immunoprecipitated with anti-MDM2 (4B11) antibodies followed by immunoblot using anti-L5, L11, L23, or MDM2 (2A10) antibodies (right panels). The lysates were also directly loaded onto a SDS gel for immunoblot analysis with above antibodies (left panels). (**B**). 5-FU treatment enhances the interaction between MDM2 and L5. Cell lysates prepared as in (A) were immunoprecipitated with anti-L5 antibodies followed by immunoblot using anti-L5 or MDM2 (2A10) antibodies (right panels). The lysates were also directly loaded onto a SDS gel for immunoblot analysis with above antibodies (left panels). (**C**). 5-FU treatment enhances the MDM2-L23 interaction. Cell lysates prepared as in (A) were immunoprecipitated with anti-L23 antibodies followed by immunoblot using anti-L23 or MDM2 (2A10) antibodies (right panels). The lysates were also directly loaded onto a SDS gel for immunoblot analysis with above antibodies (left panels). (**D**). MDM2 specifically co-immunoprecipitated with L23 in 5-FU treated cells. U2OS cells were treated with 10 μg/ml of 5-FU for 12 hrs and the clear cell lysates were immunoprecipitated with anti-L23 antibody or preimmune serum followed by immunoblot using anti-L23 or MDM2 (2A10) antibodies. (**E**). The enhancement of the MDM2-L23 interaction is not due to the increased levels of MDM2 induced by 5-FU. U2OS cells were treated with 10 μg/ml of 5-FU for 12 hrs. Six hours before harvest the cells were incubated with or without MG132 (20 μM) as indicated. The clear cell lysates were immunoprecipitated with anti-L23 antibodies followed by immunoblot using anti-L23 or MDM2 (2A10) antibodies (right panels). The lysates were

also directly loaded onto a SDS gel for immunoblot analysis with above antibodies (left panels). (**F**). Interaction between MDM2 and L11 in cells treated with 5-FU and MG132. U2OS cells were treated with or without 10 μg/ml of 5-FU for 12 hrs together with 20 μM of MG132 for 6 hrs as indicated. The clear cell lysates were immunoprecipitated with anti-MDM2 (4B11) antibodies followed by immunoblot using anti-L11 or MDM2 (2A10) antibodies (right panels). The lysates were also directly loaded onto a SDS gel for immunoblot analysis with above antibodies (left panels). Panels **C** and **D** were done by Mu-shui Dai.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0 4 8 12 hours
α-MDM2
MDM2
α-L5
L5
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L11
α-L23
L23
B
Lysate
IP: α-L5
5-FU
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L5
C
Lysate
IP: α-L23
5-FU
0 4 8 12
0 4 8 12 hours
α-MDM2
MDM2
α-L23
L23
D
IP:
IgG
α-L23
α-MDM2
MDM2
α-L23
L23
E
Lysate
IP: α-L23
- + - +
- + - +
5-FU
- - + +
- - + +
MG132
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MDM2
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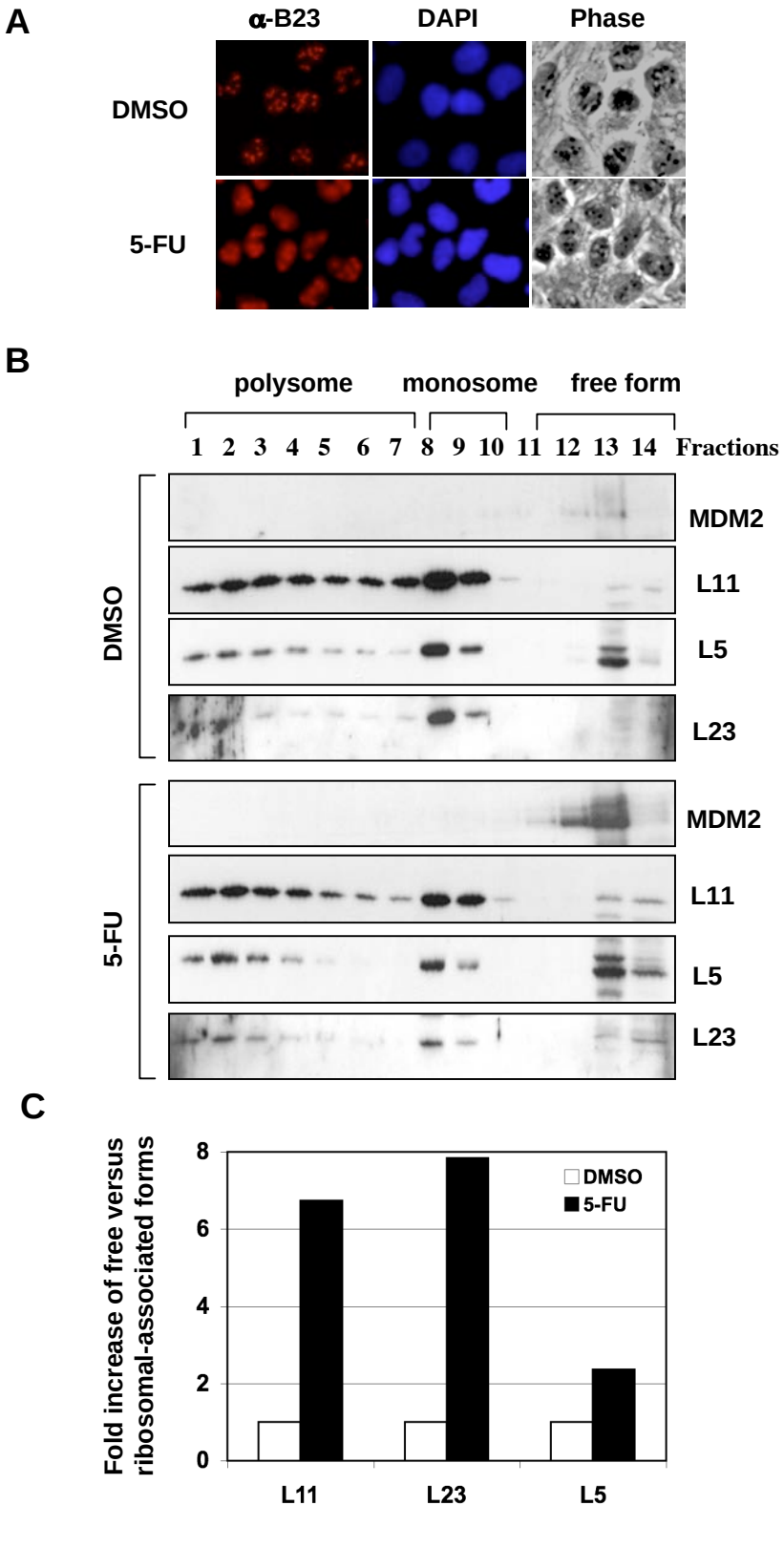
L23
F
Lysate
IP: α-MDM2
- + - +
+ + + +
5-FU
MG132
α-MDM2
MDM2
α-L11
L11

Fig. 4.3. 5-FU treatment increases ribosome-free form of L5, L11 and L23. (**A**). Immunofluorescence staining of the nucleolar marker B23. U2OS cells were treated with or without 10 μg/ml of 5-FU for 12 hrs. The cells were stained with anti-B23 monoclonal antibody, followed by staining with goat anti-mouse secondary antibody (red), as well as DAPI for DNA. (**B**). Polysome profile assays. Cytoplasmic extracts containing polysomes from U2OS cells treated with or without 10 μg/ml of 5-FU for 12 hrs were subjected to a 15%-47% linear sucrose gradient sedimentation centrifugation. Fourteen fractions were collected and 30 μl of each fraction was used for immunoblot with anti-L11, anti-L5, anti-L23, or anti-MDM2 antibodies. The distribution of polysome, monosome, and mRNPs are indicated. (**C**). The fold increase of the ratio of free versus ribosome-associated forms of L11, L5, and L23. The density of each band in above (B) was determined. The relative ratio was calculated by dividing total density of L11, L5, or L23 in fractions 11-14 by that of fractions 1-10.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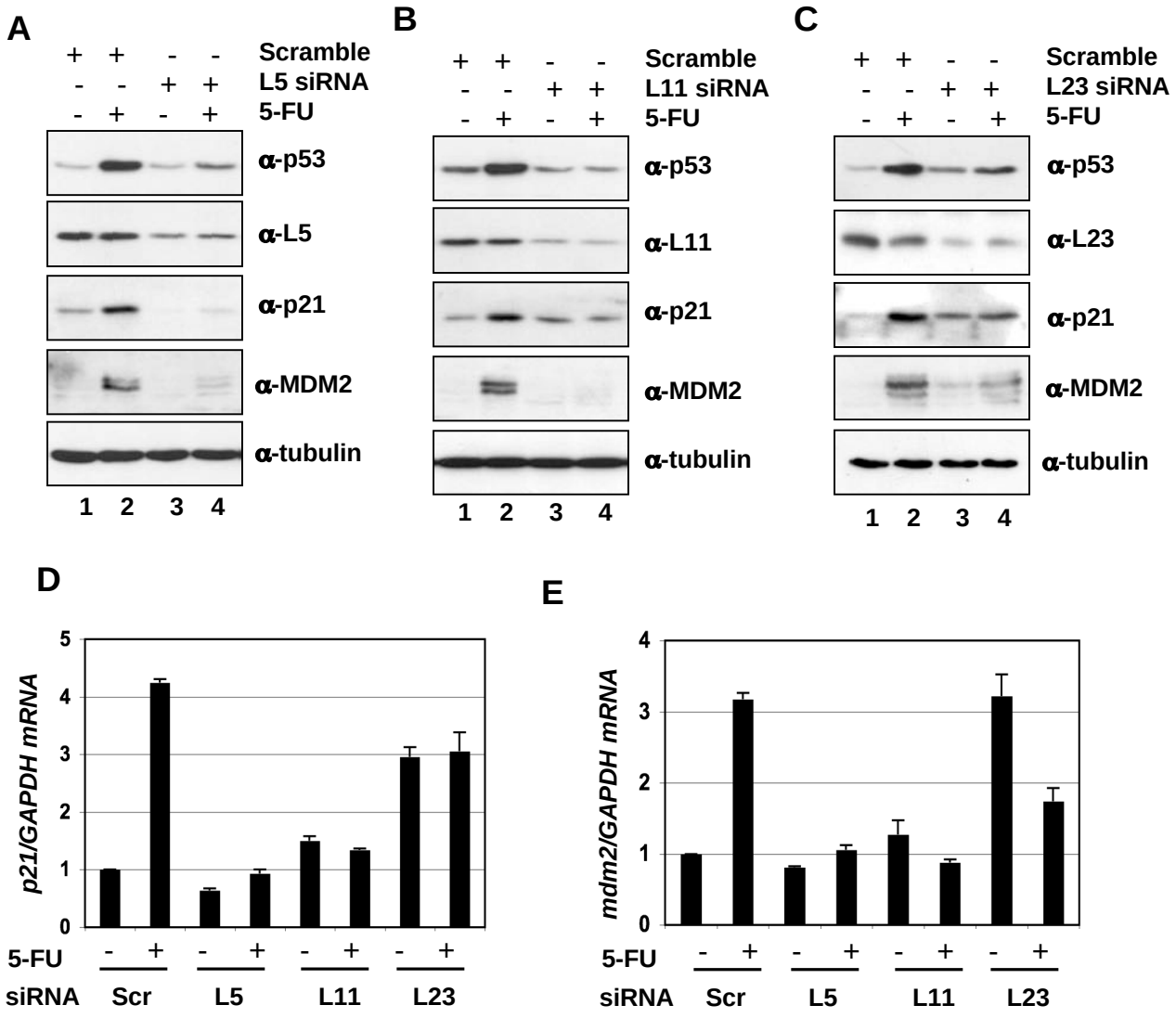
α-B23
DAPI
Phase
DMSO
5-FU
B
polysome
monosome
free form
1 2 3 4 5 6 7 8 9 10 11 12 13 14 Fractions
DMSO
MDM2
L11
L5
L23
5-FU
MDM2
L11
L5
L23
C
Fold increase of free versus ribosomal-associated forms
8
6
4
2
0
DMSO
5-FU
L11
L23
L5

Fig. 4.4. Knockdown of endogenous L5, L11, or L23 by siRNA inhibits the effect of 5-FU to induce p53 levels and activation. (**A**). Ablation of endogenous L5 by siRNA inhibits 5-FU-induced p53. U2OS cells were transfected with L5 siRNA (0.2 μM, lanes 3 and 4) or with scrambled RNA duplex (0.2 μM, lanes 1 and 2) for 48 hrs. Twelve hours before harvest the cells were treated with (lanes 2 and 4) or without (lanes 1 and 3) 10 μg/ml of 5-FU. The clear cell lysates were assayed for protein expression of p53, p21, and MDM2 by immunoblot with specific antibodies. (**B**). Ablation of endogenous L11 by siRNA inhibits 5-FU-induced p53 induction. U2OS cells were transfected with L11 siRNA (0.2 μM, lanes 3 and 4) or with scrambled RNA duplex (0.2 μM, lanes 1 and 2) for 48 hrs. Twelve hours before harvesting the cells were treated with (lanes 2 and 4) or without (lanes 1 and 3) 10 μg/ml of 5-FU. The clear cell lysates were assayed for protein expression of p53, p21, and MDM2 by immunoblot with specific antibodies. (**C**). Ablation of endogenous L23 by siRNA inhibits 5-FU-induced p53 induction. U2OS cells were transfected with L23 siRNA (0.2 μM, lanes 3 and 4) or with scrambled RNA duplex (0.2 μM, lanes 1 and 2) for 48 hrs. Twelve hours before harvesting the cells were treated with (lanes 2 and 4) or without (lanes 1 and 3) 10 μg/ml of 5-FU. The clear cell lysates were assayed for protein expression of p53, p21, and MDM2 by immunoblot with specific antibodies. (**D**), **(E)** Ablation of endogenous L5, L11, or L23 by siRNA inhibits the *p21* (D) and *mdm2* (E) mRNA levels induced by 5-FU. Total RNAs was prepared from cells transfected with L5, L11, or L23 siRNA, or scrambled RNA followed by treatment with or without 5-FU as above (A), (B), or (C) and retrotranscribed. Real-time PCR analysis was then conducted to determine the expression of the *p21* (D) and *mdm2*

(E) mRNA levels. The expression of *GAPDH* mRNA was used as control. Panels **D** and **E** were done by Mu-shui Dai.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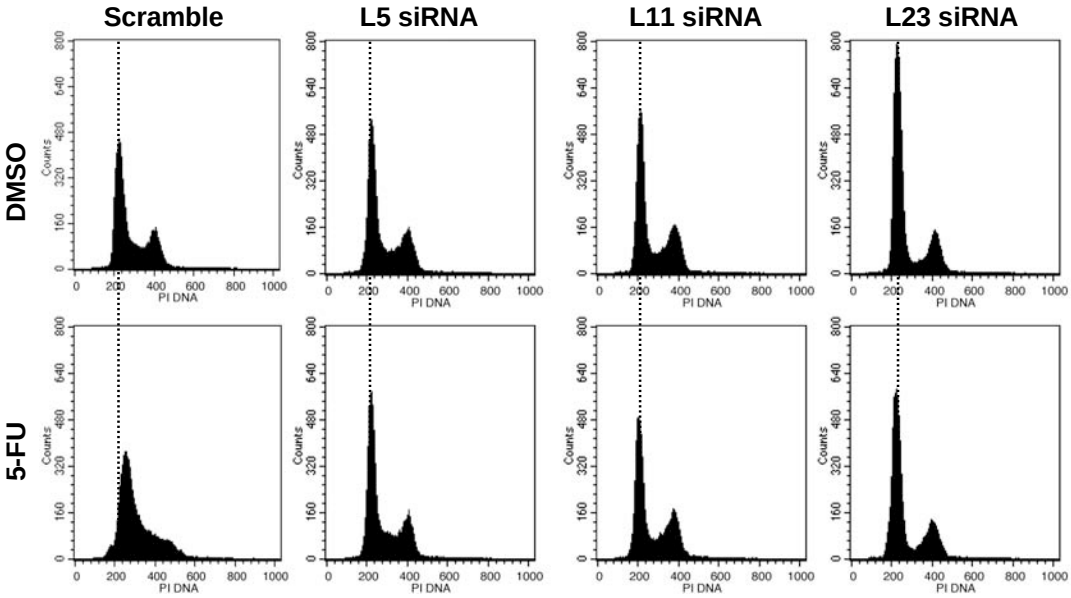
Scramble
L5 siRNA
5-FU
α-p53
α-L5
α-p21
α-MDM2
α-tubulin
1 2 3 4
B
Scramble
L11 siRNA
5-FU
α-p53
α-L11
α-p21
α-MDM2
α-tubulin
1 2 3 4
C
Scramble
L23 siRNA
5-FU
α-p53
α-L23
α-p21
α-MDM2
α-tubulin
1 2 3 4
D
p21/GAPDH mRNA
5-FU
siRNA Scr L5 L11 L23
E
mdm2/GAPDH mRNA
5-FU
siRNA Scr L5 L11 L23

Fig. 4.5. Knockdown of endogenous L5, L11, or L23 by siRNA attenuates the effect of 5-FU on cell cycle arrest. (**A**). U2OS cells were transfected with L5, L11, or L23 siRNAs or with scrambled RNA duplex for 48 hrs. Cells were also incubated with or without 10 μg/ml of 5-FU for 12 hrs before harvest. The cells were stained with PI followed by flow cytometry analysis for cell cycle profile. The dashed lines indicate G0/G1 (2N DNA content) phase cells. (**B**) The percentage of cells in G0/G1 and G2/M phases analyzed from (A). (**C**). A schematic model illustrating the role of L5, L11, and L23 in p53 activation by 5-FU. Arrows indicate activation and bars indicate inhibition. See discussion for details.

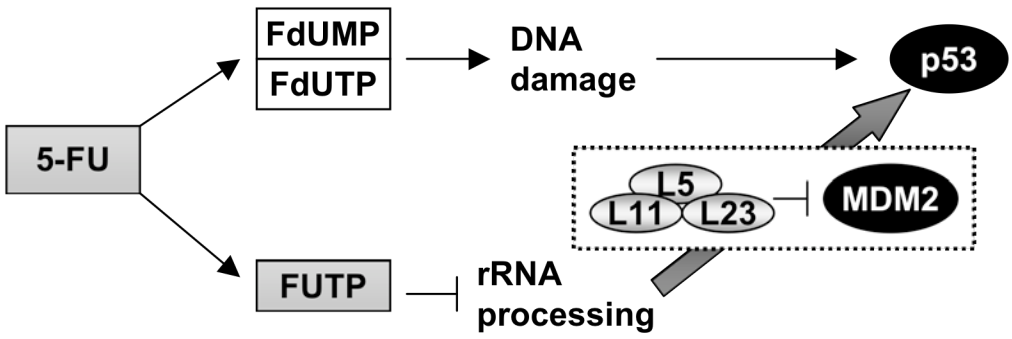
A



B

		Scramble	L5 siRNA	L11 siRNA	L23 siRNA
G0/G1	DMSO	48.2	49.1	49.5	70.7
	5-FU	64.2	48.6	48.1	66.3
G2/M	DMSO	14.1	19.7	19	16.6
	5-FU	7.5	18	21.6	20.5

C



CHAPTER FIVE

SUMMARY AND CONCLUSION

Since the MDM2-p53 feedback loop is vital for cell growth control, it is subjected to multiple forms of regulation. One form of regulation involves the nucleolus and the nucleolar proteins. Diverse cellular stress agents induce nucleolar disruption and trigger p53 stabilization in the absence of DNA damage or p53 phosphorylation. How does the nucleolus transmit the cellular stress signal to p53? This intriguing question has not been answered until recently, when it was shown that, in response to stresses including ribosomal or nucleolar stress, some nucleolar proteins could be released after nucleolus disruption and target MDM2, a potent p53 inhibitor. This indicates that these nucleolar proteins might play a crucial role in the regulation of the MDM2-p53 feedback loop. This thesis dissertation seeks to study the regulation of the MDM2-p53 feedback loop by a novel nucleolar protein called nucleostemin (NS) and several ribosomal proteins including L11, L5 and L23 in response to nucleotide depletion-induced cellular stress. Results from these studies suggest a model as illustrated in Figure 5.1 and discussed below.

NS is a recently identified novel nucleolar protein with putative GTPase ativity. It dynamically shuttles between the nucleolus and the nucleoplasm, which is highly regulated by the intracellular level of GTP. Although NS is essential for cell growth and animal development, both aberrant overexpression and reduction of its levels impair normal cell proliferation. However, the mechanisms underlying this regulation are unclear. In this dissertation, I show that unbalanced levels of NS hinders cell cycle

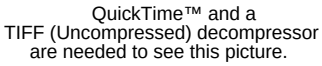


Figure 5.1. Regualtion of the MDM2-p53 feedback loop by NS and ribosomal proteins. A schematic model illustrating the role of NS and ribosomal proteins in p53 activation. Arrows indicate activation and bars indicate inhibition. See text for details.

progression by activating p53 through mechanisms involving MDM2 inhibition. At high levels, NS interacts with and inhibits the E3 ubiquitin ligase activity of MDM2, whereas at low levels nucleolar stress occurs, leading to the enhanced interaction of MDM2 with L5 and L11 that also leads to inhibition of MDM2 function. Consequently, p53 is activated in both cases. These results suggest that NS is an important regulator of the p53-MDM2 feedback loop.

5-FU is a widely used chemotherapeutic drug for the treatment of a variety of solid tumors. Its metabolite could incorporate into RNA molecules, particularly rRNA, leading to inhibition of rRNA processing. MMF, a prodrug for MPA, is highly effective immunosuppressive agent that has been used in organ and stem cell transplantation and in

autoimmune diseases. MPA selectively inhibits inosine monophosphate dehydrogenase, a rate-limiting enzyme for the *de novo* synthesis of guanine nucleotides, leading to depletion of the GTP pool. Interestingly, both drugs inhibit ribosome biogenesis, disrupt the nucleolus, activate p53, and induce cell growth arrest and apoptosis. However, how p53 serves as a sensor of nucleotide pool perturbation is unclear. In this dissertation, we provide the first evidence demonstrating that ribosomal proteins L5, L11and L23 play crucial roles in mediating 5-FU-induced p53 activation. 5-FU disrupts the nucleolus accompanied by release of the three ribosomal proteins. As a result, these ribosomal proteins bind to MDM2 and inhibit MDM2 suppression of p53 activity. Similarly, MPA treatment also disrupts the nucleolus and enhances the interaction of MDM2 with ribosomal proteins L5 and L11. Knocking down either L5 or L11 drastically retarded the p53 activation induced by both 5-FU and MPA. Thus, these ribosomal proteins are essential for 5-FU or MPA-induced p53 response. Interestingly, MPA treatment also enhances the interaction of MDM2 with NS at earlier time points, but leads to a significant reduction of NS levels at later time points. Because knock down of NS induces p53 activation as well, these results suggest that NS also play a role in the MPA-induced p53 activation pathway.

In conclusion, the results presented in this dissertation uncover a new p53-activating pathway mediated by the novel nucleolar protein NS. This dissertation also provides evidence showing that several ribosomal proteins are essential for p53 activation induced by the anti-metabolite agents 5-FU and MPA. Therefore, our results further support the idea that the nucleolus and certain nucleolar proteins play a central role in p53 activation in response to nucleolar (ribosomal) stress.

REFERENCE

Adimoolam, S., and Ford, J. M. (2002). p53 and DNA damage-inducible expression of the xeroderma pigmentosum group C gene. Proc Natl Acad Sci U S A *99*, 12985-12990.

Adimoolam, S., and Ford, J. M. (2003). p53 and regulation of DNA damage recognition during nucleotide excision repair. DNA Repair (Amst) *2*, 947-954.

Ahnen, D. J., Feigl, P., Quan, G., Fenoglio-Preiser, C., Lovato, L. C., Bunn, P. A., Jr., Stemmerman, G., Wells, J. D., Macdonald, J. S., and Meyskens, F. L., Jr. (1998). Ki-ras mutation and p53 overexpression predict the clinical behavior of colorectal cancer: a Southwest Oncology Group study. Cancer Res *58*, 1149-1158.

Allison, A. C. (2005). Mechanisms of action of mycophenolate mofetil. Lupus *14 Suppl 1*, s2-8.

Allison, A. C., and Eugui, E. M. (2005). Mechanisms of action of mycophenolate mofetil in preventing acute and chronic allograft rejection. Transplantation *80*, S181-190.

Allison, A. C., Hovi, T., Watts, R. W., and Webster, A. D. (1977). The role of de novo purine synthesis in lymphocyte transformation. Ciba Found Symp, 207-224.

Amsterdam, A., Sadler, K. C., Lai, K., Farrington, S., Bronson, R. T., Lees, J. A., and Hopkins, N. (2004). Many ribosomal protein genes are cancer genes in zebrafish. PLoS Biol *2*, E139.

Ashcroft, M., Taya, Y., and Vousden, K. H. (2000). Stress signals utilize multiple pathways to stabilize p53. Mol Cell Biol *20*, 3224-3233.

Avkin, S., Sevilya, Z., Toube, L., Geacintov, N., Chaney, S. G., Oren, M., and Livneh, Z. (2006). p53 and p21 regulate error-prone DNA repair to yield a lower mutation load. Mol Cell *22*, 407-413.

Baddoo, M., Hill, K., Wilkinson, R., Gaupp, D., Hughes, C., Kopen, G. C., and Phinney, D. G. (2003). Characterization of mesenchymal stem cells isolated from murine bone marrow by negative selection. J Cell Biochem *89*, 1235-1249.

Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y. (1998). Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science *281*, 1674-1677.

Barak, Y., Juven, T., Haffner, R., and Oren, M. (1993). mdm2 expression is induced by wild type p53 activity. Embo J *12*, 461-468.

Barlev, N. A., Liu, L., Chehab, N. H., Mansfield, K., Harris, K. G., Halazonetis, T. D., and Berger, S. L. (2001). Acetylation of p53 activates transcription through recruitment of coactivators/histone acetyltransferases. Mol Cell *8*, 1243-1254.

Bartek, J., and Lukas, J. (2003). Chk1 and Chk2 kinases in checkpoint control and cancer. Cancer Cell *3*, 421-429.

Bates, S., Ryan, K. M., Phillips, A. C., and Vousden, K. H. (1998). Cell cycle arrest and DNA endoreduplication following p21Waf1/Cip1 expression. Oncogene *17*, 1691-1703.

Bates, S., and Vousden, K. H. (1999). Mechanisms of p53-mediated apoptosis. Cell Mol Life Sci *55*, 28-37.

Beekman, C., Nichane, M., De Clercq, S., Maetens, M., Floss, T., Wurst, W., Bellefroid, E., and Marine, J. C. (2006). Evolutionarily conserved role of nucleostemin: controlling proliferation of stem/progenitor cells during early vertebrate development. Mol Cell Biol *26*, 9291-9301.

Bergamaschi, D., Samuels, Y., Jin, B., Duraisingham, S., Crook, T., and Lu, X. (2004). ASPP1 and ASPP2: common activators of p53 family members. Mol Cell Biol *24*, 1341-1350.

Bernardi, R., Scaglioni, P. P., Bergmann, S., Horn, H. F., Vousden, K. H., and Pandolfi, P. P. (2004). PML regulates p53 stability by sequestering Mdm2 to the nucleolus. Nat Cell Biol *6*, 665-672.

Bertrand, P., Saintigny, Y., and Lopez, B. S. (2004). p53's double life: transactivation-independent repression of homologous recombination. Trends Genet *20*, 235-243.

Bhat, K. P., Itahana, K., Jin, A., and Zhang, Y. (2004). Essential role of ribosomal protein L11 in mediating growth inhibition-induced p53 activation. Embo J *23*, 2402-2412.

Bode, A. M., and Dong, Z. (2004). Post-translational modification of p53 in tumorigenesis. Nat Rev Cancer *4*, 793-805.

Bond, G. L., Hu, W., Bond, E. E., Robins, H., Lutzker, S. G., Arva, N. C., Bargonetti, J., Bartel, F., Taubert, H., Wuerl, P., *et al.* (2004). A single nucleotide polymorphism in the MDM2 promoter attenuates the p53 tumor suppressor pathway and accelerates tumor formation in humans. Cell *119*, 591-602.

Boyd, S. D., Tsai, K. Y., and Jacks, T. (2000). An intact HDM2 RING-finger domain is required for nuclear exclusion of p53. Nat Cell Biol *2*, 563-568.

Boyer, J., McLean, E. G., Aroori, S., Wilson, P., McCulla, A., Carey, P. D., Longley, D. B., and Johnston, P. G. (2004). Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin, and irinotecan. Clin Cancer Res *10*, 2158-2167.

Brooks, C. L., and Gu, W. (2006). p53 ubiquitination: Mdm2 and beyond. Mol Cell *21*, 307-315.

Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J. P., Sedivy, J. M., Kinzler, K. W., and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science *282*, 1497-1501.

Bunz, F., Hwang, P. M., Torrance, C., Waldman, T., Zhang, Y., Dillehay, L., Williams, J., Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1999). Disruption of p53 in human cancer cells alters the responses to therapeutic agents. J Clin Invest *104*, 263-269.

Burley, S. K., and Roeder, R. G. (1996). Biochemistry and structural biology of transcription factor IID (TFIID). Annu Rev Biochem *65*, 769-799.

Candau, R., Scolnick, D. M., Darpino, P., Ying, C. Y., Halazonetis, T. D., and Berger, S. L. (1997). Two tandem and independent sub-activation domains in the amino terminus of p53 require the adaptor complex for activity. Oncogene *15*, 807-816.

Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998). Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. Science *281*, 1677-1679.

Carmo-Fonseca, M., Mendes-Soares, L., and Campos, I. (2000). To be or not to be in the nucleolus. Nat Cell Biol *2*, E107-112.

Carr, S. F., Papp, E., Wu, J. C., and Natsumeda, Y. (1993). Characterization of human type I and type II IMP dehydrogenases. J Biol Chem *268*, 27286-27290.

Chen D, Z. Z., Li M, Wang W, Li Y, Rayburn ER, Hill DL, Wang H, Zhang R. (2007). Ribosomal protein S7 as a novel modulator of p53-MDM2 interaction: binding to MDM2, stabilization of p53 protein, and activation of p53 function. Oncogene.

Chen, J., Marechal, V., and Levine, A. J. (1993). Mapping of the p53 and mdm-2 interaction domains. Mol Cell Biol *13*, 4107-4114.

Chipuk, J. E., Bouchier-Hayes, L., Kuwana, T., Newmeyer, D. D., and Green, D. R. (2005). PUMA couples the nuclear and cytoplasmic proapoptotic function of p53. Science *309*, 1732-1735.

Chipuk, J. E., Kuwana, T., Bouchier-Hayes, L., Droin, N. M., Newmeyer, D. D., Schuler, M., and Green, D. R. (2004). Direct activation of Bax by p53 mediates mitochondrial membrane permeabilization and apoptosis. Science *303*, 1010-1014.

Choesmel, V., Bacqueville, D., Rouquette, J., Noaillac-Depeyre, J., Fribourg, S., Cretien, A., Leblanc, T., Tchernia, G., Da Costa, L., and Gleizes, P. E. (2007). Impaired ribosome biogenesis in Diamond-Blackfan anemia. Blood *109*, 1275-1283.

Ciarmatori, S., Scott, P. H., Sutcliffe, J. E., McLees, A., Alzuherri, H. M., Dannenberg, J. H., te Riele, H., Grummt, I., Voit, R., and White, R. J. (2001). Overlapping functions of the pRb family in the regulation of rRNA synthesis. Mol Cell Biol *21*, 5806-5814.

Da Costa, L., Tchernia, G., Gascard, P., Lo, A., Meerpohl, J., Niemeyer, C., Chasis, J. A., Fixler, J., and Mohandas, N. (2003). Nucleolar localization of RPS19 protein in normal cells and mislocalization due to mutations in the nucleolar localization signals in 2 Diamond-Blackfan anemia patients: potential insights into pathophysiology. Blood *101*, 5039-5045.

Dai, M. S., Jin, Y., Gallegos, J. R., and Lu, H. (2006a). Balance of Yin and Yang: ubiquitylation-mediated regulation of p53 and c-Myc. Neoplasia *8*, 630-644.

Dai, M. S., and Lu, H. (2004). Inhibition of MDM2-mediated p53 ubiquitination and degradation by ribosomal protein L5. J Biol Chem *279*, 44475-44482.

Dai, M. S., Shi, D., Jin, Y., Sun, X. X., Zhang, Y., Grossman, S. R., and Lu, H. (2006b). Regulation of the MDM2-p53 pathway by ribosomal protein L11 involves a post-ubiquitination mechanism. J Biol Chem *281*, 24304-24313.

Dai, M. S., Zeng, S. X., Jin, Y., Sun, X. X., David, L., and Lu, H. (2004). Ribosomal protein L23 activates p53 by inhibiting MDM2 function in response to ribosomal perturbation but not to translation inhibition. Mol Cell Biol *24*, 7654-7668.

Daigle, D. M., Rossi, L., Berghuis, A. M., Aravind, L., Koonin, E. V., and Brown, E. D. (2002). YjeQ, an essential, conserved, uncharacterized protein from Escherichia coli, is an unusual GTPase with circularly permuted G-motifs and marked burst kinetics. Biochemistry *41*, 11109-11117.

Danial, N. N., and Korsmeyer, S. J. (2004). Cell death: critical control points. Cell *116*, 205-219.

Danovi, D., Meulmeester, E., Pasini, D., Migliorini, D., Capra, M., Frenk, R., de Graaf, P., Francoz, S., Gasparini, P., Gobbi, A., *et al.* (2004). Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. Mol Cell Biol *24*, 5835-5843.

Dawson, S., Apcher, S., Mee, M., Higashitsuji, H., Baker, R., Uhle, S., Dubiel, W., Fujita, J., and Mayer, R. J. (2002). Gankyrin is an ankyrin-repeat oncoprotein that interacts with CDK4 kinase and the S6 ATPase of the 26 S proteasome. J Biol Chem *277*, 10893-10902.

De Angelis, P. M., Svendsrud, D. H., Kravik, K. L., and Stokke, T. (2006). Cellular response to 5-fluorouracil (5-FU) in 5-FU-resistant colon cancer cell lines during treatment and recovery. Mol Cancer *5*, 20.

de Graaf, P., Little, N. A., Ramos, Y. F., Meulmeester, E., Letteboer, S. J., and Jochemsen, A. G. (2003). Hdmx protein stability is regulated by the ubiquitin ligase activity of Mdm2. J Biol Chem *278*, 38315-38324.

Draptchinskaia, N., Gustavsson, P., Andersson, B., Pettersson, M., Willig, T. N., Dianzani, I., Ball, S., Tchernia, G., Klar, J., Matsson, H., *et al.* (1999). The gene encoding ribosomal protein S19 is mutated in Diamond-Blackfan anaemia. Nat Genet *21*, 169-175.

Du, X., Rao, M. R., Chen, X. Q., Wu, W., Mahalingam, S., and Balasundaram, D. (2006). The homologous putative GTPases Grn1p from fission yeast and the human GNL3L are required for growth and play a role in processing of nucleolar pre-rRNA. Mol Biol Cell *17*, 460-474.

el-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817-825.

Elenbaas, B., Dobbelstein, M., Roth, J., Shenk, T., and Levine, A. J. (1996). The MDM2 oncoprotein binds specifically to RNA through its RING finger domain. Mol Med *2*, 439-451.

Espinosa, J. M., and Emerson, B. M. (2001). Transcriptional regulation by p53 through intrinsic DNA/chromatin binding and site-directed cofactor recruitment. Mol Cell *8*, 57-69.

Fakharzadeh, S. S., Trusko, S. P., and George, D. L. (1991). Tumorigenic potential associated with enhanced expression of a gene that is amplified in a mouse tumor cell line. Embo J *10*, 1565-1569.

Fan, Y., Liu, Z., Zhao, S., Lou, F., Nilsson, S., Ekman, P., Xu, D., and Fang, X. (2006). Nucleostemin mRNA is expressed in both normal and malignant renal tissues. Br J Cancer *94*, 1658-1662.

Fang, S., Jensen, J. P., Ludwig, R. L., Vousden, K. H., and Weissman, A. M. (2000). Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. J Biol Chem *275*, 8945-8951.

Fields, S., and Jang, S. K. (1990). Presence of a potent transcription activating sequence in the p53 protein. Science *249*, 1046-1049.

Flygare, J., Aspesi, A., Bailey, J. C., Miyake, K., Caffrey, J. M., Karlsson, S., and Ellis, S. R. (2007). Human RPS19, the gene mutated in Diamond-Blackfan anemia, encodes a ribosomal protein required for the maturation of 40S ribosomal subunits. Blood *109*, 980-986.

Fonagy, A., Swiderski, C., Dunn, M., and Freeman, J. W. (1992). Antisense-mediated specific inhibition of P120 protein expression prevents G1- to S-phase transition. Cancer Res *52*, 5250-5256.

Francoz, S., Froment, P., Bogaerts, S., De Clercq, S., Maetens, M., Doumont, G., Bellefroid, E., and Marine, J. C. (2006). Mdm4 and Mdm2 cooperate to inhibit p53 activity in proliferating and quiescent cells in vivo. Proc Natl Acad Sci U S A *103*, 3232-3237.

Freedman, D. A., and Levine, A. J. (1998). Nuclear export is required for degradation of endogenous p53 by MDM2 and human papillomavirus E6. Mol Cell Biol *18*, 7288-7293.

Freedman, D. A., and Levine, A. J. (1999). Regulation of the p53 protein by the MDM2 oncoprotein--thirty-eighth G.H.A. Clowes Memorial Award Lecture. Cancer Res *59*, 1-7.

Ganguli, G., and Wasylyk, B. (2003). p53-independent functions of MDM2. Mol Cancer Res *1*, 1027-1035.

Geoffroy, F. J., Allegra, C. J., Sinha, B., and Grem, J. L. (1994). Enhanced cytotoxicity with interleukin-1 alpha and 5-fluorouracil in HCT116 colon cancer cells. Oncol Res *6*, 581-591.

Geyer, R. K., Yu, Z. K., and Maki, C. G. (2000). The MDM2 RING-finger domain is required to promote p53 nuclear export. Nat Cell Biol *2*, 569-573.

Gharehbaghi, K., Burgess, G. S., Collart, F. R., Litz-Jackson, S., Huberman, E., Jayaram, H. N., and Boswell, H. S. (1994). p210 bcr-abl confers overexpression of inosine monophosphate dehydrogenase: an intrinsic pathway to drug resistance mediated by oncogene. Leukemia *8*, 1257-1263.

Ghoshal, K., and Jacob, S. T. (1994). Specific inhibition of pre-ribosomal RNA processing in extracts from the lymphosarcoma cells treated with 5-fluorouracil. Cancer Res *54*, 632-636.

Gilkes, D. M., Chen, L., and Chen, J. (2006). MDMX regulation of p53 response to ribosomal stress. Embo J *25*, 5614-5625.

Ginisty, H., Sicard, H., Roger, B., and Bouvet, P. (1999). Structure and functions of nucleolin. J Cell Sci *112 (Pt 6)*, 761-772.

Gronroos, E., Terentiev, A. A., Punga, T., and Ericsson, J. (2004). YY1 inhibits the activation of the p53 tumor suppressor in response to genotoxic stress. Proc Natl Acad Sci U S A *101*, 12165-12170.

Grossman, S. R., Deato, M. E., Brignone, C., Chan, H. M., Kung, A. L., Tagami, H., Nakatani, Y., and Livingston, D. M. (2003). Polyubiquitination of p53 by a ubiquitin ligase activity of p300. Science *300*, 342-344.

Grummt, I. (1999). Regulation of mammalian ribosomal gene transcription by RNA polymerase I. Prog Nucleic Acid Res Mol Biol *62*, 109-154.

Gu, J., Kawai, H., Nie, L., Kitao, H., Wiederschain, D., Jochemsen, A. G., Parant, J., Lozano, G., and Yuan, Z. M. (2002). Mutual dependence of MDM2 and MDMX in their functional inactivation of p53. J Biol Chem *277*, 19251-19254.

Gu, J. J., Gathy, K., Santiago, L., Chen, E., Huang, M., Graves, L. M., and Mitchell, B. S. (2003). Induction of apoptosis in IL-3-dependent hematopoietic cell lines by guanine nucleotide depletion. Blood *101*, 4958-4965.

Gu, W., and Roeder, R. G. (1997). Activation of p53 sequence-specific DNA binding by acetylation of the p53 C-terminal domain. Cell *90*, 595-606.

Hannan, K. M., Hannan, R. D., Smith, S. D., Jefferson, L. S., Lun, M., and Rothblum, L. I. (2000). Rb and p130 regulate RNA polymerase I transcription: Rb disrupts the interaction between UBF and SL-1. Oncogene *19*, 4988-4999.

Harms, K. L., and Chen, X. (2005). The C terminus of p53 family proteins is a cell fate determinant. Mol Cell Biol *25*, 2014-2030.

Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805-816.

Harper, J. W., Elledge, S. J., Keyomarsi, K., Dynlacht, B., Tsai, L. H., Zhang, P., Dobrowolski, S., Bai, C., Connell-Crowley, L., Swindell, E., and et al. (1995). Inhibition of cyclin-dependent kinases by p21. Mol Biol Cell *6*, 387-400.

Haupt, Y., Maya, R., Kazaz, A., and Oren, M. (1997). Mdm2 promotes the rapid degradation of p53. Nature *387*, 296-299.

Heinschink, A., Raab, M., Daxecker, H., Griesmacher, A., and Muller, M. M. (2000). In vitro effects of mycophenolic acid on cell cycle and activation of human lymphocytes. Clin Chim Acta *300*, 23-28.

Hernandez-Vargas, H., Ballestar, E., Carmona-Saez, P., von Kobbe, C., Banon-Rodriguez, I., Esteller, M., Moreno-Bueno, G., and Palacios, J. (2006). Transcriptional profiling of MCF7 breast cancer cells in response to 5-Fluorouracil: relationship with cell cycle changes and apoptosis, and identification of novel targets of p53. Int J Cancer *119*, 1164-1175.

Higashitsuji, H., Itoh, K., Nagao, T., Dawson, S., Nonoguchi, K., Kido, T., Mayer, R. J., Arii, S., and Fujita, J. (2000). Reduced stability of retinoblastoma protein by gankyrin, an oncogenic ankyrin-repeat protein overexpressed in hepatomas. Nat Med *6*, 96-99.

Higashitsuji, H., Itoh, K., Sakurai, T., Nagao, T., Sumitomo, Y., Masuda, T., Dawson, S., Shimada, Y., Mayer, R. J., and Fujita, J. (2005). The oncoprotein gankyrin binds to MDM2/HDM2, enhancing ubiquitylation and degradation of p53. Cancer Cell *8*, 75-87.

Honda, R., Tanaka, H., and Yasuda, H. (1997). Oncoprotein MDM2 is a ubiquitin ligase E3 for tumor suppressor p53. FEBS Lett *420*, 25-27.

Honda, R., and Yasuda, H. (1999). Association of p19(ARF) with Mdm2 inhibits ubiquitin ligase activity of Mdm2 for tumor suppressor p53. Embo J *18*, 22-27.

Hori, T., Kato, S., Saeki, M., DeMartino, G. N., Slaughter, C. A., Takeuchi, J., Toh-e, A., and Tanaka, K. (1998). cDNA cloning and functional analysis of p28 (Nas6p) and p40.5 (Nas7p), two novel regulatory subunits of the 26S proteasome. Gene *216*, 113-122.

Horn, H. F., and Vousden, K. H. (2007). Coping with stress: multiple ways to activate p53. Oncogene *26*, 1306-1316.

Houghton, J. A., Tillman, D. M., and Harwood, F. G. (1995). Ratio of 2'-deoxyadenosine-5'-triphosphate/thymidine-5'-triphosphate influences the commitment of human colon carcinoma cells to thymineless death. Clin Cancer Res *1*, 723-730.

Huang, M., Ji, Y., Itahana, K., Zhang, Y., and Mitchell, B. (2007). Guanine nucleotide depletion inhibits pre-ribosomal RNA synthesis and causes nucleolar disruption. Leuk Res.

Hwang, B. J., Ford, J. M., Hanawalt, P. C., and Chu, G. (1999). Expression of the p48 xeroderma pigmentosum gene is p53-dependent and is involved in global genomic repair. Proc Natl Acad Sci U S A *96*, 424-428.

Inai, K., Tsutani, H., Yamauchi, T., Fukushima, T., Iwasaki, H., Imamura, S., Wano, Y., Nemoto, Y., Naiki, H., and Ueda, T. (2000). Differentiation induction in non-lymphocytic leukemia cells upon treatment with mycophenolate mofetil. Leuk Res *24*, 761-768.

Inai, K., Tsutani, H., Yamauchi, T., Nakamura, T., and Ueda, T. (1998). Differentiation and reduction of intracellular GTP levels in HL-60 and U937 cells upon treatment with IMP dehydrogenase inhibitors. Adv Exp Med Biol *431*, 549-553.

Ishitsuka, K., Hideshima, T., Hamasaki, M., Raje, N., Kumar, S., Podar, K., Le Gouill, S., Shiraishi, N., Yasui, H., Roccaro, A. M., *et al.* (2005). Novel inosine monophosphate dehydrogenase inhibitor VX-944 induces apoptosis in multiple myeloma cells primarily via caspase-independent AIF/Endo G pathway. Oncogene *24*, 5888-5896.

Ito, A., Lai, C. H., Zhao, X., Saito, S., Hamilton, M. H., Appella, E., and Yao, T. P. (2001). p300/CBP-mediated p53 acetylation is commonly induced by p53-activating agents and inhibited by MDM2. Embo J *20*, 1331-1340.

Jackson, R. C., Weber, G., and Morris, H. P. (1975). IMP dehydrogenase, an enzyme linked with proliferation and malignancy. Nature *256*, 331-333.

Jeffers, J. R., Parganas, E., Lee, Y., Yang, C., Wang, J., Brennan, J., MacLean, K. H., Han, J., Chittenden, T., Ihle, J. N., *et al.* (2003). Puma is an essential mediator of p53-dependent and -independent apoptotic pathways. Cancer Cell *4*, 321-328.

Jin, A., Itahana, K., O'Keefe, K., and Zhang, Y. (2004). Inhibition of HDM2 and activation of p53 by ribosomal protein L23. Mol Cell Biol *24*, 7669-7680.

Jin, S., Tong, T., Fan, W., Fan, F., Antinore, M. J., Zhu, X., Mazzacurati, L., Li, X., Petrik, K. L., Rajasekaran, B., *et al.* (2002a). GADD45-induced cell cycle G2-M arrest associates with altered subcellular distribution of cyclin B1 and is independent of p38 kinase activity. Oncogene *21*, 8696-8704.

Jin, Y., Lee, H., Zeng, S. X., Dai, M. S., and Lu, H. (2003). MDM2 promotes p21waf1/cip1 proteasomal turnover independently of ubiquitylation. Embo J *22*, 6365-6377.

Jin, Y., Zeng, S. X., Dai, M. S., Yang, X. J., and Lu, H. (2002b). MDM2 inhibits PCAF (p300/CREB-binding protein-associated factor)-mediated p53 acetylation. J Biol Chem *277*, 30838-30843.

Jones, S. N., Roe, A. E., Donehower, L. A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature *378*, 206-208.

Juven-Gershon, T., Shifman, O., Unger, T., Elkeles, A., Haupt, Y., and Oren, M. (1998). The Mdm2 oncoprotein interacts with the cell fate regulator Numb. Mol Cell Biol *18*, 3974-3982.

Kaeser, M. D., Pebernard, S., and Iggo, R. D. (2004). Regulation of p53 stability and function in HCT116 colon cancer cells. J Biol Chem *279*, 7598-7605.

Kafienah, W., Mistry, S., Williams, C., and Hollander, A. P. (2006). Nucleostemin is a marker of proliferating stromal stem cells in adult human bone marrow. Stem Cells *24*, 1113-1120.

Kallstrom, G., Hedges, J., and Johnson, A. (2003). The putative GTPases Nog1p and Lsg1p are required for 60S ribosomal subunit biogenesis and are localized to the nucleus and cytoplasm, respectively. Mol Cell Biol *23*, 4344-4355.

Kamijo, T., Weber, J. D., Zambetti, G., Zindy, F., Roussel, M. F., and Sherr, C. J. (1998). Functional and physical interactions of the ARF tumor suppressor with p53 and Mdm2. Proc Natl Acad Sci U S A *95*, 8292-8297.

Kanamaru, R., Kakuta, H., Sato, T., Ishioka, C., and Wakui, A. (1986). The inhibitory effects of 5-fluorouracil on the metabolism of preribosomal and ribosomal RNA in L-1210 cells in vitro. Cancer Chemother Pharmacol *17*, 43-46.

Kawai, H., Wiederschain, D., Kitao, H., Stuart, J., Tsai, K. K., and Yuan, Z. M. (2003a). DNA damage-induced MDMX degradation is mediated by MDM2. J Biol Chem *278*, 45946-45953.

Kawai, H., Wiederschain, D., and Yuan, Z. M. (2003b). Critical contribution of the MDM2 acidic domain to p53 ubiquitination. Mol Cell Biol *23*, 4939-4947.

Ko, L. J., and Prives, C. (1996). p53: puzzle and paradigm. Genes Dev *10*, 1054-1072.

Kobet, E., Zeng, X., Zhu, Y., Keller, D., and Lu, H. (2000). MDM2 inhibits p300-mediated p53 acetylation and activation by forming a ternary complex with the two proteins. Proc Natl Acad Sci U S A *97*, 12547-12552.

Korgaonkar, C., Zhao, L., Modestou, M., and Quelle, D. E. (2002). ARF function does not require p53 stabilization or Mdm2 relocalization. Mol Cell Biol *22*, 196-206.

Kubbutat, M. H., Jones, S. N., and Vousden, K. H. (1997). Regulation of p53 stability by Mdm2. Nature *387*, 299-303.

Kurki, S., Peltonen, K., Latonen, L., Kiviharju, T. M., Ojala, P. M., Meek, D., and Laiho, M. (2004). Nucleolar protein NPM interacts with HDM2 and protects tumor suppressor protein p53 from HDM2-mediated degradation. Cancer Cell *5*, 465-475.

Kussie, P. H., Gorina, S., Marechal, V., Elenbaas, B., Moreau, J., Levine, A. J., and Pavletich, N. P. (1996). Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. Science *274*, 948-953.

Lafontaine, D. L., and Tollervey, D. (1998). Birth of the snoRNPs: the evolution of the modification-guide snoRNAs. Trends Biochem Sci *23*, 383-388.

Laliberte, J., Yee, A., Xiong, Y., and Mitchell, B. S. (1998). Effects of guanine nucleotide depletion on cell cycle progression in human T lymphocytes. Blood *91*, 2896-2904.

Lane, D. P. (1992). Cancer. p53, guardian of the genome. Nature *358*, 15-16.

Leipe, D. D., Wolf, Y. I., Koonin, E. V., and Aravind, L. (2002). Classification and evolution of P-loop GTPases and related ATPases. J Mol Biol *317*, 41-72.

Leu, J. I., Dumont, P., Hafey, M., Murphy, M. E., and George, D. L. (2004). Mitochondrial p53 activates Bak and causes disruption of a Bak-Mcl1 complex. Nat Cell Biol *6*, 443-450.

Leveillard, T., Andera, L., Bissonnette, N., Schaeffer, L., Bracco, L., Egly, J. M., and Wasylyk, B. (1996). Functional interactions between p53 and the TFIIH complex are affected by tumour-associated mutations. Embo J *15*, 1615-1624.

Leveillard, T., and Wasylyk, B. (1997). The MDM2 C-terminal region binds to TAFII250 and is required for MDM2 regulation of the cyclin A promoter. J Biol Chem *272*, 30651-30661.

Levine, A. J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323-331.

Li, G., Segu, V. B., Rabaglia, M. E., Luo, R. H., Kowluru, A., and Metz, S. A. (1998). Prolonged depletion of guanosine triphosphate induces death of insulin-secreting cells by apoptosis. Endocrinology *139*, 3752-3762.

Li, M., Brooks, C. L., Wu-Baer, F., Chen, D., Baer, R., and Gu, W. (2003). Mono- versus polyubiquitination: differential control of p53 fate by Mdm2. Science *302*, 1972-1975.

Li, M., Chen, D., Shiloh, A., Luo, J., Nikolaev, A. Y., Qin, J., and Gu, W. (2002). Deubiquitination of p53 by HAUSP is an important pathway for p53 stabilization. Nature *416*, 648-653.

Li, M. H., Ito, D., Sanada, M., Odani, T., Hatori, M., Iwase, M., and Nagumo, M. (2004). Effect of 5-fluorouracil on G1 phase cell cycle regulation in oral cancer cell lines. Oral Oncol *40*, 63-70.

Liang, J. T., Huang, K. C., Lai, H. S., Lee, P. H., Cheng, Y. M., Hsu, H. C., Cheng, A. L., Hsu, C. H., Yeh, K. H., Wang, S. M., *et al.* (2002). High-frequency microsatellite instability predicts better chemosensitivity to high-dose 5-fluorouracil plus leucovorin chemotherapy for stage IV sporadic colorectal cancer after palliative bowel resection. Int J Cancer *101*, 519-525.

Lin, A. W., and Lowe, S. W. (2001). Oncogenic ras activates the ARF-p53 pathway to suppress epithelial cell transformation. Proc Natl Acad Sci U S A *98*, 5025-5030.

Linares, L. K., Hengstermann, A., Ciechanover, A., Muller, S., and Scheffner, M. (2003). HdmX stimulates Hdm2-mediated ubiquitination and degradation of p53. Proc Natl Acad Sci U S A *100*, 12009-12014.

Lindstrom, M. S., Deisenroth, C., and Zhang, Y. (2007). Putting a finger on growth surveillance: insight into MDM2 zinc finger-ribosomal protein interactions. Cell Cycle *6*, 434-437.

Linke, S. P., Clarkin, K. C., Di Leonardo, A., Tsou, A., and Wahl, G. M. (1996). A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. Genes Dev *10*, 934-947.

Linke, S. P., Sengupta, S., Khabie, N., Jeffries, B. A., Buchhop, S., Miska, S., Henning, W., Pedeux, R., Wang, X. W., Hofseth, L. J., *et al.* (2003). p53 interacts with hRAD51 and hRAD54, and directly modulates homologous recombination. Cancer Res *63*, 2596-2605.

Liu, M. T., Chang, Y. T., Chen, S. C., Chuang, Y. C., Chen, Y. R., Lin, C. S., and Chen, J. Y. (2005). Epstein-Barr virus latent membrane protein 1 represses p53-mediated DNA repair and transcriptional activity. Oncogene *24*, 2635-2646.

Liu, S. J., Cai, Z. W., Liu, Y. J., Dong, M. Y., Sun, L. Q., Hu, G. F., Wei, Y. Y., and Lao, W. D. (2004). Role of nucleostemin in growth regulation of gastric cancer, liver cancer and other malignancies. World J Gastroenterol *10*, 1246-1249.

Liu, Y., and Kulesz-Martin, M. (2001). p53 protein at the hub of cellular DNA damage response pathways through sequence-specific and non-sequence-specific DNA binding. Carcinogenesis *22*, 851-860.

Llanos, S., Clark, P. A., Rowe, J., and Peters, G. (2001). Stabilization of p53 by p14ARF without relocation of MDM2 to the nucleolus. Nat Cell Biol *3*, 445-452.

Lohrum, M. A., Ashcroft, M., Kubbutat, M. H., and Vousden, K. H. (2000). Identification of a cryptic nucleolar-localization signal in MDM2. Nat Cell Biol *2*, 179-181.

Lohrum, M. A., Ludwig, R. L., Kubbutat, M. H., Hanlon, M., and Vousden, K. H. (2003). Regulation of HDM2 activity by the ribosomal protein L11. Cancer Cell *3*, 577-587.

Longley, D. B., Boyer, J., Allen, W. L., Latif, T., Ferguson, P. R., Maxwell, P. J., McDermott, U., Lynch, M., Harkin, D. P., and Johnston, P. G. (2002). The role of thymidylate synthase induction in modulating p53-regulated gene expression in response to 5-fluorouracil and antifolates. Cancer Res *62*, 2644-2649.

Longley, D. B., Harkin, D. P., and Johnston, P. G. (2003). 5-fluorouracil: mechanisms of action and clinical strategies. Nat Rev Cancer *3*, 330-338.

Loughran, O., and La Thangue, N. B. (2000). Apoptotic and growth-promoting activity of E2F modulated by MDM2. Mol Cell Biol *20*, 2186-2197.

Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., Housman, D. E., and Jacks, T. (1994). p53 status and the efficacy of cancer therapy in vivo. Science *266*, 807-810.

Lowe, S. W., Ruley, H. E., Jacks, T., and Housman, D. E. (1993). p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell *74*, 957-967.

Lowe, S. W., and Sherr, C. J. (2003). Tumor suppression by Ink4a-Arf: progress and puzzles. Curr Opin Genet Dev *13*, 77-83.

Lu, H., and Levine, A. J. (1995). Human TAFII31 protein is a transcriptional coactivator of the p53 protein. Proc Natl Acad Sci U S A *92*, 5154-5158.

Marine, J. C., and Jochemsen, A. G. (2005). Mdmx as an essential regulator of p53 activity. Biochem Biophys Res Commun *331*, 750-760.

Martin, K., Trouche, D., Hagemeier, C., Sorensen, T. S., La Thangue, N. B., and Kouzarides, T. (1995). Stimulation of E2F1/DP1 transcriptional activity by MDM2 oncoprotein. Nature *375*, 691-694.

Maya, R., Balass, M., Kim, S. T., Shkedy, D., Leal, J. F., Shifman, O., Moas, M., Buschmann, T., Ronai, Z., Shiloh, Y., *et al.* (2001). ATM-dependent phosphorylation of Mdm2 on serine 395: role in p53 activation by DNA damage. Genes Dev *15*, 1067-1077.

Maybaum, J., Ullman, B., Mandel, H. G., Day, J. L., and Sadee, W. (1980). Regulation of RNA- and DNA-directed actions of 5-fluoropyrimidines in mouse T-lymphoma (S-49) cells. Cancer Res *40*, 4209-4215.

McKinney, K., Mattia, M., Gottifredi, V., and Prives, C. (2004). p53 linear diffusion along DNA requires its C terminus. Mol Cell *16*, 413-424.

Medema, R. H., Klompmaker, R., Smits, V. A., and Rijksen, G. (1998). p21waf1 can block cells at two points in the cell cycle, but does not interfere with processive DNA-replication or stress-activated kinases. Oncogene *16*, 431-441.

Meek, D. W., and Knippschild, U. (2003). Posttranslational modification of MDM2. Mol Cancer Res *1*, 1017-1026.

Meng, L., Yasumoto, H., and Tsai, R. Y. (2006). Multiple controls regulate nucleostemin partitioning between nucleolus and nucleoplasm. J Cell Sci *119*, 5124-5136.

Messina, E., Barile, L., Lupi, F., and Giacomello, A. (2004a). Potential role of mycophenolate mofetil in the management of neuroblastoma patients. Nucleosides Nucleotides Nucleic Acids *23*, 1545-1549.

Messina, E., Gazzaniga, P., Micheli, V., Guaglianone, M. R., Barbato, S., Morrone, S., Frati, L., Agliano, A. M., and Giacomello, A. (2004b). Guanine nucleotide depletion triggers cell cycle arrest and apoptosis in human neuroblastoma cell lines. Int J Cancer *108*, 812-817.

Messina, E., Micheli, V., and Giacomello, A. (2005). Guanine nucleotide depletion induces differentiation and aberrant neurite outgrowth in human dopaminergic neuroblastoma lines: a model for basal ganglia dysfunction in Lesch-Nyhan disease. Neurosci Lett *375*, 97-100.

Meulmeester, E., Frenk, R., Stad, R., de Graaf, P., Marine, J. C., Vousden, K. H., and Jochemsen, A. G. (2003). Critical role for a central part of Mdm2 in the ubiquitylation of p53. Mol Cell Biol *23*, 4929-4938.

Midgley, C. A., Desterro, J. M., Saville, M. K., Howard, S., Sparks, A., Hay, R. T., and Lane, D. P. (2000). An N-terminal p14ARF peptide blocks Mdm2-dependent ubiquitination in vitro and can activate p53 in vivo. Oncogene *19*, 2312-2323.

Migliorini, D., Lazzerini Denchi, E., Danovi, D., Jochemsen, A., Capillo, M., Gobbi, A., Helin, K., Pelicci, P. G., and Marine, J. C. (2002). Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development. Mol Cell Biol *22*, 5527-5538.

Mihara, M., Erster, S., Zaika, A., Petrenko, O., Chittenden, T., Pancoska, P., and Moll, U. M. (2003). p53 has a direct apoptogenic role at the mitochondria. Mol Cell *11*, 577-590.

Minsky, N., and Oren, M. (2004). The RING domain of Mdm2 mediates histone ubiquitylation and transcriptional repression. Mol Cell *16*, 631-639.

Momand, J., Jung, D., Wilczynski, S., and Niland, J. (1998). The MDM2 gene amplification database. Nucleic Acids Res *26*, 3453-3459.

Momand, J., Zambetti, G. P., Olson, D. C., George, D., and Levine, A. J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell *69*, 1237-1245.

Montes de Oca Luna, R., Wagner, D. S., and Lozano, G. (1995). Rescue of early embryonic lethality in mdm2-deficient mice by deletion of p53. Nature *378*, 203-206.

Murphy, M., Ahn, J., Walker, K. K., Hoffman, W. H., Evans, R. M., Levine, A. J., and George, D. L. (1999). Transcriptional repression by wild-type p53 utilizes histone deacetylases, mediated by interaction with mSin3a. Genes Dev *13*, 2490-2501.

Nagai, M., Natsumeda, Y., Konno, Y., Hoffman, R., Irino, S., and Weber, G. (1991). Selective up-regulation of type II inosine 5'-monophosphate dehydrogenase messenger RNA expression in human leukemias. Cancer Res *51*, 3886-3890.

Nagai, M., Natsumeda, Y., and Weber, G. (1992). Proliferation-linked regulation of type II IMP dehydrogenase gene in human normal lymphocytes and HL-60 leukemic cells. Cancer Res *52*, 258-261.

Nakano, K., and Vousden, K. H. (2001). PUMA, a novel proapoptotic gene, is induced by p53. Mol Cell *7*, 683-694.

Natsumeda, Y., Ohno, S., Kawasaki, H., Konno, Y., Weber, G., and Suzuki, K. (1990). Two distinct cDNAs for human IMP dehydrogenase. J Biol Chem *265*, 5292-5295.

Nazar, R. N. (2004). Ribosomal RNA processing and ribosome biogenesis in eukaryotes. IUBMB Life *56*, 457-465.

Niculescu, A. B., 3rd, Chen, X., Smeets, M., Hengst, L., Prives, C., and Reed, S. I. (1998). Effects of p21(Cip1/Waf1) at both the G1/S and the G2/M cell cycle transitions: pRb is a critical determinant in blocking DNA replication and in preventing endoreduplication. Mol Cell Biol *18*, 629-643.

Nowak, M. A., Komarova, N. L., Sengupta, A., Jallepalli, P. V., Shih Ie, M., Vogelstein, B., and Lengauer, C. (2002). The role of chromosomal instability in tumor initiation. Proc Natl Acad Sci U S A *99*, 16226-16231.

O'Connor, P. M., Jackman, J., Bae, I., Myers, T. G., Fan, S., Mutoh, M., Scudiero, D. A., Monks, A., Sausville, E. A., Weinstein, J. N., *et al.* (1997). Characterization of the p53 tumor suppressor pathway in cell lines of the National Cancer Institute anticancer drug screen and correlations with the growth-inhibitory potency of 123 anticancer agents. Cancer Res *57*, 4285-4300.

Oda, E., Ohki, R., Murasawa, H., Nemoto, J., Shibue, T., Yamashita, T., Tokino, T., Taniguchi, T., and Tanaka, N. (2000). Noxa, a BH3-only member of the Bcl-2 family and candidate mediator of p53-induced apoptosis. Science *288*, 1053-1058.

Oliner, J. D., Kinzler, K. W., Meltzer, P. S., George, D. L., and Vogelstein, B. (1992). Amplification of a gene encoding a p53-associated protein in human sarcomas. Nature *358*, 80-83.

Oliner, J. D., Pietenpol, J. A., Thiagalingam, S., Gyuris, J., Kinzler, K. W., and Vogelstein, B. (1993). Oncoprotein MDM2 conceals the activation domain of tumour suppressor p53. Nature *362*, 857-860.

Oren, M. (2003). Decision making by p53: life, death and cancer. Cell Death Differ *10*, 431-442.

Palmero, I., Pantoja, C., and Serrano, M. (1998). p19ARF links the tumour suppressor p53 to Ras. Nature *395*, 125-126.

Pan, Y., and Chen, J. (2003). MDM2 promotes ubiquitination and degradation of MDMX. Mol Cell Biol *23*, 5113-5121.

Panic, L., Montagne, J., Cokaric, M., and Volarevic, S. (2007). S6-haploinsufficiency activates the p53 tumor suppressor. Cell Cycle *6*, 20-24.

Panic, L., Tamarut, S., Sticker-Jantscheff, M., Barkic, M., Solter, D., Uzelac, M., Grabusic, K., and Volarevic, S. (2006). Ribosomal protein S6 gene haploinsufficiency is associated with activation of a p53-dependent checkpoint during gastrulation. Mol Cell Biol *26*, 8880-8891.

Parant, J., Chavez-Reyes, A., Little, N. A., Yan, W., Reinke, V., Jochemsen, A. G., and Lozano, G. (2001). Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. Nat Genet *29*, 92-95.

Perry, M. E., Piette, J., Zawadzki, J. A., Harvey, D., and Levine, A. J. (1993). The mdm-2 gene is induced in response to UV light in a p53-dependent manner. Proc Natl Acad Sci U S A *90*, 11623-11627.

Pestov, D. G., Strezoska, Z., and Lau, L. F. (2001). Evidence of p53-dependent cross-talk between ribosome biogenesis and the cell cycle: effects of nucleolar protein Bop1 on G(1)/S transition. Mol Cell Biol *21*, 4246-4255.

Picksley, S. M., and Lane, D. P. (1993). The p53-mdm2 autoregulatory feedback loop: a paradigm for the regulation of growth control by p53? Bioessays *15*, 689-690.

Pizzorno, G., Sun, Z., and Handschumacher, R. E. (1995). Aberrant cell cycle inhibition pattern in human colon carcinoma cell lines after exposure to 5-fluorouracil. Biochem Pharmacol *49*, 553-557.

Politz, J. C., Polena, I., Trask, I., Bazett-Jones, D. P., and Pederson, T. (2005). A nonribosomal landscape in the nucleolus revealed by the stem cell protein nucleostemin. Mol Biol Cell *16*, 3401-3410.

Pritchard, D. M., Watson, A. J., Potten, C. S., Jackman, A. L., and Hickman, J. A. (1997). Inhibition by uridine but not thymidine of p53-dependent intestinal apoptosis initiated by 5-fluorouracil: evidence for the involvement of RNA perturbation. Proc Natl Acad Sci U S A *94*, 1795-1799.

Quemeneur, L., Gerland, L. M., Flacher, M., Ffrench, M., Revillard, J. P., and Genestier, L. (2003). Differential control of cell cycle, proliferation, and survival of primary T lymphocytes by purine and pyrimidine nucleotides. J Immunol *170*, 4986-4995.

Raska, I., Shaw, P. J., and Cmarko, D. (2006a). New insights into nucleolar architecture and activity. Int Rev Cytol *255*, 177-235.

Raska, I., Shaw, P. J., and Cmarko, D. (2006b). Structure and function of the nucleolus in the spotlight. Curr Opin Cell Biol *18*, 325-334.

Raycroft, L., Wu, H. Y., and Lozano, G. (1990). Transcriptional activation by wild-type but not transforming mutants of the p53 anti-oncogene. Science *249*, 1049-1051.

Reeder, R. H. (1999). Regulation of RNA polymerase I transcription in yeast and vertebrates. Prog Nucleic Acid Res Mol Biol *62*, 293-327.

Reynaud, E. G., Andrade, M. A., Bonneau, F., Ly, T. B., Knop, M., Scheffzek, K., and Pepperkok, R. (2005). Human Lsg1 defines a family of essential GTPases that correlates with the evolution of compartmentalization. BMC Biol *3*, 21.

Roth, J., Dobbelstein, M., Freedman, D. A., Shenk, T., and Levine, A. J. (1998). Nucleo-cytoplasmic shuttling of the hdm2 oncoprotein regulates the levels of the p53 protein via a pathway used by the human immunodeficiency virus rev protein. Embo J *17*, 554-564.

Rubbi, C. P., and Milner, J. (2003a). Disruption of the nucleolus mediates stabilization of p53 in response to DNA damage and other stresses. Embo J *22*, 6068-6077.

Rubbi, C. P., and Milner, J. (2003b). p53 is a chromatin accessibility factor for nucleotide excision repair of DNA damage. Embo J *22*, 975-986.

Ruggero, D., Grisendi, S., Piazza, F., Rego, E., Mari, F., Rao, P. H., Cordon-Cardo, C., and Pandolfi, P. P. (2003). Dyskeratosis congenita and cancer in mice deficient in ribosomal RNA modification. Science *299*, 259-262.

Ruggero, D., and Pandolfi, P. P. (2003). Does the ribosome translate cancer? Nat Rev Cancer *3*, 179-192.

Ryan, K. M., and Vousden, K. H. (1998). Characterization of structural p53 mutants which show selective defects in apoptosis but not cell cycle arrest. Mol Cell Biol *18*, 3692-3698.

Sakaguchi, K., Herrera, J. E., Saito, S., Miki, T., Bustin, M., Vassilev, A., Anderson, C. W., and Appella, E. (1998). DNA damage activates p53 through a phosphorylation-acetylation cascade. Genes Dev *12*, 2831-2841.

Saxena, A., Rorie, C. J., Dimitrova, D., Daniely, Y., and Borowiec, J. A. (2006). Nucleolin inhibits Hdm2 by multiple pathways leading to p53 stabilization. Oncogene *25*, 7274-7288.

Schnapp, A., Pfleiderer, C., Rosenbauer, H., and Grummt, I. (1990). A growth-dependent transcription initiation factor (TIF-IA) interacting with RNA polymerase I regulates mouse ribosomal RNA synthesis. Embo J *9*, 2857-2863.

Schon, O., Friedler, A., Bycroft, M., Freund, S. M., and Fersht, A. R. (2002). Molecular mechanism of the interaction between MDM2 and p53. J Mol Biol *323*, 491-501.

Scoumanne, A., Harms, K. L., and Chen, X. (2005). Structural basis for gene activation by p53 family members. Cancer Biol Ther *4*, 1178-1185.

Seger, Y. R., Garcia-Cao, M., Piccinin, S., Cunsolo, C. L., Doglioni, C., Blasco, M. A., Hannon, G. J., and Maestro, R. (2002). Transformation of normal human cells in the absence of telomerase activation. Cancer Cell *2*, 401-413.

Sengupta, S., and Harris, C. C. (2005). p53: traffic cop at the crossroads of DNA repair and recombination. Nat Rev Mol Cell Biol *6*, 44-55.

Sengupta, S., Linke, S. P., Pedeux, R., Yang, Q., Farnsworth, J., Garfield, S. H., Valerie, K., Shay, J. W., Ellis, N. A., Wasylyk, B., and Harris, C. C. (2003). BLM helicase-dependent transport of p53 to sites of stalled DNA replication forks modulates homologous recombination. Embo J *22*, 1210-1222.

Sherr, C. J., and Weber, J. D. (2000). The ARF/p53 pathway. Curr Opin Genet Dev *10*, 94-99.

Shirangi, T. R., Zaika, A., and Moll, U. M. (2002). Nuclear degradation of p53 occurs during down-regulation of the p53 response after DNA damage. Faseb J *16*, 420-422.

Shvarts, A., Steegenga, W. T., Riteco, N., van Laar, T., Dekker, P., Bazuine, M., van Ham, R. C., van der Houven van Oordt, W., Hateboer, G., van der Eb, A. J., and

Jochemsen, A. G. (1996). MDMX: a novel p53-binding protein with some functional properties of MDM2. Embo J *15*, 5349-5357.

Siliciano, J. D., Canman, C. E., Taya, Y., Sakaguchi, K., Appella, E., and Kastan, M. B. (1997). DNA damage induces phosphorylation of the amino terminus of p53. Genes Dev *11*, 3471-3481.

Smith, M. L., and Seo, Y. R. (2002). p53 regulation of DNA excision repair pathways. Mutagenesis *17*, 149-156.

Stad, R., Little, N. A., Xirodimas, D. P., Frenk, R., van der Eb, A. J., Lane, D. P., Saville, M. K., and Jochemsen, A. G. (2001). Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms. EMBO Rep *2*, 1029-1034.

Stommel, J. M., Marchenko, N. D., Jimenez, G. S., Moll, U. M., Hope, T. J., and Wahl, G. M. (1999). A leucine-rich nuclear export signal in the p53 tetramerization domain: regulation of subcellular localization and p53 activity by NES masking. Embo J *18*, 1660-1672.

Stott, F. J., Bates, S., James, M. C., McConnell, B. B., Starborg, M., Brookes, S., Palmero, I., Ryan, K., Hara, E., Vousden, K. H., and Peters, G. (1998). The alternative product from the human CDKN2A locus, p14(ARF), participates in a regulatory feedback loop with p53 and MDM2. Embo J *17*, 5001-5014.

Sui, G., Affar el, B., Shi, Y., Brignone, C., Wall, N. R., Yin, P., Donohoe, M., Luke, M. P., Calvo, D., and Grossman, S. R. (2004). Yin Yang 1 is a negative regulator of p53. Cell *117*, 859-872.

Sun, X. X., Dai, M. S., and Lu, H. (2007). 5-fluorouracil activation of p53 involves an MDM2-ribosomal protein interaction. J Biol Chem *282*, 8052-8059.

Takebe, N., Cheng, X., Fandy, T. E., Srivastava, R. K., Wu, S., Shankar, S., Bauer, K., Shaughnessy, J., and Tricot, G. (2006). IMP dehydrogenase inhibitor mycophenolate mofetil induces caspase-dependent apoptosis and cell cycle inhibition in multiple myeloma cells. Mol Cancer Ther *5*, 457-466.

Tao, W., and Levine, A. J. (1999). Nucleocytoplasmic shuttling of oncoprotein Hdm2 is required for Hdm2-mediated degradation of p53. Proc Natl Acad Sci U S A *96*, 3077-3080.

Taylor, W. R., and Stark, G. R. (2001). Regulation of the G2/M transition by p53. Oncogene *20*, 1803-1815.

Thiry, M., and Lafontaine, D. L. (2005). Birth of a nucleolus: the evolution of nucleolar compartments. Trends Cell Biol *15*, 194-199.

Toledo, F., and Wahl, G. M. (2006). Regulating the p53 pathway: in vitro hypotheses, in vivo veritas. Nat Rev Cancer *6*, 909-923.

Tsai, R. Y., and McKay, R. D. (2002). A nucleolar mechanism controlling cell proliferation in stem cells and cancer cells. Genes Dev *16*, 2991-3003.

Tsai, R. Y., and McKay, R. D. (2005). A multistep, GTP-driven mechanism controlling the dynamic cycling of nucleostemin. J Cell Biol *168*, 179-184.

Uchida, C., Miwa, S., Kitagawa, K., Hattori, T., Isobe, T., Otani, S., Oda, T., Sugimura, H., Kamijo, T., Ookawa, K., *et al.* (2005). Enhanced Mdm2 activity inhibits pRB function via ubiquitin-dependent degradation. Embo J *24*, 160-169.

van Steensel, B., and de Lange, T. (1997). Control of telomere length by the human telomeric protein TRF1. Nature *385*, 740-743.

Villunger, A., Michalak, E. M., Coultas, L., Mullauer, F., Bock, G., Ausserlechner, M. J., Adams, J. M., and Strasser, A. (2003). p53- and drug-induced apoptotic responses mediated by BH3-only proteins puma and noxa. Science *302*, 1036-1038.

Vogelstein, B., Lane, D., and Levine, A. J. (2000). Surfing the p53 network. Nature *408*, 307-310.

Volarevic, S., Stewart, M. J., Ledermann, B., Zilberman, F., Terracciano, L., Montini, E., Grompe, M., Kozma, S. C., and Thomas, G. (2000). Proliferation, but not growth, blocked by conditional deletion of 40S ribosomal protein S6. Science *288*, 2045-2047.

Vousden, K. H., and Lu, X. (2002). Live or let die: the cell's response to p53. Nat Rev Cancer *2*, 594-604.

Wang, L., Wu, Q., Qiu, P., Mirza, A., McGuirk, M., Kirschmeier, P., Greene, J. R., Wang, Y., Pickett, C. B., and Liu, S. (2001). Analyses of p53 target genes in the human genome by bioinformatic and microarray approaches. J Biol Chem *276*, 43604-43610.

Wang, Q. E., Zhu, Q., Wani, M. A., Wani, G., Chen, J., and Wani, A. A. (2003). Tumor suppressor p53 dependent recruitment of nucleotide excision repair factors XPC and TFIIH to DNA damage. DNA Repair (Amst) *2*, 483-499.

Wang, X. W., Yeh, H., Schaeffer, L., Roy, R., Moncollin, V., Egly, J. M., Wang, Z., Freidberg, E. C., Evans, M. K., Taffe, B. G., and et al. (1995). p53 modulation of TFIIH-associated nucleotide excision repair activity. Nat Genet *10*, 188-195.

Wang, Y., Reed, M., Wang, P., Stenger, J. E., Mayr, G., Anderson, M. E., Schwedes, J. F., and Tegtmeyer, P. (1993). p53 domains: identification and characterization of two autonomous DNA-binding regions. Genes Dev *7*, 2575-2586.

Weber, J. D., Taylor, L. J., Roussel, M. F., Sherr, C. J., and Bar-Sagi, D. (1999). Nucleolar Arf sequesters Mdm2 and activates p53. Nat Cell Biol *1*, 20-26.

Weigel, G., Griesmacher, A., Zuckermann, A. O., Laufer, G., and Mueller, M. M. (2001). Effect of mycophenolate mofetil therapy on inosine monophosphate dehydrogenase induction in red blood cells of heart transplant recipients. Clin Pharmacol Ther *69*, 137-144.

Wilkinson, D. S., and Pitot, H. C. (1973). Inhibition of ribosomal ribonucleic acid maturation in Novikoff hepatoma cells by 5-fluorouracil and 5-fluorouridine. J Biol Chem *248*, 63-68.

Wu, X., Bayle, J. H., Olson, D., and Levine, A. J. (1993). The p53-mdm-2 autoregulatory feedback loop. Genes Dev *7*, 1126-1132.

Xiao, Z. X., Chen, J., Levine, A. J., Modjtahedi, N., Xing, J., Sellers, W. R., and Livingston, D. M. (1995). Interaction between the retinoblastoma protein and the oncoprotein MDM2. Nature *375*, 694-698.

Xiong, S., Van Pelt, C. S., Elizondo-Fraire, A. C., Liu, G., and Lozano, G. (2006). Synergistic roles of Mdm2 and Mdm4 for p53 inhibition in central nervous system development. Proc Natl Acad Sci U S A *103*, 3226-3231.

Xirodimas, D., Saville, M. K., Edling, C., Lane, D. P., and Lain, S. (2001a). Different effects of p14ARF on the levels of ubiquitinated p53 and Mdm2 in vivo. Oncogene *20*, 4972-4983.

Xirodimas, D. P., Saville, M. K., Bourdon, J. C., Hay, R. T., and Lane, D. P. (2004). Mdm2-mediated NEDD8 conjugation of p53 inhibits its transcriptional activity. Cell *118*, 83-97.

Xirodimas, D. P., Stephen, C. W., and Lane, D. P. (2001b). Cocompartmentalization of p53 and Mdm2 is a major determinant for Mdm2-mediated degradation of p53. Exp Cell Res *270*, 66-77.

Yap, D. B., Hsieh, J. K., and Lu, X. (2000). Mdm2 inhibits the apoptotic function of p53 mainly by targeting it for degradation. J Biol Chem *275*, 37296-37302.

Yogosawa, S., Miyauchi, Y., Honda, R., Tanaka, H., and Yasuda, H. (2003). Mammalian Numb is a target protein of Mdm2, ubiquitin ligase. Biochem Biophys Res Commun *302*, 869-872.

Yu, J., and Zhang, L. (2003). No PUMA, no death: implications for p53-dependent apoptosis. Cancer Cell *4*, 248-249.

Yu, J., Zhang, L., Hwang, P. M., Kinzler, K. W., and Vogelstein, B. (2001). PUMA induces the rapid apoptosis of colorectal cancer cells. Mol Cell *7*, 673-682.

Yuan, X., Zhou, Y., Casanova, E., Chai, M., Kiss, E., Grone, H. J., Schutz, G., and Grummt, I. (2005). Genetic inactivation of the transcription factor TIF-IA leads to nucleolar disruption, cell cycle arrest, and p53-mediated apoptosis. Mol Cell *19*, 77-87.

Zhan, Q., Antinore, M. J., Wang, X. W., Carrier, F., Smith, M. L., Harris, C. C., and Fornace, A. J., Jr. (1999). Association with Cdc2 and inhibition of Cdc2/Cyclin B1 kinase activity by the p53-regulated protein Gadd45. Oncogene *18*, 2892-2900.

Zhang, Y., Wolf, G. W., Bhat, K., Jin, A., Allio, T., Burkhart, W. A., and Xiong, Y. (2003). Ribosomal protein L11 negatively regulates oncoprotein MDM2 and mediates a p53-dependent ribosomal-stress checkpoint pathway. Mol Cell Biol *23*, 8902-8912.

Zhang, Y., Xiong, Y., and Yarbrough, W. G. (1998). ARF promotes MDM2 degradation and stabilizes p53: ARF-INK4a locus deletion impairs both the Rb and p53 tumor suppression pathways. Cell *92*, 725-734.

Zhou, J., Ahn, J., Wilson, S. H., and Prives, C. (2001). A role for p53 in base excision repair. Embo J *20*, 914-923.

Zhu, J., Zhou, W., Jiang, J., and Chen, X. (1998). Identification of a novel p53 functional domain that is necessary for mediating apoptosis. J Biol Chem *273*, 13030-13036.

Zhu, Q., Yasumoto, H., and Tsai, R. Y. (2006). Nucleostemin delays cellular senescence and negatively regulates TRF1 protein stability. Mol Cell Biol *26*, 9279-9290.

Zilfou, J. T., Hoffman, W. H., Sank, M., George, D. L., and Murphy, M. (2001). The corepressor mSin3a interacts with the proline-rich domain of p53 and protects p53 from proteasome-mediated degradation. Mol Cell Biol *21*, 3974-3985.

Zindy, F., Eischen, C. M., Randle, D. H., Kamijo, T., Cleveland, J. L., Sherr, C. J., and Roussel, M. F. (1998). Myc signaling via the ARF tumor suppressor regulates p53-dependent apoptosis and immortalization. Genes Dev *12*, 2424-2433.

Zurer, I., Hofseth, L. J., Cohen, Y., Xu-Welliver, M., Hussain, S. P., Harris, C. C., and Rotter, V. (2004). The role of p53 in base excision repair following genotoxic stress. Carcinogenesis *25*, 11-19.