REGULATION OF THE MDM2-P53 FEEDBACK LOOP BY RIBOSOMAL PROTEINS

by

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Act D	actinomycin D
ARF	alternative reading frame
ATM	ataxia telangiectasia mutated
ATR	ataxia telangiectasia related
C-	carboxy-terminus
CDK	cyclin-dependent kinase
CK2	casein kinase 2
CHX	cyclohexamide
DBA	Diamond-Blackfan anemia
DC	dyskeratosis congenital
E2	ubiquitin carrier protein
E3	ubiquitin-protein isopeptide ligase
GFP	green fluorescence protein
GST	glutathione-S-transferase
HIPK2	homeodomain-interacting protein kinase 2
His	6× histidine tag
IB	immunoblot
IgG	immunoglobin type G
IP	immunoprecipitation
IR	ionizing radiation
ITS	internal transcribed spacer sequence
MDM2	gene amplified on the mouse double minute chromosome
mRNA	message RNA
N-	amino-terminus
NE	nuclear extract
NES	nuclear export signal
NLS	nuclear localization signal
NoLS	nucleolus localization signal
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
rRNA	ribosomal RNA
RT	reverse transcription
SDS-PAGE	sodium-dodecyl-sulfate polyacrylamide gel electrophoresis
siRNA	small interference RNA
snoRNA	small nucleolar RNA
tRNA	transfer RNA
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 vital for cell growth control. Therefore, it is subjected to multiple forms of regulation. This dissertation sought to identify proteins that regulate the MDM2-p53 feedback loop through association with MDM2. Using a stable human 293 cell line that constitutively expresses MDM2, I purified MDM2-associated complexes that contains ribosomal proteins L5, L11, and L23. Ribosomal biogenesis tightly coordinates with cell growth. Perturbation of ribosomal biogenesis induced by inhibiting rRNA synthesis, processing, or assembly leads to ribosomal stress that activates the p53 pathway and stalls cell growth. However, the detailed mechanism underlying this pathway is still unknown. Here we show that ribosomal proteins L5, L11, and L23 form a complex or complexes with MDM2 independently of ribosomes. Functionally, overexpression of L5, L11, or L23 induces p53 transcriptional activity and p53-dependent G1 cell cycle arrest by inhibiting MDM2-mediated p53 ubiquitination and degradation. Interestingly, a low dose (5 nM) of actinomycin D, but not γ irradiation or translation inhibitors, enhances the MDM2-L proteins interaction. Reduction of L23 and L5 by siRNA inhibits actinomycin D-induced p53 activation. Furthermore, we show that L11, but not L5 and L23, leads to a dramatic accumulation of the ubiquitinated species and the steady-state level of MDM2, indicating that while L11 inhibits MDM2 activity towards p53, it enhances MDM2 auto-ubiquitination and also stabilizes MDM2 through a potential post-ubiquitination mechanism. In summary, these results demonstrate that ribosomal proteins L5, L11, and L23 are important regulators of the MDM2-p53 feedback loop in response to ribosomal stress.

CHAPTER ONE

INTRODUCTION

1.1 The p53 tumor suppressor

The tumor suppressor protein p53 is a critical transcription factor activated in response to stress to induce the expression of its target genes (44, 240, 303). The proteins encoded by these genes mediate multiple biological functions, including cell cycle arrest, apoptosis, cell senescence, differentiation, and accelerated DNA repair (224). Thus, p53 provides a crucial surveillance mechanism to allow the cell to either recover from the insults or to be eliminated from the replicative pool if damage is sustained and irrepairable, thereby preventing the cells with aberrant growth from producing malignant progenies.

The importance of p53 in tumor suppression is mirrored by the fact that more than half of human tumors harbor mutations or deletions in the p53 gene and many others retain impaired function of the p53 pathway through indirect mechanisms (112, 160, 277, 303). Indeed, gene knockout studies show that mice homozygous for inactivated p53 alleles develop normally, but are highly susceptible to spontaneous tumorigenesis (66). Furthermore, germ-line mutations of p53 occur in individuals with the cancer-prone Li-Fraumeni syndrome (181, 278). Therefore, p53 is recognized as the principal guardian of the genome that prevents the initiation and progression of tumors.

The p53 protein has typical structural domains of a transcription factor (Fig. 1.1A). The central DNA-binding domain mediates sequence-specific DNA binding (12, 232, 308). The majority of p53 gene mutations are located in this domain, highlighting the

importance of this region for p53 function (139). The N-terminal bi-partite acidic transactivation domain makes contacts with basal transcription factors and co-activators, thus initiating transcriptional activation of target genes (81, 243). The C-terminal tetramerization domain allows p53 to form a homotetramer, and is required for transcriptional activation of its target genes (308). The basic regulatory region on the extreme C-terminus is thought to regulate the sequence-specific binding activity of the central core DNA-binding domain and contributes to the ability of p53 to recognize several forms of DNA that resemble DNA-damaging agent-induced structures. (14, 116, 120, 156, 172, 245, 308). Finally, a proline-rich domain containing five copies of the sequence PXXP is found at its N terminus and might be necessary for p53 to induce apoptosis in response to DNA-damaging agents (10, 259, 302, 306, 336, 337).

The p53 pathway can be activated by a wide variety of cellular stresses, including DNA damaging agents such as irradiation and chemical mutagens, oncogenes, hypoxia, reactive oxygen species, telomere erosion, and the loss of survival signals (90, 226) (Fig. 1.2). There are at least three independent pathways that can activate the p53 network. First, DNA damage, such as that caused by ionizing radiation (IR), triggers an ATM (for ataxia telangiectasia mutated) and Chk2- dependent p53 activation pathway (38). Second, aberrant growth signals, such as those resulting from the expression of the oncogenes Ras or c-Myc, trigger an ARF (alternative reading frame, $p14^{ARF}$ in human, $p19^{ARF}$ in mouse) dependent p53 activation pathway (179, 268). Third, a wide range of chemotherapeutic drugs, ultraviolet light (UV), and protein-kinase inhibitors can activate p53 through ATR (ataxia telangiectasia related) or casein kinase (CK) 2-dependent pathways (192). All of

these pathways stabilize and activate p53 by modifying p53 and/or inhibiting MDM2 function (see below for details).

Upon activation, p53 binds to its cognate p53 responsive elements (p53RE) within the genome and activates the transcription of genes residing in the vicinity of these binding sites (90). More than one hundred of these genes have been described (303) and many more are expected to be identified with the development of advanced molecular technology (209). The proteins encoded by these genes contribute in multiple ways to diverse biological functions of p53, with most roles on regulating the cell cycle and apoptosis (224) (Fig 1.2).

Proper cell cycle checkpoints ensure cell division with genomic integrity (130, 147, 191). The p53 protein plays an important role in the G1 and G2 checkpoints of the cell cycle. This is accomplished in part by induction of its target genes $p21^{WAF1}$, 14-3-3-σ and GADD45. The p21 gene product is an inhibitor of cyclin D-associated cell cycle dependent kinases (CDK) (73). As a result, p21 maintains the Rb-E2F complex and consequently prevents the G1-S transition (95, 139). The cyclin B-cdc2 kinase is essential for the G2-M transition. Induction of 14-3-3-σ and GADD45 by p53 inhibits this kinase complex, thus mediating the G2 cell cycle arrest (108, 290). In response to DNA damage, the 14-3-3-σ protein binds to phosphorylated Cdc25 and sequesters Cdc25 in the cytoplasm where it cannot activate Cdc2. GADD45 dissociates cdc2 from cyclin B, thus blocking G2-M phase transition (123, 327).

Depending on the type and duration of the stress and the growth condition of the cell, p53 selectively activates a different subset of target genes with proapoptotic activity (17, 224). One group of these genes encodes proteins that localize to the cell membrane

such as Fas/CD95, KILLER/DR5, and PERP (7, 206, 227, 315, 316). These proteins trigger the death-receptor-mediated apoptotic pathway (287, 317). The second group of genes encodes cytoplasmic proteins, including PIDD and PIGs (p53-inducible genes) (169, 237). These proteins control mitochondrial membrane integrity, and reactive oxygen species (ROS) produced by the PIGs subsequently cause damage to the mitochondria and initiate apoptosis (237). The third group of genes encodes mitochondrial proteins such as BAX, NOXA (217), PUMA (210), p53AIP1 (218), BID, etc. These gene products promote the loss of mitochondrial membrane potential and cytochrome C release, resulting in the formation of the apoptosome complex with Apaf-1 and caspase. In addition, it has recently been proposed that p53 can interact directly with antiapoptotic proteins such as Bcl-XL and Bcl-2 to exert its apoptogenic function in the mitochondria independently of transcription activation (46, 69, 198), Thus p53 initiates apoptosis through multiple ways.

Given that p53 is toxic to the cell due to its inhibitory effect on cell growth and initiation of apoptosis, it must be precisely regulated in the cell. Indeed, nature has developed a mechanism for the quick response of p53 to stress, largely through inhibition of its degradation instead of increase of its transcription and translation. P53 degradation is mainly performed by its negative regulator MDM2 and involves changes in cellular localization and posttranslational modification of p53.

p53 constantly shuttles between the nucleus and the cytoplasm directed by several nuclear localization signals (NLS) and nuclear export signals (NES) (Fig. 1.1B). Three monopartite NLSs were originally found in the C-terminus of p53 (267). The NLS I is a bipartite nuclear localization signal which is the most active in directing p53 nuclear

import (166, 167). The NLS II and NLS III appear to be less effective and less conserved (267). Two nuclear export signals (NES) confer on p53 the ability to export from the nucleus (280, 332).

The nucleo-cytoplasmic shuttling is important for the regulation of p53 function and is regulated by MDM2. In order to be functional, p53 must be retained in the nucleus where it activates transcription of its target genes. It is believed that MDM2 binds to p53 and transports p53 from the nucleus to the cytoplasm where it is degraded (87, 216, 252, 288). Phosphorylation of p53 at the N-terminal MDM2 binding domain in response to DNA damage blocks the function of the N-terminal NES of p53 (332). Also, p53 forms a tetramer upon activation that blocks the C-terminal NES (280), thus retaining p53 in the nucleus. Therefore, the function of the NESs are highly regulated and contribute greatly to p53 activation in response to cellular stresses. However, whether p53 is degraded in the cytoplasm or the nucleus is still under debate (127, 271, 281). Recently, it was shown that degradation of endogenous p53 and MDM2 during down-regulation of the p53 response can also occur in the nucleus (127, 271, 281). Thus, both nuclear and cytoplasmic proteasomes are possibly able to efficiently degrade the elevated p53 and MDM2 protein levels after stress. In addition to MDM2, a recently reported protein Parc, a Parkin-like protein, was shown to function as a cytoplasmic anchor for p53. Abnormal cytoplasmic sequestration of wild-type p53 in several neuroblastoma cell lines correlated with elevated Parc levels. RNAi-mediated reduction of endogenous Parc relocated p53 to the nucleus and sensitized these cells to a p53-mediated DNA damage response (214), further emphasizing the importance of cellular localization in the regulation of p53 function.

Posttranslational modifications of p53 play a crucial role in regulating its stability and activity. These include phosphorylation, acetylation, ubiquitination, sumoylation, methylation, glycosolytion, and ribosylation (3, 240, 305, 314). p53 is phosphorylated on numerous serines (Ser) and threonine (Thr) residues in both the N- and C-terminal domains by a number of kinases in response to stress (Figure. 1.3) (90, 305, 314). For example, in response to IR, p53 is phosphorylated by ATM kinase at Ser 15 (9, 35, 208, 273). ATM also phosphorylates and activates Chk2 kinase, which in turn phosphorylates p53 at Ser 20 (42, 109, 269). In response to UV, p53 is phorsphorylated at Ser 15 by ATR kinase (129, 293) and at Ser 20 by Chk1 kinase, which is phosphorylated and activated by ATR (334). Following UV, the p53 protein is also phosphorylated at Ser 392 by CK2 kinase (28, 102, 193). Other kinases that phosphorylate p53 are summarized in Fig. 1.3. These include CK1 (199, 258), human vaccinia-related kinase 1 (VRK1) (300), DNA-PK (157, 270), CDK-activating kinase (CAK) (140), the homeodomain-interacting protein kinase 2 (HIPK2) (55), Jun NH2-terminal kinase (JNK), CDK2 and cdc2 (19, 239), protein kinase C (PKC) (13), and aurora kinase A (131).

The phosphorylation status of the p53 protein can have profound consequences upon its function. For example, phosphorylation within the N-terminal MDM2 binding domain (Ser 15, Ser 20, and Thr 18) clearly regulates p53 stability by interfering with its ability to bind to MDM2 (9, 35, 42, 152, 258, 264, 269, 273, 296). In addition, phosphorylation also regulates the recruitment of transcriptional co-activators such as p300/CBP to p53 (31), thus enhancing transcriptional activity of p53. Interestingly, phosphorylation of p53 at specific sites allows differential transcriptional activation of its target genes. For example, phosphorylation of p53 at Ser 46 by HIPK2 selectively drives the expression of

an apoptotic target gene, p53AIP1, but not other apoptotic genes such as PIG3 (55, 111, 218). Not all phosphorylation events lead to enhanced p53 stability and activation. For example, phosphorylation of p53 at Thr 55 by TAF-1 (the largest subunit of TFIID) has recently been shown to promote degradation of p53 and this phosphorylation was reduced following DNA damage (162). Thus, phosphorylation has a diverse impact on p53 stability and transcriptional activity.

p53 function is also regulated by acetylation. Members of the histone acetylase family, p300/CBP, bind to p53 and enhance p53-mediated transcription (8, 101, 168, 265). p300 was subsequently found to directly acetylate p53 at Lysine (Lys) 382 and activate the latent sequence-specific DNA-binding activity of p53 (101, 171, 257). Furthermore, the acetyltransferase PCAF acetylates p53 at Lys 320 (171, 257) and enhances sequence-specific binding of p53. Importantly, these acetylation events are responsive to DNA damage in cells. DNA damage-induced N-terminal phosphorylation of p53 enhances p300 binding and the C-terminal acetylation (171, 257). These results suggest that a series of posttranslational modifications may occur interdependently to activate p53.

Furthermore, p53 is sumoylated at one of the Lys residues that is also ubiquitinated. The sumoylation does not regulate p53 stability, but does enhance its transcriptional activity (94, 207, 249). Glycosylation and ribosylation by PARP have also been described to regulate both stability and transcriptional activity of p53 (148, 299). Interestingly, this modification is also regulated by phosphorylation, further illustrating the complex codependence of regulatory modifications of p53. Recently, p53 was shown to be

methylated at Lys 372 by Set9 methyltransferase. This modification restricts p53 to the nucleus and positively affects its stability (47).

Finally, p53 protein stability is controlled by ubiquitination. Although several other ubiquitin ligases, such as Pirh2 (158) and COP1 (67) have been recently shown to ubiquitinate p53, the central player to ubiquitinate p53 is the oncoprotein called MDM2.

1.2. The oncoprotein MDM2.

The oncoprotein MDM2, encoded by the *mdm2* gene originally identified on a mouse double minute chromosome in the 3T3DM cell line (33), is a key negative regulator of p53. MDM2 can immortalize and, in cooperation with Ras, transform rat embryonic fibroblasts (83). Also, overexpression of MDM2 potentiates the tumorigenesis of NIH 3T3 cells (75), implying its transforming activity. Consistently, amplification and overexpression of MDM2 has been shown in a variety of human tumors, particularly in soft tissue sarcomas, carcinomas, leukemias, lymphomas, breast and lung cancers (30, 53, 63, 71, 201, 311). The tumorigenic potential of MDM2 is closely linked to its ability to inhibit the tumor suppressor function of p53.

MDM2 is a nuclear phosphoprotein that possesses several important functional domains (Figure 1.4A). The N-terminal p53-interacting domain of MDM2 mediates its binding to the N-terminal transcriptional activation domain of p53 (43, 221). The central acidic domain of MDM2 has recently been shown to be pivotal for MDM2-mediated p53 degradation, but not ubiquitination (4, 133, 195, 338). In the C-terminal side of the acidic domain are a zinc finger domain with unknown function and the RING domain, which is required for its E3 ligase activity (76). The MDM2 protein also contains an NLS and an

NES, which shuttle MDM2 between the cytoplasm and the nucleus and provide another means by which p53 activity is tightly regulated (87, 252). Within the RING domain, amino acids 464–471 can function as a nucleolar localization signal (NoLS) (176).

MDM2 inhibits p53 function through the following mechanisms. MDM2 specifically binds, through its N-terminal p53-binding domain, to the N-terminal transcription activation domain of p53 (43, 221). This binding conceals the N-terminal transcription domain of p53, directly blocking its transcriptional activity (202, 221). In addition, this binding initiates p53 ubiquitination, thus leading to its degradation by the proteasome system, as MDM2 is a Ring-finger-containing E3 ubiquitin ligase (76, 113) . MDM2 also contains an NES and relocalizes p53 to the cytoplasm where p53 is unable to access target DNA (25, 89, 178, 288). Finally, it has been shown that MDM2 associates with p53 on the promoter of target genes and inhibits p53 transcriptional activation (125, 200). MDM2 can also interact directly with histones and promote monoubiquitination of histone H2B in the vicinity of a p53 binding site within the p21 promoter, leading to transcriptional repression (200). Thus MDM2 is a central negative regulator of p53.

Of note, the ubiquitin E3 ligase activity of MDM2 is crucial for negatively regulating p53 stability. It is known that MDM2 ubiquitinates p53 at a cluster of Lys residues in the C-terminus of p53 (248). However, recent data indicate that MDM2 mediates multiple monoubiquitinations on these residues instead of polyubiquitination (153). p300 acts as a potential E4 enzyme and mediates subsequent polyubiquitination and degradation by cooperating with MDM2 (97). Interestingly, it was further shown that low levels of MDM2 lead to multiple monoubiquitination events while high levels of

MDM2 cause polyubiquitnation (164). It was proposed that monoubiquitnation targets p53 for nuclear export while polyubiquitination is the signal for degradation (164).

Given that MDM2 is a critical inhibitor of p53, its activity must be tightly regulated in cells. Similar to the case of p53, posttranslational modifications of MDM2 also play a role in its regulation. MDM2 can be phosphorylated at multiple sites (Fig. 1.4B), most of which are clustered within the N-terminal p53-binding domain and the central acidic domain. For example, MDM2 is phosphorylated by DNA-PK at Ser 17. This phosphorylation might play a role in blocking the MDM2-p53 interaction (189). ATM phosphorylates MDM2 at Ser 395 and impairs the ability of MDM2 to promote p53 degradation possibly through phosphorylation-dependent inhibition of nuclear export of p53 by MDM2 (62, 135, 186). Mitogen-induced activation or ectopic expression of constitutively active AKT kinase phosphorylates MDM2 on Ser 166 and Ser 186 and stimulates nuclear entry of MDM2 and diminishes cellular levels of p53 (187, 188, 219, 335). Consistently, HER-2/neu, which is expressed at a higher level in breast tumors, can protect against p53-induced apoptosis by inducing p53 ubiquitination via AKT-mediated MDM2 phosphorylation (335). The c-Abl protein tyrosine kinase can block ubiquitination and nuclear export of p53 in response to DNA damage (274, 275) by phosphorylating MDM2 at tyrosine (Tyr) 394 (92), thereby contributing to DNA damage-induced apoptosis. Other kinases that phosphorylate MDM2 include cyclin A-CDK1/2 kinases (328) and CK2 (96, 110).

Like other RING domain containing E3 ligases, MDM2 also mediates its own ubiquitination and degradation (114). Thus, under normal conditions, cellular MDM2 is extremely short-lived (with half-life only 15-20 minutes) due to its fast turnover rate.

However, how the MDM2-mediated autoubiquitination is regulated and whether it coordinates with MDM2-mediated p53 ubiquitination is still unknown. Interestingly, MDM2 can be conjugated with other ubiquitin-like moieties such as SUMO-1. Sumoylation of MDM2 may modulate its E3 ligase activity in a manner that favors MDM2-mediated p53 ubiquitination (32).

Although the major function of MDM2 is to negatively regulate p53, increasing evidence indicates that MDM2 also performs p53-independent functions relevant to transformation such as cell cycle regulation (88). For example, MDM2 directly interacts with the RB tumor suppressor protein and inhibits the ability of RB to block E2F1 function, thus perturbing Rb-mediated G1 cell cycle arrest (319). It was recently shown that RB is efficiently ubiquitinated by wild-type MDM2 (295). MDM2 interacts with the general transcription factor TFIID (98, 159) and activates the promoter of cyclin A, a gene that is important for S-phase entry (159). MDM2 has been shown to interact with E2F1 and stimulate E2F1-dependent activation of E2F promoters, and DNA synthesis (183). The growth-promoting activity of MDM2 though E2F1 could be crucial for the p53-independent oncogenic activities of MDM2. These data indicate that MDM2 promotes cell proliferation by regulating other components of the cell cycle in addition to regulating p53. Finally, MDM2 has been shown to bind to a number of other proteins involved in cell cycle control, differentiation, DNA synthesis, RNA biosynthesis, transcription, and cell surface receptor turnover through ubiquitination-dependent or ubiquitination-independent manner. These proteins include MDM2 binding protein (MTBP), ß-arrestin, Numb, DNA polymerase ε, and the androgen receptor (AR) (88).

1.3. The MDM2-p53 feedback loop and its regulation

The *mdm2* gene itself is a downstream target gene of p53 (11, 233) and MDM2, in turn, inhibits p53 function, thus forming an elegant autoregulatory feed back loop (235, 318). Indeed, genetic disruption of the p53 gene completely rescues the lethal phenotype of the *mdm2* knockout mice, suggesting that the embryonic lethality is due to active p53, and firmly validating the notion of the MDM2-p53 feedback loop (126, 204). Again, this tight feedback loop requires physical interaction between MDM2 and p53.

Because the MDM2-p53 feedback loop is critical for cells to maintain a low level of p53 as well as quickly respond to cellular stress, it must be tightly regulated in cells. There are two mechanisms to regulate the pathway in order to activate p53: disruption of the MDM2-p53 interaction and direct inhibition of the E3 ligase activity.

The first mechanism acts to dissociate the MDM2-p53 complex and involves posttranslational modification of both p53 and MDM2, particularly phosphorylation. This is applied to the DNA damage-triggered p53 activation. The inhibition of p53 function by the MDM2-p53 interaction is described in Part 1.2. The crystal structure of the MDM2-p53 binding has been solved (150). MDM2 contains a well-defined relatively deep hydrophobic pocket at the N- terminus (residues 25-109) where the transactivation domain of p53 binds thereby concealing p53 from interacting with the transcriptional machinery (150). The minimal MDM2-binding site on the p53 protein was subsequently mapped within residues 18–26 (22, 23, 43). The pocket is filled primarily by three side chains (Phe 19, Trp 23, and Leu 26) from the helical region of the p53 peptide (22, 150). Thr 18 is very important for the stability of the p53 helix (150). Accordingly, p53 mutants at the MDM2-binding site are resistant to degradation by MDM2 (105, 146).

Similarly, mutations of MDM2 at residues Gly 58, Glu 68, Val 75, or Cys 77 result in a lack of p53 binding (86). Phosphorylation of Ser 15 and Ser 20 does not directly affect MDM2-p53 binding, but Thr 18 phosphorylation weakens the binding by 10-fold, indicating that phosphorylation of Thr 18 is directly responsible for abrogating MDM2-p53 binding (264). However, phosphorylation of Thr 18 requires the prior phosphorylation of p53 at S15 (258).

The second mechanism involves factors that directly inhibit the E3 ligase activity of MDM2 towards p53 apparently without disruption of the MDM2-p53 complex. This mechanism is largely independent of p53 modifications (5, 20, 135), as testified by the oncogene-mediated p53 activation pathway that is independent of phosphorylation of p53 (61). The best characterized example is the ARF-induced p53 activation in response to activation of viral and cellular oncoproteins such as Ras and c-Myc (115, 128, 333). The ARF protein prevents MDM2 from targeting p53 probably through two mechanisms: separating the two proteins from different cellular compartments and inhibiting MDM2-mediated p53 ubiquitination. The interaction of ARF with MDM2 blocks MDM2 shuttling between the nucleus and cytoplasm via the nucleolus (289, 313). Sequestration requires the combined nucleolar localization signals (NoLS) of ARF and MDM2 (176). Sequestration of MDM2 in the nucleolus thus results in activation of p53 (115, 289, 333). Mutations in human ARF exon 2 disrupts its nucleolar localization and impairs its ability to block nuclear export of MDM2 and p53 (333). However, there is some disagreement as to whether sequestration of MDM2 by ARF takes place in the nucleolus or in the nucleoplasm (174), because some data indicates that ARF can stabilize p53 without

relocalization of MDM2 to the nucleolus (174, 196). In addition, ARF can directly inhibit MDM2 ubiquitin ligase activity *in vitro* (115).

Additionally, the MDM2-p53 feedback loop is subjected to regulation by the transcriptional coactivators p300 and CBP. Intriguingly, p300 appears to exert a dual function on this loop (132). As stated above, p300 acetylates p53 and stimulates its activity. This acetylation can be inhibited by MDM2 (141). On the other hand, the p300/CBP protein interacts with MDM2 in nuclear body-like structures where MDM2 might be protected from proteasomal degradation (323) and cooperates with MDM2 to degrade p53 (98, 132, 292). Consistently, MDM2 mutants lacking the p300/CBP-binding domain within the central acidic domain failed to degrade p53 but still promoted monoubiquitination of p53 (4, 338). More recently, p300/CBP was shown to act as an E4 enzyme to assist MDM2 in polyubiquitinating p53 (97). It is as yet unclear under what physiological conditions p300 regulates this loop, either positively or negatively.

Another regulator of MDM2 is its homolog MDMX, which assists MDM2 in down-regulating p53 function (272). As in the case of MDM2, genetically targeting the p53 gene also rescues the lethal phenotype of *mdmx* knockout mice, suggesting that MDMX is critical for the MDM2-p53 feedback regulation as well (82, 197, 230). Consistently, increased expression of MDMX has already been observed in human tumors (241, 246, 247). However, MDMX itself is not an E3 ligase (82, 272) nor does it have NLSs and NESs. MDMX does bind to p53 with similar requirements to MDM2. In the absence of MDMX, MDM2 is relatively ineffective in downregulating p53 because of its extremely short half-life. MDMX renders MDM2 protein sufficiently stable to function at its full potential for p53 degradation by interacting through their RING finger domains (100).

MDMX is also degraded by MDM2 (60, 229). Moreover, ARF blocks MDM2 from degrading p53 and shifts MDM2 activity to degrade MDMX instead (229). Therefore, MDM2 and MDMX may in fact have different roles in inhibiting p53.

Finally, HAUSP, a ubiquitin hydrolase, is a direct antagonist of MDM2 activity and acts by specifically deubiquitinating p53 after stimulation by DNA damage, thus protecting p53 from MDM2-mediated degradation (165). Therefore, even MDM2-mediated p53 ubiquitination is precisely regulated in cells.

All of the above studies firmly support the idea that the MDM2-p53 feedback loop is tightly regulated by a variety of proteins. Therefore, identifying other factors that regulate this pathway will benefit our understanding of p53 activation and ultimately contribute to treatment of cancer. One strategy is to identify other MDM2-associated proteins in cells. In order to do so, in this study, I have generated a stable human embryonic kidney epithelial 293 cell line that constitutively expresses MDM2. Using this cell line, I purified an MDM2-associated complex through immunoaffinity purification followed by mass spectrometric analysis. Surprisingly, several ribosome proteins, including L23, L11, and L5, were found in this complex (57, 58).

The interaction between MDM2 and L5 was reported ten years ago (182). Further studies have shown that the central acidic domain of MDM2 interacts with L5 and the C-terminal RING domain of MDM2 interacts with 5S rRNA (74, 182). Given that MDM2 has an NLS and is found in the nucleolus (176) and that L5 is a component of the large subunit of ribosomes and also a nucleolus protein, it has been speculated that MDM2 may play a role in ribosome assembly, transport, or RNA synthesis, or that MDM2 "rides the ribosome" to the cytoplasm. However, no further functional data has been shown

since these initial studies. The findings in this thesis not only confirmed the MDM2-L5 interaction, but also redrew attention to this interaction, because MDM2 actually associates with multiple ribosomal proteins. When I was finishing the functional analysis of the MDM2-L23 interaction in regulation of MDM2-p53 feedback loop, the functional interaction between MDM2 and L11 was also reported by other groups (177, 329). Interestingly, the regulation of the MDM2-p53 feedback loop by these MDM2-L protein interactions falls into the second model of p53 activation as mentioned above. These interactions, like ARF, inhibit MDM2-mediated p53 ubiquitination and subsequently block the proteasome-mediated degradation of p53 without apparent disruption of the MDM2-p53 interaction. Importantly, this regulation is responsive to ribosomal stress triggered by perturbation of ribosomal biogenesis (57, 58, 122, 177, 329).

1.4. Ribosomal Biogenesis and its implication in tumors.

Cell growth requires the synthesis of proteins, and the synthesis of proteins requires ribosomes. The ribosome is a finely tuned cellular machine that translates cellular mRNA through a static, higher-ordered cellular process (254, 256). Synthesis of ribosomes, a process called ribosomal biogenesis (Fig. 1.7), consumes a vast portion of cellular energy and metabolites, and plays a key role in cell growth and proliferation (103, 309). To produce a ribosome, eukaryotic cells must assemble about 79 ribosomal proteins with four different ribosomal RNA (rRNA) species (28S, 18S, 5.8S and 5S), which account for up to 50~80% of the total steady-state cellular RNAs (103, 309). Also, ribosomal biogenesis requires a number of accessory factors. These factors include numerous small nucleolar RNAs (snoRNAs) and non-ribosomal proteins that process and modify the pre-

rRNAs (endonucleases, exonucleases, pseudouridine synthases, and methyltransferases), mediate RNP folding/remodeling (RNA helicases, RNA chaperones), or facilitate protein association/dissociation (GTPases, ATPases) (145). In principle, ribosomal biogenesis requires the coordination of several events, including the synthesis and import of ribosomal proteins, synthesis and processing of rRNA, the concomitant assembly of ribosomal proteins into the pre-ribosomal subunits and their subsequent transport (77, 276, 310). Notably, all three RNA polymerases (I, II and III) are involved and coordinated to ensure the high efficiency and accuracy of ribosome production. The 18S, 5.8S, and 28S rRNA species are derived from a single 47S rRNA precursor that is transcribed by RNA polymerase I (Pol I) from multiple copies of the genes for pre-ribosomal RNA (rDNA) and then processed by sequential endonucleolytic and exonulceolytic cleavages (72, 103, 297). The 5S rRNA is synthesized separately by Pol III and is associated with the 60S pre-ribosomal subunit early in assembly. Pol II transcribes the mRNAs for the ribosomal proteins. In mammalian cells, the mature 40S ribosomal subunit contains the 18S rRNA and approximately 32 ribosomal proteins, whereas the 60S subunit is composed of the 5S, 5.8S, and 28S rRNAs and approximately 47 ribosomal proteins (145, 301).

In eukaryotic cells, the nucleolus is a critical cellular workshop in which the rRNAs are transcribed and processed and the ribosomal subunits are assembled (223, 242). It is a membrane-free nuclear subcompartment containing three distinct subcompartments based on their morphology in the electron microscope: the fibrillar centers (FC), dense fibrillar components (DFC), and granular components (GC). It is believed that rRNA transcription is restricted to the periphery of the FC, while transient accumulation,

modification, and processing of primary rRNA transcripts occurs in the DFC, and later processing and rRNA assembly occurs in the GC (37, 70, 262, 301). Newly synthesized ribosomal proteins, imported into the nucleolus, associate with precursor rRNA intermediates, snoRNAs, and accessory proteins to form maturing ribonucleoprotein particles (77, 104, 161, 215, 276). Precursor rRNA first assembles into a 90S pre-ribosomal particle, after which several rapid RNA cleavage steps separate the precursors to the large and small subunits. Families of pre-60S particles containing different accessory molecules undergo progressive changes in the nucleolus and then are released into the nucleoplasm and exported to the cytoplasm, where remaining nonstructural proteins (accessory factors) are released. Maturation of the 40S subunit is also initiated in the nucleolus, but factors controlling its export are less well characterized.

Ribosomal biogenesis is tightly coordinated with cell growth and proliferation. The synthesis of rRNA is linked to cell cycle progression. It is maximal in S and G2 phases, repressed in mitosis and increased in G1 (99, 138). Cells that exit the division cycle into a quiescent state greatly limit ribosome production and overall protein synthesis (72, 231). These fluctuations in cell-cycle-mediated rRNA synthesis are dependent on the activity of Pol I. Also, the transcription factor UBF (the HMG1 box containing upstream binding factor) regulates rRNA synthesis by modulating Pol I transcriptional activity (15, 39). UBF binds to two regions of the rDNA promoter, the upstream control element (UCE) and the core, which are also recognized by Pol I (16, 244). Another basal transcriptional factor called SL-1 or TIF-IB is also essential for Pol I transcription. Although the mechanism underlying this precise coordination between ribosomal biogenesis and cell growth remains obscure, several proto-oncogenes and tumor suppressors have been

shown to regulate rRNA synthesis by modulating UBF and SL-1 activity, respectively, thus directly regulating ribosomal biogenesis (78).

In response to environmental stresses, active tumor suppressors such as Rb and p53 negatively regulate Pol I transcription as cells exit the cycle (29, 39, 48, 304, 326). The RB protein directly binds to UBF and blocks the interaction of UBF with SL-1, leading to inhibition of Pol I-mediated rRNA transcription (39, 103, 304). Another RB-family member p130, but not p107, also represses rRNA transcription through its ability to bind to and inactivate UBF (48, 103). The inhibition of rRNA synthesis by RB is correlated with cell growth and proliferation (103). p53 has also been shown to repress Pol I transcription through direct binding to SL-1 and blocking the UBF-SL-1-Pol I initiation complex formation on the rRNA promoter (29, 326). In addition to inducing p53 and limiting rRNA biosynthesis, the ARF tumor suppressor protein can directly inhibit rRNA processing independent of p53 (284). Finally, p53 and RB-family members have been shown to control Pol III transcription (34, 54, 155, 285), which transcribes 5S rRNA, various snoRNAs as well as tRNA. Both of the tumor suppressors negatively regulate Pol-III-mediated transcription through direct inactivating interactions with TF-IIIB, a co-activator that is responsible for Pol-III-mediated transcription. Therefore, overproduction of both p53 and RB in response to stresses can inhibit cell growth by suppressing the biosynthesis of tRNA and rRNA through direct inhibition of both Pol I and Pol III activity.

On the other hand, the proto-oncogene product MYC has been shown to directly regulate ribosome biogenesis through the transcription of ribosomal proteins and the regulation of S6K kinase activity. Using serial analysis of gene expression (SAGE) and

oligonucleotide microarray analysis, many ribosomal proteins, ribosome assembly factors, translation initiation and elongation factors were found to be MYC target genes (21, 51, 194). These results support a role for MYC-family members as key regulators of ribosome biogenesis and translation control. Consistently, transgenic mice that constitutively expressed c-Myc under the control of the immunoglobulin heavy-chain enhancer show increases in cell size corresponding to increased ribosome synthesis (118, 136). Recently, it was shown that c-Myc directly activates Pol III transcription by association with TFIIIB, a Pol III-specific general transcription factor (93). Therefore, several tumor suppressor genes and proto-oncogenes execute their function in a manner directly connected to ribosomal biogenesis.

Consistent with the effect of MYC on ribosomal biogenesis, several ribosomal proteins have been shown to be overexpressed in cancers (80, 143, 175). For example, overexpression of the ribosomal protein S3a was able to induce transformation of NIH 3T3 cells and induce formation of tumors in nude mice by inhibiting apoptosis (212). Kondoh, *et al* (143) showed that ribosomal proteins S8, L12, L23a, L27, and L30 are up-regulated in human hepatocellular carcinomas. Many other ribosomal proteins are also reported to be deregulated in various tumors (211). However, how the overexpression of individual ribosomal proteins contributes to tumorigenesis is not known, given that the ribosome synthesis is a highly coordinated process and needs almost 79 ribosomal proteins and many accessory factors. A reasonable explanation is that certain ribosomal proteins have extra-ribosomal functions, for example, S3a has a possible anti-apoptotic effect (212). This is particularly supported by a recent study showing that ribosomal protein L13a is released from ribosome in response to interferon (IFN) γ, binds to the 3'-

UTR (untranslated region) of a gene called IFN γ-activated inhibitor of translation (GAIT) of ceruloplasmin (Cp) mRNA and silences the translation of this gene (190). Interestingly, *drosophila* S3 protein has a β,δ-elimination AP lyase activity to efficiently repair 8-oxo-7, 8-dihydroquanine (8-oxoG) DNA lesion (36). Another ribosomal protein called S6 is also of particular interest because a phosphorylation cascade pathway leads to the phsophorylation and activation of S6. The AKT kinase, in response to extracellular signaling pathways, phosphorylates mTOR (mammalian target of rapamycin) kinase. mTOR phosphorylates S6 kinase (S6K). Finally, S6K phosphorylates and activates S6 (106). Activation of S6 by phosphorylation leads to an upregulation in the translation of a specific class of mRNAs termed TOP (a terminal oligopyrimidine tract in the 5'-UTR) mRNA. TOP mRNAs include transcripts for ribosomal proteins, elongation factors and several other proteins that are involved in ribosome biogenesis (121, 291). Therefore, S6 could be an important regulator of cell growth, through the regulation of translation of TOP mRNAs.

In contrast to the overexpression or activation of ribosomal proteins, the reduction of ribosomal proteins or perturbation of ribosomal biogenesis may also contribute to tumorigenesis. The expression of ribosomal protein genes is a coordinated process, leading to the precise equimolar production of 79 proteins (309). Gene-targeting experiments in yeast have shown that the depletion of a single ribosomal protein such as L16, results in a decrease of the 60S ribosomal subunit and a defect in cellular growth (251). The control of ribosome biogenesis by individual ribosomal proteins has been further validated in *Drosophila*. Mutations in individual ribosomal proteins in *Drosophila* led to a class of mutants collectively known as minute, characterized by reductions in

body size, diminished fertility and recessive lethality as a result of reduced protein synthesis (144). The first knockout study of a ribosomal protein gene in mammals has been recently reported for ribosomal protein S19. Homozygous disruption of the S19 allele in mice was embryonic lethal (184), further indicating the profound effect of ribosomal biogenesis on cell growth and development.

There are several lines of evidence that indicate the direct connection between perturbation of ribosomal biogenesis and human cancers. First, mutations in the gene that encodes ribosomal protein S19 have been identified in approximately 25% of patients with Diamond-Blackfan anemia (DBA), a cancer susceptibility syndrome characterized by anemia and an increased susceptibility to haematopoietic malignancies (68). The molecular mechanisms by which mutation of S19 causes the cancer predisposition are unknown. While homozygous for the disrupted S19 allele in mice was embryonic lethal, the heterozygous mice are normal for cell growth and organ development, indicating that one normal S19 allele is sufficient to maintain normal ribosomal and possibly extraribosomal functions (184). However, this study does not exclude the possibility that DBA is due to mutations of S19. It is possible that one normal S19 allele may perform normal function in ribosome through an allele compensation mechanism but may not sufficiently perform tumor suppressor function. Therefore, long-term followup of the heterozygous mice is needed to examine if the mice have increased tumorigenesis compared to wild-type animals (184). Second, mutations in the *DKC1* gene are associated with dyskeratosis congenita (DC), a disease characterized by premature aging, bone-marrow failure, hyperkeratosis of the skin, and an increased susceptibility to tumor formation (65, 107). *DKC1* encodes a putative pseudouridine synthase, dyskerin, which

mediates post-transcriptional modification of rRNA through the site-specific conversion of uridine to pseudouridine (151). 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 are impaired in rRNA processing (255). 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. Third, it has been shown that the primary structure of ribosomal protein L10 is closely related to a putative Wilms' tumor suppressor (41). Fourth, the S29 ribosomal protein increases the tumor suppressor activity of the K rev-1 gene on v-K ras-transformed NIH3T3 cells (142), indicating that certain ribosomal proteins might play a tumor suppressor function. Lastly, this idea is further supported by a recent study in zebrafish showing that 11 of the 12 lines with elevated cancer incidence (malignant peripheral nerve sheath tumors) were each heterozygous for a mutation in a different ribosomal protein gene, suggesting that many ribosomal proteins may act as haploinsufficient tumor suppressors (1).

1.5. The ribosomal stress and p53 activation pathway.

The next question to ask is how the loss of a particular ribosomal protein or perturbation of ribosomal biogenesis, due to defects in its accessory factors, ultimately leads to tumor formation. Do these defects funnel into a stress signal to activate tumor suppressor pathways in cells?

The concept of "ribosomal stress" has therefore been recently proposed by several studies, and it leads to activation of p53 (6, 234, 282, 283). For example, overexpression of dominant negative mutants of Bop1, a nucleolar protein critical for rRNA processing

and ribosome assembly (282), inhibited 28S and 5.8S rRNA formation and led to a deficiency of newly synthesized 60S ribosomal subunits in 3T3 fibroblast cells. Consequently, the cells underwent p53-dependent G1 cell cycle arrest (234, 283). Also, a low dose of actinomycin D, which inhibits RNA polymerase I at the concentration of 5 nM, can stall rRNA synthesis and ribosome assembly. By doing so, this compound activates p53 function without triggering N-terminal phosphorylation of p53 (5, 6). Furthermore, the tumor suppressor ARF also directly inhibits rRNA processing, and may generate ribosomal stress thus contributing to p53 activation in addition to its inhibitory effect on MDM2-mediated p53 ubiquitination and degradation, and again without N-terminal phosphorylation of p53 as mentioned above. Therefore, these studies suggest an elaborate coordination between p53-mediated cell growth regulation and cellular responses to the malfunction of ribosomal biogenesis through a novel signaling pathway.

In addition to ARF, several other nucleolar proteins have also been shown to be closely connected to the p53 pathway. First, nucleophosmin/B23, a ubiquitiously expressed nucleolar phosphoprotein and clinical marker of highly proliferative cells, has been proposed to function in ribosomal protein assembly and transport, and also as a molecular chaperone that prevents proteins from aggregating in the crowded enviroment of the nucleolus (286). It also acts as an endonuclease to direct endonucleolytic cleavage of rRNA precursors at a site within the second internal transcribed spacer sequence (ITS-2) located 3' to the 5.8 S domain in 32 pre-rRNA (ITS-2 specific endoribonuclease) (260), thereby inhibiting the processing of 32S rRNA precursor into 28S rRNA. Colombo *et al* first reported that B23 interacts directly with p53 and increases its stability and transcriptional activity after different types of stresses, including UV-induced DNA

damage. Consequently, it induces p53-dependent premature senescence upon overexpression in fibroblasts (52). This is further verified by a recent study showing that B23 interacts directly with MDM2 and inhibits MDM2-mediated p53 ubiquitination and degradation in response to UV (149), thus inhibiting the MDM2-p53 feedback loop. Second, another abundant nucleolar protein nucleolin (also called C23), which plays a critical role in rRNA processing (91), has also been shown to affect p53 activity. Upon exposure to certain stresses including heat shock, IR and a radiomemetic agent camptothecin, C23 relocalized from the nucleolus to the nucleoplasm whereupon it bound to replication protein A (RPA) and inhibits DNA replication initiation. This response was dependent on association of C23 with p53, implying a novel p53-dependent, C23-mediated transient replication inhibition and DNA repair (59). Third, another newly identified novel nucleolar protein called nucleostemin was shown to modulate p53 activity during cell cycle progression in the central nerve system and cancer cells. Overexpression or reduction of this protein prevented cells from entering mitosis and caused p53-dependent apoptosis, though its exact function on ribosomal biogenesis is unknown (294).

Given that all the above proteins are primarily nucleolar proteins and that the major task of the nucleolus is in ribosomal biogenesis, it is speculated that perturbation of ribosomal biogenesis will trigger "nucleolar stress" which activates the p53 pathway. Loss of this p53 response can result in unrestrained cellular proliferation (205, 254). Alternatively, the nucleolus participates as a natural regulator in controlling the abundance of p53 in cells (180). In addition, the nucleolus could perform diverse nontraditional cellular duties including signal recognition, particle assembly, cell cycle

regulation, control of aging, modification of small nuclear RNAs, and modulation of telomerase function (222). These functions may be also connected to, or act as a result of p53 activation. Recent proteomic analyses of the nucleolus allowed the identification of a significant number of the nucleolar proteins that are ribosomal proteins and rRNA processing and ribosome assembly factors (2, 263). These studies broaden our interest in the regulation of p53 by the nucleolus.

It is worth noting that Rubbi and Milner (253) recently proposed that the nucleolus is a major cellular stress sensor and transmits signals to p53 activation. They showed that 11 of 13 different agents that induced p53 stabilization cause disruption of the nucleolus. The disruption is usually accompanied by release of nucleolar components as evident by a measurement of the translocation of B23 to the nucleoplasm (40, 122). They also showed that direct disruption of the nucleoli in the absence of DNA damage results in stabilization of p53, and UV lesions do not directly cause the p53 response unless the nucleolus is irradiated and destroyed. Therefore, nucleolar disruption alone is able to trigger p53 stabilization in the absence of DNA damage. Although the nucleolar-disruption model does not apply to every p53 activation pathway, the model is still plausible because the data shown in this paper and many others strongly indicates the relationship between perturbation of ribosomal biogenesis and cellular response and its correlation with tumorigenesis.

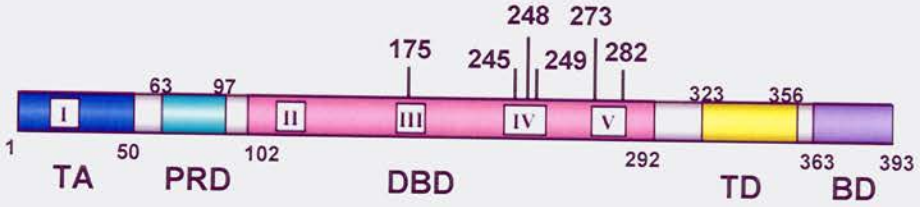
Finally, the central question is how the nucleolus transmits cellular stress signals to the p53 pathway. ARF is the most likely candidate. Perturbation of the nucleolus may lead to the release of ARF, which can then inhibit MDM2 activity in the nucleoplasm. However, ARF is not present in all species or cell types (330) and in ARF-null cells p53

response to nucleolar stress, such as that induced by treatment of a low dose actinomycin D, is still intact (57, 58, 122, 177). Therefore, searching for the nucleolar candidates that regulate the MDM2-p53 feedback loop is critical for better understanding the mechanisms by which the nucleolus modulates p53 activity in response to ribosomal or nucleolar stress.

Therefore, in this thesis, I have focused on the regulation of the MDM2-p53 feedback loop by ribosomal proteins in response to ribosomal stress.

Figure 1.1. Schematic diagrams of the functional domains of p53. (A). Functional domains of p53. The transcriptional activation domain (TAD) is located within residues 1-50 and the proline-rich domain (PRD) is located within residues 63-97. The central core DNA binding domain (DBD) encompasses residues 102-292. The tetramerization domain (TD) is located within residues 323-356 while the basic regulatory region at the extreme C-terminus between residues 363-393 is labeled as basic domain (BD). Evolutionarily conserved boxes are indicated as I–V. The mutation hot-spots are located within DBD and residues R175, G245, R248, R249, R273, and R282 are the six most frequently mutated residues in human tumors. These residues directly contact both DNA. (B). The nuclear localization signals (NLS) and nuclear export signals (NES) of p53. The three NLSs (red bars) are located at C-terminus of p53 as indicated. The N-terminal NES (green bar) is located within residues 11-27, and the C-terminal NES (green bar) encompasses residues 340-351.

A


248
273
175
245
249
282
63
97
323
356
I
II
III
IV
V
1
50
102
292
363
393
TA
PRD
DBD
TD
BD

B


NES
NLS
NES
NLS
NLS
11
27
305
322
340
351
369
375
379
384

Figure 1.2. The upsteam signals and the transcriptional target genes of p53. p53 can be stabilized in posttranslational level and activated in response to DNA damage caused by IR, UV and mutagens, oncogenic overexpression such as Ras, c-Myc, ribosomal stress such as treatment of a low dose of actinomycin D, loss of survival signals, mitotubule inhibitors, ribonucleotide depletion, hypoxia, and telomere erosion, etc. Activated p53 then acts as a transcriptional factor to induce the expression of its down stream target genes. Depending the cellular context and type and duration of the encountered stress, different subsets of the target genes are transciptionally activated. These genes are included in at least five categories involving in (1) inhibition of cell cycle progress, (2) initiation of apoptosis, (3) accelerating DNA repair, (4) cell senescence, (5) inhibition of angiogenesis.

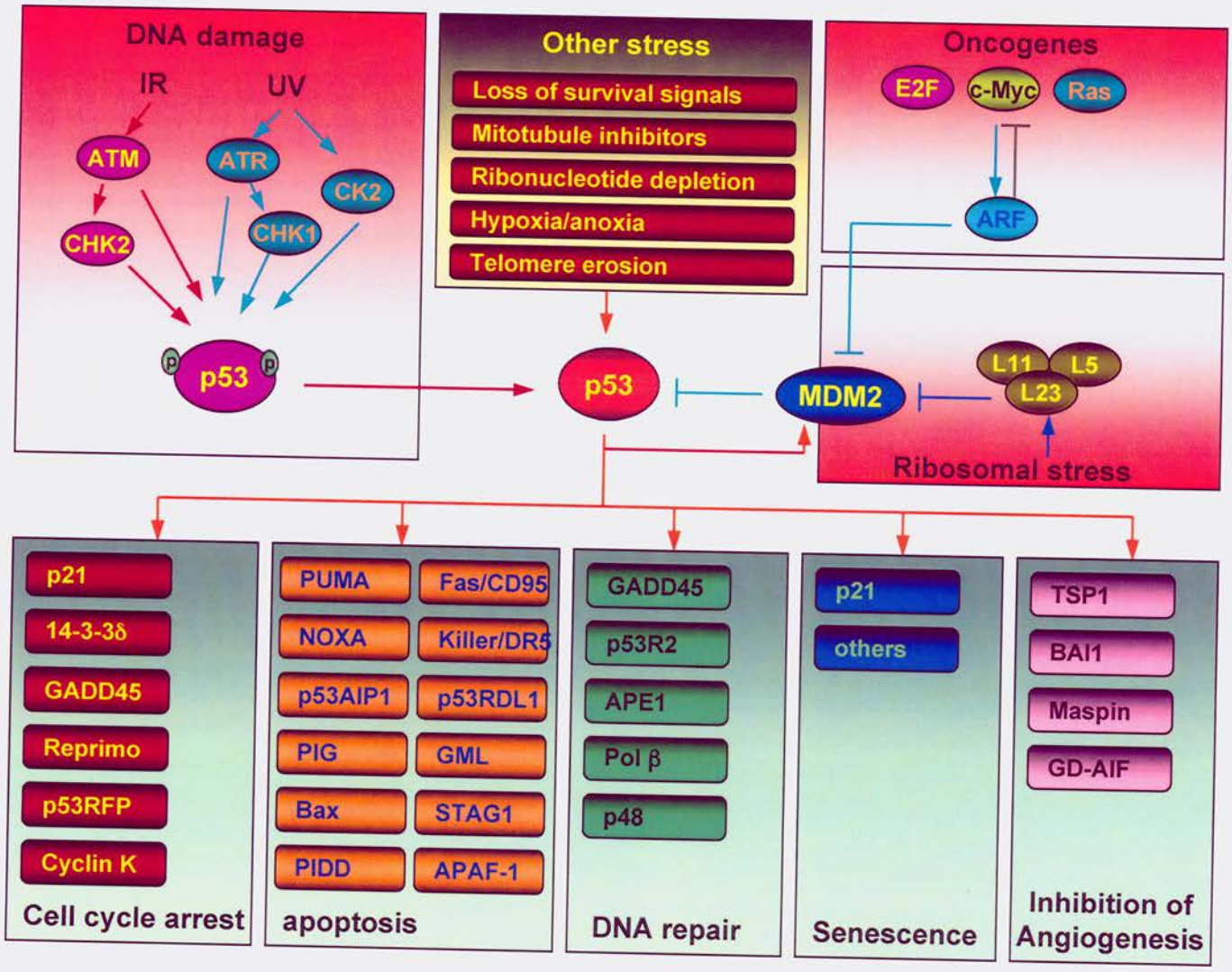

DNA damage
IR
UV
ATM
ATR
CK2
CHK2
CHK1
p
p53
p
Other stress
Loss of survival signals
Mitotubule inhibitors
Ribonucleotide depletion
Hypoxia/anoxia
Telomere erosion
Oncogenes
E2F
c-Myc
Ras
ARF
p53
MDM2
L11
L5
L23
Ribosomal stress
p21
14-3-3δ
GADD45
Reprimo
p53RFP
Cyclin K
Cell cycle arrest
PUMA
Fas/CD95
NOXA
Killer/DR5
p53AIP1
p53RDL1
PIG
GML
Bax
STAG1
PIDD
APAF-1
apoptosis
GADD45
p53R2
APE1
Pol β
p48
DNA repair
p21
others
Senescence
TSP1
BAI1
Maspin
GD-AIF
Inhibition of Angiogenesis

Figure 1.3. Posttranslational modification of p53. In response to DNA damaging agents, the p53 protein can be posttranslationally modified by many different kinases, acetytransferases, methytransferase as indicated at different Serine (S), Threonine (T), or Lysine (K) residues (the number indicates the position of the residues). The arrows indicate modifying. "p" indicates phosphorylation. "Ac" indicates acetylation. "CH3" indicates methylation. "s" indicates sumoylation. MDM2 can ubiquitinate p53 at a cluster of Lysine residues (not shown) and mediate p53 degradation through proteasome system. Also, MDM2 can inhibits p300 or PCAF-mediated p53 acetylation.

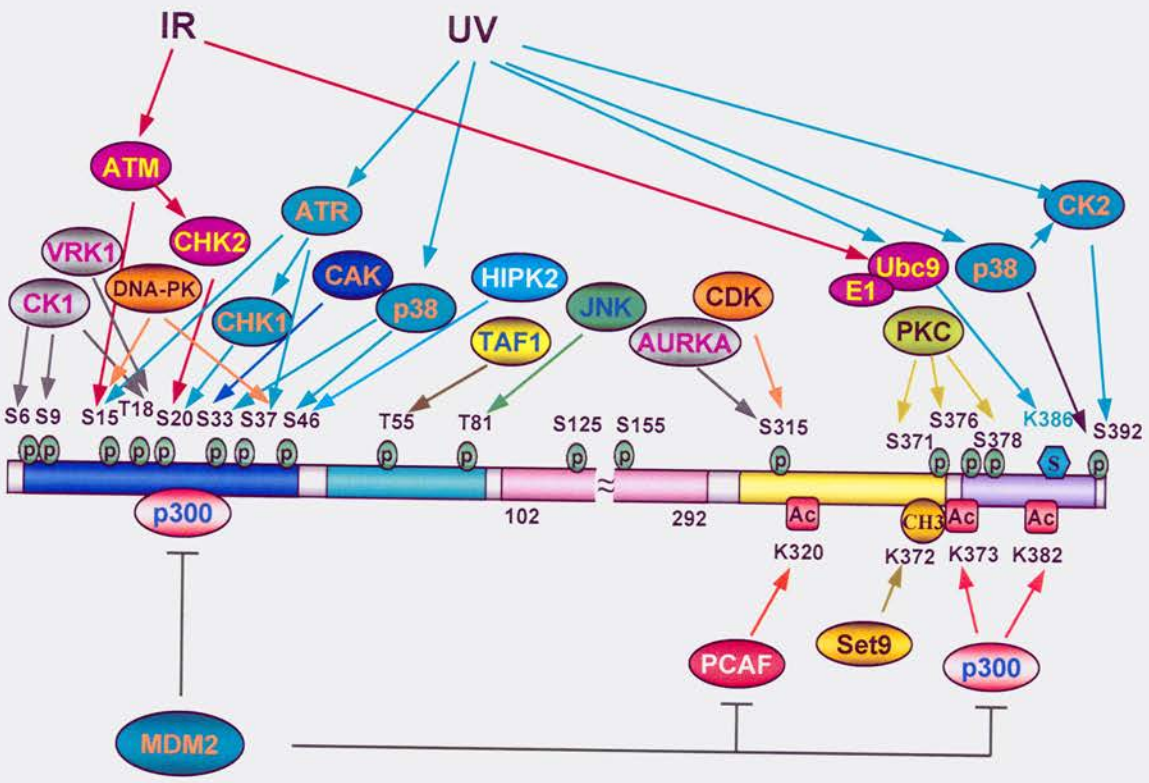

IR
UV
ATM
ATR
CHK2
VRK1
DNA-PK
CK1
CHK1
CAK
p38
HIPK2
TAF1
JNK
CDK
AURKA
Ubc9
E1
p38
CK2
PKC
S6 S9
S15
T18
S20
S33
S37
S46
T55
T81
S125
S155
S315
S371
S376
S378
K386
S392
p300
102
292
Ac
K320
CH3
K372
Ac
K373
Ac
K382
PCAF
Set9
p300
MDM2

Figure 1.4. Schematic diagrams of the functional domains of MDM2, its posttranslational modifications, and MDM2-interacting proteins. (A). Functional domains of MDM2. The p53-binding domain is located within residues 26–108. The zinc finger domain is located within residues 305–332. The RING finger domain encompasses residues 437–491. The central acidic domain is located within residues 221-274 while the C-terminal acidic domain is located within residues 390-428. Two NLSs (red and green bars) and C-terminal nucleolus localization signal (NoLS) are indicated. (B). Modifications of MDM2. MDM2 can be phosphorylated by a number of kinases at different Serine (S), Threonine (T), or Tyrosine (Y) as indicated (the number indicates the position of the residues). The arrows indicate modifying. "p" indicates phosphorylation. MDM2 can also be ubiquitinated by its self. The ubiquitination residues are not defined (not shown). (C) MDM2-interacting proteins. p53 binds to the N-terminal p53 binding domain of MDM2. MDMX binds to the RING finger domain of MDM2. p300 binds to the central acidic domain of MDM2. Other proteins binds to different regions of MDM2 as indicated.

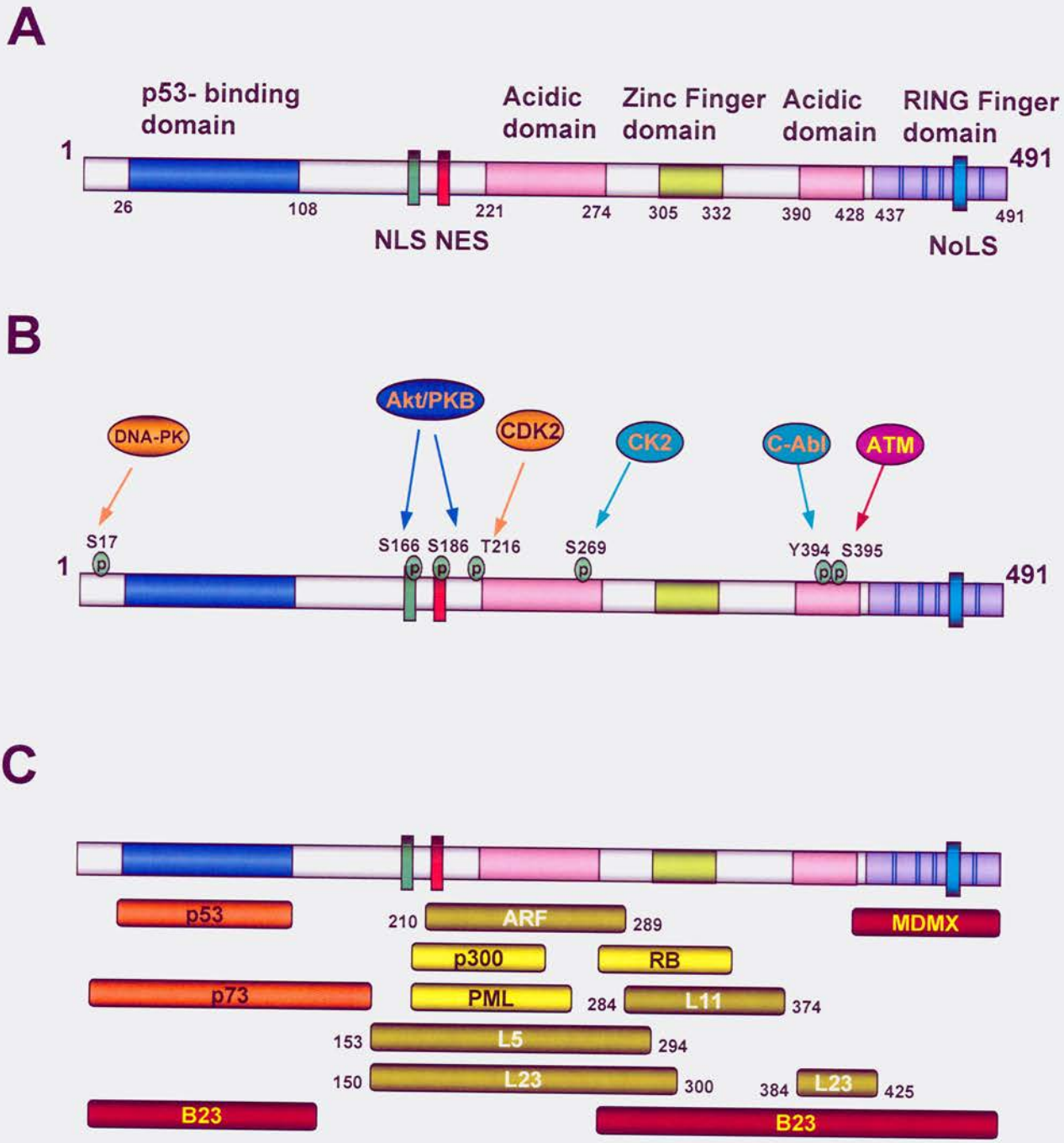

A
p53- binding domain
Acidic domain
Zinc Finger domain
Acidic domain
RING Finger domain
1
491
26
108
221
274
305
332
390
428
437
491
NLS NES
NoLS
B
Akt/PKB
CDK2
CK2
C-Abl
ATM
DNA-PK
S17
S166 S186 T216
S269
Y394 S395
1
491
C
p53
210
ARF
289
MDMX
p300
RB
p73
PML
284
L11
374
153
L5
294
150
L23
300
384
L23
425
B23
B23

Figure 1.5 The MDM2-p53 feedback loop. Upon activation the tumor suppressor p53 protein stimulates transcriptional expression of mdm2 gene. MDM2, in turn, inhibits p53 function, thus forming a tight autoregulatory feedback loop. MDM2 inhibits p53 stabilization and activation by multiple mechanisms: (1) MDM2 ubiquitinates and targets p53 for proteasome-mediated degradation. HAUSP, an ubiquitin phydrolase, deubiquitinates p53. (2) 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. (3) MDM2 transports p53 from the nucleus to the cytoplasm facilitating the degradation of p53 (not shown). (4) MDM2 could binds to p53 in p53 target gene promoter and inhibits its transcriptional activity possibly by mono-ubiquitinating histones in the promoter. (5) MDM2 also inhibits p300/CBP and PCAF-mediated p53 acetylation thus inhibiting its transcriptional activity.

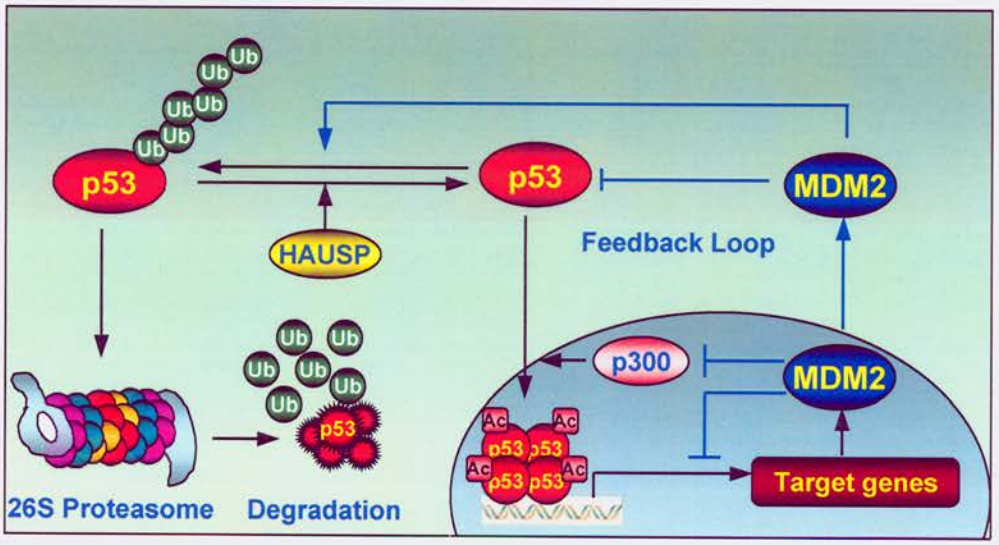

Ub
p53
HAUSP
p53
MDM2
Feedback Loop
p300
MDM2
Ac
p53
Target genes
26S Proteasome
Degradation

Figure 1.6 The regulation of the MDM2-p53 feedback loop. Inhibition of the MDM2-p53 feedback loop thus stabilizing and activating p53 can be obtained through two different pathways: (1) DNA damage, such as those induced by IR and UV, leads to phosphorylation of Ser/Thr residues on the N-terminal MDM2 binding domain of p53 and the N-terminal p53 binding domain of MDM2, therefore disrupting the interaction between MDM2 and p53, while this interaction is essential for MDM2-mediated p53 inhibition. Also, the N-terminal phosphorylation of p53 could block the function of N-terminal NES, thus inhibiting the nuclear export and degradation of p53. (2) The MDM2-p53 feedback loop can also be inhibited by aberrant growth signals, such as those induced by overexpression and activation of oncogenes (c-Myc, Ras, E2F), through induction of ARF. The ARF tumor supressor protein inhibits MDM2-mediated p53 ubiquitination and degradation, thus stabilizing and activating p53. This pathway does not involve N-terminal phosphorylation of p53. ARF could also inhibit degradation of the ubiquitinated p53 and MDM2 through a potential post-ubiquitination mechanism.

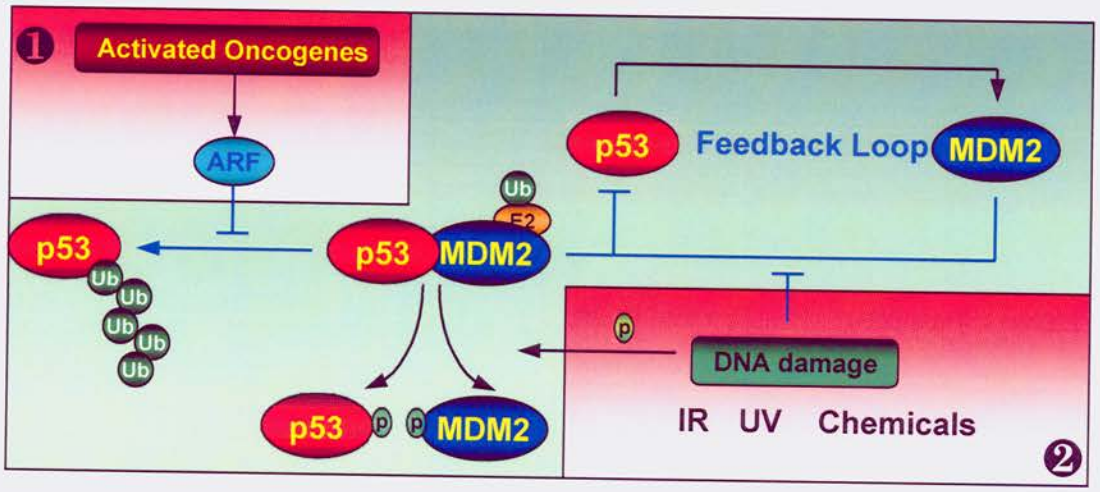
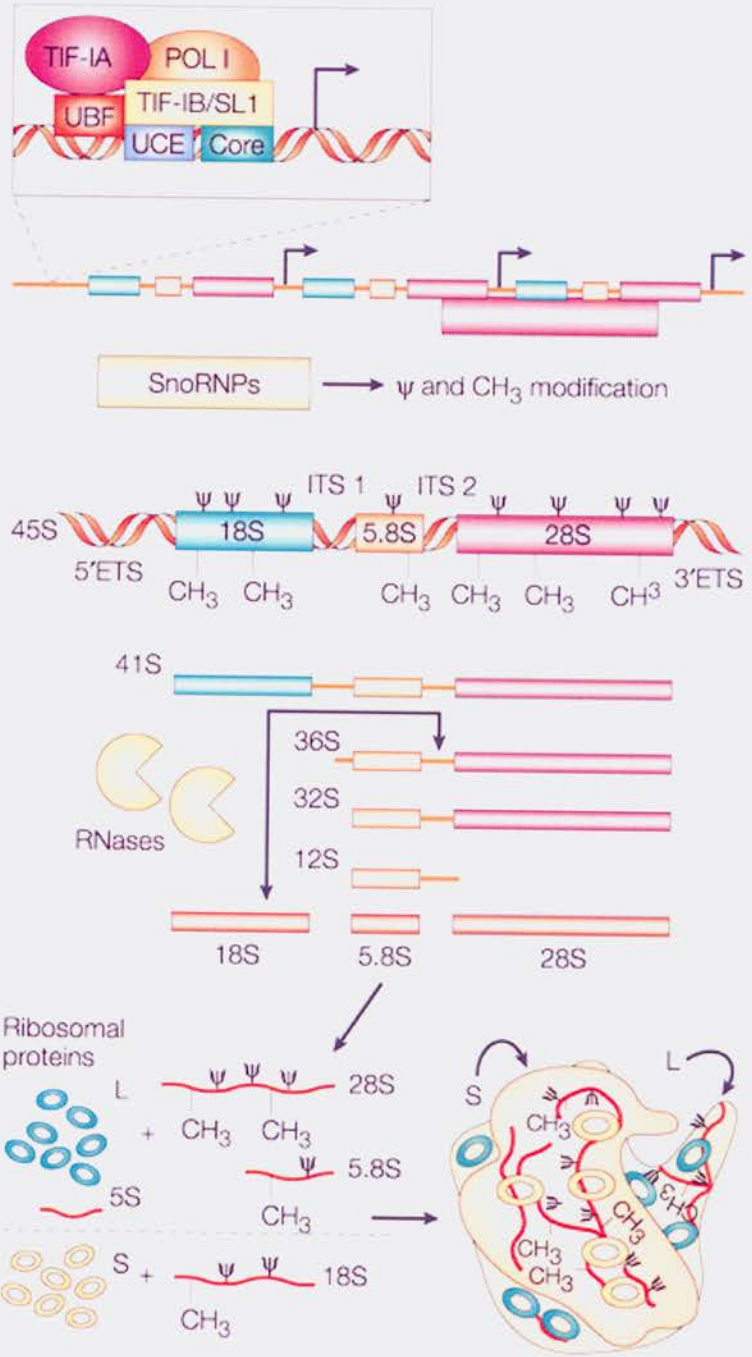

1
Activated Oncogenes
ARF
p53
Feedback Loop
MDM2
Ub
E2
p53
MDM2
Ub
Ub
Ub
Ub
Ub
p
DNA damage
IR UV Chemicals
p53
p
p
MDM2
2

Figure 1.7. Ribosomal biogenesis. All ribosomal RNAs except the 5S are transcribed as a polycistronic transcript known as 45S pre-ribosomal RNA in the nucleolus. This transcription is dependent on three basal transcription factors: the 'selectivity complex' (SL1 or TIF-IB), the HMG1 box architectural upstream binding factor (UBF) and the DNA-dependent RNA polymerase I (Pol I). UBF is thought to bind the promoter first, enabling subsequent recruitment of SL1 and Pol I. Concomitant with rRNA transcription, the rRNA sequences are extensively modified. A large family of small nucleolar RNAs (snoRNAs) guides the site-specific conversion of uridine to pseudouridine (ψ) in rRNA. This is accomplished through direct base-pairing of snoRNAs with specific rRNA sequences, leaving a single uridine exposed to the enzymatic activity of dyskerin, the pseudouridine synthase that mediates the modification of this residue. In addition, other snoRNAs also guide the formation of 2'-*O*-methylated nucleosides (-CH3) in rRNA. The pre-rRNA precursor is then cleaved at specific sites by RNases to produce a series of characteristic intermediates (41S), and finally mature rRNAs — 18S, 5.8S and 28S. During rRNA processing, the rRNA species must associate with about 79 ribosomal proteins, as well as the 5S rRNA in the nucleolus, to form the 40S small and the 60S large ribosomal subunits, which are assembled and transported to the cytoplasm to initiate protein synthesis.

(adapted from Ruggero D & Pandolfi PP. Nat Rev Cancer 3:179-192, 2003)


TIF-IA
POL I
UBF
TIF-IB/SL1
UCE
Core
SnoRNPs
ψ and CH3 modification
45S
ITS 1
ITS 2
18S
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CHAPTER TWO

Ribosomal Protein L23 Activates p53 by Inhibiting Mdm2 Function in Response to Ribosomal Perturbation but not to Translation Inhibition

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SUMMARY

The p53-MDM2 feedback loop is vital for cell growth control and is subjected to multiple regulations in response to various stress signals. Here we report another regulator of this loop. Using an immuno-affinity method, we purified an MDM2-associated protein complex that contains the ribosomal protein L23. L23 interacted with MDM2, forming a complex independent of the 80S ribosome and polysome. The interaction of L23 with MDM2 was enhanced by treatment of actinomycin D but not γ irradiation, leading to p53 activation. This activation was inhibited by siRNA against L23. Ectopic expression of L23 reduced MDM2-mediated p53 ubiquitination and also induced p53 activity and G1 arrest in p53-proficient U2OS, but not in p53-deficient Saos2 cells. These results reveal L23 as another regulator of the p53-MDM2 feedback regulation.

INTRODUCTION

The tumor suppressor function of p53 is primarily attributed to its ability to activate transcriptional expression of many genes whose protein products induce cell growth arrest, apoptosis, senescence, or inhibition of angiogenesis in response to various stresses, thus protecting cells from transformation and tumorigenesis (266, 303). Cells also develop a negative feedback mechanism to monitor p53 function because p53 activation is toxic to the cells (11, 235, 318). A crucial player in this feedback regulation is an oncoprotein called MDM2 (202). MDM2 specifically binds, through its N-terminal domain, to the N-terminus of p53 (43, 221). On one hand, this binding conceals the N-terminal transcription domain of p53, directly blocking its transcriptional activity (43, 202). On the other hand, this binding initiates p53 ubiquitination, thus leading to its

degradation by the proteasome system (105, 146), as MDM2 is a Ring-finger-containing E3 ubiquitin ligase (76, 113). Additionally, MDM2 contains a nuclear export signal (NES) and induces p53 nuclear export through direct interaction (25, 89, 178, 288), thus preventing p53 from accessing its responsive DNA elements. Consequently, MDM2 suppresses p53-mediated cell growth arrest and apoptosis. This regulation presents an elegant auto-regulatory feedback loop, because MDM2 is also induced by p53 and in turn inhibits p53 function (11, 235, 318). Indeed, genetic disruption of the *p53* gene rescues the lethal phenotype of the *mdm2* knock out mouse, firmly validating the notion of the MDM2-p53 feedback loop (126, 204).

This MDM2-p53 feedback loop is subjected to multiple regulations in response to different signals, because of its importance in cell growth control and transformation. For instance, DNA damage-induced phosphorylation of p53 or MDM2 suppresses p53-MDM2 binding, thus inhibiting MDM2-mediated p53 suppression (9, 35, 134, 186, 273). Also, viral and cellular oncoproteins, such as Ras and c-Myc, can activate p53 by alleviating the function of MDM2 through induction of an MDM2 inhibitor called $p14^{arf}$ (mouse $p19^{arf}$) (228, 339). The ARF protein prevents MDM2 from targeting p53, probably through two mechanisms: separating the two proteins from different cellular compartments and inhibiting MDM2-mediated p53 ubiquitination (115, 289, 312, 313, 331). MDM2 is also regulated by its homolog MDMX (272), which assists MDM2 in down-regulating p53 function (100, 119, 170, 279). Furthermore, phosphorylation of MDM2 at serines 166 and 186 by Akt in response to the Her2-mediated cell growth signaling enhances the nuclear localization of MDM2 and as a result, inactivates p53

(335). Therefore, the MDM2-p53 loop is tightly regulated by distinct proteins in response to different signals.

The p53-MDM2 feedback loop is also regulated by stress on ribosomal biogenesis. Proper ribosome assembly is essential for the health of a cell. Therefore, it is logical that impairment to this function would require cell growth arrest or apoptosis to facilitate repair or to remove affected cells, probably mediated by p53. For example, overexpression of a dominant negative mutant of Bop1, a nucleolar protein critical for rRNA processing and ribosome assembly, inhibited 28S and 5.8S rRNA formation and led to deficiency of newly synthesized 60S ribosomal subunits in 3T3 fibroblast cells (234). Consequently, the cells underwent p53 dependent G1 arrest (234). Also, actinomycin D, which inhibits RNA polymerase I at the concentration of 5 nM, can stall rRNA synthesis and ribosome assembly. By doing so, this compound activates p53 function without triggering N-terminal phosphorylation of p53 (5, 6). These studies suggest a potential signaling pathway that may mediate p53 activation by sensing stresses on ribosome biogenesis. Indeed, a ribosomal protein L11 has been recently shown to be a regulator of this pathway (177, 329). Hence, it is important to uncover other regulators in this pathway.

In order to do so, we have generated a stable human embryonic kidney epithelial 293 cell line that constitutively expresses MDM2. Using this cell line, we purified a cytoplasmic MDM2-associated complex through an immuno-affinity purification followed by a mass spectrometric analysis. Surprisingly, several ribosome proteins including L23, L11, and L5 were found in the complex. This finding not only confirms the previously reported interaction of MDM2 with L5 (182) and most recently with L11

(177, 329), but also identified L23 as a new regulator of MDM2. Our further study shows that L23, like L5 and L11, bound to MDM2, but unlike L5 and L11, preferentially interacted with a distinct domain of MDM2, independently of the 80S ribosome and polysome. L23 activated p53 by preventing MDM2 from targeting and ubiquitinating p53. Interestingly, the RNA polymerase I inhibitor actinomycin D, but not γ irradiation or translation inhibitors, enhanced the MDM2-L23 binding. Furthermore, ablation of L23 by siRNA inhibited actinomycin D-induced p53 activation. Consistently, ectopic expression of L23 induces p53 transcriptional activity and G1 arrest in p53-containing U2OS, but not in p53 null Saos-2 cells. These results suggest that L23 in response to ribosomal stress may activate p53 by inhibiting the MDM2-mediated feedback regulation of p53.

MATERIALS AND METHODS

Plasmids and antibodies. To generate human L23 expression construct pcDNA3-2Flag-L23, the full-length L23 cDNA was amplified by RT-PCR from Hela cell mRNA using primers (restriction enzyme sites are underlined): P1, 5'-CGCGGATCCATGTCG-AAGCGAGGACGTGGTG-3'; P2, 5'-CCGGAATTCTCATGCAATCCTGCCAGCAT-TG-3'. The PCR product was subcloned into pcDNA3-2Flag vector. The pcDNA3-2Flag-L23ΔN vector deleted for amino acids 1-65 was constructed by PCR amplification using primers: P2 and P3: 5'-CGCGGATCCAAGAAAGGCAAAC-CACAGCTC-3'. The GFP-L23 expression vector was cloned by inserting PCR product using primers P2 and P4: 5'-ACAGAAGATCTATGTCGAAGCGAGGACGTGG-3' into pEGFP-C1 (Clontech). GST-L23 bacterial expression vector was constructed by subcloning the full length L23 from pcDNA3-2Flag-L23 into pGEX.4T.1 (Pharmacia Biotech). The GST

fusion L23 fragments pGEX.4T.1-L23/1-65, pGEX.4T.1-L23/66-140, pGEX.4T.1-L23/1-105 and pGEX.4T.1-L23/35-105 were subsequently constructed by cloning PCR products into pGEX.4T.1 vector. GST-MDM2 and GST-MDM2 deletion mutants were described (125). His-MDM2 bacterial expression vector was constructed by inserting a PCR product into pet24a vector (Novagen), the primers are 5'-CGCGGATCCATGTGC-AATACCAACATGTCTG-3' and 5'-CCGGAATTCGAGGGGGAAATAAGTTAGC-AC-3'.

For generation of polyclonal anti-L23 antibody, the full length L23 was amplified by PCR using primers P1 and P5, 5'-CCGGAATTCCGTGCAATCCTGCCAGCATTG-3'. The PCR product was subcloned into pet24a-His vector to generate pet24a-His-L23. The His-tagged L23 protein was expressed in *E. Coli* and purified using Ni-NTA beads as an antigen to raise rabbit polyclonal anti-L23 antisera. Anti-L11 antibodies were kindly provided by Dr. Yangping Zhang (The University of Texas at Houston, M.D. Anderson Cancer Institute, Houston). Anti-Flag (Sigma), anti-p21 (NeoMarkers), and anti-p53 (DO-1, Santa Cruz) were purchased. Anti-MDM2 (2A10) and anti-HA (12CA5) have been described (125).

Buffers and reagents. Lysis buffer consisted of 50 mM Tris/HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, and 1 mM phenylmethylsulfonyl fluoride (PMSF). SNNTE buffer contained 50 mM Tris/HCl (pH 7.4), 5 mM EDTA, 1% Nonidet P-40, 500 mM NaCl, and 5% sucrose. RIPA was comprised of 50 mM Tris/HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, and 1% (w/v) sodium deoxycholate. Buffer C 100 (BC100) included 20 mM Tris/HCl (pH 7.9), 0.1 mM EDTA, 10% glycerol,

100 mM KCl, 4 mM $MgCl_2$, 0.2 mM PMSF, 1 mM dithiothreitol, and 0.25 μg/ml pepstatin A.

Cell culture. Human embryonic kidney epithelial 293 cells, human lung small cell carcinoma H1299 cells, human p53-proficient oesteosarcoma U2OS cells, and human p53 null osteosarcoma Saos-2 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 (124, 125, 322).

Establishment of HA-MDM2 Expression cell lines. 293 cells were transfected with pcDNA3-HA-MDM2 or pcDNA3 vector. Transfected cells expressing HA-MDM2 were selected in the presence of neomycin (0.5 mg/ml) and screened by immunoprecipitation with anti-HA antibodies followed by Western blot with the monoclonal anti-MDM2 antibody 2A10.

Affinity purification of human MDM2 associated protein complexes. Approximately $1x10^9$ of 293 cells were used for preparation of nuclear extract (NE) and cytoplasm (S100) using a method described previously (64). The 12CA5 affinity beads were prepared by conjugating anti-HA monoclonal antibodies (12CA5) to protein A agarose beads as described (154). The beads were washed with phosphate buffered saline (PBS) and suspended in PBS as a 50% slurry. Fifty mg of S100 protein fractions from either 293-HA-MDM2 cells or empty vector expressing 293 cells as control were incubated with 0.2 ml of 12CA5-affinity beads at 4°C for 4 hours. The beads were washed four times in lysis buffer containing protein inhibitors. The bead-bound proteins were eluted in 0.2 ml lysis buffer containing 4 mg/ml of synthetic HA peptides. Eluted

proteins were loaded onto a 5%-17% gradient SDS-PAGE gel for colloidal blue staining. Specific bands from 293-HA-MDM2 fractions as compared to the 293 control fractions were excised and subjected to mass spectrometric analysis.

Cotransfection, immunoblot and co-immunoprecipitation analyses. H1299, U2OS, or Saos-2 cells were transfected with plasmids as indicated in each figure legends using Lipofectin in light of the manufacturer's protocol (Invitrogen). Cells were harvested at 48 hours posttransfection and lysed in lysis buffer. Equal amounts of clear cell lysate were used for immunoblot analysis as described previously (325). Immunoprecipitation was conducted using antibodies as indicated in figure legends and described previously (322). Beads were washed with lysis buffer twice, once with SNNTE buffer and once with RIPA buffer. Bound proteins were detected by immunoblot using antibodies as indicated in figure legends.

Transient transfection and luciferase assays. U2OS, Saos-2, or H1299 cells were transfected with the pCMV-β-galactoside reporter plasmid (0.1μg) and a luciferase reporter plasmid (0.1 μg) driven by two copies of the p53RE motif derived from the MDM2 promoter (318), together with a combination of different plasmids (total plasmid DNA 1 μg/well) as indicated in Figure 6, using Lipofectin (Invitrogen). At 48 hours posttransfection, cells were harvested for luciferase assays as described previously (322, 324). Luciferase activity was normalized by a factor of β-gal activity in the same assay.

Glycerol gradient sedimentation centrifugation. Whole cell lysates were prepared from 293 cells as described (325). 2 mg of lysates mixed with molecular weight markers were loaded onto the surface of a 12.5% -25% glycerol gradient solution containing 150 mM NaCl, 10 mM Tris-HCl (pH 7.5) in a 12 ml centrifuge tube. The

samples were subjected to centrifugation in a Beckman SW41 rotor at 32K rpm at 4°C for 20 hrs. 200 μl per fraction were collected from each tube. 30 μl of each fraction were loaded onto an SDS gel for electrophoresis, followed by immunoblot analysis.

Polysomes/mRNPs distribution analysis. Postmitochondrial supernatant (PMS) extractions, sucrose gradient sedimentation of polysomes, and analysis of the polysomes/mRNPs distribution of proteins and RNAs were carried out as previously described with minor modifications (79, 321). Briefly, cells were incubated with 100 μg/ml of cycloheximide (CHX) for 15 minutes, which arrests polysome migration prior to the isolation of PMS. 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% NP40, 50 μg/ml CHX, 30 U/ml RNasin inhibitor, 1 mM dithiothreitol (DTT), 1mM PMSF, 1 mM pepstatin, 1 mM leupeptin. After incubation on ice for 5 minutes, the lysates were centrifuged at 12,000 g at 4°C for 8 min. 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 for 2 hrs. Fourteen fractions were collected from each tube. RNAs were extracted from the fractions by phenol/chloroform extraction.

Cell cycle analysis. U2OS or Saos-2 cells were transfected with plasmids encoding GFP or GFP–L23 or GFP-L23ΔC. Thirty-two hours post-transfection, cells were treated with 200 μg/ml nocodazole for additional 16 hours. Cells were harvested and suspended in 100 μl of PBS, and transferred to a polystyrene tube. Cells were 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 min,

and then analyzed for DNA content using a Becton Dickinson FACScan flow cytometer. Data were collected using the ModFit software program. GFP-positive cells were gated for cell cycle analysis.

GST fusion protein association assays. His-tagged L23 and MDM2 proteins were expressed in *E. coli* and purified through a Ni-NTA (Qiagen) column and eluted by 0.5 M imidazole. Protein–protein interaction assays were conducted as described using fusion protein-containing glutathione beads (125). Purified L23 proteins were incubated with the glutathione–Sepharose 4B beads (Sigma) containing 200 ng of GST–MDM2/1-491, GST–MDM2/1-301, GST–MDM2/1-150, GST–MDM2/151-301, GST–MDM2/294-491, GST–MDM2/384-491, GST–MDM2/425-491, or GST, respectively. Purified MDM2 proteins were incubated with GST-L23/1-140, GST-L23/1-105, GST-L23/1-65, GST-L23/66-140, GST-L23/36-105, or GST, respectively. Thirty minutes after incubation at room temperature, the mixtures were washed once in BC100 containing 0.1% Nonidet P-40, twice in SNNTE and once in RIPA. Bound proteins were analyzed on a 10% or 15% SDS gel and detected by immunoblot using anti-L23 and anti-MDM2 (2A10) monoclonal antibodies.

***In vivo* ubiquitination assay.** *In vivo* ubiquitination assay was conducted as previously described with minor modifications (320). H1299 cells (60% confluence/100 mm plate) were transfected with His_6-ubiquitin (2 μg), p53 (2 μg), L23 (2 μg) or Ha-MDM2 (2 μg) expression plasmids using Lipofectin. 48 hours after transfection, cells from each plate were harvested and split into two aliquots, one for Western blot and the other for ubiquitination assays. Cell pellets were lysed in buffer I (6 M guanidinium-HCl, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl, pH8.0, 10 mM β-mecaptoethanol) and

incubated with Ni-NTA beads (Qiagen) at room temperature for 4 hrs. Beads were washed once with buffer I, buffer II (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl, pH8.0, 10 mM β-mecaptoethanol), and buffer III (8 M urea, 0.1 M Na_2HPO_4/NaH_2PO_4, 10 mM Tris-HCl, pH6.3, 10 mM β-mecaptoethanol), respectively. Proteins were eluted from beads in buffer IV (200 mM imidazole, 0.15 M Tris-HCl pH6.7, 30% glycerol, 0.72 M β-mecaptoethanol and 5% SDS). Eluted proteins were analyzed by Western blot with monoclonal p53 (DO-1, Santa Cruz) or anti-HA antibodies.

Immunofluorescent staining. 293-HA-MDM2 cells were transfected with Flag-L23 expression plasmid. Fourty-eight hours after transfection, cells were fixed for immunofluorescent staining with monoclonal anti-Flag antibodies and polyclonal anti-MDM2 antibodies, as well as for DNA staining with 4',6-diamidino-2-penylindole (DAPI). The Alexa Fluor 488 (green) goat anti-mouse antibody and the Alexa Fluor 546 (red) goat anti-rabbit antibody (Molecular Probes, OR) were used for Flag-L23 and MDM2, respectively. Stained cells were analyzed under an Zeiss Axiovert 25 fluorescent microscope.

Inhibition of L23 by siRNA and treatment of cells with actinomycin D. U2OS cells were maintained in DMEM plus 10% fetal bovine serum. RNAi-mediated ablation of endogenous L23 was performed essentially as previously described (214). A 21-nucleotide siRNA duplex with a 3' dTdT overhang, corresponding to L23 mRNA (AATTCCGGATTTCCTTGGGTC), or the scramble II RNA duplex (AAGCGCGCTTTGTAGGATTC) as a control were synthesized (Dhamacon). These siRNA duplexes (0.2 μM) were introduced into cells using Oligofectamine (Invitrogen),

following the manufacturer's protocol. Cells were then treated with or without 5 nM of actinomycin D for 8 hours before harvesting. Cells were harvested 72 hrs after transfection for immunoblot, RT-PCR, cell cycle, and luciferase activity analyses. For cell cycle analysis, cells were treated with 200 μg/ml nocodazole for additional 16 hours before harvesting. For luciferase assay, cells were transfected with pCMV-β-galactoside reporter plasmid (0.2μg) and a luciferase reporter plasmid (0.2 μg) driven by two copies of the p53RE motif derived from the MDM2 promoter as mentioned above before siRNA transfections.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. U2OS cells were transfected with or without L23 siRNA and treated with or without 5 nM actinomycin D as described above. RNA was isolated from cells using Qiagen RNeasy Mini Kits (Qiagen, Valencia, CA). Reverse transcriptions were performed as described (322). PCR reactions were performed in a 20 μl mixture containing 1 × PCR buffer, 60 μmol/L of dNTPs, 1 U Taq polymerase (Roche), 0.5 μmol/L of each prime and 0.2 μCi ^{32}P-dCTP for 18 to 20 cycles as described (322). PCR products were resolved onto 6% polyacrylamide gel. The gel was dried followed by autoradiography. The primers for amplifying p21$^{waf1/cip1}$, MDM2 and GAPDH were described (322). The other primers were: p53: 5'-TACAGTCAGAGCCAACCTCAG-3', 5'-AGATGAAGCTCCCAGA-ATGCC-3'; L23: 5'-ATGTCGAAGCGAGGACGTGGTG-3'; 5'-TCATGCAATCCTG-CCAGCATTG-3'.

RESULTS

Isolation of a human MDM2 associated protein complex. In order to understand how nuclear and cytoplasmic proteins regulate the MDM2-p53 pathway, we previously purified a nuclear complex containing MDM2, p300 and p53 from HeLa nuclear extracts and identified a cytoplasmic MDM2-associated protein complex free of p53 and p300 from HeLa cytoplasmic extracts via biochemical fractionation (141). To further illustrate the identity of the cytoplasmic MDM2-associated proteins, we have tried to purify the complex using conventional chromatography. However, due to the unstable nature of the endogenous MDM2 (data not shown), this purification failed to yield sufficient amounts of proteins for further analysis. Thus to surmount this obstacle, we have generated an MDM2 stably overexpressing cell line (293-HA-MDM2) by using human 293 cells and neomycin selection. We used an affinity purification to isolate the MDM2 associated proteins in this cell line. Immunoprecipitation was performed using cytoplasmic fractions (S100) from 293-HA-MDM2 cells as well as 293 cells expressing the empty vector pcDNA3. MDM2 and associated proteins were eluted with HA peptides and visualized on an SDS-PAGE gel by Colloidal Blue staining (Fig. 2.1A). Several bands appeared specifically in the HA-MDM2 expressing sample, but not in the control sample. Three proteins were revealed by mass spectrometric analysis to be ribosomal proteins L5, L11, and L23, respectively (Figs. 2.1A and 1B). Two doublet bands were MDM2. This result not only identifies L23 as another potential regulator of MDM2, but also suggests a possible MDM2-ribosomal protein complex.

MDM2 binds to L23 in cells. To verify the association of MDM2 with L23, Flag-L23 was expressed in 293-HA-MDM2 cells. Whole cell lysates were prepared and

subjected to co-immunoprecipitation with either anti-HA or anti-Flag antibodies, followed by immunoblot analysis. Immunoblot results revealed that Flag-L23 and HA-MDM2 were specifically co-immunoprecipitated by either anti-HA (Fig. 2.2A) or anti-Flag antibodies (Fig. 2.2B), but not by control antibodies, indicating that MDM2 binds to L23 in cells. Further, to determine whether this interaction is also true with endogenous MDM2 and L23 proteins, we generated rabbit polyclonal antisera against the full length L23 and employed them in coimmunoprecipitation assays using U2OS cells. Indeed, the endogenous MDM2 and L23 proteins were specifically coimmunoprecipitated by anti-L23 antisera, but not by preimmune sera (Figure 2.2E). Using the same approaches, we also verified the previously reported interaction between L5 and MDM2 (182) and the recently reported interaction between L11 and MDM2 (177, 329) (data not shown).

To determine where the L23-MDM2 binding may occur in cells, several experiments were conducted. First, 293-HA-MDM2 cells were transfected with Flag-L23 and immunostained with anti-Flag (green) and anti-MDM2 (red) antibodies. As shown in Fig. 2.2C, stably expressed MDM2 in 293 cells were localized in both the cytoplasm and the nucleus, but not in the nucleolus, while Flag-L23 was detected in the cytoplasm, the nucleoplasma, and the nucleolus. Second, we introduced wild type MDM2 and its deletion mutant that lacks the nuclear localization sequence (Δ150-230) into 293 cells and carried out a co-immunoprecipitation assay with an anti-L23 antibody followed by an immunoblot assay. As shown in Fig. 2.2D, either wild type or mutant MDM2 was co-immunoprecipitated by the anti-L23 antibody. This result indicates that L23 can bind to MDM2 in the cytoplasm, because L23 bound to the Δ150-320 mutant of MDM2 that has been shown to locate only in the cytoplasm (124). Also, using both cytoplasmic and

nuclear fractions from 293-HA-MDM2 cells transfected with Flag-L23, we were able to immunprecipitate MDM2 by anti-Flag antibody from both fractions (data not shown). Taken together, L23 may bind to MDM2 in both the nucleoplasm and the cytoplasm.

MDM2 binds to L23 *in vitro*. Next, we wanted to determine whether the interaction between MDM2 and L23 is direct or indirect through other ribosome proteins L11 (177, 329) or L5 (182). To do so, glutathione S-transferase (GST)-fusion protein-protein association assays were conducted using His-L23 and GST-MDM2 deletion fusion proteins purified from bacteria (Figs. 2.3A-3C). As shown in Fig. 2.3B, GST-MDM2, but not GST alone, interacted directly with His-L23. It was shown previously that L5 binds to a central acidic domain of MDM2 (residues 153-294) (74), and recently reported that L11 binds to the residues 212-296 of MDM2 (177). Different from L5 and L11, L23 appeared to bind to the aa 150-301 and aa 384-425 regions of MDM2 with a strong preference to the latter *in vitro* (Fig. 2.3C). A close examination of the MDM2 sequence revealed a second acidic domain in this region, which contains 35% acidic amino acids. These residues may be important for L23-binding. Thus, the central region of MDM2 may possess two L23-binding sites.

To map the MDM2 binding site in L23, we performed a similar GST-pull down assay using His-MDM2 and GST-L23 fusion proteins purified from bacteria (Figs. 2.3D and 2.3F). As shown in Fig. 3E, His-MDM2 bound to the GST-full length L23 protein, but not its N-terminal or C-terminal half fused with GST, nor GST alone, indicating the central portion of L23 may be essential for the binding. Indeed, His-MDM2 bound to GST-L23/1-105 or GST-L23/35-105 fragment, although less efficiently. These results demonstrate that MDM2 can physically bind to L23 *in vitro*. The observation that L23,

L11 and L5 bind to different regions of MDM2 (Fig. 2.3G) suggests that MDM2 may form a complex with these ribosome proteins.

MDM2 forms a complex with L5, L11 and L23 in cells. To determine whether the three ribosomal proteins identified from our immunoprecipitation purification interact with MDM2 in the same complex in cells, we fractionated 293-HA-MDM2 cell lysates using glycerol gradient sedimentation centrifugation and analyzed fractions using an immunoblot assay. As shown in Fig. 2.4A, some L5, L11, and L23 proteins were co-eluted with HA-MDM2 (lanes 6-10). MDM2 may form a complex with the ribosomal proteins in fractions 28-30 (lanes 9-10) because these fractions eluted around where a 670 Kd molecular weight marker was eluted. To test this idea, we performed a coimmonoprecipitation assay using fraction 30. As shown in Fig. 2.4B, indeed, L5, L11, and L23 were all specifically co-immunoprecipitated with HA-MDM2 by the anti-HA antibody, but not a control antibody. Noticeably, there were some fractions, such as fractions 22-26, which also possessed all the tested proteins and were eluted before the 670 Kd marker. Two possibilities may account for this observation. First, the protein samples might be overloaded. Alternatively, MDM2 may form a larger complex that contains not only the identified ribosomal proteins but also other yet unidentified proteins (Fig. 2.1). In summary, these results indicate that MDM2 may form a complex at least with L5, L11 and L23 in cells.

MDM2 is not associated with 80S ribosomes or polysomes. The observation that MDM2 associated with three ribosomal proteins L5, L11, and L23 in one complex raises the question of whether MDM2 may associate with 80S ribosomes or polysomes through these proteins. To address this issue, we performed a polysome profile analysis.

Cytoplasmic extracts prepared from 293-HA-MDM2 cells were subjected to a linear sucrose gradient sedimentation centrifugation. Fourteen fractions were collected and subjected to immunoblot assays for detection of MDM2, L11 and L23 as well as rRNA analysis with ethidium bromide (Fig. 2.5A). The result showed that MDM2 was not co-eluted with either polysomes or 80S ribosomes both of which contain L11 and L23 (fractions 1-7 and 8-10), instead stayed near the top of the gradient where the ribosome-free ribosomal proteins L11 and L23 were also detected by blotting with anti-L11 and anti-L23 antibodies. The polysome and 80S ribosome profile was verified by determining the distribution of rRNAs (the lower panel of Fig. 2.5A). The distribution of rRNAs together with L11 and L23 coordinated well with polysomes/ribosomes and mRNP (small ribonuclear protein) profiles as expected (79, 321). Consistent with the result in Fig. 5A, endogenous MDM2 proteins associated with free L11 and L23 but not with intact 80S ribosomes and polysomes (Fig. 2.5B). Thus we conclude that MDM2 does not associate with the 80S ribosomes or polyribosomes.

L23 activates p53 by overcoming MDM2-mediated suppression. The finding that L23 associates with MDM2 in cells suggests that L23 may regulate the MDM2-p53 feedback loop. We first examined whether overexpression of L23 affects MDM2-mediated p53 degradation by introducing exogenous proteins into p53-deficient human non-small cell carcinoma H1299 cells, because MDM2 mediates ubiquitination and proteasome-mediated degradation of p53 (76, 105, 113, 146). As expected, overexpression of MDM2 remarkably reduced p53 levels (lane 3 of Fig. 2.6A). By contrast, further overexpression of L23 partially rescued MDM2-mediated p53 degradation (lane 4). This rescue appeared to be dependent on the interaction of L23 with

MDM2, as the C-terminal domain of L23, which did not bind to MDM2 (Fig. 2.3), was unable to stabilize p53 (data not shown). Overexpression of L23 also slightly stabilized HA-MDM2 (top panel, lane 4). Consistent with these results, overexpression of L23 led to marked inhibition of MDM2-mediated p53 ubiquitination and MDM2 ubiquitination (Fig. 2.6B). Thus L23 can stabilize p53 by alleviating MDM2-mediated p53 ubiquitination and degradation.

Next, we examined the effect of L23 on endogenous p53 by introducing Flag-L23 into human osteosarcoma U2OS cells that contain wild-type p53. Interestingly, overexpression of Flag-L23, but not a Flag-L23 deletion mutant that does not bind to MDM2 (Flag-L23ΔN), markedly induced p53 in a dose-dependent fashion (second panel from top of Fig. 2.6C). Correspondingly, the levels of the p53 targets $p21^{cip1}$ and MDM2 were also induced (middle and top panels). This result together with the results above suggests that ectopic expression of L23 induces the level of the endogenous p53 as well as its targets $p21^{cip1}$ and MDM2 by blocking MDM2-mediated p53 degradation.

L23 stimulates p53-dependent transcription and G1 arrest. The finding that overexpression of L23 led to the induction of p53 and $p21^{cip1}$ levels suggests that the high level of cellular L23 may enhance p53-dependent transcription and cell growth arrest. To test this concept, we transfected U2OS cells with Flag-L23 or Flag-L23ΔN together with a luciferase reporter plasmid driven by the p53RE derived from the MDM2 promoter (318), and then carried out luciferase assays. Consistent with the result of Fig. 6C, ectopic expression of full length L23 markedly stimulated p53RE-driven transcription as presented in luciferase activity in a dose-dependent manner (Fig. 2.7A). This stimulation was dependent on p53, as no significant change of luciferase activity was detected in

human osteosarcoma Saos-2 cells that are deficient in p53 (Fig. 2.7B). This stimulation might also require the interaction of L23 with MDM2, as the L23 mutant Flag-L23ΔN (aa 66-140) which was unable to bind to MDM2 (Fig. 2.3E; data not shown), failed to enhance p53-dependent transcription activity (Fig. 2.7A) and induction of $p21^{cip1}$ (Fig. 2.6C). Hence, these results suggest that L23 may stimulate p53 activity and this stimulation requires the MDM2-L23 interaction.

Next, we determined whether induction of $p21^{cip1}$ by L23 through p53 activation could result in G1 arrest, because activated p53 triggers $p21^{cip1}$-dependent cell cycle arrest (303). To do so, p53-proficient U2OS or p53-deficient Saos2 cells were transiently transfected with either the GFP-fused L23 (GFP-L23) or the GFP-L23 C-terminus deletion (GFP-L23ΔC) (retaining only aa 1-65). Cells were then treated with the mitotic inhibitor nocodazole before FACS analysis, thus leading to G2/M arrest (24). 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. 2.7C, 28.9% of GFP-L23 expressing U2OS cells were arrested in the G1 phase, while only 7.5% and 9.5% of U2OS cells expressing GFP and GFP-L23ΔC were detected in the G1 phase. The G1 arrest induced by L23 was dependent on p53 because only a marginal change was observed in p53-null Saos2 cells (Figs. 2.7C and 2.7D). These results were reproducible as summarized in Fig. 2.7D. Therefore, ectopic expression of L23 induced p53-dependent G1 arrest and this induction is also dependent the MDM2-L23 interaction because the L23 N-terminal domain (aa 1-65) was unable to exert such an effect (Figs. 2.7C and 2.7D).

Actinomycin D, but not pactamycin or g irradiation, enhances L23-MDM2 interaction and activates p53. It has been shown that a low dose of actinomycin D (5 nM) specifically inhibits RNA polymerase I and consequently reduces ribosomal RNA synthesis, leading to perturbation of ribosomal biogenesis (6). Intriguingly, this biogenesis perturbation activates p53 without inducing phosphorylation at its N-terminal domain in cells, suggesting a previously uncharacterized p53 signaling pathway (6). The identification of the MDM2-L23 interaction led us to determine whether L23 may play a role in this pathway. Consistent with previous studies (6), our result from the experiment using U2OS cells treated with different doses of actinomycin D showed that low doses of actinomycin D (1 and 5 nM) markedly induced p53, as well as MDM2 and $p21^{cip1}$ (Fig. 2.8A). However, higher doses (50 and 400 nM) of the drug, which inhibit all RNA polymerases including RNA polymerase II (173), only induced p53 but not MDM2 or p21 levels (Fig. 2.8A). We wanted to test whether actyinomycin D inhibition of ribosomal biogenesis may affect the interaction between L23 and MDM2. The activation of p53 by a low dose (5 nM) of actinomycin D is time-dependent. It was induced as early as 2 hours after treatment followed by MDM2 and p21 induction in U2OS cells (Fig. 2.8B). Of note, L23 level slightly increased 8 hours after treatment of U2OS or WI38 cells with 5 nM of actinomycin D, and then decreased 24 hours after the treatment. By contrast, L11 level was not affected by actinomycin D treatment of both the cell lines (Fig. 2.8B and C). Next, we wanted to determine if actinomycin could affect the interaction between MDM2 and L23. U2OS cells were treated with 5 nM of actinomycin D and harvested at different time points posttreatment for immunoprecipitation-immunoblot assays. As shown in Fig. 2.8D, MDM2-L23 interaction was drastically

increased after actinomycin D treatment in a time-dependent manner; this increase began from 2 hrs and peaked at 8 hrs after this treatment (lower panels of Fig. 2.8D), consistent with p53 induction in response to this stress (upper left panels of Fig. 2.8D). The enhancement of the MDM2-L23 interaction was not merely due to the increase of MDM2 levels, as this interaction was not increased when MDM2 was stabilized by the proteasome inhibitor MG132 (Fig. 2.8D, compare lane 1 with lane 5). The decrease of L23 24 hours after the drug treatment would not underscore the importance of L23 in regulating MDM2 function in response to this stress, as the induction of L23-MDM2 interaction and p53 level was detected much earlier (approximately 2 hrs after actinomycin D treatment, Fig. 2.8D). In contrast to actynomycin D, the enhancement of the L23-MDM2 binding was not observed when U2OS cells were treated with γ irradiation regardless of high or low levels of MDM2 (Fig. 2.8E). These results suggest that the L23-MDM2 binding is highly related with p53 induction by 5 nM of actinomycin D but not by γ irradiation.

To test if the induction of p53 and MDM2-L23 interaction by actinomycin D is due to general inhibition of translation machinery, we also assayed the consequences of translation inhibition by protein synthesis inhibitors. Pactamycin, a translation initiation inhibitor acting on inhibition of Met-tRNA binding (117), did not activate p53 and induce p21 and MDM2. Instead, these proteins decreased after pactamycin treatment as shown in Fig. 2.8F. Consequently, MDM2-L23 interaction was reduced after pactamycin treatment (Fig. 2.8F). The same result was also obtained from cells treated with cyclohexamide, which inhibits ribosomal translocation and thus translation elongation (data not shown).

These data demonstrated that the L23-MDM-p53 pathway is specifically activated in response only to the ribosomal stress, but not to translation inhibition.

Removal of L23 by siRNA activates p53 but inhibits actinomycin D-induced p53 activation. To demonstrate the physiological relevance of L23 in this signaling pathway, we employed siRNA against L23 and determined whether ablation of L23 by its siRNA could affect p53 induction by actinomycin D. Indeed as shown in Fig. 2.9, this was the case, as reduction of L23 levels by its siRNA, but not by the scrambled siRNA duplex, correlated well with a decrease of actinomycin D-induced p53 levels (compare lane 3 with lane 4 of Fig. 2.9A). Consistently, actinomycin D-induced p53-dependent transcription, as represented in induction of its targets p21, MDM2, and luciferase activity driven by the p53RE-containing MDM2 promoter (Figs. 2.9A, 2.9B, and 2.9E), and G1 arrest (Figs. 2.9C and D) were also reduced by L23 siRNA. These results were reproducible and suggest that L23 may mediate p53 activation in response to this drug. Noticeably, in the absence of actinomycin D, siRNA against L23 (lane 2 of Fig. 2.9A), but not the scramble siRNA (lane 1), also induced p53 as well as the protein and mRNA levels of MDM2 and $p21^{cip1}$ (Figs. 2.9A, and 2.9B). Consistently, p53 transcriptional activity and p53-dependent G1 arrest were also induced by L23 siRNA (Figs. 2.9C, 2.9D and 2.9E). The reason for p53 activation by reducing endogenous L23 is currently unknown. One possibility would be that lowering L23 level might cause another ribosomal biogenesis stress that in turn induces p53 probably through a yet unidentified pathway. In summary, the results as described above demonstrate that ribosomal biogenesis stress caused by the low dose of actinomycin D, but not by γ irradiation or

direct inhibition of translation, can activate p53 possibly by inducing the association of L23 with MDM2 and inhibiting MDM2-mediated p53 degradation.

DISCUSSION

The p53-MDM2 feedback loop is regulated by distinct pathways in response to different stress signals (225). Here we have described a ribosomal protein that regulates this loop in response to ribosomal biogenesis stress. First, we have purified an MDM2-associated cytoplasmic complex by immuno-affinity purification from human 293 cells. This complex contains multiple ribosomal proteins, including the previously reported L5 (182), the recently reported L11 (177, 329), and a new component-the ribosomal protein L23 (Fig. 2.1). Our further characterization of this complex indicates that L23, unlike L5 which bind to the first acidic region (aa 221-274) of MDM2 (74) and L11 which bind to aa 284-374 of MDM2 (177, 329), preferentially binds to the second acidic domain (aa 384-425) of MDM2 with a minor binding site at first acidic domain (Fig. 2.3). Interestingly, L23 appears to interact with MDM2 in both the nucleus and the cytoplasm when they were overexpressed (Fig. 2.2). In response to actinomycin D, this interaction might mostly occur in the nucleus of U2OS cells (data not shown). Functionally, ectopic expression of L23 inhibits MDM2-mediated p53 degradation and thus induces p53 levels as well as its activity (Fig. 2.6). L23 also leads to p53-dependent G1 arrest (Fig. 2.7) by inducing p53-dependent $p21^{cip1}$ production (Fig. 2.6). Finally, the interaction between L23 and MDM2 is dramatically enhanced by a low dose of actinomycin D that only inhibits rRNA synthesis. Further, ablation of endogenous L23 molecules by siRNA alleviates p53 induction by this drug (Fig. 2.9). Hence our study documents L23 as

another possible regulator of the p53-MDM2 feedback pathway in response to the ribosomal biogenesis perturbation.

Association of MDM2 with multiple ribosomal proteins. Another finding from our study is that the three ribosomal proteins associate with MDM2 in one complex although MDM2-L5 and MDM2-L11 interactions were individually reported (177, 182). Several lines of evidence support this notion. First, an MDM2-associated protein complex has been purified and has been found to contain all the three ribosomal proteins from 293 cells (Fig. 2.1), whose native mass is similar to our previously identified MDM2-associated complex free of p53 and p300 from HeLa cytoplasmic extracts (141). Second, MDM2 co-sediments with L5, L11, and L23 by glycerol gradient sedimentation centrifugation with a molecular mass arranging from ~200 Kd to ~800 Kd (Fig. 2.4A). The simultaneous association of MDM2 with L23, L5, and L11 in one of the peak fractions (fraction 30) is confirmed by a co-immunoprecipitation analysis with anti-HA antibodies (Fig. 2.4B). Consistent with this result is that MDM2 directly interacts with L23 (Fig. 2.3), L11 and L5 *in vitro* through its different domains (74) (data not shown). Although whether these ribosomal proteins could work in concert to inhibit the function of MDM2 remains to be studied, overexpression of L11 (177, 329), L23 (this study) and L5 (our unpublished observations) alone is able to activate p53 by preventing MDM2-mediated p53 ubiquitination and degradation. The MDM2-associated complex may contain other yet unidentified proteins besides these ribosomal proteins because of its large native molecular weight (Fig. 2.4) and some MDM2-associated polypeptides remain to be identified (Fig. 2.1). Despite its interaction with three ribosomal proteins, MDM2 does not appear to bind to the 80S ribosome and polysomes, as our sucrose

gradient sedimentation centrifugation separates MDM2 from the 80S ribosome and polysomes (Fig. 2.5). Hence, MDM2 forms a complex with ribosomal proteins L5, L11 and L23 independently of the 80S ribosome and polysome.

L23 activates p53 by blocking MDM2 feedback regulation. Our study suggests that L23 induces p53 through direct binding to MDM2 (Figs. 2.1-4) and inhibition of MDM2-mediated p53 ubiquitination and MDM2 ubiquitination (Fig. 2.6). This mode of action seems similar to that mediated by the ARF protein (115, 176, 196, 289, 312, 313, 331) or by L11 (177, 329). Both ARF and L11 could re-localize MDM2 into the nucleolus. However, our data show that the interaction of L23 with MDM2 appears to occur in the nucleus as well as the cytoplasm but not in the nucleolus. Four lines of evidence support this assumption. First, the ribosomal proteins containing MDM2 complex was isolated from cytoplasmic fraction of the cells. Second, the MDM2 deletion mutation which lacks the NLS sequence (Δ150-230) and stays in the cytoplasm (124) can interact with L23 as well as wild type MDM2 (Fig. 2.2E). Third, MDM2 was co-immunoprecipitated by anti-L23 in both the cytoplasm and the nucleus (data not shown). Finally, in our 293-HA-MDM2 cells transfected with Flag-L23, MDM2 was expressed in both the cytoplasm and the nucleus, but not in nucleolus, while L23 was expressed in all three compartments (Fig. 2.2D). Therefore, MDM2 is probably retained in the cytoplasm and the nucleus as a complex and this complex formation may represent one mechanism by which L23 suppresses the MDM2 feedback regulation. Alternatively, L23 inhibits MDM2-mediated p53 ubiquitination and thus stabilizes p53 most likely by simply binding to MDM2, while the L23 deletion, which is defective in MDM2 binding (Fig. 2.3), is unable to induce p53 (Fig. 2.6) and its activity (Fig. 2.7). Although these

possibilities need to be investigated further, it seems that L23 does not directly bind to p53 (data not shown) and the cytoplasmic MDM2-associated complex is free of p53 (141), suggesting that it is less likely that L23 directly targets p53. Therefore, L23 may activate p53 by preventing the MDM2 feedback suppression.

Role of L23 in the ribosome biogenesis-p53 pathway. In mammals, ribosomal biogenesis is well coordinated by cell growth signals (72, 256). Although the mechanism underlying this precise coordination remains obscure, several studies suggest that some cell cycle regulators and tumor suppressors may be involved in this regulation. For example, p53 and Rb have been shown to inhibit rRNA synthesis, thus lessening ribosome assembly (29, 39, 48, 304, 326). Also, the other regulator of the MDM2-p53 loop, $p19^{arf}$, has recently been reported to inhibit rRNA processing (284). These studies suggest that overproduction of these tumor suppressors would inhibit protein synthesis while stopping cell growth in response to stress signals. Remarkably, p53 can also sense the perturbation of ribosomal biogenesis, such as inhibition of rRNA synthesis and processing or disruption of ribosome assembly (5, 6, 234, 283). For instance, overexpression of dominant negative mutants of a nucleolar protein Bop1, an inhibitor of rRNA processing, induced p53-dependent G1 arrest (234, 283). Another newly identified nucleolar protein called nucleostemin prevented cells from entering mitosis and caused p53-dependent apoptosis (294), though its exact function on ribosomal biogenesis is unknown. Also, as aforementioned, a low dose (5nM) of actinomycin D rapidly induced p53 activity independently of the p53 N-terminal phosphorylation (5, 6), which is critical for p53 activation by γ irradiation (9, 35, 134, 273). These studies suggest an elaborate

coordination between p53-mediated cell growth regulation and cellular responses to malfunction of ribosomal biogenesis through a novel signaling pathway.

The findings that L23 and L11 activate p53 by binding to MDM2 and inhibiting MDM2-mediated p53 degradation process as described here and by others (177) suggest a plausible model for the role of these ribosomal proteins in the ribosomal biogenesis-p53 pathway (Fig. 2.10). Inhibition of ribosomal biogenesis such as caused by actinomycin D, but not direct inhibition of translation by protein synthesis inhibitors such as pactamycin or cycloheximide, would interfere with the assembly of the 80S ribosome complex and hence release free ribosomal proteins such as L23, L11, or L5. These free ribosomal proteins could then bind to MDM2, probably forming a multiple subunit complex in the cytoplasm or the nucleus. In doing so, they could either prevent MDM2 from being transported into the nucleus to target p53 or inhibit MDM2-mediated p53 ubiquitination. Consequently, p53 would become stabilized and activated to induce $p21^{cip1}$-dependent G1 arrest and to inhibit protein synthesis. Strongly supporting this model are two lines of evidence. For instance, a low dose of actinomycin D induces the interaction of L23 with MDM2, leading to p53 activation (Fig. 2.8B). Also, ablation of L23 by siRNA markedly reduced actinomycin D-caused p53 induction (Fig. 2.9). Hence L23 may play a role in the ribosomal biogenesis-p53 pathway, although other pathological signals that turn on this pathway remain to be uncovered.

Emerging evidence implies that alterations of the ribosomal biogenesis pathway might contribute to tumorigenesis (256). For instance, mutations in a ribosomal gene encoding S19 lead to a cancer susceptibility syndrome called Diamond-Blackfan anemia (68). Also, mutations of the dyskerin gene encoding a crucial pseudouridine synthase that

mediates posttranscriptional modification of ribosomal RNA are frequent in the dyskeratosis congenital disease characterized by premature aging and an increased susceptibility to cancer (255). Moreover, experimentally generated tumor mice harbor characteristic mutations in the ribosomal protein L11 (185) or L9 (203). Inversely, overexpression of some ribosomal proteins has been shown to induce cell cycle arrest (211, 213). Therefore, it would be interesting and worthwhile to investigate whether L23 is altered in human cancers.

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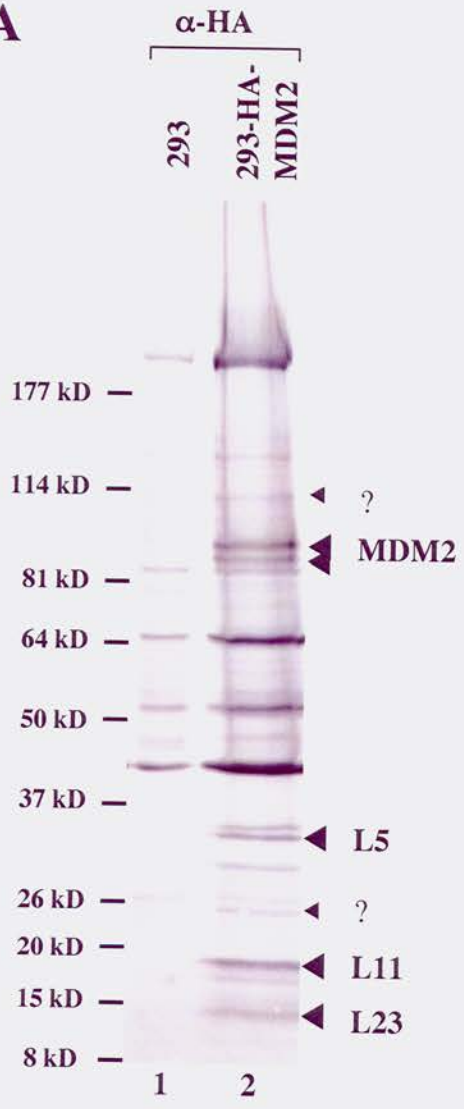
Fig. 2.1. Isolation of a human MDM2-associated protein complex by immuno-affinity purification. (**A**). Collodial-blue staining analysis of proteins eluted from 12CA5 beads loaded with either empty vector expressing 293 cytoplasmic extracts (293) or the cytoplasmic extracts from the 293-HA-MDM2 cell line (lane 2). MDM2-associated polypeptides were digested and subjected to sequence analysis by mass spectrometry. The MDM2, L5, L11, and L23 bands are indicated. The question markers denote unidentified polypeptides. (**B**). Peptide sequences for L5, L11, and L23 bands obtained from mass spectrometry analysis.

A



B **Peptide sequences**

L5: (27% by amino acid count)

DIICQIAYAR
EFNAEVHR
FPGYDSESK
GAVDGGLSIPHSTK
QFSQYIK
VGLTNYAAAYCTGLLLAR
YLMEEDEDAYKK

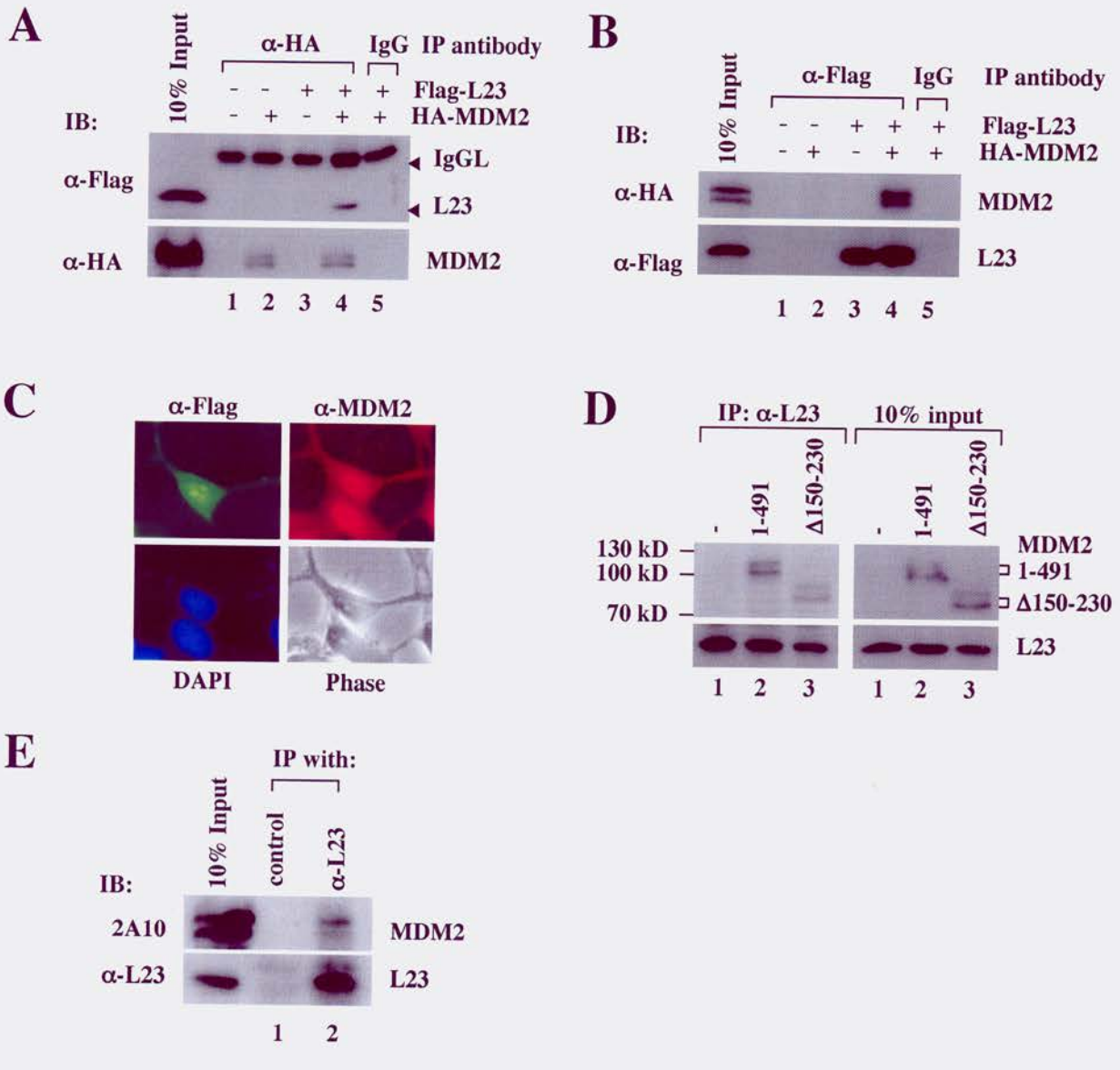
L11: (49% by amino acid count)

AEEILEK
IAVHCTVR
NNFSDTGNFGFGIQEHIDLGIK
PGFSIADK
VLEQLTGQTPVFSK
YDGIILPGK
YDPSIGIYGLDFYVVLGR

L23: (20% by amino acid count)

ISLGLPVGAVINCADNTGAK
NLYIISVK

Fig. 2.2. L23 interacts with MDM2 in cells. (**A**). Exogenous MDM2 and L23 interact with each other in 293 cells. HA-MDM2 (1.5 μg), Flag-L23 (1.5 μg), or both vectors (1.5 μg each) were used for transfection, as indicated at the top. Whole-cell lysates (500 μg) were subjected to immunoprecipitation (IP) with anti-HA (α-HA) or a control antibody followed by immunoblotting (IB) with anti-Flag (α-Flag) (upper) or anti-HA (lower) antibodies. IgG, immunoglobulin G. **(B)**. The same transfections as shown in panel A were conducted except that anti-Flag antibodies were used for immunoprecipitation. **(C)**. MDM2 colocalized with L23 in both the nucleus and the cytoplasm but not in the nucleolus. 293-HA-MDM2 cells were transfected with Flag-L23 and immunostained with both polyclonal anti-MDM2 (red) and monoclonal anti-Flag (green) antibodies. **(D)**. L23 binds to MDM2 deletion mutants in cells. 293 cells were transfected with 6 μg of wild-type MDM2, MDM2150-230, or empty (–) plasmids, as indicated at the top. Whole-cell lysates (500 μg) were immunoprecipitated with anti-L23 (α-L23) antibodies followed by immunoblotting with anti-MDM2 (2A10) and anti-L23 antibodies. Ten percent of the lysates loaded as input are shown in the panels on the right side. **(E)**. Endogenous L23 interacts with endogenous MDM2 in U2OS cells. Whole-cell lysate (500 μg) was used for immunoprecipitation with either rabbit polyclonal anti-L23 antibody or preimmune serum (control), followed by immunoblotting with anti-MDM2 (2A10) (top panel) or anti-L23 (bottom panel) antibody.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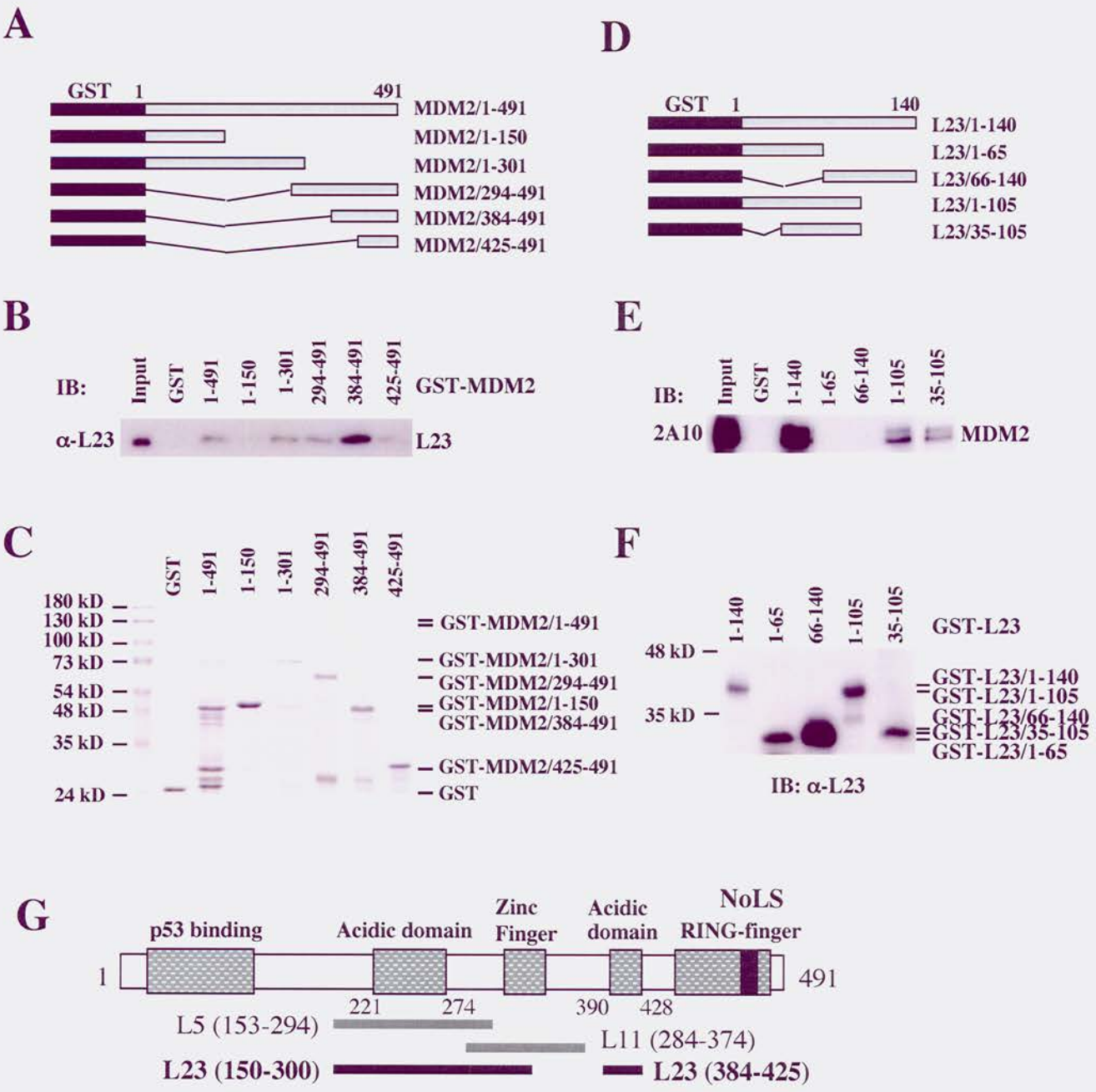
10% Input
α-HA
IgG
IP antibody
Flag-L23
HA-MDM2
IB:
α-Flag
IgGL
L23
α-HA
MDM2
1 2 3 4 5
B
10% Input
α-Flag
IgG
IP antibody
Flag-L23
HA-MDM2
IB:
α-HA
MDM2
α-Flag
L23
1 2 3 4 5
C
α-Flag
α-MDM2
DAPI
Phase
D
IP: α-L23
10% input
-
1-491
Δ150-230
130 kD
100 kD
70 kD
MDM2
1-491
Δ150-230
L23
1 2 3 1 2 3
E
IP with:
10% Input
control
α-L23
IB:
2A10
α-L23
MDM2
L23
1 2

Fig. 2.3. The ribosomal protein L23 interacts with MDM2 *in vitro*. (**A**). Schematic presentation of recombinant full length MDM2 and its fragments fused to GST. The black rectangles indicate GST. The gray rectangles indicate MDM2 or its fragments. (**B**). L23 preferentially binds to the acidic domains of MDM2. About 200 ng of purified GST alone, full length GST-MDM2, or GST-MDM2 deletion mutants including MDM2/1-150, MDM2/1-301, MDM2/294-491, MDM2/384-491 and MDM2/425-491 immobilized on glutathione beads were used in GST pull-down assays with 200 ng of His-L23 purified from bacteria. Bound L23 was detected by immunoblotting (IB) with anti-L23 (α-L23) antibodies. (**C**). Commassie blue staining of GST-MDM2 fusion proteins used in panel B. (**D**). Schematic presentation of recombinant full length L23 and its fragments fused to GST. (**E**). MDM2 binds to the middle domain of L23 *in vitro*. The same GST-fusion protein pull-down assay as that in panel B was conducted except that purified 200 ng of GST-L23 and 200 ng of GST-L23 deletion mutants were incubated with 200 ng of His-MDM2 purified from bacteria, as indicated. Bound MDM2 were detected by immunoblotting with anti-MDM2 antibodies (2A10). (**F**). Immunoblot of GST-L23 fusion proteins used in panel E with anti-L23 antibodies. (**G**). Schematic presentation of MDM2 domains that bind to L23, L5, and L11.


A
GST 1 491
MDM2/1-491
MDM2/1-150
MDM2/1-301
MDM2/294-491
MDM2/384-491
MDM2/425-491
D
GST 1 140
L23/1-140
L23/1-65
L23/66-140
L23/1-105
L23/35-105
B
IB:
Input
GST
1-491
1-150
1-301
294-491
384-491
425-491
GST-MDM2
α-L23
L23
E
IB:
Input
GST
1-140
1-65
66-140
1-105
35-105
2A10
MDM2
C
GST
1-491
1-150
1-301
294-491
384-491
425-491
180 kD
130 kD
100 kD
73 kD
54 kD
48 kD
35 kD
24 kD
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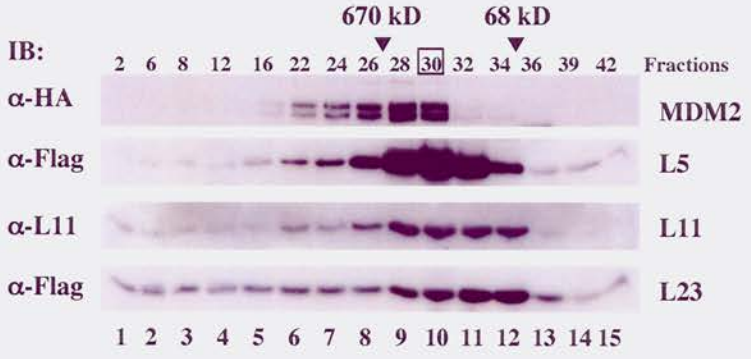
GST-MDM2/1-150
GST-MDM2/384-491
GST-MDM2/425-491
GST
F
1-140
1-65
66-140
1-105
35-105
GST-L23
48 kD
35 kD
GST-L23/1-140
GST-L23/1-105
GST-L23/66-140
GST-L23/35-105
GST-L23/1-65
IB: α-L23
G
p53 binding
Acidic domain
Zinc Finger
Acidic domain
NoLS
RING-finger
1
491
221
274
390
428
L5 (153-294)
L11 (284-374)
L23 (150-300)
L23 (384-425)

Fig. 2.4. MDM2 forms a complex with L23, L5, and L11 in cells. (**A**). MDM2 cosedimented with L23, L5, and L11. Whole-cell lysates (2 mg) prepared from HA-MDM2-expressing 293 cells were subjected to a 12.5 to 25% glycerol gradient sedimentation centrifugation. Fractions were collected for immunoblot (IB) analysis with antibodies indicated to the left of each panel. Molecular markers coeluted with fractions are indicated at the top. Fraction 30, boxed, was used for immunoprecipitation and immunoblot analysis shown in panel B. (**B**). MDM2 is coimmunoprecipitated with L23, L5, and L11 in fraction 30. Fraction 30 (200 μl) was used for immunoprecipitation (IP) with the anti-HA (α-HA) or control antibody as indicated at the top, followed by immunoblotting with the antibodies indicated to the left. IgG, immunoglobulin G; -Flag, anti-Flag; -L11, anti-L11.

A


670 kD
68 kD
IB:
2 6 8 12 16 22 24 26 28 30 32 34 36 39 42 Fractions
α-HA
MDM2
α-Flag
L5
α-L11
L11
α-Flag
L23
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15

B

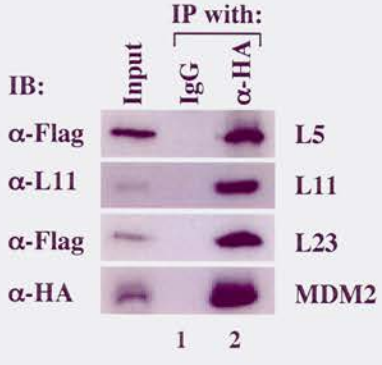

IP with:
Input
IgG
α-HA
IB:
α-Flag
L5
α-L11
L11
α-Flag
L23
α-HA
MDM2
1 2

Fig. 2.5. MDM2 does not associate with the 80S ribosome and polysomes. (**A**). Ectopically expressed MDM2 does not associate with polysomes. Cytoplasmic extracts (5 mg) containing polysomes from HA-MDM2-expressing 293 cells were subjected to a 15 to 47% linear sucrose gradient sedimentation centrifugation. Fourteen fractions were collected, and 30 μl of each fraction was used for immunoblotting (IB) with anti-HA (α-HA), anti-L11 (α-L11), or anti-L23 (α-L23) antibodies as indicated to the left. Total RNAs were isolated from each fraction and subjected to electrophoresis on a 1% agarose gel and stained with ethidium bromide as shown in the bottom panel. 28S, 18S, 5.8S, and 5S rRNAs are indicated to the right. The fractions containing polysomes and mRNPs are indicated on the top. The 80S ribosome is indicated at the bottom. **(B)**. Endogenous MDM2 does not associate with polysomes. The same fractionation as that shown in panel A was performed with U2OS cell extracts. The distributions of polysomes and mRNPs are indicated. Thirty microliters of each fraction was subjected to immunoblot analysis with anti-MDM2 (2A10, α-MDM2), anti-L11, or anti-L23 antibodies as indicated to the left.

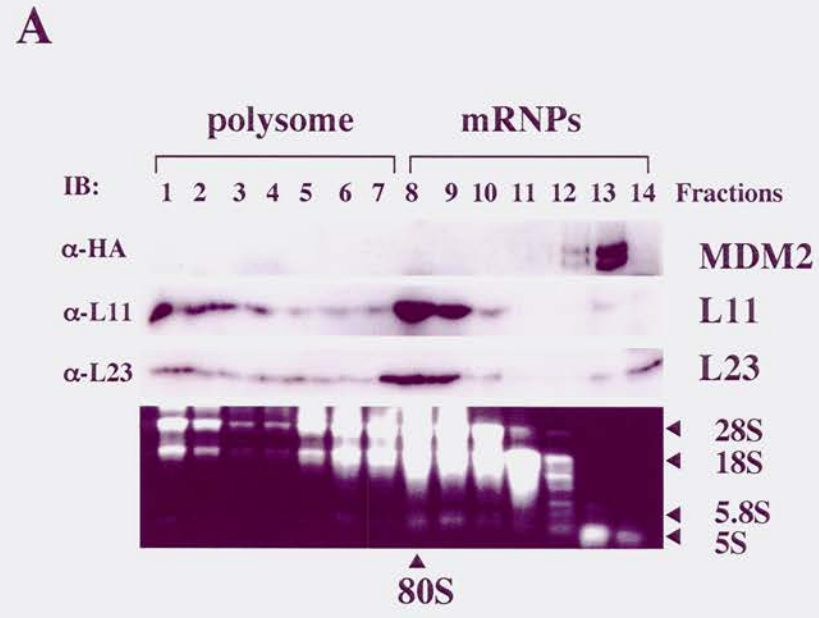
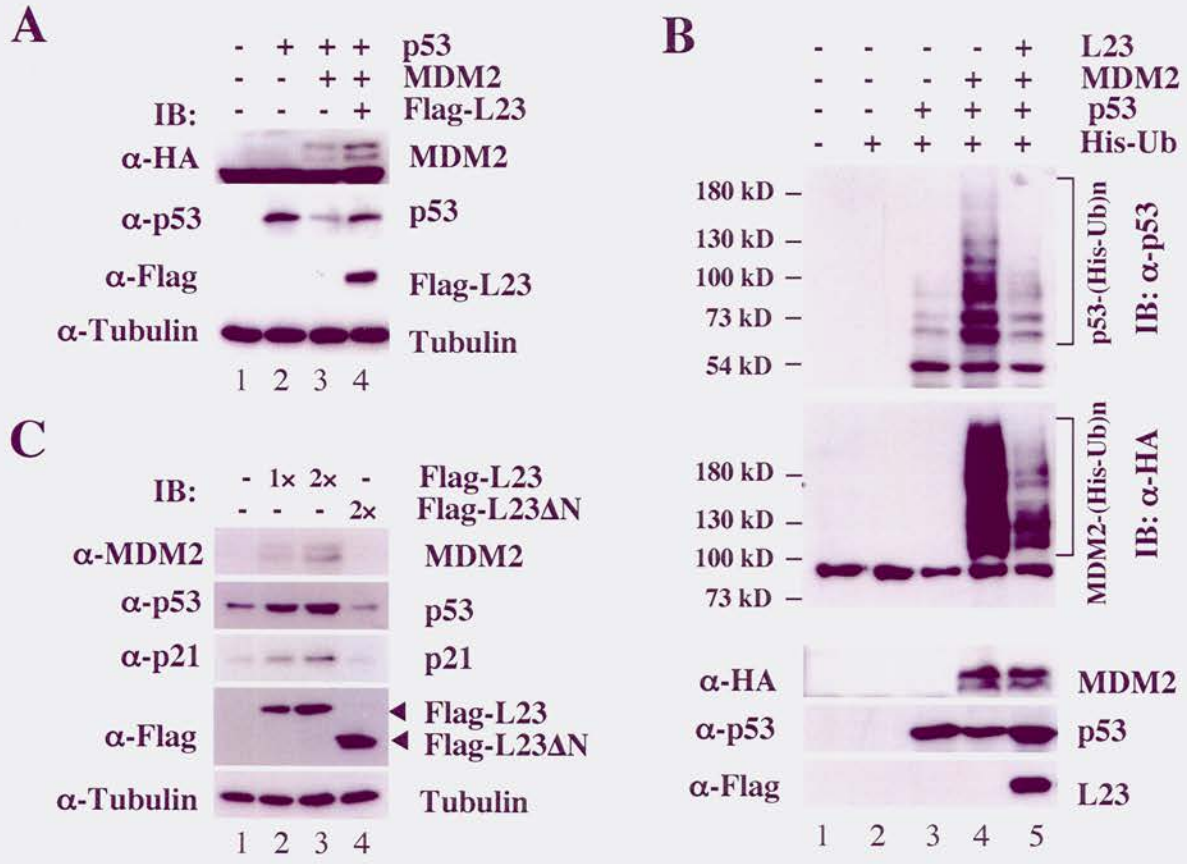

A
polysome
mRNPs
IB:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 Fractions
α-HA
MDM2
α-L11
L11
α-L23
L23
28S
18S
5.8S
5S
80S
B
polysome
mRNPs
IB:
1 2 3 4 5 6 7 8 9 10 11 12 13 14 Fractions
α -MDM2
MDM2
α-L11
L11
α-L23
L23
80S

Fig. 2.6. Ectopic expression of L23 stabilizes p53 and inhibits MDM2-mediated p53 ubiquintination. (**A**) Ectopic expression of L23 reverses MDM2-mediated p53 degradation. H1299 cells were transfected with 1.0 μg of Flag-L23 in the presence of p53 (0.5 μg) with (+) or without (-) MDM2 (1.0 μg) as indicated. Cell lysates (50 μg) were immunoblotted (IB) with anti-MDM2 (α-MDM2), anti-p53 (α-p53), anti-Flag (α-Flag), or antitubulin (α-tubulin) antibodies as indicated to the left. **(B)**. L23 inhibits MDM2-mediated p53 ubiquitination in cells. H1299 cells were transfected with combinations of L23 (2 μg)-, p53 (1 μg)-, or MDM2 (1 μg)-encoding plasmids in the presence of the His-ubiquitin (His-Ub) (2 μg) plasmid as indicated at the top. The cells were treated with MG132 (20 μM) for 8 h before harvesting. The in vivo ubiquitination assay was performed as described in Materials and Methods. Ubiquitinated proteins were detected by immunoblotting with the anti-p53 (DO-1) and anti-HA (α-HA) antibodies. Ubiquitinated p53s [p53-(His-Ub)n] and ubiquitinated MDM2s [MDM2-(His-Ub)n] are indicated to the right of the upper and middle panels. The expression levels of MDM2, p53, and L23 are shown in the lower panels. **(C)**. Ectopic expression of L23 induces endogenous p53. U2OS cells were transfected with 1.0 μg (lane 2) or 2.0 μg (lane 3) of Flag-L23 or 2.0 μg of Flag-L23ΔN (lane 4) plasmids. Cell lysates (50 μg) were used for immunoblot analysis with antibodies as indicated to the right. α-p21, anti-p21.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α-HA
MDM2
α-p53
p53
α-Flag
Flag-L23
α-Tubulin
Tubulin
1 2 3 4
B
- - - - + L23
- - - + + MDM2
- - + + + p53
- + + + + His-Ub
180 kD
130 kD
100 kD
73 kD
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p53-(His-Ub)n
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180 kD
130 kD
100 kD
73 kD
MDM2-(His-Ub)n
IB: α-HA
α-HA
MDM2
α-p53
p53
α-Flag
L23
1 2 3 4 5
C
IB: - 1× 2× - Flag-L23
- - - 2× Flag-L23ΔN
α-MDM2
MDM2
α-p53
p53
α-p21
p21
α-Flag
Flag-L23
Flag-L23ΔN
α-Tubulin
Tubulin
1 2 3 4

Fig. 2.7. Ectopic expression of L23 stimulates p53-dependent transcription and G1 arrest. (**A**). Ectopic expression of L23 increases p53RE-dependent luciferase activity in p53-proficient U2OS cells. U2OS cells were trnasfected with increasing amounts of Flag-L23 (0.4 μg, 1x and 0.8 μg, 2x) or Flag-L23ΔN (0.4 μg, 1x and 0.8 μg, 2x) in the presence of a luciferase reporter plasmid driven by the p53RE (BP100 luc, 0.1μg) or a control luciferase reporter plasmid (TB luc, 0.1 μg). Luciferase activity was presented in arbitrary units. (**B**). L23 does not affect p53RE-dependent luciferase activity in p53-deficient Saos-2 cells. The same transfection followed by a luciferase assay as that in panel A was conducted except Saos-2 cells were used here. (**C**). Ectopic expression of L23 leads to p53-dependent G1 cell cycle arrest. U2OS or Saos2 cells were transfected with GFP (2 μg), GFP-L23 (2 μg), or GFP-L23ΔC (2 μg) plasmids and treated with 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. Percentage indicates the cells that were arrested in G1 phase. (**D**). The mean percentage of the cells arrested in G1 phase obtained from four separate experiments is presented. Bars indicate the standard deviation. +, present; - absent.

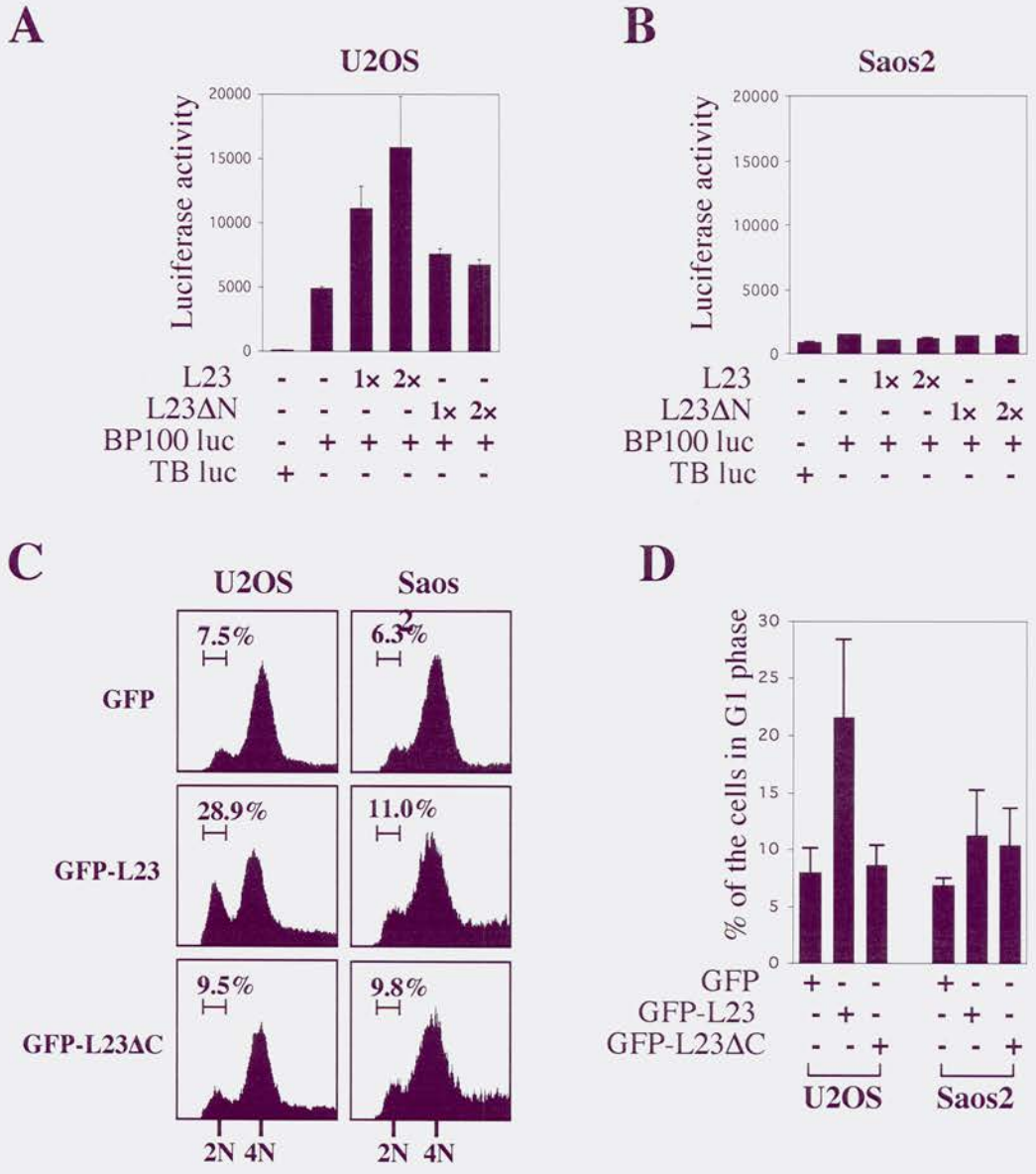

A
U2OS
Luciferase activity
20000
15000
10000
5000
0
L23 - - 1× 2× - -
L23ΔN - - - - 1× 2×
BP100 luc - + + + + +
TB luc + - - - - -
B
Saos2
Luciferase activity
20000
15000
10000
5000
0
L23 - - 1× 2× - -
L23ΔN - - - - 1× 2×
BP100 luc - + + + + +
TB luc + - - - - -
C
U2OS
Saos
GFP
7.5%
6.3%
GFP-L23
28.9%
11.0%
GFP-L23ΔC
9.5%
9.8%
2N 4N
2N 4N
D
% of the cells in G1 phase
30
25
20
15
10
5
0
GFP + - - + - -
GFP-L23 - + - - + -
GFP-L23ΔC - - + - - +
U2OS
Saos2

Fig. 2.8. A low dose of actinomycin D enhances MDM2-L23 interaction and p53 activation. (**A**). Low doses of actinomycin D induce p53 and its function whereas high doses of actinomycin D induce p53 but not its function. U2OS cells were treated with increasing amounts of actinomycin D (Act D) as indicated on top. Cell lysates (50 μg) were used for an immunoblot analysis with antibodies indicated on right. (**B**). Time-dependent effect of actinomycin D on p53 and L23 levels in U2OS cells. U2OS cells were treated with 5 nM of actinomycin D and harvested at different time points as indicated on top. Cell lysates (50 μg) were used for an immunoblot analysis with antibodies indicated on left of each panels. (**C**). Time-dependent effect of actinomycin D on p53and L23 levels in WI38 cells. WI38 cells were treated with 5 nM of actinomycin D, harvested and blotted with antibodies as described in panel B. (D). Five nanomolar actinomycin D enhances MDM2-L23 interaction. U2OS cells were treated with 5nM of actinomycin D and harvested at different time points as indicated on top. Cells were incubated with (+) or without (-) MG132 (20 μM) for 6 hours before harvesting. Cell lysates (500 μg) were subjected to an immunoprecipitation with anti-L23 antibodies and immunoblot with anti-MDM2 or anti-L23 antibodies (lower panels). The lysates were also directly loaded on an SDS gel for an immunoblot analysis with anti-MDM2, anti-p53, or anti-L23 antibodies (upper panels). (**E**). Ionizing irradiation does not affect L23-MDM2 interaction. U2OS cells were treated with g irradiation (10 gray) and harvested at different time points as indicated. Cell lysates (50 μg) were subjected to an immunoblot analysis with anti-p53, anti-MDM2, or anti-L23 antibodies (up panels). The cell lysates (500 μg) were subjected to an immunoprecipitation analysis with anti-L23 antibodies, followed by an immunoblot analysis with anti-MDM2 or anti-L23 antibodies (bottom

panels). (F). Pactamycin treatment does not induce p53 and the MDM2-L23 interaction. U2OS cells were treated with 0.2 μg/ml of pactamycin for different hours as indicated on the top. The cells were harvested for immunoblot with the indicated antibodies (top panels). The cell lysates were also subjected to immunoprecipitation with anti-L23 followed by immunoblot with anti-MDM2 and anti-L23 antibodies (bottom panels). α-L23, anti-L23; α-p53, anti-p53; α-MDM2, anti-MDM2; α-p21, anti-p21; α-tubulin, antitubulin; α-L11, anti-L11.

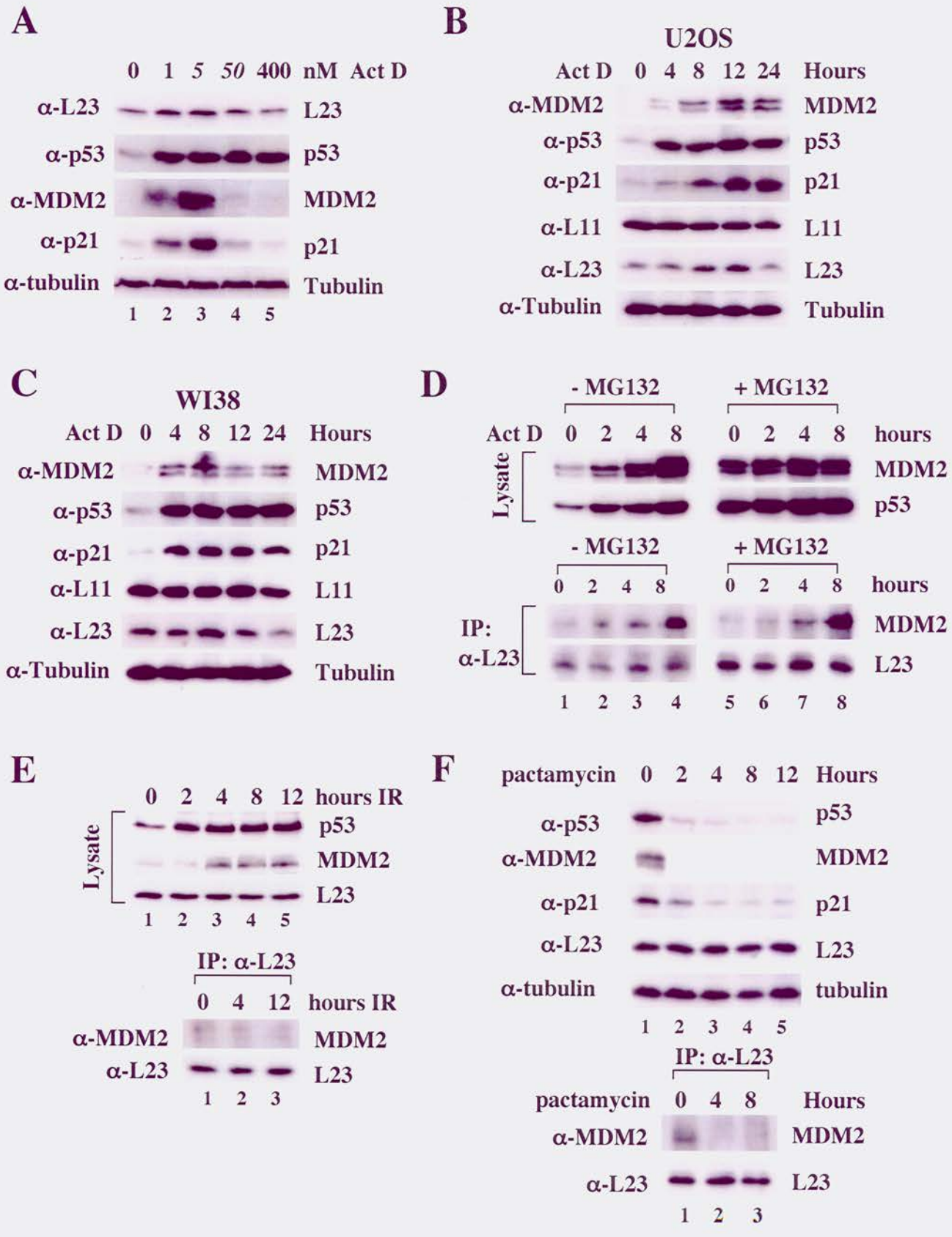
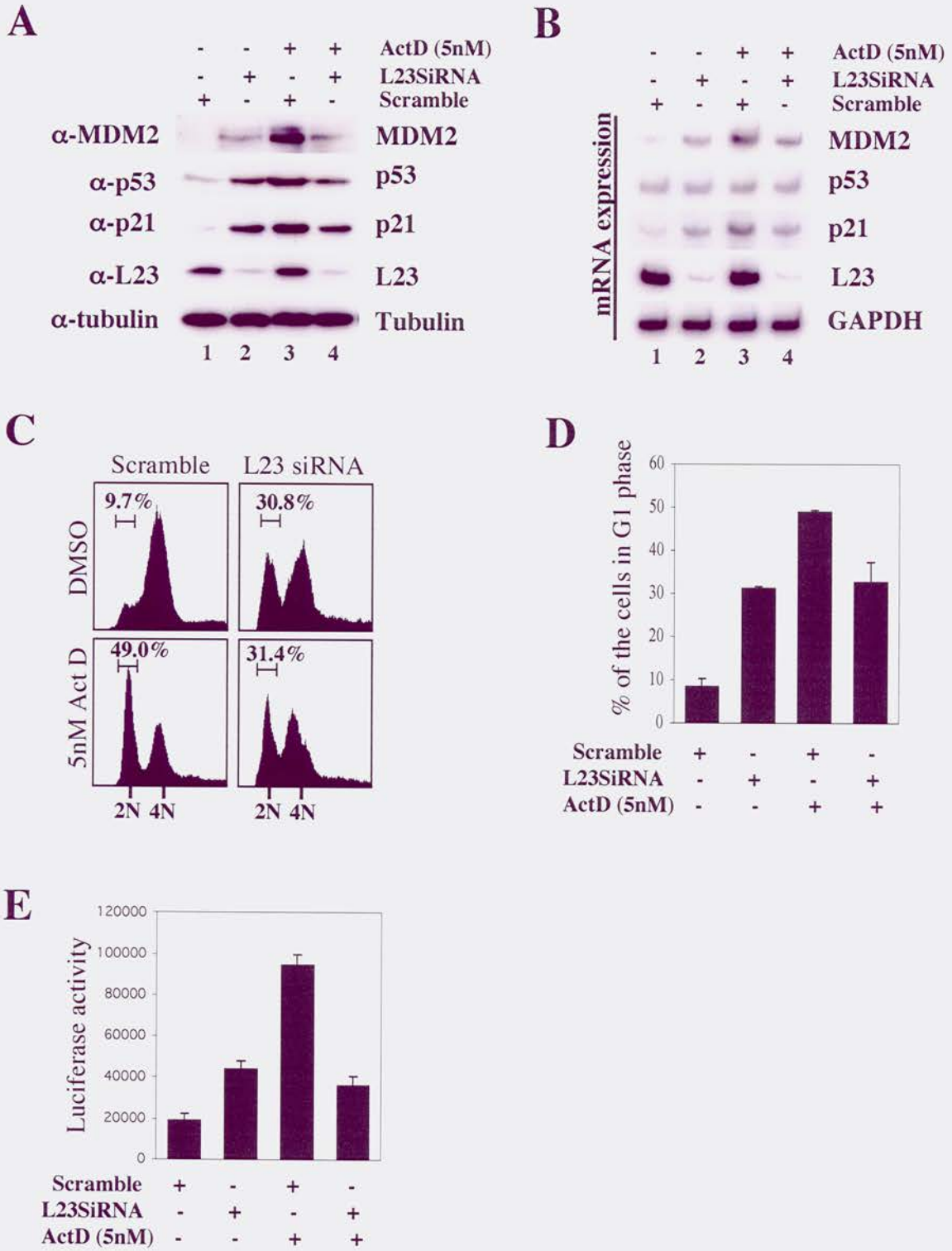

A
0 1 5 50 400 nM Act D
α-L23
L23
α-p53
p53
α-MDM2
MDM2
α-p21
p21
α-tubulin
Tubulin
1 2 3 4 5
B
U2OS
Act D 0 4 8 12 24 Hours
α-MDM2
MDM2
α-p53
p53
α-p21
p21
α-L11
L11
α-L23
L23
α-Tubulin
Tubulin
C
WI38
Act D 0 4 8 12 24 Hours
α-MDM2
MDM2
α-p53
p53
α-p21
p21
α-L11
L11
α-L23
L23
α-Tubulin
Tubulin
D
- MG132
+ MG132
Act D 0 2 4 8 0 2 4 8 hours
Lysate
MDM2
p53
- MG132
+ MG132
0 2 4 8 0 2 4 8 hours
IP:
α-L23
MDM2
L23
1 2 3 4 5 6 7 8
E
0 2 4 8 12 hours IR
Lysate
p53
MDM2
L23
1 2 3 4 5
IP: α-L23
0 4 12 hours IR
α-MDM2
MDM2
α-L23
L23
1 2 3
F
pactamycin 0 2 4 8 12 Hours
α-p53
p53
α-MDM2
MDM2
α-p21
p21
α-L23
L23
α-tubulin
tubulin
1 2 3 4 5
IP: α-L23
pactamycin 0 4 8 Hours
α-MDM2
MDM2
α-L23
L23
1 2 3

Fig. 2.9. Ablation of endogenous L23 by siRNA induces p53 but inhibits actinomycin D-induced p53 activation and G1 arrest. (A). Ablation of endogenous L23 by siRNA induces p53 level but inhibits actinomycin D-caused p53 induction. U2OS cells were transfected with L23 siRNA oligonucleotides (0.2 μM, lanes 2 and 4) or with scrambled RNA duplex (0.2 μM, lanes 1 and 3). Cells were then incubated with (+) (lanes 3 and 4) or without (-) (lanes 1 and 2) 5 nM of actinomycin D for 8 hours before harvesting. Cell lysates (50 μg) were then immunoblotted with anti-MDM2, anti-p53, anti-p21, anti-L23 or anti-tubulin antibodies. (B) Ablation of endogenous L23 by siRNA inhibits actinomycin D-induced p53 transcriptional activity. Cells were prepared as described in panel A and total RNA was extracted. RT-PCR analysis was performed to detect MDM2, p53, p21, L23, and GAPDH mRNA levels as indicated. (C). Actinomycin-induced G1 arrest is inhibited by ablation of endogenous L23 by siRNA. U2OS cells were treated as described in panel A except that cells were treated with nocodazole for 16 hrs before harvesting. Cells were then stained with PI for FACS analysis. The histograms of PI staining from one representative experimental are shown. (D). A summary from three independent experiments as described in panel C. The mean percentage of the cells arrested in G1 phase was presented in this graph. Bars indicate the standard deviation. (E). Ablation of endogenous L23 by siRNA inhibits actinomycin D-induced p53RE-dependent luciferase activity. U2OS cells were transfected with a luciferase reporter plasmid driven by the p53RE (BP100 luc, 0.2μg) and β-gal plasmid (0.2 μg) followed by treatment with L23 siRNA and actinomycin D as described in panel A. Luciferase activity is presented in arbitrary units. α-L23, anti-L23; α-p53, anti-p53; α-MDM2, anti-MDM2; α-p21, anti-p21; α-tubulin, antitubulin; DMSO, dimethyl sulfoxide.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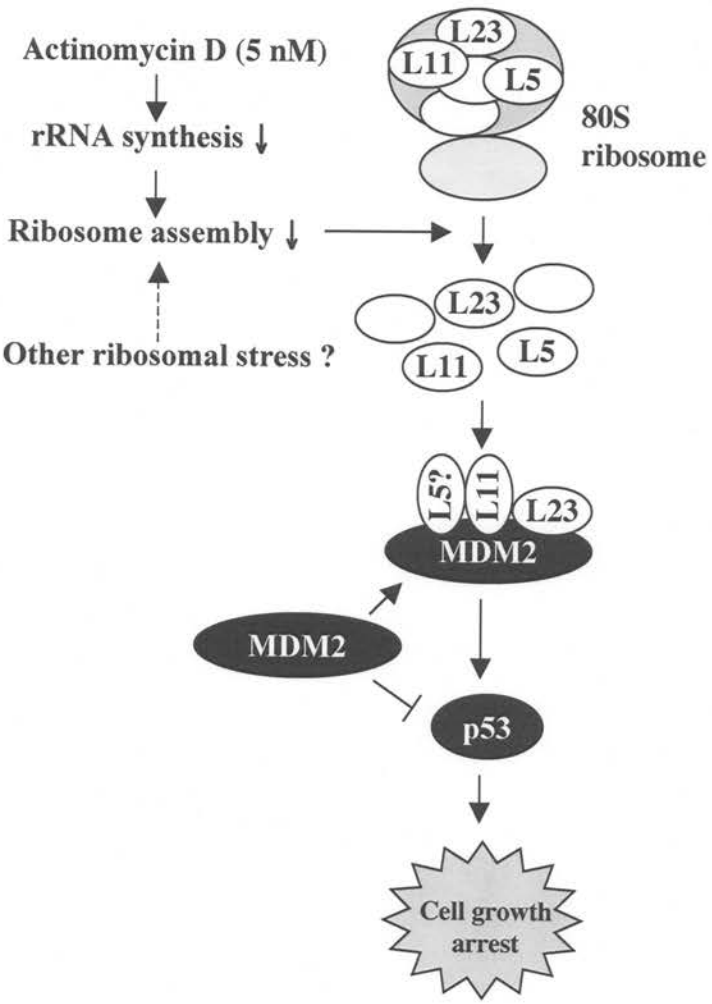
- - + + ActD (5nM)
- + - + L23SiRNA
+ - + - Scramble
α-MDM2
MDM2
α-p53
p53
α-p21
p21
α-L23
L23
α-tubulin
Tubulin
1 2 3 4
B
- - + + ActD (5nM)
- + - + L23SiRNA
+ - + - Scramble
mRNA expression
MDM2
p53
p21
L23
GAPDH
1 2 3 4
C
Scramble
L23 siRNA
DMSO
5nM Act D
9.7%
30.8%
49.0%
31.4%
2N 4N
2N 4N
D
% of the cells in G1 phase
60
50
40
30
20
10
0
Scramble + - + -
L23SiRNA - + - +
ActD (5nM) - - + +
E
Luciferase activity
120000
100000
80000
60000
40000
20000
0
Scramble + - + -
L23SiRNA - + - +
ActD (5nM) - - + +

Fig. 2.10. A model for p53 activation by interfering with the MDM2-p53 feedback loop in response to perturbation of ribosomal biogenesis. Bars indicate inhibition whereas arrows denote activation.


Actinomycin D (5 nM)
rRNA synthesis ↓
Ribosome assembly ↓
Other ribosomal stress ?
L23
L11
L5
80S
ribosome
L23
L11
L5
L5?
L11
L23
MDM2
MDM2
p53
Cell growth
arrest

CHAPTER THREE

Inhibition of MDM2-Mediated p53 Ubiquitination and Degradation by Ribosomal Protein L5

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Running Title: Regulation of the MDM-p53 loop by L5.

Key Words: L5, L11, L23, MDM2, p53, ribosome proteins, transcription, and cell cycle.

Abbreviations: Act D: actinomycin D; siRNA: small interference RNA. GST: Glutathione S-transferase; GFP: green fluorescence protein; E2: ubiquitin carrier protein; E3: ubiquitin-protein isopeptide ligase.

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SUMMARY

The oncoprotein MDM2 associates with ribosomal proteins L5, L11, and L23. Both L11 and L23 have been shown to activate p53 by inhibiting MDM2-mediated p53 suppression. Here we show that L5 also activates p53. Overexpression of L5 stabilized ectopic p53 in H1299 cells and endogenous p53 in U2OS cells. Consequently, L5 enhanced p53 transcriptional activity and induced p53-dependent G1 cell cycle arrest. Furthermore, like L11 and L23, L5 also remarkably inhibited MDM2-mediated p53 ubiquitination. The interaction of L5 with MDM2 was also enhanced by treatment with a low dose of actinomycin D. Actinomycin D-induced p53 was inhibited by siRNA against L5. By reciprocal co-immunoprecipitation, we further showed that there were at least two MDM2-ribosomal protein complexes in cells: MDM2-L5-L11-L23 and p53-MDM2-L5-L11-L23. We propose that the MDM2-L5-L11-L23 complex functions to inhibit MDM2-mediated p53 ubiquitination and thus activates p53.

INTRODUCTION

The oncoprotein MDM2 is a crucial feedback regulator of the tumor suppressor protein p53 (236). Under physiological conditions, p53 is short-lived mainly due to MDM2-mediated ubiquitination and degradation. Under pathological conditions, the half-life of p53 is prolonged because multiple cellular pathways are activated to prevent MDM2-mediated p53 ubiquitination and degradation, consequently leading to p53 activation (27). In addition to induction of many p53 responsive genes including those involved in cell cycle and apoptotic regulation, activated p53 also induces transcription of MDM2, which in turn suppresses p53 function. Hence, tight regulation of this MDM2-p53 feedback pathway is critically important for a cell to respond to various stresses.

An example of such a stress is ribosomal biogenesis stress. In response to the ribosomal biogenesis stress caused by either a low dose (5 nM) of actinomycin D^1 (Act D) or overexpression of the mutant Bop1, a nucleolar protein critical for rRNA processing and ribosome assembly, p53 is activated and induces cell growth arrest at the G1 phase (5, 234, 283). Ironically, this activation does not appear to require posttranslational modifications of p53 such as phosphorylation (6). Instead, recent studies by several groups including ours suggest that association of ribosomal proteins L11 and L23 with MDM2 might be responsible for p53 activation after Act D treatment (56, 177, 329). L11 and L23 can bind to MDM2 directly and this binding is enhanced in response to Act D treatment. RNAi against L23 can block Act D-induced p53 activation, demonstrating that L23 is required for the p53 activation in response to ribosomal biogenesis stress (56). Another ribosomal protein L5 has also been shown to bind to MDM2 *in vitro* and in cells (74, 182). However, it is still puzzling whether L5, like L11 and L23, can activate p53 by inhibiting MDM2-mediated p53 ubiquitination. It is also unclear whether these three ribosomal proteins can form a single complex with MDM2 to inhibit its ubiquitin ligase activity toward p53 in cells. To address these issues, we have performed a series of cellular experiments. Our studies demonstrate that L5 can also activate p53 and induce p53-dependent G1 arrest in response to Act D-induced ribosomal biogenesis stress. Additionally, a set of reciprocal immunoprecipitation experiments reveals that L5, L11, and L23 appear to bind to MDM2 simultaneously, forming one quadruple complex in cells. The steady state level of the complex is reduced in the presence of p53, suggesting that the ribosomal proteins may compete with p53 for

MDM2. Thus our study shows that the three ribosomal proteins inhibit MDM2 function by forming a complex with MDM2.

MATERIALS AND METHODS

Cell lines, plasmids and antibodies. Human 293, H1299, U2OS, Saos-2, and SJSA cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (vol/vol) fetal bovine serum (FBS) as previously described (56, 124).
To generate human L5 expression construct pcDNA3-2Flag-L5, the full-length L5 cDNA was amplified by RT-PCR from Hela cell mRNA using primers: P1, 5'-CGCGG-ATCCATGGGGTTTGTTAAAGTTG-3'; P2, 5'-CCGGAATTCTTAGCTCTCAGCAG-CCCGCTC-3'. The PCR product was cloned into pcDNA3-2Flag vector in BamH I and EcoR I sites. The pEGFP-L5 was cloned by inserting PCR product using primers P3, 5'-CCGGAATTCATGGGGTT-TGTTAAAGTTG-3'and P4: 5'-CGCGGATCCTTAGCTC-TCAGCAGCCCGCTC-3' into pEGFP-C1 (Clontech) in EcoR I and BamH I sites. GST-MDM2 and His-MDM2 bacterial expression vectors were described previously (56, 125). The His-tagged L5 bacteria expression vector pet24a-His-L5 was cloned by PCR using primers P1 and P5, 5'- CCGGAATTCCGGCTCTCA-GCAGCCCGCTC-3' into pet24a-His vector in BamH I and EcoR I sites.

For generation of the polyclonal anti-L5 antibody, His-tagged full-length L5 protein was expressed in *E. Coli*, purified using Ni-NTA beads, and used as an antigen to raise rabbit polyclonal anti-L5 antisera. Anti-L23 and anti-L11 polyclonal antibody antibodies were described (56, 329). Anti-Flag (Sigma), anti-$p21^{cip1}$ (NeoMarkers), anti-p53 (DO-1, Santa Cruz) and polyclonal anti-MDM2 (Santa Cruz) were purchased.

Monoclonal anti-MDM2 (2A10 and 4B11) and anti-HA (12CA5) have been previously described (56, 125).

Cotransfection, immunoblot and co-immunoprecipitation analyses. H1299, U2OS, or Saos2 cells were transfected with plasmids as indicated in figure legends using Lipofectin following the manufacturer's protocol (Invitrogen). Cells were harvested at 48 hours posttransfection and lysed in lysis buffer. Equal amounts of clear cell lysates were used for immunoblot analysis as described previously (56, 322). Immunoprecipitation was conducted using antibodies as indicated in the figure legends and described previously (56, 322). Beads were washed with lysis buffer twice, once with SNNTE buffer and once with RIPA buffer. Bound proteins were detected by immunoblot using antibodies as indicated in the figure legends. Lysis buffer, SNNTE, RIPA, and Buffer C 100 (BC100) were described. (56, 322)

GST fusion protein association assays. His-tagged L5, L11, L23 and MDM2 proteins were expressed in *E. coli* and purified through a Ni-NTA column and eluted by 0.5 M imidazole. Protein–protein interaction assays were conducted as described (125). Briefly, purified His-tagged L5, L11, or L23 proteins were incubated with the glutathione–Sepharose 4B beads (Sigma) containing 200 ng of GST–MDM2 or GST, respectively, for 30 minutes at room temperature. The mixtures were then washed once in BC100 containing 0.1% Nonidet P-40, twice in SNNTE and once in RIPA. Bound proteins were analyzed on a 10% SDS gel and detected by immunoblot using anti-His, anti-L11, anti-L23, or anti-MDM2 (2A10) antibody.

Transient transfection and luciferase assays. U2OS or Saos-2 cells were transfected with the pCMV-β-galactoside reporter plasmid (0.1μg) and a luciferase

reporter plasmid (0.1 μg) driven by two copies of the p53RE motif derived from the MDM2 promoter (318), together with a combination of different plasmids (total plasmid DNA 1 μg/well) as indicated in Figure 2B, using Lipofectin (Invitrogen). At 48 hours posttransfection, cells were harvested for luciferase assays as described previously (56, 322). Luciferase activity was normalized by a factor of β-gal activity in the same assay.

Cell cycle analysis. U2OS or Saos-2 cells were transfected with plasmids encoding GFP or GFP-L5. 32 hrs post-transfection, cells were treated with 200 μg/ml of nocodazole for additional 16 hours. Cells were stained in 500 μl of propidium iodide (PI, Sigma) stain buffer and analyzed for DNA content using a Becton Dickinson FACScan flow cytometer as described (56, 322). Data were collected using the ModFit software program. GFP-positive cells were gated for cell cycle analysis.

Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis. Total RNA was isolated using Qiagen RNeasy Mini Kits (Qiagen, Valencia, CA). Reverse transcriptions were performed as described (322). The PCR reactions were performed in a 20 μl mixture containing 1 × PCR buffer, 60 μmol/L of dNTPs, 1 U Taq polymerase (Roche), 0.5 μmol/L of each primer and 0.2 μCi ^{32}P-dCTP for 18 to 20 cycles as described (322). PCR products were resolved onto a 6% polyacrylamide gel. The gel was dried followed by autoradiography. The primers for amplifying $p21^{cip1}$, GAPDH were described (322). The primers for amplifying p53 were 5'-TACAGTCAGA-GCCAACCTCAG-3' and 5'-AGATGAAG-CTCCCAGAATGCC-3'.

***In vivo* ubiquitination assays.** H1299 cells were transfected with His_6-ubiquitin (2 mg), p53 (2 mg), Ha-MDM2 (2 mg) and L5 (1, 2mg) expression plasmids as indicated in the figure legend using Lipofectin. 48 hours after transfection, cells were harvested and

split into two aliquots, one for immunoblot and the other for ubiquitination assays. The *in vivo* ubiquitination assay was conducted as previously described (56, 124). Eluted proteins were analyzed by immunoblot with monoclonal p53 antibodies (DO-1, Santa Crutz).

Introduction of siRNA against L5 into human cells followed by treatment with Act D. RNAi-mediated ablation of endogenous L5 was performed essentially as previously described (56, 214). The 21-nucleotide siRNA duplexes with a 3' dTdT overhang, corresponding to L5 mRNA (AAGGGAGCTGTGGATGGAGGC), or the scramble II RNA duplex (AAGCGCGCTTTGTAGGATTC) as a control, were synthesized (Dhamacon). These siRNA duplexes (0.2 μM) were introduced into U2OS cells using Oligofectamine (Invitrogen) following the manufacturer's protocol. The cells were then treated either with or without 5 nM of Act D for 8 hours before harvesting. Cells were harvested 48 hours after transfection for immunoblot analysis. To determine the global protein synthesis after L5 siRNA treatment, U2OS cells were transfected with either L5 siRNA or scramble RNA as above. The cells were directly lysed and equal amounts (10 μg) of total protein were loaded on a 10% SDS-PAGE gel followed by a silver staining. Alternatively, the cells were starved in a methionine-free medium for 30 minutes followed by pulse labeling with 50 μCi/ml of ^{35}S-methionine for 30 minutes. The cells were lysed and equal amounts of total proteins were then loaded onto a 10% SDS-PAGE gel. The gel was incubated in an Amplify solution (Amersham Pharmacia Biotech) for 10 minutes, dried, and exposed to X-ray film.

RESULTS

L5 interacts with MDM2 in cells and *in vitro*. We previously identified an MDM2 complex that contains ribosomal proteins L5, L11, and L23 from cytoplasmic fractions of a stable HA-MDM2-expressing 293 cell line (293-HA-MDM2) using immuno-affinity chromatography followed by mass spectrometry (56). Consistent with an early report (182), we also found that L5 directly bound to MDM2. Ectopically expressed Flag-L5 and HA-MDM2 were co-immunoprecipitated in 293 cells with antibodies against HA or Flag but not the control antibody (Fig. 3.1A). Because it has not been determined whether endogenous L5 and MDM2 proteins bind to each other, we generated polyclonal anti-L5 antibodies to test this unsolved issue. The antibody specifically recognized a band at around 35 kD, the predicted size for L5, in 293 cells (Fig. 3.1B, lane 1), as well as the ectopically expressed Flag-L5 (Fig. 3.1B, lane 2). This antibody also specifically detected endogenous L5 co-immunoprecipitated by anti-MDM2, but not control antibody, in SJSA cells, which expressed a relatively high level of endogenous MDM2 (Fig. 3.1C, lane 2). Also, L5, like L11 (Fig. 3.1F, second panel) and L23 (56), directly interacted with MDM2 *in vitro,* as shown in Fig. 3.1F using glutathione S-transferase (GST)-fusion MDM2-protein association assays.

To determine where the L5-MDM2 binding occurs in cells, we prepared both cytoplasmic and nuclear fractions of lysates from 293-HA-MDM2 cells. HA-MDM2 was expressed in both the cytoplasm and the nucleus as shown by immunoblot (Fig. 3.1D, lanes 3 and 4) and by immunofluorescence staining (7). Equal amounts of both fractions were immunoprecipitated with anti-L5 antibody. As shown in Fig. 3.1D, HA-MDM2 was co-immunoprecipitated by anti-L5 in both fractions. Furthermore, we introduced Flag-L5

together with wild type MDM2 or its nuclear localization sequence (NLS) deletion mutant (MDM2Δ150-230) into 293 cells. Cell lysates were co-immunoprecipitated with an anti-Flag antibody. As shown in Fig. 3.1E, both MDM2Δ150-230 and wild type MDM2 were co-immunoprecipitated with the anti-Flag antibody. It is worth noting that the level of this deletion mutant was apparently lower than that of wild type MDM2, due to its rapid degradation in the cytoplasm (12). Because the Δ150-320 mutant of MDM2 is localized only in the cytoplasm (124) and L5 is localized in both the cytoplasm and the nucleus (data not shown), these results indicate that L5 can bind to MDM2 in both the cytoplasm and the nucleus.

Ectopic expression of L5 induces p53 transcriptional activity and G1 cell cycle arrest. We (56) and others (177, 329) have shown that overexpression of either L11 or L23 induces p53 transcriptional activity. To determine if L5 has a similar effect, we introduced Flag-L5 into human osteosarcoma U2OS cells that contain endogenous wild-type p53. Interestingly, overexpression of Flag-L5 also markedly induced p53 in a dose-dependent fashion (Fig. 3.2A). Correspondingly, the levels of the p53 targets $p21^{cip1}$ and MDM2 were also induced. Overexpression of GFP-L5 had a similar effect on p53 and $p21^{cip1}$ induction (Fig. 3.3C). The induction of p53 by L5 occurred at the posttranscriptional level, as the mRNA level of p53 was not affected, while $p21^{cip1}$ induction occurred at the transcriptional level (Fig. 3.3D). This result suggests that overexpression of L5 stimulates p53 transcriptional activity. To further confirm this stimulatory effect on p53 transcriptional activity, we carried out luciferase assays using a luciferase reporter plasmid driven by a p53RE. Indeed, ectopic expression of L5 markedly stimulated p53RE-driven transcription in a dose-dependent manner (Fig. 3.2B)

in p53-proficient U2OS cells, but not in p53-deficient human osteosarcoma Saos-2 cells. This result indicates that the enhanced luciferase activity is p53-dependent.

To test whether induction of p53 and $p21^{cip1}$ levels by L5 leads to cell growth arrest, we introduced GFP-fused L5 or GFP alone into U2OS and Saos2 cells. Cells were then subjected to FACS analysis after treatment with the mitotic inhibitor nocodazole (24). GFP-positive cells were then gated for cell cycle analysis. A representative result in Fig. 3A shows that 26.6% of GFP-L5 expressing U2OS cells were arrested in the G1 phase, while only 5.9% of U2OS cells expressing GFP were detected in the G1 phase (Fig. 3.3A, 3.3B). The G1 arrest induced by L5 was dependent on p53 because no significant change was observed in p53-null Saos2 cells that contained ectopically expressed GFP-L5 (Figs. 3.3A, 3.3B). Correspondingly, both p53 and $p21^{cip1}$ protein levels (Fig. 3.3C) as well as the mRNA level of $p21^{cip1}$ (Fig. 3.3D) were induced by GFP-L5 but not GFP in U2OS cells. Taken together, these results demonstrate that ectopic expression of L5 induces p53 transcriptional activity and p53-dependent G1 arrest.

Overexpression of L5 inhibits MDM2-mediated p53 ubiquitination. To determine if p53 induction by L5 is due to inhibition of MDM2-mediated p53 ubiquitination and degradation, we introduced exogenous MDM2, p53 and L5 into p53-deficient human non-small cell carcinoma H1299 cells. Transfected cells were harvested 48 hours after transfection and 6 hours after treatment with the proteasome inhibitor MG132 for ubiquitination and immunoblot assays. As shown in Fig. 3.4A and by others (76, 105, 113), MDM2 ubiquitinated p53 (lane 4). By contrast, expression of L5 (lanes 5 and 6) remarkably inhibited p53 ubiquitination in a dose-dependent manner. This inhibition was not generated by sample loading, as the levels of all the proteins were

approximately equivalent (bottom panels of Fig. 3.4A). Consistently, expression of L5 also partially rescued MDM2-mediated p53 degradation in the absence of MG132 (compare lane 3 with lane 4 of Fig. 3.4B). These results indicate that L5 can stabilize p53 by alleviating MDM2-mediated p53 ubiquitination and degradation.

Actinomycin D induces L5-MDM2 interaction and activates p53, which is inhibited by L5 siRNA. Our recent study suggests that L23 may be involved in p53 activation induced by a low dose (5 nM) of Act D (56). Because 5 nM of Act D has been shown to specifically inhibit RNA polymerase I and thus to lead to perturbation of ribosomal biogenesis and p53 activation (6), we wanted to determine whether L5, like L11 (177, 329) and L23 (56), also plays a role in this ribosomal biogenesis stress-p53 pathway. To this end, siRNA against L5 was introduced into U2OS cells prior to treatment with 5 nM of ActD. As shown in Fig. 3.5A, similar to the case of L23 (56), siRNA against L5 also drastically inhibited Act D-induced p53 level (5.5 fold reduction, compare lane 4 to lane 3 of top panel) and activation evident by the reduced MDM2 and $p21^{cip1}$ levels (lane 4 of second and third panels), suggesting that L5 might also be an important regulator of the Act D-p53 pathway. However, unlike in the case of L23, in which L23 reduction by its siRNA drastically induced both p53 and MDM2 (56), ablation of L5 by its siRNA slightly reduced the p53 level in Act D-untreated cells (2.1 fold reduction, compare lane 2 to lane 1 of top panel). This slight reduction of p53 by L5 siRNA does not contradict the result above showing that siRNA against L5 inhibited Act D-induced p53 level, because the reduction (5.5 fold) of p53 by L5 siRNA in cells treated with Act D was much greater than that in cells without Act D treatment (2.1 fold). Of note, the slight decrease of p53 in L5 siRNA treated cells was specific to p53, it was not

due to global translational inhibition after reduction of L5 by its siRNA. First, the level of another short-living cyclin-dependent kinase inhibitor $p27^{kip1}$ was not affected by L5 siRNA treatment (Fig. 3.5A, lanes 2 and 4 of fourth panel) compared to scramble RNA transfected cells (lanes 1 and 3). Second, as shown in Fig. 3.5B, L5 siRNA treatment did not significantly reduce the overall protein level (compare lane 2 to lane 1) and protein synthesis (compare lane 4 to lane 3) as determined by silver staining and ^{35}S-methionine pulse labeling experiments, respectively. These results indicate that partial reduction of L5 in a short time (slightly more than half reduction of L5 level in less than 48 hours in our assays) may not significantly affect global translation (Fig. 3.5A, third lower panel). This phenotype is also supported by a recent knock out study, showing that mice heterozygous for the disrupted ribosomal s19 allele displayed normal growth and organ development though homozygous mice were embryonic lethal (184).

Next, we also examined the interaction between L5 and MDM2 after Act D treatment in U2OS cells. The cells were treated with or without 5 nM of Act D for 8 hours and used for immunoprecipitation with either monoclonal anti-MDM2 4B11 or polyclonal anti-L5 antibodies followed by immunoblotting with anti-MDM2 2A10 or anti-L5 antibodies. As shown in Fig. 3.5C, indeed, the interaction between endogenous L5 and MDM2 was observed in cells treated with Act D, as detected by co-immunoprecipitation with either anti-L5 or anti-MDM2 antibodies, followed by immunoblot with both of the antibodies (lanes 3-6). The Act D-induced MDM2-L5 interaction was not due to the induced level of MDM2 by Act D, because when we treated the cells with a proteasome inhibitor MG132 to normalize the MDM2 levels between Act D-treated and untreated cells 6 hours before harvesting, the enhanced

MDM2-L5 interaction was still observed in Act D treated cells compared to cells without Act D treatment (data not shown). Taken together, these data show that there is an Act D-induced association of L5 and MDM2 in cells, indicating that like L23 and L11 (56) (data not shown), L5 may also play a role in Act D-induced p53 activation.

MDM2 complexes with L5, L11 and L23 in cells in the presence or absence of p53. We have thus far learned that all of the L5, L11 and L23 proteins can activate p53 by alleviating MDM2-mediated p53 ubiquitination and degradation (56, 329). Because all three ribosomal proteins are present in our initially purified MDM2-associated complex (56), it is highly likely that MDM2 simultaneously associates with these ribosomal proteins in the same complex. To test this idea, a set of reciprocal co-immunoprecipitation assays were performed. H1299 cells were transfected with HA-MDM2, Flag-L5, and p53 as indicated in Fig. 3.6A. Cell lysates were co-immunoprecipitated with anti-HA, anti-Flag, anti-p53, anti-L11, or anti-L23 antibody, respectively. As shown in Fig. 3.6A, L5, L11, and L23 were all specifically co-immunoprecipitated with HA-MDM2 by the anti-HA antibody in the absence of p53 (lane 2). The same complex was also immunoprecipitated by anti-Flag (lane 6), anti-L23 (lane 10), and anti-L11 (lane 14) antibodies, respectively. These results indicate that overexpressed MDM2 can form a complex with L5, L11, and L23 (MDM2-L5-L11-L23 complex) independently of p53 in cells. Interestingly, when p53 was co-expressed, the level of this MDM2-L5-L11-L23 complex was reduced, although p53 also associated with this quadruple complex as co-immunoprecipitated by almost all of the corresponding antibodies (lanes 3, 7, 11, and 15). Also, the anti-p53 antibody co-immunoprecipitated MDM2, L5, L11, and L23 (lane 19). The p53-MDM2-L5-L11-L23

complex may represent an intermediate complex, as the stoichiometry of the components in this complex was different from that of the quadruple complex (compare lanes 2, 6, 10 and 14 with lanes 3, 7, 11 and 15). This difference was not due to varying expression levels of these proteins, as all of these proteins were expressed equivalently in each transfection (lanes 21-24). Also this difference suggests that the ribosomal proteins may partially compete with p53 for binding to MDM2, though they bind to different domains of MDM2 (43, 221). These L proteins did not appear to directly interact with one another as none of them was co-immunoprecipitated with the antibody against each of these proteins in the absence of overexpressed MDM2 (lanes 5, 9 and 13). In addition, p53 did not appear to bind to these L proteins directly, as p53 was not co-immunoprecipitated by antibodies against Flag, L11 and L23 (lanes 8, 12 and 16), nor were the L proteins pulled down by the anti-p53 antibody in the absence of overexpressed MDM2 (lane 20). In summary, these results indicate that L5, L11 and L23 can associate with overexpressed MDM2 simultaneously.

To determine whether endogenous MDM2 and L proteins form a complex in response to Act D treatment, we performed similar reciprocal co-immunoprecipitation assays using antibodies against L23 and MDM2. U2OS cells were treated with 5 nM Act D for 8 hours. Cell lysates were co-immunoprecipitated with anti-MDM2 (4B11) or anti-L23 antibodies followed by immunoblot with anti-MDM2 (2A10), anti-L5, anti-L11, or anti-L23 antibodies. As shown in Fig. 3.6B, MDM2, L5, L11, and L23 were all co-immunoprecipitated with anti-MDM2 and anti-L23 antibodies (lanes 2 and 4), respectively, after Act D treatment. These results demonstrate that MDM2, L5, L11, and

L23 form a complex in cells in response to Act D treatment, consequently leading to inhibition of MDM2-mediated p53 ubiquitination and degradation.

DISCUSSION

The p53-MDM2 feedback loop is critical for maintaining low levels of p53, therefore blocking this loop is necessary for p53 induction and activation in response to stresses (225). Ribosomal biogenesis stresses, such as deregulation of ribosomal RNA synthesis by Act D, processing, and assembly, also activate p53 (5, 6, 234, 283). Recently, two ribosomal proteins L11 and L23 have been shown to mediate p53 activation by blocking the MDM2-p53 loop (56, 177, 329). Our study described here reveals another ribosomal protein L5 that also plays a crucial role in p53 activation after the stress induced by 5 nM of Act D. Several lines of evidence support this notion. First, we verified the previously reported interaction between L5 and MDM2 *in vitro* and in cells (182) (Fig. 3.1). Because we were able to produce anti-L5 antibody, we also demonstrated the interaction between endogenous MDM2 and L5 *in vivo*. Further we found that overexpression of L5 induced endogenous p53 and stabilized exogenously expressed p53 (Figs. 3.2 and 3.3). Consequently, p53 transcriptional activity was stimulated to induce $p21^{cip1}$ and G1 arrest (Figs. 3.2 and 3.3). Like L11 and L23, L5 also inhibited MDM2-mediated p53 ubiquitination (Fig. 3.4). Consistently, ribosomal stress caused by exposure to a low dose of Act D enhanced the L5-MDM2 interaction and p53 activation, which was impaired by siRNA ablation of L5 (Fig. 3.5). Although a recent report by others (9) did not show this L5 effect, their results may be due to the possibility that L5 was not well expressed in their transient transfection experiments because there

was not a figure showing L5 expression. Our results strongly demonstrate that L5 is also an important player in the ribosomal biogenesis stress-p53 pathway.

Interestingly, all the three large ribosomal proteins L5, L11 and L23 have been found to activate p53 by negating the MDM2-mediated feedback inhibition of p53 in response to the same type of stresses caused by Act D (56, 177, 329). These studies suggest a common pathway to activate p53 in response to ribosomal stress. This stress, such as inhibition of rRNA synthesis or ribosomal assembly, releases free ribosomal L5, L11 and L23 proteins, which in turn associate with MDM2 and thus inhibit its ubiquitin ligase activity toward p53, and as a result, lead to p53 induction and activation. Although each of these three L proteins when overexpressed can inhibit MDM2 function and activate p53 (7-9) (and this study), these ribosomal proteins can also form a quadruple complex with MDM2 in response to Act D treatment (Fig. 3.6), probably working in concert to negate the MDM2 feedback regulation of p53. Supporting this model is the fact that decreasing the level of each of these L proteins by siRNA markedly reduces Act D-induced p53 activation. This result also suggests that each of these ribosomal proteins is essential for activating p53 as the absence of any one of them impaired Act D-induced p53 activation (56, 329) (data not shown). The finding that the quadruple complex is markedly reduced in the presence of high levels of p53 (Fig. 3.6A) suggests that these ribosomal proteins may compete with p53 for binding to MDM2. Although a low level of the L5-L11-L23-MDM2-p53 complex was also detected, this complex might be dynamic or transient (Fig. 3.7). Because all the ribosomal proteins can inhibit MDM2-mediated p53 ubiquitination (56, 329) (and this study), it is likely that they execute such inhibitory effects by physically interacting with MDM2 (E3) and thus causing a steric hindrance

that prevents the transfer of the ubiquitin moiety from E2 to p53 (Fig. 3.7). Of note, all of these ribosomal proteins tend to bind to the MDM2 middle region containing the acidic domain (56, 74, 177, 329), and this acidic domain has been recently shown to be critical for MDM2-mediated p53 degradation (133, 195). It is also possible that p53 stabilization by L5, L11 and L23 in response to ribosomal stress may partly attribute to concealing the acidic domain of MDM2 by these L proteins, although how exactly the acidic domain of MDM2 contributes to the regulation of p53 stability is unclear (133, 195).

The finding that L5 induces p53 by suppressing the MDM2-p53 feedback loop raises another important question of whether L5 affects the MDM2 and p53 subcellular localization because L5 has been reported to be an intracellular 5S rRNA transport factor (5S rRNA-L5 ribonucleoprotein particle) (50). In addition to binding to MDM2, L5 also binds to the eukaryotic initiation factor 5A (261), which is a critical cofactor of the Rev transactivator of human immunodeficiency virus type 1 (HIV-1). Both MDM2 and Rev are nucleocytoplasmic shuttle proteins (85, 330) and the nuclear export pathways for Rev and 5S rRNA share common components (85). Furthermore, L5 itself contains a defined nuclear localization signal (NLS) and a nuclear export signal (NES) (250). Therefore, it is likely that L5 may modulate MDM2 nuclearcytoplasmic shuttling. We have shown that the MDM2-L5 (this study) and MDM2-L23 (56) interaction can occur in both the cytoplasm and the nucleus because an NLS deletion mutant of MDM2 (MDM2Δ150-320) that is exclusively expressed in the cytoplasm was efficiently co-immunoprecipitated with L5 and L23. Another speculation would be that L5, L11, or L23 may serve as cytoplasmic anchor proteins for MDM2.

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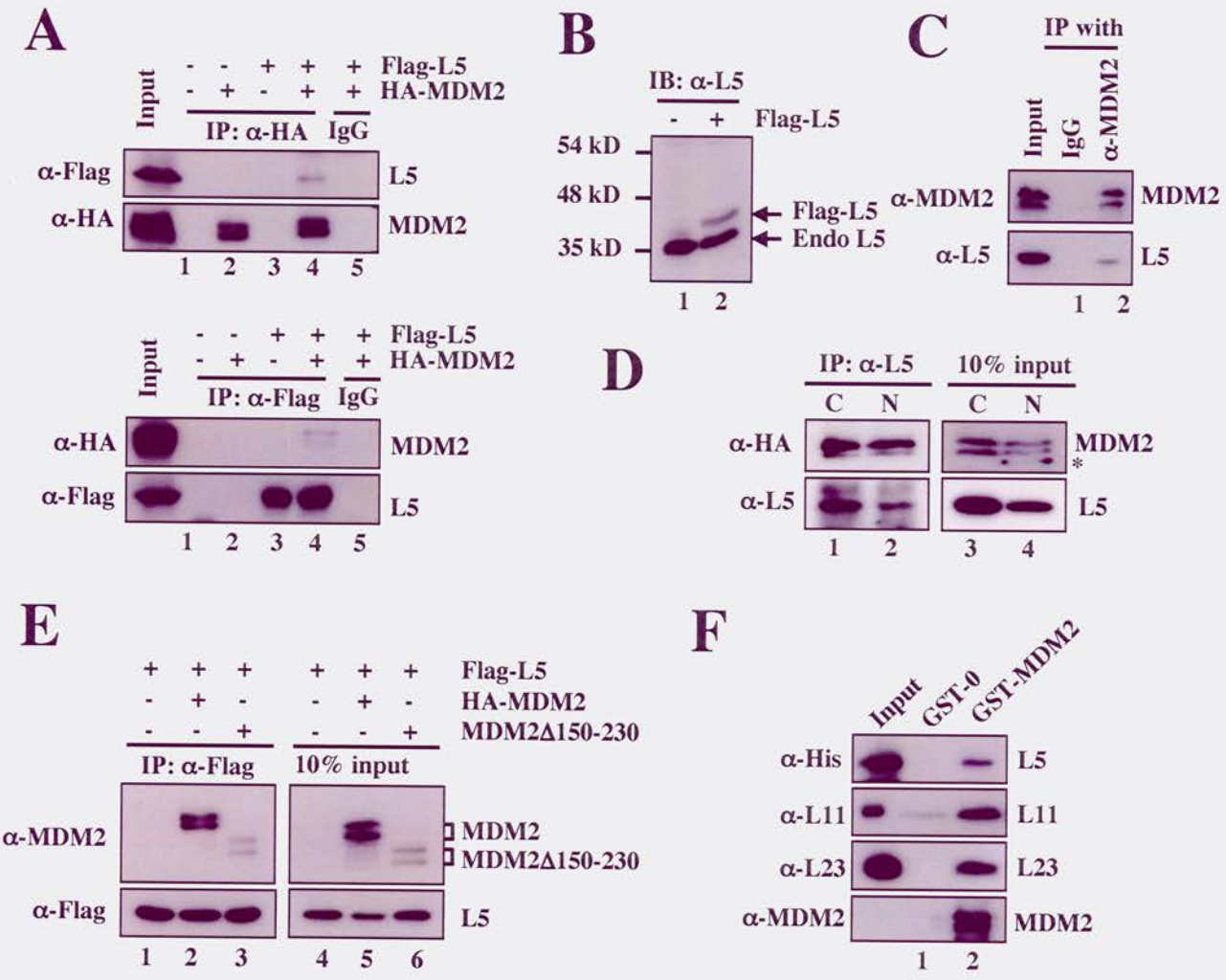
Fig. 3.1. L5 interacts with MDM2 in cells and in vitro. (**A**). MDM2 interacts with L5 in 293 cells. The cells were transfected with HA-MDM2 (1.5 μg), or Flag-L5 (1.5 μg), or both vectors (1.5 μg each). Whole cell lysates were immunoprecipitated with anti-HA, anti-Flag, or control antibodies followed by immunoblot with anti-Flag or anti-HA antibodies. (**B**). The anti-L5 antibody. 293 cells were transfected with Flag-L5 (lane 2) or empty vector (lane 1). The cell lysates were subjected to immunoblot with polyclonal anti-L5 antibody. (C). Endogenous MDM2 associates with endogenous L5 in cells. The whole cell lysates prepared from SJSA cells were immunoprecipitated with polyclonal anti-MDM2 antibody (lane 2) or control rabbit serum (lane 1). The immunoprecipitates were immunoblotted with monoclonal anti-MDM2 (2A10) or anti-L5 antibodies. (D). MDM2 interacts with L5 in both the nucleus and the cytoplasm. The 293-HA-MDM2 cells were fractionated into both cytoplasmic (C) and nuclear (N) fractions. Equal amounts of both reactions were immunoprecipitated with anti-L5 antibody followed by immunoblot with anti-HA or anti-L5 antibodies. A nonspecific band recognized by anti-HA antibody is specifically shown in nuclear fraction (asterisk), indicating that cytoplasmic fraction was not contaminated by nuclear fraction. (E). L5 binds to MDM2 deletion mutant (MDM2Δ150-230) in cells. 293 cells were transfected with 3 μg of empty vector (-), wild type MDM2 or MDM2Δ150-230 together with 3 μg of Flag-L5 plasmids, respectively, as indicated. Whole cell lysates were immunoprecipitated with anti-Flag antibodies followed by immnoblotting with anti-MDM2 (2A10). (F). MDM2 directly binds to L5 in vitro. Purified GST or GST-MDM2 immobilized on glutathione beads was incubated with 200 ng of bacterially purified His-L5, or His-L11, or His-L23. Bound proteins were blotted with corresponding antibodies.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Input
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IgG
Flag-L5
HA-MDM2
α-Flag
α-HA
L5
MDM2
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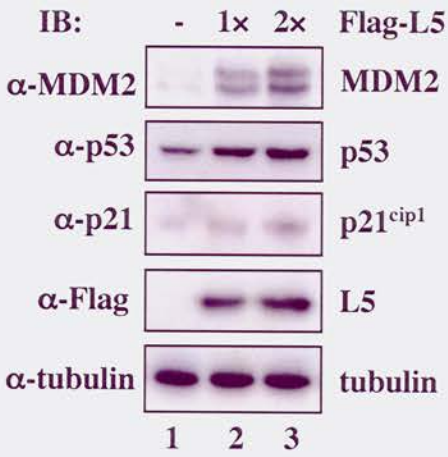
B
IB: α-L5
54 kD
48 kD
35 kD
Flag-L5
Endo L5
C
IP with
α-MDM2
D
IP: α-L5
10% input
C N
E
MDM2Δ150-230
F
GST-0
GST-MDM2
α-His
α-L11
α-L23
L11
L23

Fig. 3.2. Ectopic expression of L5 induces p53 and stimulates p53-dependent transcription. (**A**). Ectopic expression of L5 induces endogenous p53. U2OS cells were transfected with empty vector (-), 1.0 μg (1×) or 2.0 μg (2×) of Flag-L5 plasmid. Cell lysates were used for immunoblot with antibodies as indicated on left. (B). Ectopic expression of L5 increases p53 and p53RE-dependent luciferase activity. U2OS cells or Saos2 cells were transfected with increasing amounts of Flag-L5 (0.4 μg, 1x and 0.8 μg, 2x) in the presence of a luciferase reporter plasmid driven by the p53RE (BP100 luc, 0.1μg) or a control luciferase reporter plasmid (TB luc, 0.1 μg). Luciferase activity was presented in arbitrary units.

A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-
1×
2×
Flag-L5
α-MDM2
MDM2
α-p53
p53
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p21cip1
α-Flag
L5
α-tubulin
tubulin
1
2
3

B

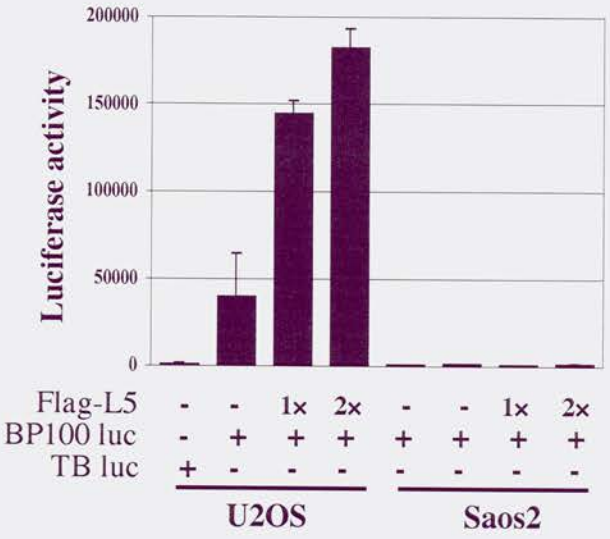
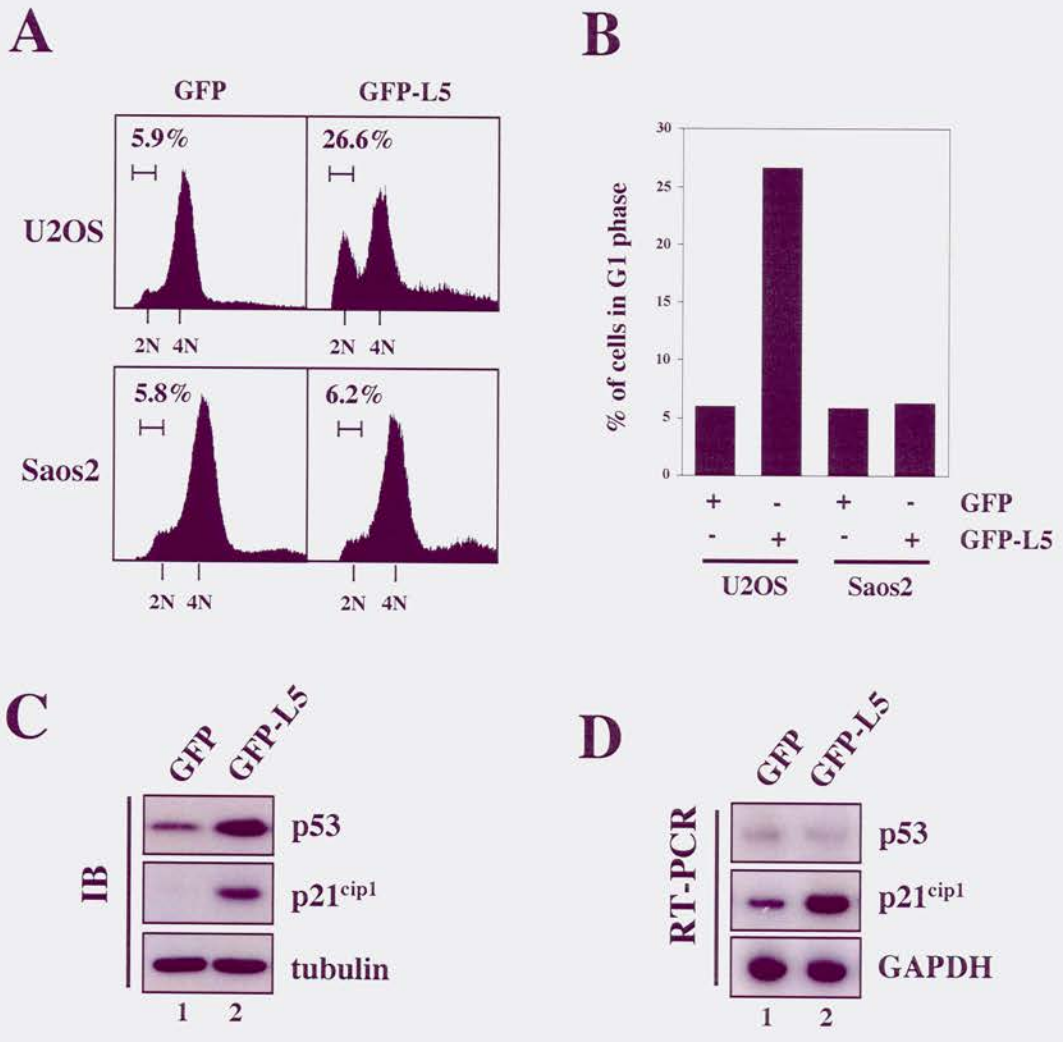

Luciferase activity
200000
150000
100000
50000
0
Flag-L5 - - 1× 2× - - 1× 2×
BP100 luc - + + + + + + +
TB luc + - - - - - - -
U2OS
Saos2

Fig. 3.3. Ectopic expression of L5 induced p53-dependent G1 cell cycle arrest. (A). Ectopic expression of L5 leads to p53-dependent G1 cell cycle arrest. U2OS and Saos2 cells were transfected with GFP (2 μg) or GFP-L5 (2 μg) plasmid and treated with nocodazole as described in the experimental procedures. GFP-expressing cells were then gated for cell cycle analysis. The histograms of PI staining from one representative experiment are shown. Percentage indicates the cells that were arrested in G1 phase and shown by bars on (B). (C). The above transfections were used for immunoblot with anti-p53, anti-$p21^{cip1}$, and anti-tubulin antibodies. (D). The above transfections were used for RT-PCR analysis to detect p53, $p21^{cip1}$ and GAPDH mRNAs.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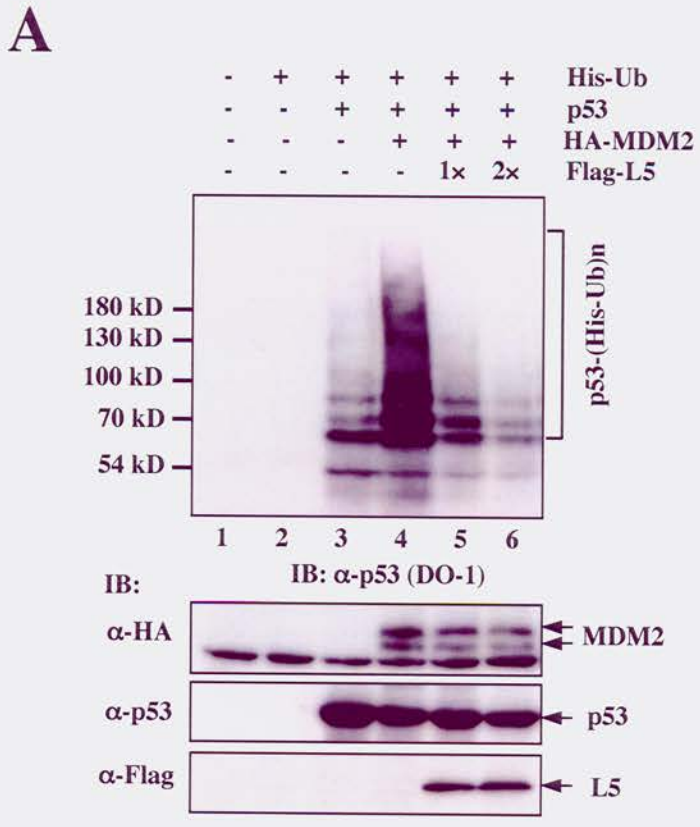
GFP
GFP-L5
5.9%
26.6%
U2OS
2N 4N
2N 4N
5.8%
6.2%
Saos2
2N 4N
2N 4N
B
% of cells in G1 phase
30
25
20
15
10
5
0
+ - + - GFP
- + - + GFP-L5
U2OS
Saos2
C
GFP
GFP-L5
IB
p53
p21cip1
tubulin
1 2
D
GFP
GFP-L5
RT-PCR
p53
p21cip1
GAPDH
1 2

Fig. 3.4. L5 inhibits MDM2-mediated p53 ubiquintination. (**A**). L5 inhibits MDM2-mediated p53 ubiquitination in cells. H1299 cells were transfected with combinations of Flag-L5 (1 μg, 1x; and 2 μg, 2x), p53 (1 μg), or MDM2 (1 μg) plasmids in the presence of the His-ubiquitin (His-Ub) (2 μg) plasmid as indicated. The cells were treated with MG132 (20 μM) for 6 hours before harvesting. The *in vivo* ubiquitination assay was performed as described in the experimental procedures. Ubiquitinated proteins were detected by immunoblot with the anti-p53 (DO-1) antibody. Ubiquitinated p53 (p53-(His-Ub)n) is indicated. The expression of MDM2, p53, and L5 was shown in the lower panels. (**B**). Ectopic expression of L5 reverses MDM2-mediated p53 degradation. H1299 cells were transfected with 1.0 μg of Flag-L5, p53 (0.5 μg), MDM2 (1.0 μg) as indicated. Cell lysates (50 μg) were immonublotted with anti-HA, anti-p53, or anti-Flag antibodies as indicated on the left.


A
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- - + + + + p53
- - - + + + HA-MDM2
- - - - 1× 2× Flag-L5
180 kD
130 kD
100 kD
70 kD
54 kD
p53-(His-Ub)n
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MDM2
α-p53
p53
α-Flag
L5

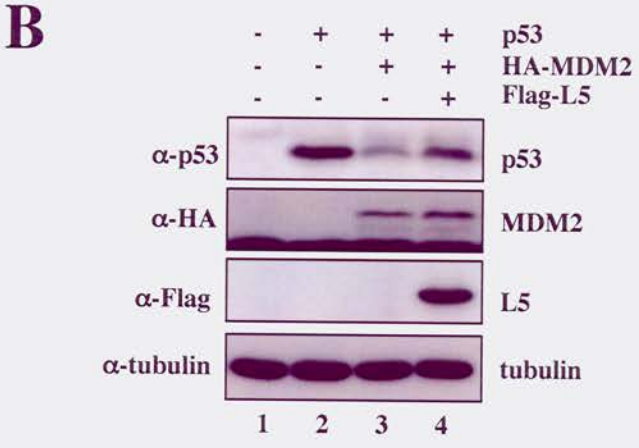
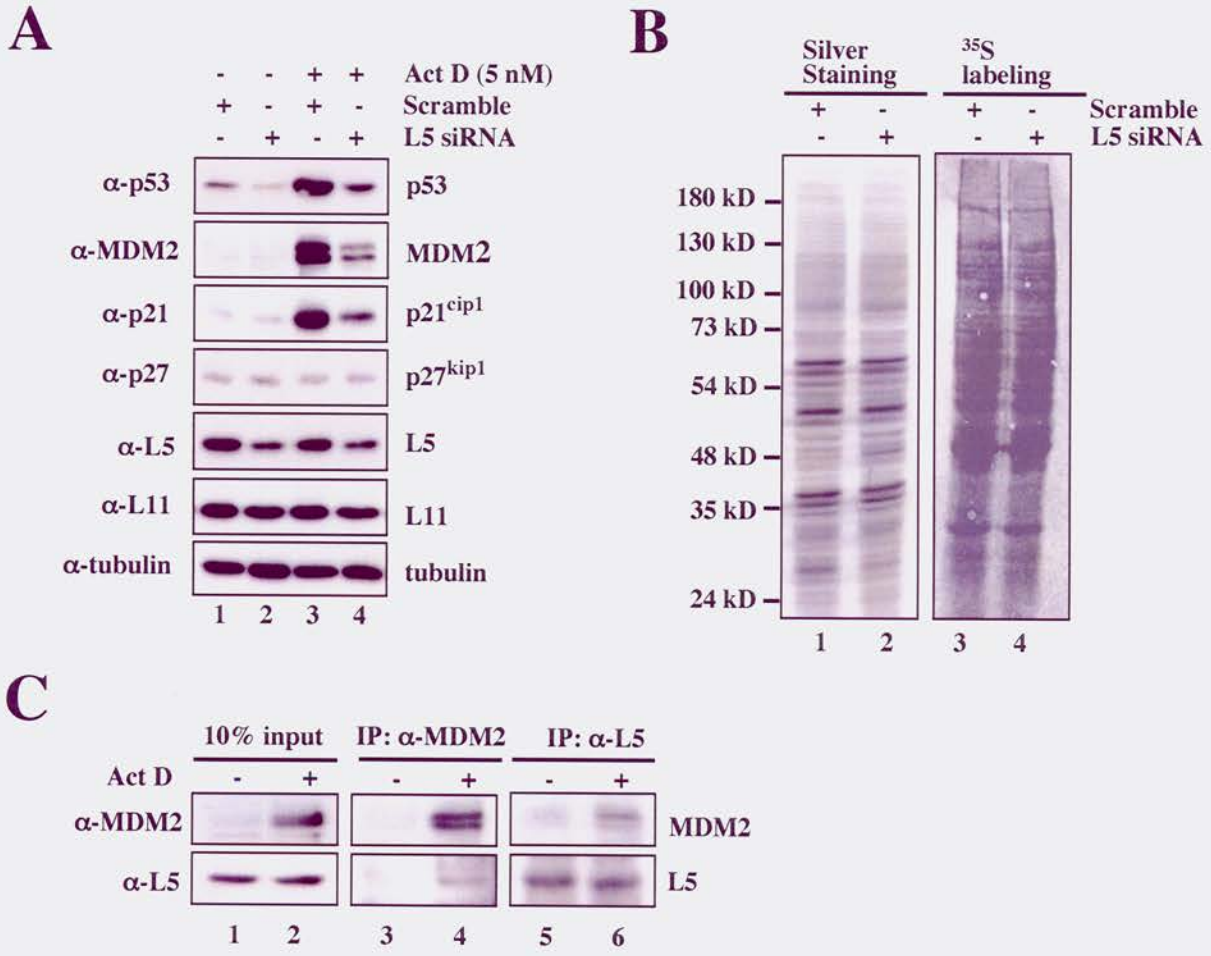

B
- + + + p53
- - + + HA-MDM2
- - - + Flag-L5
α-p53
p53
α-HA
MDM2
α-Flag
L5
α-tubulin
tubulin
1 2 3 4

Fig. 3.5. Act D-induced p53 activation is inhibited by siRNA ablation of L5 and MDM2-L5 interaction is enhanced by Act D treatment. (**A**). Ablation of endogenous L5 by siRNA inhibits Act D-induced p53. U2OS cells were transfected with L5 siRNA oligonucleotides (0.2 μM, lanes 2 and 4) or with scrambled RNA duplex (0.2 μM, lanes 1 and 3). Cells were then incubated with (lanes 3 and 4) or without (lanes 1 and 2) 5 nM of Act D for 8 hours before harvesting. The cells were then harvested after 48 hours. Cell lysates were immunoblotted with anti-MDM2, anti-p53, anti-$p21^{cip1}$, $p27^{kip1}$, anti-L5, anti-L11, or anti-tubulin antibodies. (**B**). Ablation of L5 by siRNA does not affect global translation. U2OS cells were transfected with L5 siRNA (lanes 2 and 4) or scramble RNA (lanes 1 and 3) as mentioned on (A). The cells were directly lysed (lanes 1 and 2), or pulse-labeled with 50 μCi/ml of ^{35}S-methionine for 30 minutes (lanes 3 and 4). Equal amounts of total proteins were loaded for either silver staining (lane 1 and 2) and exposed to film for detecting labeled radioactive proteins, respectively (lanes 3 and 4). (C). A low dose of Act D enhances the MDM2-L5 interaction. U2OS cells were treated with Act D (5 nM) for 8 hours. Cell lysates were immunoprecipitated with anti-MDM2 (4B11) antibody (lanes 3 and 4) or anti-L5 antibody (lanes 5 and 6) and immunobloted with anti-MDM2 (2A10) or anti-L5 antibodies. The lysates were also directly loaded onto a SDS gel for immunoblot analysis with anti-MDM2 or anti-L5 antibodies as loading controls (lanes 1 and 2).


A
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+ - + - Scramble
- + - + L5 siRNA
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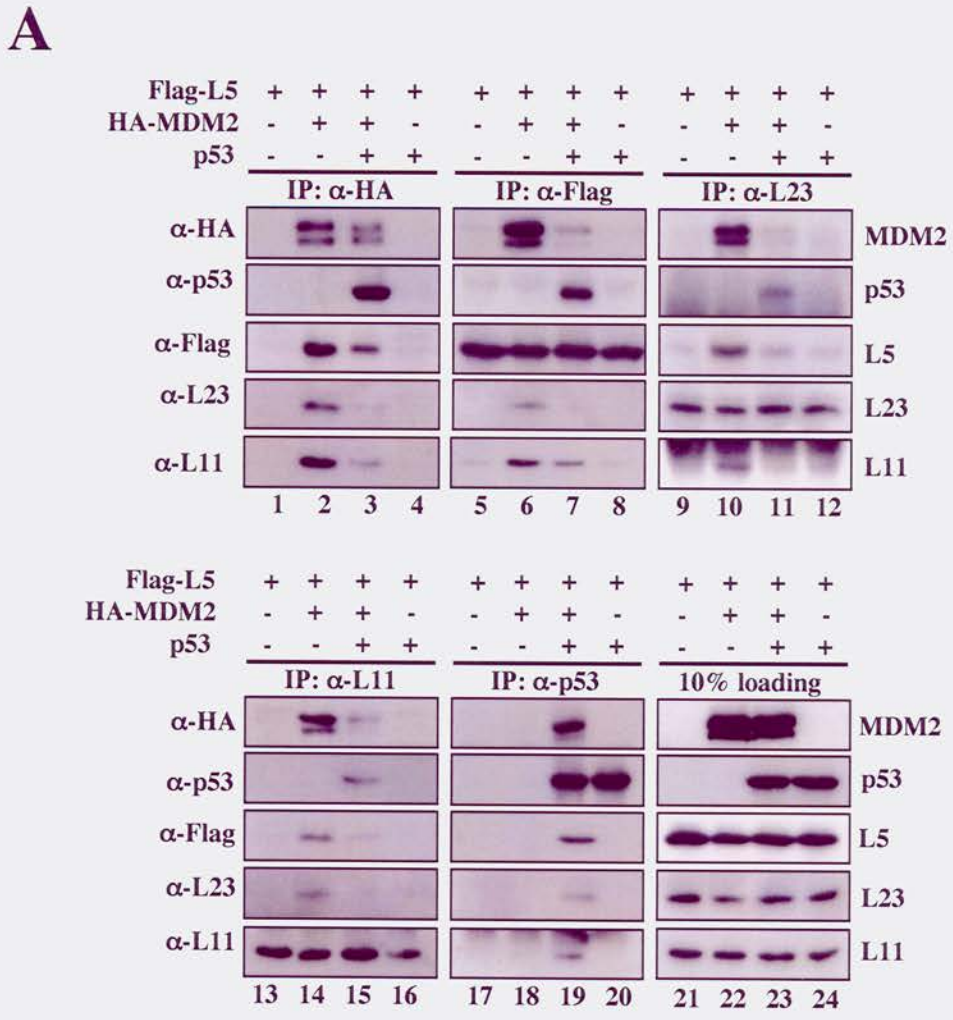
p53
α-MDM2
MDM2
α-p21
p21cip1
α-p27
p27kip1
α-L5
L5
α-L11
L11
α-tubulin
tubulin
1 2 3 4
B
Silver Staining
35S labeling
+ - + - Scramble
- + - + L5 siRNA
180 kD
130 kD
100 kD
73 kD
54 kD
48 kD
35 kD
24 kD
1 2 3 4
C
10% input
IP: α-MDM2
IP: α-L5
Act D - + - + - +
α-MDM2
α-L5
MDM2
L5
1 2 3 4 5 6

Fig. 3.6. MDM2 associates with L5, L11, and L23 in the presence or in the absence of p53. (A). Exogenously expressed L5 associates with MDM2, L11, and L23 in the presence or in the absence of p53. H1299 cells were transfected with Flag-L5, MDM2, and p53 as indicated. The cell lysates were immunoprecipitated with anti-HA (lanes 1-4), anti-Flag (lanes 5-8), anti-L23 (lanes 9-12), anti-L11 (lanes 13-16), and anti-p53 (lanes 17-20). The immunoprecipitates were blotted with anti-HA, anti-Flag, anti-L23, anti-L11, and anti-p53 antibodies as indicated. The cell lysates were also directly loaded on SDS gel and blotted with above antibodies as loading controls. (B). Endogenous MDM2 associates with endogenous L5, L11, and L23 in the presence of 5 nM Act D. U2OS cells were treated with (lanes 2, 4, and 6) or without (lanes 1, 3, and 5) Act D (5 nM) for 8 hours. Cell lysates were immunoprecipitated with anti-MDM2 (4B11) antibody (lanes 1 and 2) or anti-L23 antibody (lanes 3 and 4) and immunobloted with anti-MDM2 (2A10), anti-L5, anti-L11 or anti-L23 antibodies. The lysates were also directly loaded onto a SDS gel for immunoblot analysis with above antibodies as loading controls (lanes 5 and 6).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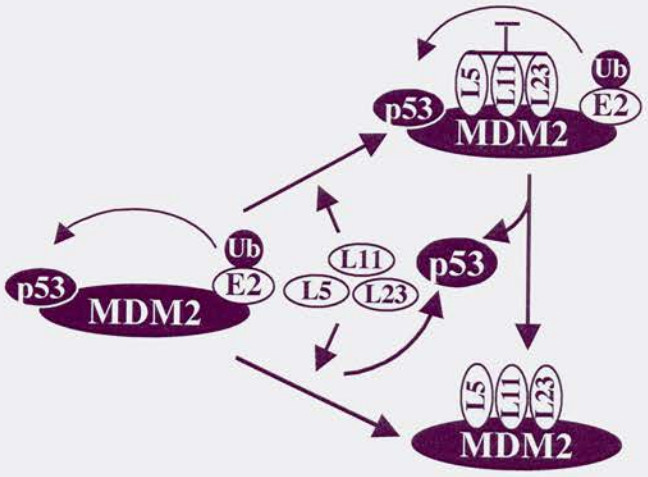
Flag-L5
HA-MDM2
p53
IP: α-HA
IP: α-Flag
IP: α-L23
α-HA
α-p53
α-Flag
α-L23
α-L11
MDM2
p53
L5
L23
L11
1 2 3 4 5 6 7 8 9 10 11 12
IP: α-L11
IP: α-p53
10% loading
13 14 15 16 17 18 19 20 21 22 23 24
B
IP: α-MDM2
IP: α-L23
10% loading
Act D
α-L5
α-L11
α-L23
α-MDM2
1 2 3 4 5 6

Fig. 3.7. A schematic model for the function of the MDM2-L5-L11-L23 complex. The MDM2-L5-L11-L23 and MDM2-L5-L11-L23-p53 complexes were discussed in the text.


L5
L11
L23
Ub
E2
p53
MDM2
L11
L5
L23
p53
Ub
E2
p53
MDM2
L5
L11
L23
MDM2

CHAPTER FOUR

Differential regulation of MDM2-mediated p53 ubiquitination and MDM2 auto-ubiquitination by ribosomal protein L11

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Key Words: L11, L5, L23, $p14^{ARF}$, MDM2, p53, ribosome proteins, ubiquitination.

Abbreviations: ARF: alternative reading frame; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; GFP, green fluorescence protein; Ub, ubiquitin.

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ABSTRACT

Inhibition of the MDM2-p53 feedback loop is critical for p53 activation in response to cellular stresses. The ribosomal proteins L5, L11, and L23 can block this loop by inhibiting MDM2-mediated p53 ubiquitination and degradation in response to ribosomal stress. Here, we show that L11, but not L5 and L23, leads to a dramatic accumulation of the ubiquitinated species and the steady-state level of MDM2. This effect is dependent on the ubiquitin ligase activity of MDM2, but not p53, and requires the central MDM2-binding domain (residues 51 to 108) of L11. Also, L11 prolongs the half-life of MDM2. Although ectopic expression of L11 was previously shown to relocalize MDM2 to the nucleolus, L11 does not appear to require nucleolus localization to regulate MDM2 and to activate p53. These results suggest that while L11 inhibits MDM2-mediated p53 ubiquitination, it, unlike L23 and L5, enhances MDM2 auto-ubiquitination and also stabilizes MDM2 perhaps through a potential post-ubiquitination mechanism.

INTRODUCTION

The tumor suppressor protein p53 is a transcription factor activated in response to stress to induce expression of its target genes. The proteins encoded by these genes then mediate multiple cellular responses, such as cell cycle arrest, apoptosis, differentiation, cell senescence, or DNA repair (224). Also, p53 can directly trigger mitochondria-apoptosis in mitochondria in response to DNA damage (46, 69, 198). The tumor suppressor role of p53 is well reflected in the fact that more than half of human tumors harbor mutations in the p53 gene and many others retain impaired function of the p53 pathway (277, 303). Because of its inhibitory effect on cell growth, p53 is maintained at a

low steady-state level and in an inert form in physiological conditions. This duty is mainly fulfilled by the E3 ubiquitin ligase MDM2 that mediates p53 constant degradation through an ubiquitin-dependent proteasome pathway (105, 146). The mdm2 gene itself is a down stream target of p53, thus forming a tight autoregulatory feedback loop (11, 235, 318). Consistently, gene amplification and overexpression of MDM2 have also been shown in a variety of tumors, particularly in soft tissue sarcomas, carcinomas, lymphomas, breast and lung cancers (53, 63, 71, 201, 311). Interfering with the MDM2-p53 feedback loop leads to activation of p53 and ultimately the prevention of neoplasia. One example of this regulation is ARF[1] (alternative reading frame, $p14^{ARF}$ in human, $p19^{ARF}$ in mouse)-mediated inhibition of this loop in response to oncogene overexpression such as c-Myc and RAS (228, 339). Also, in response to DNA damaging agents, NH_2-terminal serine/threonine phosphorylation at the MDM2 binding domain of p53 interrupts the MDM2-p53 interaction and activates p53 (9, 35, 186, 264, 273). Hence, the MDM2-p53 loop presents as a central regulatory point in response to various stress.

Increasing evidence shows that the MDM2-p53 feedback loop can also be regulated by ribosomal stress. Fine coordination between ribosomal biogenesis and other cellular functions, such as the cell cycle and differentiation, is important for normal cell growth (205, 254). Impeding ribosomal biogenesis would generate ribosomal stress that activates p53 to stop cell growth. Such stress has been exampled as either the inhibition of ribosomal RNA (rRNA) synthesis and processing by a low dose (<5 nM) of actinomycin D or overexpression of a dominant-negative mutant of the rRNA processing factor Bop1 (6, 234). In accordance with these cellular studies, malfunctions of ribosomal biogenesis have been proposed to correlate to human cancers (256). Recently, we and others

reported that ribosomal proteins L5, L11, and L23 interacted with MDM2 and inhibited the MDM2-p53 feedback loop in response to ribosomal stress, such as treatment of a low dose of actinomycin D, serum starvation, or possibly small interference RNA-induced reduction of L23 (18, 57, 58, 122, 177, 329). Interestingly, these L proteins as well as the tumor suppressor protein ARF are primarily nucleolar proteins. Disruption of the nucleolus appears to be a common event in stress-induced p53 activation pathways (253). Thus, releasing small protein molecules such as the ribosomal L proteins from the nucleolus leads to p53 activation in response to ribosomal stress.

While ectopic expression of L5, L11, and L23 has been shown to inhibit MDM2-mediated p53 ubiquitination and degradation (57, 58, 122, 177, 329), detailed mechanisms underlying this effect remain largely indeterminate. Also, little is known about the effect of these L proteins on MDM2 stability and ubiquitination. We have begun to address these issues by performing a series of cellular and biochemical analyses. Here, we report that these ribosomal L proteins differentially regulate MDM2 ubiquitination. We found that unlike L5 and L23, which drastically inhibited ubiquitination of both p53 and MDM2, L11 slightly inhibited MDM2-mediated p53 ubiquitination but remarkably increased the ubiquitinated species and the steady-state level of MDM2. This effect was dependent on the ubiquitin ligase activity of MDM2, but not p53, and required the central MDM2-binding domain of L11. Consistently, L11 prolonged the half-life of MDM2. In summary, these results suggest that MDM2-mediated p53 ubiquitination and MDM2 auto-ubiquitination are differentially regulated by L11, and that L11, unlike L5 and L23, may block the MDM2-dependent proteasomal degradation pathway through a potential post-ubiquitination mechanism.

MATERIALS AND METHODS

Cell lines, plasmids and antibodies. Human lung nonsmall cell adenocarcinoma H1299 cells, human oesteosarcoma U2OS cells, and mouse $p53^{-/-}/mdm2^{-/-}$ MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 50 U/ml penicillin and 0.1 mg/ml streptomycin at 37°C in a 5% CO_2 humidified atmosphere as previously described (58).

The Flag-tagged L5, L11, and L23 expression plasmids have been described previously (57, 58, 177). All the deletion mutants of L11 were generated using PCR and cloned into pcDNA3-2Flag vector. The HA-MDM2 expression vector has been described (58). The full-length $p14^{ARF}$ (ARF, hereafter) was amplified using PCR and inserted into pcDNA3-2Flag or pcDNA3-V5 vectors to generate pcDNA3-2Flag-ARF and pcDNA3-V5-ARF, respectively. The MDM2 mutant with a point mutation at position 464 from cystidine to analine ($MDM2^{C464A}$) was generously provided by Dr. Karen H. Vousden (76).

Anti-L11 antibodies were described previously (26). Anti-Flag (Sigma), anti-p21 (NeoMarkers), and anti-p53 (DO-1, Santa Cruz) were purchased. Anti-MDM2 (2A10) and anti-HA (12CA5) have been described (58).

Cotransfection, immunoblot and co-immunoprecipitation analyses. H1299, U2OS, or $p53^{-/-}/mdm2^{-/-}$ MEFs cells were transfected with plasmids as indicated in each figure legend using Lipofectin following the manufacturer's protocol (Invitrogen). Cells were harvested at 48 hours posttransfection and lysed in lysis buffer consisted of 50 mM Tris/HCl (pH 8.0), 0.5% Nonidet P-40, 1 mM EDTA, 150 mM NaCl, and 1 mM

phenylmethylsulfonyl fluoride (PMSF). Equal amounts of clear cell lysate were used for immunoblot analysis as described previously (58). Immunoprecipitation was conducted using antibodies as indicated in the figure legends and described previously (58). Beads were washed with lysis buffer twice, once with SNNTE buffer (50 mM Tris/HCl (pH 7.4), 5 mM EDTA, 1% Nonidet P-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.

***In vivo* ubiquitination assay.** *In vivo* ubiquitination assays were conducted as previously described (58). Briefly, H1299 cells or $p53^{-/-}/mdm2^{-/-}$ MEFs (60% confluence/100 mm plate) were transfected with combinations of the following plasmids as indicated in the figure legends: His_6-ubiquitin (2 μg), p53 (2 μg), HA-MDM2 (2 μg), Flag-L5 (2 μg), Flag-L11 (2 μg), Flag-L23 (2 μg), Flag- or V5-ARF using Lipofectin (for H1299 cells) or Lipofectamine 2000 (for $p53^{-/-}/mdm2^{-/-}$ MEFs, Invitrogen). For inhibition of proteasome-mediated protein degradation, the cells were treated with 20 nmol/L of MG132 for 8 hours prior to harvest. Forty-eight hours after transfection, cells from each plate were harvested and split into two aliquots, one for immunoblot and the other for ubiquitination assays. Cell pellets were lysed in buffer I (6 M guanidinium-HCl, 0.1 mol/L Na_2HPO_4/NaH_2PO_4, 10 mmol/L Tris-HCl, pH8.0, 10 mmol/L β-mecaptoethanol) and incubated with Ni-NTA beads (Qiagen) at room temperature for 4 hrs. Beads were washed once each with buffer I, buffer II (8 mol/L urea, 0.1 mol/L Na_2HPO_4/NaH_2PO_4, 10 mmol/L Tris-HCl, pH 8.0, 10 mmol/L β-mecaptoethanol), and buffer III (8 mol/L urea, 0.1 mol/L Na_2HPO_4/NaH_2PO_4, 10 mmol/L Tris-HCl, pH 6.3, 10 mmol/L β-

mecaptoethanol), respectively. Proteins were eluted from the beads in buffer IV (200 mmol/L imidazole, 0.15 mol/L Tris-HCl pH 6.7, 30% (v/v) glycerol, 0.72 mol/L β-mecaptoethanol and 5% (w/v) SDS). Eluted proteins were analyzed by immunoblot with monoclonal anti-p53 (DO-1), anti-HA or anti-MDM2 (2A10) antibodies.

Immunofluorescent staining. U2OS cells were transfected with the wild-type Flag-L11 or Flag-L11 deletion mutant expression plasmids. Forty-eight hours after transfection, cells were fixed on 4% (w/v) paraformaldehyde for 15 minutes and permeabilized in ice-cold 1× phosphate buffered saline containing 0.2% (v/v) Triton X-100. The cells were then stained with monoclonal anti-Flag antibodies followed by staining with Alexa Fluor 488 (green) goat anti-mouse secondary antibody (Molecular Probes, OR) as well as 4', 6-diamidino-2-phenylindole (DAPI). Stained cells were analyzed under a Zeiss Axiovert 25 fluorescent microscope.

RESULTS

Differential regulation of L11, L5, L23, and ARF on MDM2 and p53 ubiquitination. In an attempt to elucidate the mechanisms by which ribosomal proteins regulate the stability of MDM2 and p53, we determined the effect of ribosomal proteins L5, L11, and L23 on ubiquitination of MDM2 and of p53 in p53-deficient H1299 cells with ARF as a control. The cells were transfected with plasmids encoding MDM2 and p53 alone or with either one of the L proteins or ARF. As a control, the cells transfected with MDM2 and p53 only were treated with 20 μM of the proteasome inhibitor MG132 for 8 hrs before harvesting. The cells were harvested 48 hours after transfection and *in vivo* ubiquitination assays were conducted as described in the Experimental Procedures.

As shown in Fig. 4.1A and expected (76, 113), cotransfection of MDM2 with p53 resulted in p53 ubiquitination and drastic reduction of its protein level (compare lane 3 to lane 2). Consistent with our previous results (57, 58), ectopic expression of L5 and L23 markedly inhibited the ubiquitination of both p53 and MDM2 and consequently protected p53 degradation by MDM2 (lanes 5 and 7 of Figs. 1A and 1B). However, overexpression of L11 dramatically increased the ubiquinated species of both p53 and MDM2 as well as their protein levels (lane 6 of Figs. 4.1A and 4.1B). This effect was similar to that of ARF (lane 4 of Figs. 4.1A and 4.1B) as reported previously (320). The enhancement of the level of uniquitinated MDM2 and p53 species as well as their protein levels by L11 and ARF was not due to the variation in transfection efficiency because the GFP protein level in each transfectant was equivalent (Fig. 4.1C). Interestingly, the effect of L11 and ARF on ubiquitination of MDM2 and p53 was similar to that of MG132 treatment (lane 8, Fig. 4.1A and 4.1B). These results suggest that these ribosomal proteins may utilize different mechanisms to regulate the MDM2 and p53 ubiquitination and proteasomal pathway though all these nucleolar proteins can stabilize p53 upon overexpression (Fig. 4.1).

However, we were unsure whether the changes of ubiquitinated p53 or MDM2 species by the ribosomal L proteins were due to the direct effect of these proteins on the ubiquitin ligase activity of MDM2, because ubiquitinated MDM2 and p53 underwent rapid proteasome-mediated degradation and the total protein levels of MDM2 and p53 varied (Fig. 4.1). To determine the effect of the L proteins and ARF on the MDM2 ubiquitin ligase activity with p53 and MDM2 as substrates, we performed a set of transfections similar to that in Fig. 4.1, but 20 μM of MG132 was used to block proteasomal degradation so that we could compare ubiquitination at the same protein

level. As shown in Fig. 4.2, L5 and L23 again dramatically inhibited ubiquitination of both p53 and MDM2 (lanes 5 and 7, Fig. 4.2A and 4.2B), indicating that these two L proteins may directly inhibit MDM2 ubiquitin ligase activity as previously described (57, 58). By striking contrast, L11, like ARF (320), while slightly inhibited MDM2-mediated p53 ubiquitination (lanes 4 and 6, Fig. 2A), markedly increased MDM2 ubiquitination (lanes 4 and 6, Fig. 4.2B). This difference was not due to different protein levels or transfection efficiency as all of the exogenous proteins were expressed equally well (Fig. 4.2C). Two conclusions can be drawn from these results: First, L11, L5 and L23 differentially regulate MDM2 ubiquitin ligase activity towards itself - L11 enhances this activity whereas L5 and L23 inhibit it; Second, L11, similar to ARF (320), enhances MDM2 auto-ubiquitination, but not MDM2-mediated p53 ubiquitination.

L11 stabilizes both MDM2 and p53 in cells. It has been shown that ectopic expression of L11 leads to elevated p53 protein level and transcriptional activity in cells (177, 329). Consistently, we also observed that both endogenous p53 and MDM2 proteins were dramatically increased upon L11 overexpression in p53 proficient U2OS cells (Fig. 4.3A). Because Figs. 4.1 and 4.2 show that L11 increases MDM2 level and ubiquitin ligase activity towards MDM2, we wanted to determine whether L11 affects the half-lives of p53 and MDM2. Flag-L11 plasmids were transfected into U2OS cells. Forty-eight hours after transfection the cells were treated with cyclohexamide to stop protein synthesis in cells. The cells were then harvested at different time points and subjected to immunoblot to determine endogenous p53 and MDM2 levels (Fig. 4.3B). The protein levels were determined by measuring the intensity of each band and normalized with expression of GFP. As shown in Figs. 4.3B and 4.3C, the half-life of p53

prolonged from ~25 minutes in empty vector transfected cells to ~90 minutes in the cells overexpressing Flag-L11. The half-life of MDM2 also increased from about 20 minutes to 45 minutes in the same assay (Figs, 4.3B and 4.3D). These results demonstrate that the increased level of p53 in the presence of ectopically expressed L11 is due to p53 stabilization by this ribosomal protein. Although transcription of MDM2 was induced by activated p53 in response to overexpression of L11 (11, 235, 318), L11 can also stabilize MDM2.

Increment of MDM2 ubiquination and stability by L11 is p53-independent. L11 can directly bind to MDM2 (57), but not p53 (data not shown). Therefore, it is convincing that stabilization of p53 by L11 is through the inhibitory effect of L11 on MDM2. However, is L11-induced MDM2 ubiquitination and stabilization dependent on p53? To address this issue, we examined the effect of L11 on the half-life of MDM2 and its autoubiquitination in p53 deficient H1299 cells. Indeed, as shown in Fig. 4.4A and 4.4B, the half-life of ectopic MDM2 protein increased from ~20 min in the cells transfected with a control vector to 2 hrs in the Flag-L11-expressing cells. Because L11 is a component of the large subunit ribosomal complex, we also tested whether overexpression of L11 could enhance the translation of MDM2. H1299 cells were transfected with HA-MDM2 with or without Flag-L11. Forty-eight hours posttransfection, the cells were starved in the medium without methionine and then pulse-labeled with ^{35}S-methionine for 15 min. Equal amounts of total protein were immunoprecipitated with the anti-HA antibody. Total MDM2 protein and ^{35}S-labeled MDM2 levels were determined by immunoblot and autoradiography, respectively. As shown in Fig. 4.4C, the de novo MDM2 protein translation was not affected by Flag-L11

overexpression (top and bottom panels) while the increment of the MDM2 protein level was clearly evident (middle panel). Consistently, L11 also elevated MDM2 ubiquitination in p53-deficient H1299 cells (Fig. 4.4D) and in p53/mdm2 double knock out MEF cells (Fig. 4.5C). Taken together, these results demonstrate that L11 can stabilize MDM2 and enhance its ubiquitination in a p53-independent manner.

Enhancement of MDM2 ubiquitination by L11 is dependent on MDM2 ubiquitin ligase activity. To determine whether the L11-induced increase of MDM2 ubiquitination is dependent on MDM2 E3 ubiquitin ligase activity, we performed *in vivo* ubiquitination assays using an MDM2 mutant with substitution of cystine 464 by alanine ($MDM2^{C464A}$). This cystine residue is essential for the ubiquitination of p53 by MDM2 and the C464A mutation abolishes the ubiquitin E3 ligase activity of MDM2 (76, 113, 114), suggesting that MDM2 ubiquitination in cells is executed through its own E3 ubiquitin ligase activity. First, we transfected H1299 cells with His-ubiquitin together with either a wild type or a C464A mutant MDM2 plasmid in the presence or absence of Flag-L11. Ubiquitinated MDM2 was pulled down with Ni-NTA beads followed by immunoblot with the anti-MDM2 antibody. As shown in Fig. 4.5A, the cells transfected with MDM2 in the absence of Flag-L11 showed a detectable level of ubiquitinated MDM2 (lane 2 of top panel). By contrast, the $MDM2^{C464A}$ mutant showed no detectable ubiquitination (lane 4), suggesting that MDM2 ubiquitination is dependent on its own functional ubiquitin ligase activity. Upon overexpression of L11, ubiquitinated species of wild-type MDM2 were remarkably accumulated (lane 3 of top panel). Surprisingly, ubiquitinated species of $MDM2^{C464A}$ mutant were also dramatically enhanced by Flag-L11 (lane 5 compared to lane 4 of top panel). Two possibilities may account for this

effect: Flag-L11 may enhance MDM2^{C464A} ubiquitination by other ubiquitin ligases; Or the enhanced MDM2^{C464A} ubiquitination may be mediated by endogenous MDM2, which is expressed at a low level in p53 free H1299 cells (307). To test these possibilities, we then determined whether endogenous MDM2 in H1299 cells could be stabilized by L11. Indeed, overexpression of Flag-L11 induced endogenous MDM2 levels independent of p53 (Fig. 4.5B). This result was also repeated in Fig. 4.4C. Further, we performed *in vivo* ubiquitination assays similar to that in Fig. 5A using p53$^{-/-}$/mdm2$^{-/-}$ MEFs. Interestingly, ubiquitinated MDM2^{C464A} was hardly detectable in this mdm2/p53 null MEF cell line regardless of the presence of Flag-L11 or not (lane 4 and 5 of top penal in Fig. 4.5C), indicating that the enhancement of ubiqutinated MDM2^{C464A} or MDM2 by L11 in H1299 cells is due to the induction of endogenous MDM2 by L11. Taken together, these results indicate that stabilization and the increased level of ubiquitinated species of MDM2 by L11 are dependent on the intrinsic ubiquitin E3 ligase activity of MDM2, although it remains to investigate whether MDM2 autoubiquitination is through an inter- or intra-molecular mechanism.

The central domain of L11 binds to MDM2 and is required for MDM2 stabilization and the enhanced level of the autoubiquitinated species of MDM2. To determine which domain of L11 is important for the above regulation of MDM2 by L11, we generated a set of deletion mutants of L11, which were Flag-tagged as illustrated in Fig. 4.6B. Because the central region (aa 63 to 125) of L11 has been shown to bind to MDM2 (329), we wanted to verify this result while also hoped to narrow down the MDM2 binding domain. To this end, we performed a series of co-immunoprecipitation assays using the newly generated L11 deletion mutants (Fig. 4.6B). As shown in Fig. 6A,

HA-MDM2 was co-immunoprecipitated with C-terminal deleted mutants ($L11^{1-108}$, $L11^{1-125}$, $L11^{1-143}$) using anti-Flag antibodies (lanes 4 to 6) though the binding efficiency of these mutants decreased in comparison with that of wild-type L11 (lane 2). However, $L11^{1-65}$ did bind to MDM2 at all (lane 3). An N-terminal deletion mutant ($L11^{51-178}$), but not other N-terminal deletion mutants ($L11^{66-178}$, $L11^{109-178}$), was also co-immunoprecipitated with MDM2 (lanes 7 to 9). These results, as summarized in Fig. 4.6B, indicate that the central domain of L11 consisting of amino acids 65-108 is essential for MDM2-binding.

To determine if the binding is required for L11 to stabilize MDM2, we co-transfected H1299 cells with MDM2 alone or together with wild type or deletion mutant L11 plasmids. As shown in Fig. 4.6C, similar to wild-type L11 (lane 3), both the MDM2-binding N-terminal ($L11^{51-178}$) and C-terminal deleted L11 mutants ($L11^{1-108}$ and $L11^{1-125}$), like wild type L11 (lane 3), effectively stabilized exogenous MDM2 (lanes 5 to 7), whereas other mutants, which did not interact with MDM2 (Figs. 4.6A and 4.6B), failed to stabilize MDM2 (lanes 4, 8, and 9). Thus, the central domain of L11 consistent of residue 51 to 108 is required for L11 to stabilize MDM2 in cells. Furthermore, we examined the effect of some of these L11 deletion mutants on MDM2 autoubiquitination. Consistently, only the MDM2-binding deletion mutant of L11 ($L11^{1-108}$), but not the MDM2-binding deficient mutants ($L1^{11-65}$ or $L^{109-178}$), enhanced the level of ubiquitinated species of MDM2 in cells (Fig. 4.6D). These results suggest that the central domain of L11 (aa 51 to 108) is required for L11 to interact with and to stabilize MDM2 as well as to enhance the ubiquitinated species of MDM2 in cells.

Nucleolus localization of L11 is not required for its function to stabilize both MDM2 and p53. Ectopically expressed L11 has been shown to relocalize MDM2 to the nucleolus, indicating that the function of L11 on the MDM2-p53 pathway may involve nucleolus localization (177). To test if the nucleolus localization of L11 is crucial for L11's function, we examined the cellular localization of the above L11 deletion mutants. The p53-proficient U2OS cells were transfected with Flag-tagged L11 and its deletion mutants and stained with anti-Flag antibody. As show in Fig. 4.7A, wild-type Flag-L11 localized predominantly in the nucleolus and the nucleoplasm, but much less in the cytoplasm. Deletion of the C-terminal 70 or more amino acids ($L11^{1-65}$ and $L11^{1-108}$) of L11 abolished the nucleolus localization of L11 and retained its nucleoplasmic localization, while led to a large cytoplasmic distribution of these mutants, suggesting that the nucleolus localization signal (NoLS) of L11 resides at its C-terminus. Indeed, the N-terminal deletion mutant ($L11^{51-178}$) showed nucleolus localization (Fig. 4.7A). A putative NoLS motif was predicted in the C-terminus at residues 144 to 147 with an array of basic residues (RRKK) (329). However, an extreme C-terminal fragment ($L11^{126-178}$) harboring this motif failed to localize in the nucleolus, instead predominantly localized in the cytoplasm (data not shown), suggesting that additional amino acids are required for nuclear and nucleolus localization. Because the $L11^{1-108}$ deletion mutant, which was excluded from the nucleolus, but stayed in the nucleus and the cytoplasm (Fig. 7A), was able to stabilize MDM2 and to enhance its ubiquitination in cells (Figs. 4.6C and 4.6D), these results indicate that the nucleolus localization of L11 is not required for its function in regulating MDM2 stability and ubiquitination. This is in agreement with that L11 may function in the nucleoplasm where it binds to MDM2 and inhibits MDM2 function (18).

To further determine if $L11^{1-108}$ can activate p53 function, we transfected U2OS cells with wild type or different deletion mutants of L11 and then examined the endogenous levels of p53, p21 and MDM2. Indeed, $L11^{1-108}$, but not $L11^{1-65}$, $L11^{109-178}$ and $L11^{126-178}$, induced the p53 level (top panel) and stimulated its transcriptional activity as measured by the induction of the p53 target genes, p21 and MDM2 (Fig. 4.7B). This result indicates that the nucleolus localization of L11 may not even be needed for activating p53.

DISCUSSION

Inhibition of the MDM2-p53 feedback loop is important for p53 induction and activation in response to cellular stress signals. This inhibition can take place by either interfering with the physical interaction between MDM2 and p53, or directly inhibiting the MDM2 E3 ligase activity (45, 240). Significantly, these cellular mechanisms have become targeting sites for identifying small molecule inhibitors of MDM2 as anti-tumor drug candidates (44, 84, 298). In cells, one of the well-studied natural inhibitors of the MDM2-p53 feedback loop is the tumor suppressor ARF in response to high levels of oncoproteins, while ARF is believed to regulate rRNA processing in the nucleolus under normal situations (115, 238, 284, 333) . Likewise, several ribosomal proteins including L5, L11, and L23, which are usually assembled into the 80S translation complex in the nucleolus and exported to the rough endoplasmic reticulum (RER) for protein synthesis, have also been recently identified as the cellular blockers of this MDM2-p53 loop in response to ribosomal stress (18, 57, 58, 122, 177, 329). Therefore, fully understanding the mechanisms by which these ribosomal proteins inhibit MDM2 function would

provide useful information for recapturing p53 activation in MDM2-highly expressed cancer cells and hence for pharmacological study.

As a result of this effort, we have described here that these three ribosomal proteins appear to utilize different mechanisms to regulate MDM2 function, in spite of that they could form a quadruple complex with MDM2 (57). Overexpression of L5, L11, or L23 has been shown to inhibit MDM2-mediated p53 ubiquitination (57, 58, 122, 329). Here, we found that L23 (58) and L5 (Fig. 4.1B and 4.2B) inhibited MDM2 autoubiquitination in cells, suggesting that both of the proteins may stall MDM2-mediated p53 ubiquitination by inhibiting the ubiquitin ligase activity of MDM2. Although the detailed biochemical mechanism underlying this inhibition remains to be investigated, it is possible that L5 or L23 could recruit certain ubiquitin hydrolases, such as the recently reported HAUSP (163, 165), leading to deubiquitination of both MDM2 and p53. Alternatively, L5 and L23 may directly inhibit the E3 ubiquitin ligase activity of MDM2 by concealing the accessibility of MDM2 or p53 to E2.

Unlike L23 and L5, but similar to ARF (320), L11 inversely enhanced MDM2 ubiquitination (Figs. 4.1-2) while slightly reducing MDM2-mediated p53 ubiquitination (Fig. 4.2). This effect is surprising as the ultimate outcome of L11 regulation of MDM2 is inhibition of its function, consequently activation of p53 (57, 58, 122, 177, 329). Yet, this effect was similar to that of the proteasome inhibitor, MG132, implying that L11 may resemble MG132 to increase ubiquitinated MDM2 molecules by preventing proteasome-mediated proteolysis of ubiquitinated MDM2 (Fig. 4.8). This potential post-ubiquitination mechanism could also explain why p53 ubiquitination is slightly, instead of drastically, reduced by L11. Supporting this speculation are at least two lines of

evidence. First, overexpression of L11 markedly increased the half-life of MDM2 in a p53-independent fashion (Figs. 4.3-4). Correspondingly, L11 also induced the level of both the exogenous (Figs 4.1 and 4.6) and endogenous MDM2 (Figs. 4.2-4) independent of p53. This induction was not due to the effect of highly and transiently expressed L11 on de novo protein synthesis (Fig. 4.4C). Another possibility to explain the increase of MDM2 ubiquitination by L11 would be that by binding to MDM2, L11 renders it into a conformation that favors its autoubiquitination but not p53 ubiquitination. Supporting this assumption is that L11 still stimulates MDM2 autoubiquitination, but slightly inhibits p53 ubiquitination, in the presence of MG132 which prevents proteolysis and thus maintains the same protein levels (Fig. 4.2B). Also, L11 prefers binding to the Zinc finger domain of MDM2 while L23 and L5 interact with the acidic domains (aa 221-274 for L5 and aa 150-300 and 384-425 for L23) (58, 122, 329). Perhaps, by binding to the Zinc finger domain of MDM2, L11 may induce the conformational change of MDM2, whereas L5 and L23 would not do so as they bind elsewhere. This difference in MDM2-binding by L11, L5 and L23 may also explain why these L proteins differentially regulate MDM2 ubiquitin ligase activity (Figs. 4.1-2). If this prediction is correct, the Zinc finger domain and acid domains of MDM2 must play distinct roles in modulating its E3 ligase activity. Finally, L11 may block nuclear export of MDM2, thus facilitating its stabilization in the nucleus, given that L11 interacts with MDM2 in both the nucleolus (177) and the nucleoplasm (18), and MDM2 has been proposed to target ubiquitnated p53 to the cytoplasm for degradation (216, 252). However, inconsistent with these results, MDM2 was found to be degraded in both the cytoplasm and the nucleus by the proteasome pathway (281). Also, nucleolus localization of L11 is not required for its

inhibitory effect on the MDM2-p53 pathway (Fig. 4.7), similar to that for ARF (174). Thus, the final assumption appears to be less likely. Recently, a member of proteasome-interacting protein family that contains ubiquitin-like and ubiquitin-associated domains (UbL-UBA proteins), hHR23, was shown to associate with MDM2 and to regulate the targeting of p53 to the proteasome for degradation (26). It would be interesting to find out whether L11 or ARF could stabilize MDM2 and p53 by altering this postubiquitination pathway.

Despite the above speculations, it is clear that direct binding of L11 to MDM2 is essential for stabilizing MDM2 and inducing p53, as the MDM2-binding defective deletion mutants were unable to affect MDM2 level and ubiquitination (Figs. 4.6-7). Conversely, the MDM2-binding domain-containing deletion mutants of L11 were able to stabilize MDM2 and to enhance its ubiquitination (Figs. 4.6-7). Our functional mapping defined the central aa 51-108 domain as the important region for regulating MDM2 function. Interestingly, the function of this domain resembles that of the N-terminal domain (aa 30) of ARF, which is also required for ARF inhibitory effect on MDM2 function (49, 196). Although sequence comparison between these two domains showed no significant homology (data not shown), it is likely that these two MDM2-binding domains may share structural similarity. Therefore, solving the crystal structure of MDM2-ARF and MDM2-L11 complexes is crucial for better understanding the detailed molecular mechanisms underlying the regulation of MDM2 function by L11 and ARF.

ACKNOWLEDGMENTS

We are grateful to Dr. Karen Vousden for providing us with the Flag-L11 and $MDM2^{C464A}$ mutant plasmids. We thank Yama Khan Kharoti for technical assistance, Mary Mac Partlin for proofreading the manuscript, and other members of the laboratory for active discussion. This work was supported by NCI, National Institutes of Health Grants CA93614, CA095441 and CA079721 to H.L.

Fig. 4.1. Regulation of MDM2-mediated p53 and MDM2 ubiquitination by ribosomal proteins L5, L11, and L23 and ARF. (A). L11 and ARF, but not L5 and L23, increase MDM2-mediated ubiquitinated species of p53. H1299 cells were transfected with combinations of p53 (1 μg), HA-MDM2 (2 μg), V5-ARF (2 μg), Flag-L5 (2 μg), Flag-11 (2 μg), or Flag-L23 (2 μg) plasmids in the presence of the His-ubiquitin (His-Ub) (2 μg) plasmid as indicated (lanes 1 to 7). As a control, cells transfected with His-Ub, p53, and HA-MDM2 plasmids were treated with MG132 (20 μM) for 6 hours before harvesting (lane 8). The GFP expression vector pEGFP-C1 was included in each transfection as indication of transfection efficiency. The *in vivo* ubiquitination assay was performed as described in the Materials and Methods. Ubiquitinated proteins were detected by immunoblot with the anti-p53 (DO-1) antibody (upper panel). Ubiquitinated p53 (p53-(His-Ub)n) is indicated. The expression of total p53 is shown in lower panel. (**B**). L11 and ARF, but not L5 and L23, increase the level of ubiquitinated species of MDM2 in cells. The same blot as in (A) was immunoblotted with anti-HA antibody. Ubiquitinated MDM2 (MDM2-(His-Ub)n) is indicated. The expression of total MDM2 is shown in lower panel. (**C**). Total cell lysates (50 μg) from above transfection were immonublotted with anti-Flag and anti-V5 antibodies to show the expression of transfected L5, L11, L23 and ARF as indicated on right (upper panel). GFP expression is shown in lower panel.

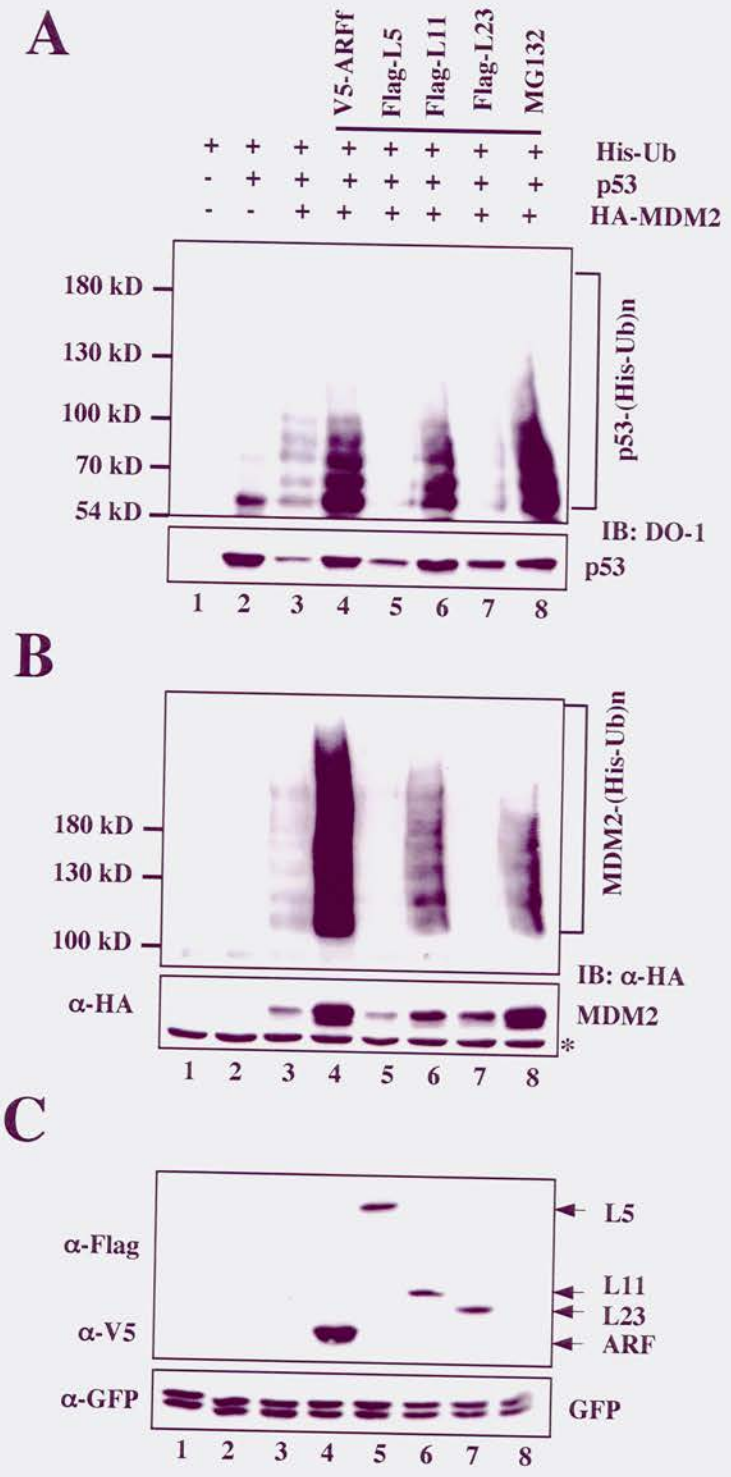

A
V5-ARFf
Flag-L5
Flag-L11
Flag-L23
MG132
His-Ub
p53
HA-MDM2
180 kD
130 kD
100 kD
70 kD
54 kD
p53-(His-Ub)n
IB: DO-1
p53
1 2 3 4 5 6 7 8
B
180 kD
130 kD
100 kD
MDM2-(His-Ub)n
IB: α-HA
α-HA
MDM2
*
1 2 3 4 5 6 7 8
C
α-Flag
α-V5
L5
L11
L23
ARF
α-GFP
GFP
1 2 3 4 5 6 7 8

Fig. 4.2. Regulation of MDM2-mediated p53 and MDM2 ubiquitination by ribosomal proteins L5, L11, L23, and ARF in the presence of MG132. (**A**). L11 and ARF slightly, but L5 and L23 dramatically, inhibit MDM2-mediated p53 unbiquitination in cells. H1299 cells were transfected with combinations of p53 (1 μg), HA-MDM2 (2 μg), V5-ARF (2 μg), Flag-L5 (2 μg), Flag-L11 (2 μg), or Flag-L23 (2 μg) plasmids as well as the His-Ub (2 μg) and pEGFP-C1 plasmids as indicated. The cells were then treated with MG132 (20 μM) for 6 hours before harvesting. The *in vivo* ubiquitination assay was performed. Ubiquitinated proteins were detected by immunoblot with the anti-p53 (DO-1) antibody (upper panel). Ubiquitinated p53 (p53-(His-Ub)n) is indicated. The expression of total p53 is shown in lower panel. (**B**). L11 and ARF, but not L5 and L23, increase ubiquitinated species of MDM2 in cells. The same blot as in (A) was immunoblotted with anti-HA antibody. Ubiquitinated MDM2 (MDM2-(His-Ub)n) is indicated. The expression of total MDM2 is shown in lower panel. (**C**). Total cell lysates (50 μg) from the above transfection were immonublotted with anti-Flag and anti-V5 antibodies to show the expression of transfected L5, L11, L23 and ARF as indicated on right of upper panel. GFP expression is shown in lower panel.

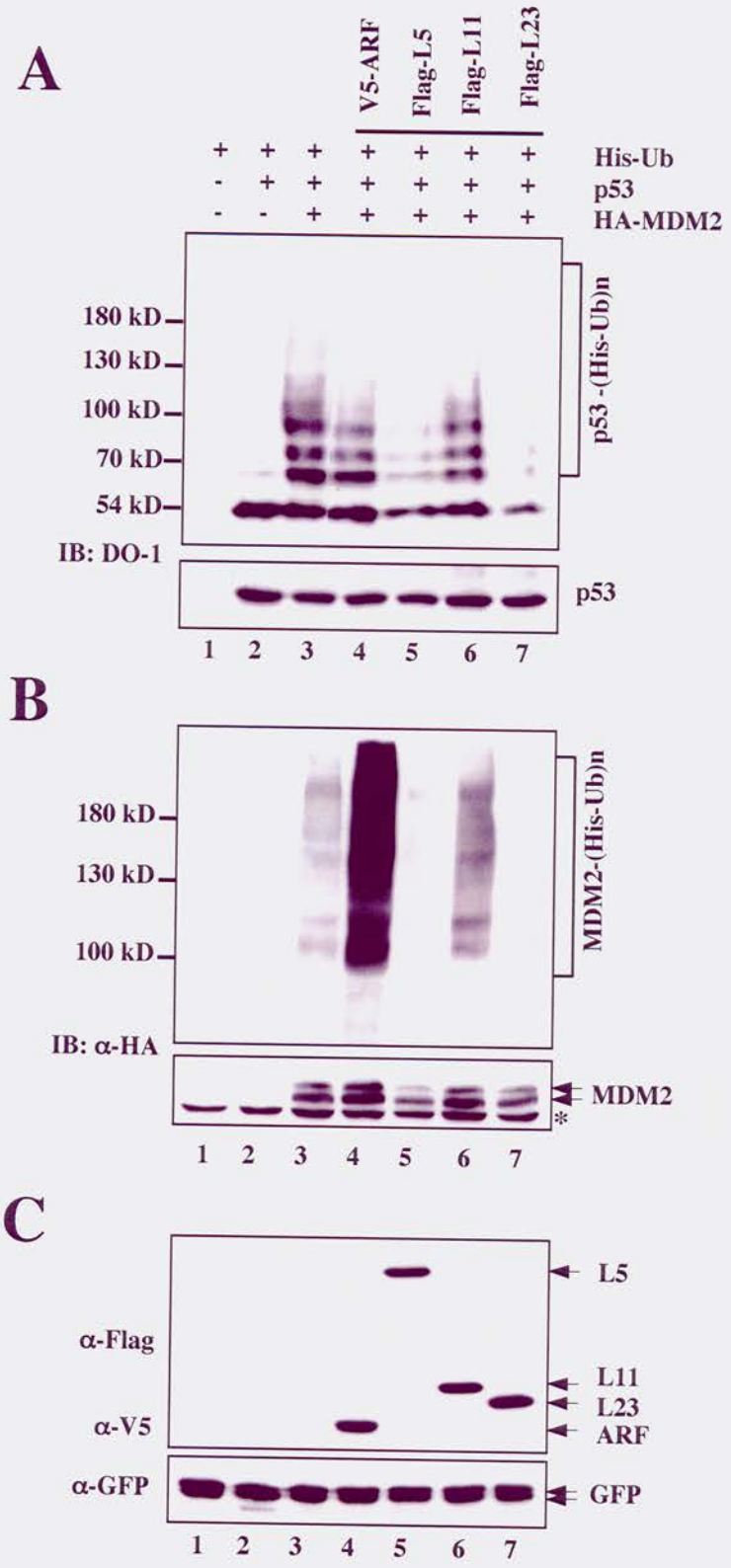

A
V5-ARF
Flag-L5
Flag-L11
Flag-L23
His-Ub
p53
HA-MDM2
180 kD
130 kD
100 kD
70 kD
54 kD
p53 -(His-Ub)n
IB: DO-1
p53
1 2 3 4 5 6 7
B
180 kD
130 kD
100 kD
MDM2-(His-Ub)n
IB: α-HA
MDM2
*
1 2 3 4 5 6 7
C
L5
α-Flag
L11
L23
α-V5
ARF
α-GFP
GFP
1 2 3 4 5 6 7

Fig. 4.3. L11 stabilizes both endogenous p53 and MDM2 in cells. (**A**). Ectopic expression of L11 increases p53 and MDM2 levels in cells. U2OS cells were transfected with Flag-L11 (3 μg, lane 2) or empty vector (3 μg, lane 1). Total cell lysates (50 μg) were immunoblotted with anti-p53 (DO-1), anti-MDM2 (2A10), anti-Flag, or anti-tubulin antibodies as indicated. (**B**). Ectopic expression of L11 increases the half-life of p53 and MDM2 in cells. U2OS cells were transfected with Flag-L11 (3 μg, right panels) or empty vector (3 μg, left panels) in the presence of pEGFP-C1 vector (0.1 μg). Forty-eight hours posttransfection, the cells were treated with 50 μg/ml of cyclohexamide (CHX) and harvested at different time points as indicated. The total cell lysates (50 μg) were immunoblotted with anti-p53 (DO-1), anti-MDM2 (2A10), anti-Flag, or anti-GFP antibodies as indicated. (**C**). The half-life of p53 in cells transfected with Flag-L11 or an empty vector. The density of p53 in each lane of panel B was quantified against the level of GFP and plotted in a graph. (**D**). The half-life of MDM2 in cells transfected with Flag-L11 or an empty vector. The density of MDM2 in each lane of panel B was quantified as that for panel C.

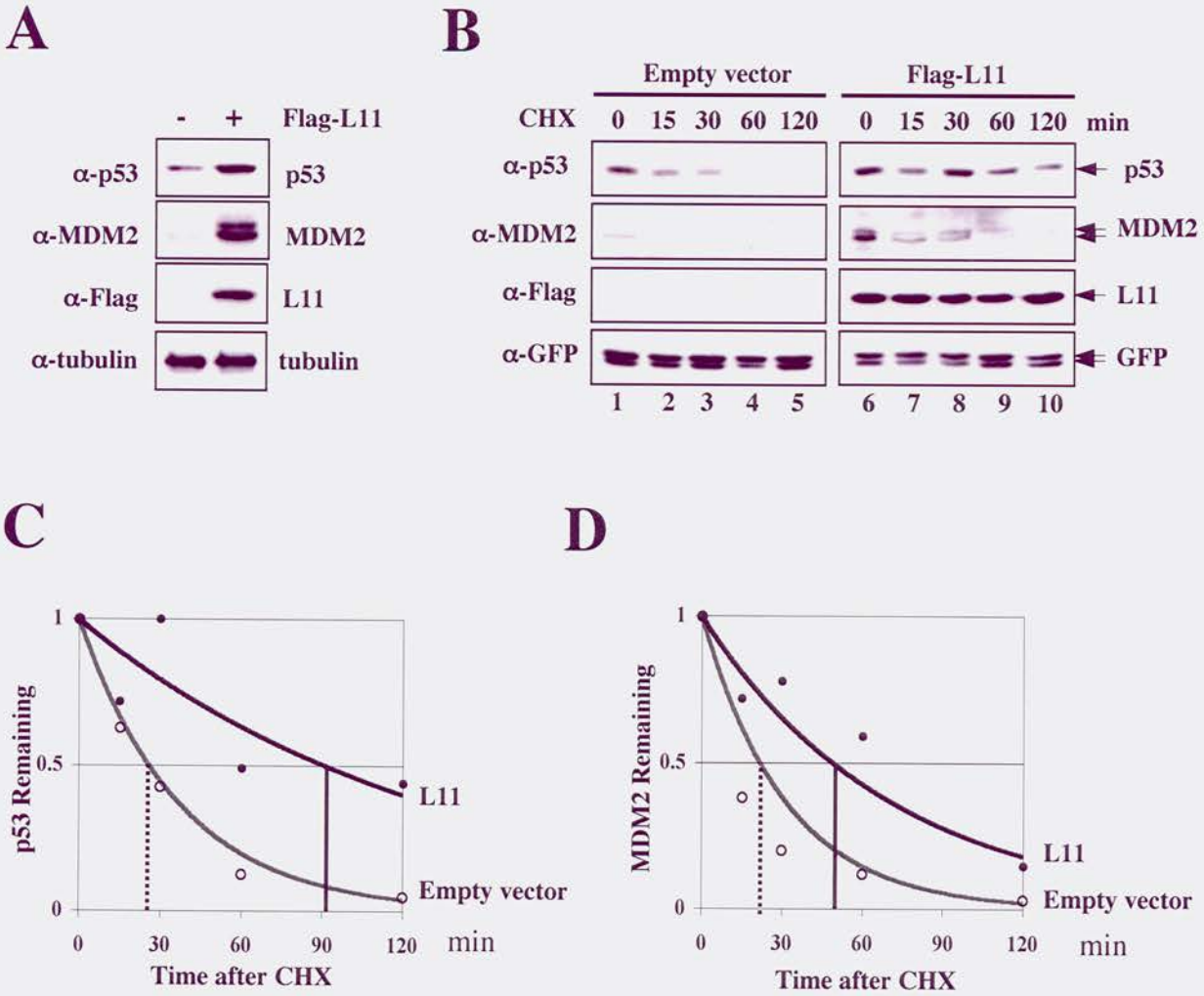

A
- + Flag-L11
α-p53
p53
α-MDM2
MDM2
α-Flag
L11
α-tubulin
tubulin
B
Empty vector
Flag-L11
CHX 0 15 30 60 120 0 15 30 60 120 min
α-p53
α-MDM2
α-Flag
α-GFP
p53
MDM2
L11
GFP
1 2 3 4 5 6 7 8 9 10
C
p53 Remaining
1
0.5
0
0 30 60 90 120 min
Time after CHX
L11
Empty vector
D
MDM2 Remaining
1
0.5
0
0 30 60 90 120 min
Time after CHX
L11
Empty vector

Fig. 4.4. L11 stabilizes MDM2 independent of p53. (**A**). Ectopic expression of L11 increases the half-life of MDM2 in the absence of p53. H1299 cells were transfected with HA-MDM2 (1 μg, left panels) or HA-MDM2 plus Flag-L11 (2 μg, right panels) in the presence of pEGFP-C1 vector (0.1 μg). Forty-eight hours posttransfection, the cells were treated with 50 μg/ml cyclohexamide (CHX) and harvested at different time points as indicated. The total cell lysates (50 μg) were immunoblotted with anti-HA, anti-Flag, or anti-GFP antibodies as indicated. (**B**). The half-life of exogenous MDM2 in H1299 cells transfected with Flag-L11 or an empty vector. The density of MDM2 in each lane of panel A was determined and the MDM2 levels were normalized against the expression of GFP and plotted in a graph. (**C**). Enhancement of MDM2 protein level by L11 is not due to increased translation. H1299 cells were transfected with HA-MDM2 (1 μg, lane 1) or HA-MDM2 plus Flag-L11 (2 μg, lane 2) plasmids. The cells were pulse-labeled with 50 μCi/ml of [^{35}S]methionine for 15 min. Equal amounts of total proteins were immunoprecipitated with anti-HA antibody for autoradiography (top panel). The lysates were also directly subjected to immunoblot using anti-HA or anti-Flag antibodies (middle and bottom panels). (**D**). L11 enhances ubiquitinated MDM2 species in the absence of p53. H1299 cells were transfected with His-Ub (2 μg), HA-MDM2 (2 μg), Flag-L11 (2 μg) plasmids as indicated. The cells were harvested after 48 hours and subjected to ubiquitination assays. Ubiquitinated MDM2 was detected by immunoblot with the anti-HA antibody (top panel). The ubiquitinated MDM2 species (MDM2-(His-Ub)n) are indicated on right. The cell lysates were also subjected to direct immunoblot with antibodies as indicated on the left of three bottom panels.

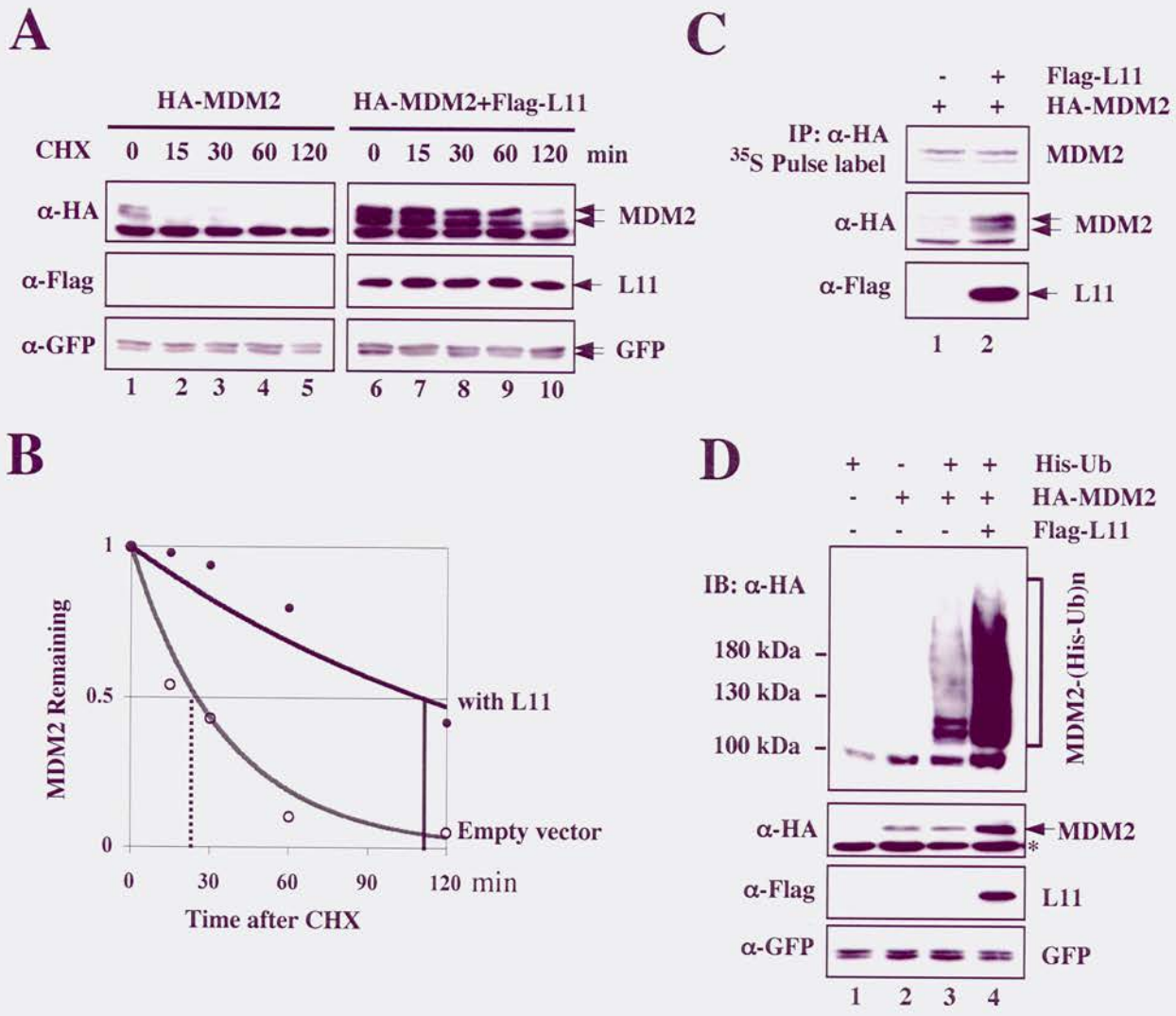

A
HA-MDM2
HA-MDM2+Flag-L11
CHX 0 15 30 60 120 0 15 30 60 120 min
α-HA
α-Flag
α-GFP
MDM2
L11
GFP
1 2 3 4 5 6 7 8 9 10
B
MDM2 Remaining
1
0.5
0
with L11
Empty vector
0 30 60 90 120 min
Time after CHX
C
- + Flag-L11
+ + HA-MDM2
IP: α-HA
35S Pulse label
MDM2
α-HA
MDM2
α-Flag
L11
1 2
D
+ - + + His-Ub
- + + + HA-MDM2
- - - + Flag-L11
IB: α-HA
180 kDa
130 kDa
100 kDa
MDM2-(His-Ub)n
α-HA
MDM2
*
α-Flag
L11
α-GFP
GFP
1 2 3 4

Fig. 4.5. The ubiquitin E3 ligase activity of MDM2 is required for L11 to enhance MDM2 ubiquitination. (**A**). L11 enhances ubiquitinated species of both wild type and C464A mutant of MDM2 in H1299 cells. H1299 cells were transfected with the wild type (wt, 2 μg) or C464A mutant MDM2 ($MDM2^{C464A}$, 2 μg) in the presence or absence of Flag-L11 (2 μg) together with His-Ub (2 μg) as indicated. The cells were harvested after 48 hours and subjected to ubiquitination assays. The ubiquitinated MDM2 species (MDM2-(His-Ub)n) were detected by immunoblot with anti-MDM2 (2A10) antibody. The ubiquitinated MDM2 is shown (top panel). The cell lysates were also subjected to direct immunoblot with anti-MDM2 (2A10), anti-Flag, or anti-GFP antibodies as indicated in the left of three bottom panels. (**B**). L11 stabilizes endogenous MDM2 in the absence of p53. H1299 cells were transfected with (lane 2) or without (lane 1) Flag-L11 (3 μg). The cell lysates were immunoblotted with anti-MDM2 (2A10), anti-L11, or anti-tubulin antibodies as indicated. (**C**). L11 does not increase ubiquitinated species of $MDM2^{C464A}$ in the absence of endogenous wild-type MDM2. Mouse $p53^{-/-}/mdm2^{-/-}$ MEFs were transfected with wild-type (wt) or $MDM2^{C464A}$ mutant (2 μg) in the presence or absence of Flag-L11 (2 μg) together with His-Ub (2 μg) as indicated. The cells lysates were subjected to ubiquitination assays. The ubiquitinated MDM2 species (MDM2-(His-Ub)n) were detected by immunoblot with the anti-MDM2 (2A10) antibody. The ubiquitinated MDM2 species were shown (top panel). The cell lysates were also subjected to direct immunoblot with 2A10, anti-Flag, or anti-GFP antibodies as indicated on the left of three bottom panels.

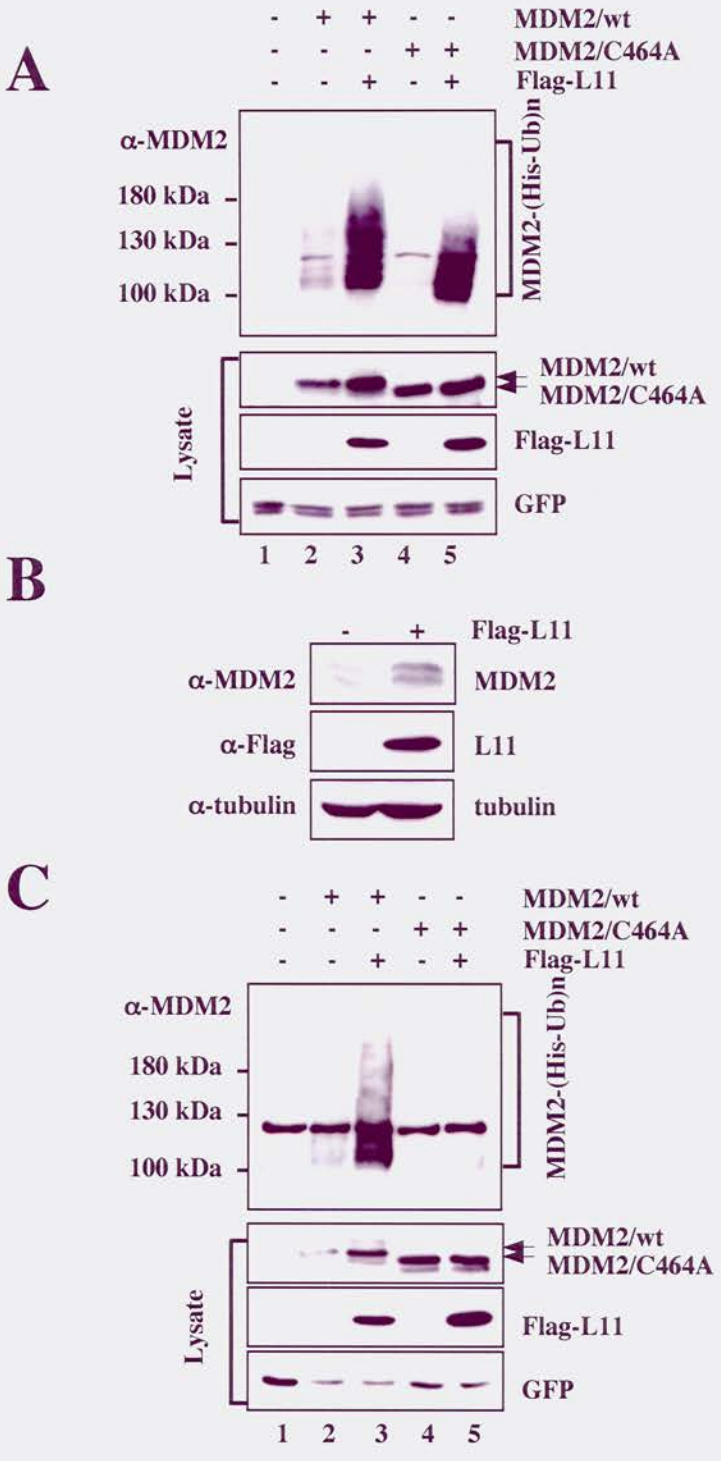

A
- + + - - MDM2/wt
- - - + + MDM2/C464A
- - + - + Flag-L11
α-MDM2
180 kDa
130 kDa
100 kDa
MDM2-(His-Ub)n
Lysate
MDM2/wt
MDM2/C464A
Flag-L11
GFP
1 2 3 4 5
B
- + Flag-L11
α-MDM2
MDM2
α-Flag
L11
α-tubulin
tubulin
C
- + + - - MDM2/wt
- - - + + MDM2/C464A
- - + - + Flag-L11
α-MDM2
180 kDa
130 kDa
100 kDa
MDM2-(His-Ub)n
Lysate
MDM2/wt
MDM2/C464A
Flag-L11
GFP
1 2 3 4 5

Fig. 4.6. The central domain of L11 binds to MDM2 and is required for L11 to stabilize MDM2 and enhance the ubiquitinated species of MDM2. (**A**). L11 binds to the central region of L11 in cells. H1299 cells were transfected with HA-MDM2 (3 μg) and Flag-tagged wild-type (wt) or its deletion mutants (3 μg) as indicated. The cell lysates were immunoprecipitated with the anti-Flag antibody and immunoblotted with anti-HA (top panel) or anti-Flag (bottom panel) antibodies. (**B**). Diagram of the Flag-tagged wild-type L11 and its deletion mutants. The co-immunoprecipitation results determined in panel A are shown on right. "+" indicates binding and "-" indicates lack of binding. (**C**). The central region-containing deletion mutants of L11 retain the ability to stabilize MDM2 in the absence of p53. H1299 cells were transfected with HA-MDM2 (1 μg) and Flag-tagged wild-type (wt) or its deletion mutants (2 μg) as indicated. The cell lysates (50 μg) were subjected to immunoblot with anti-HA and anti-Flag antibodies as indicated on the left of each panel. (**D**). The central region-containing deletion mutants of L11 ($L11^{1-108}$) can partially enhance the ubiquitinated species of MDM2 in the absence of p53. H1299 cells were transfected with His-Ub (2 μg), HA-MDM2 (2 μg) with Flag-tagged wild type (wt) or its deletion mutants (2 μg) as indicated. The cells lysates were subjected to ubiquitination assays. The ubiquitinated MDM2 species (MDM2-(His-Ub)n) were detected by immunoblot with the anti-HA antibody. The ubiquitinated MDM2 species are shown (top panel). The cell lysates were also subjected to direct immunoblot with anti-HA, anti-Flag, or anti-GFP antibodies as indicated on the left of three bottom panels.

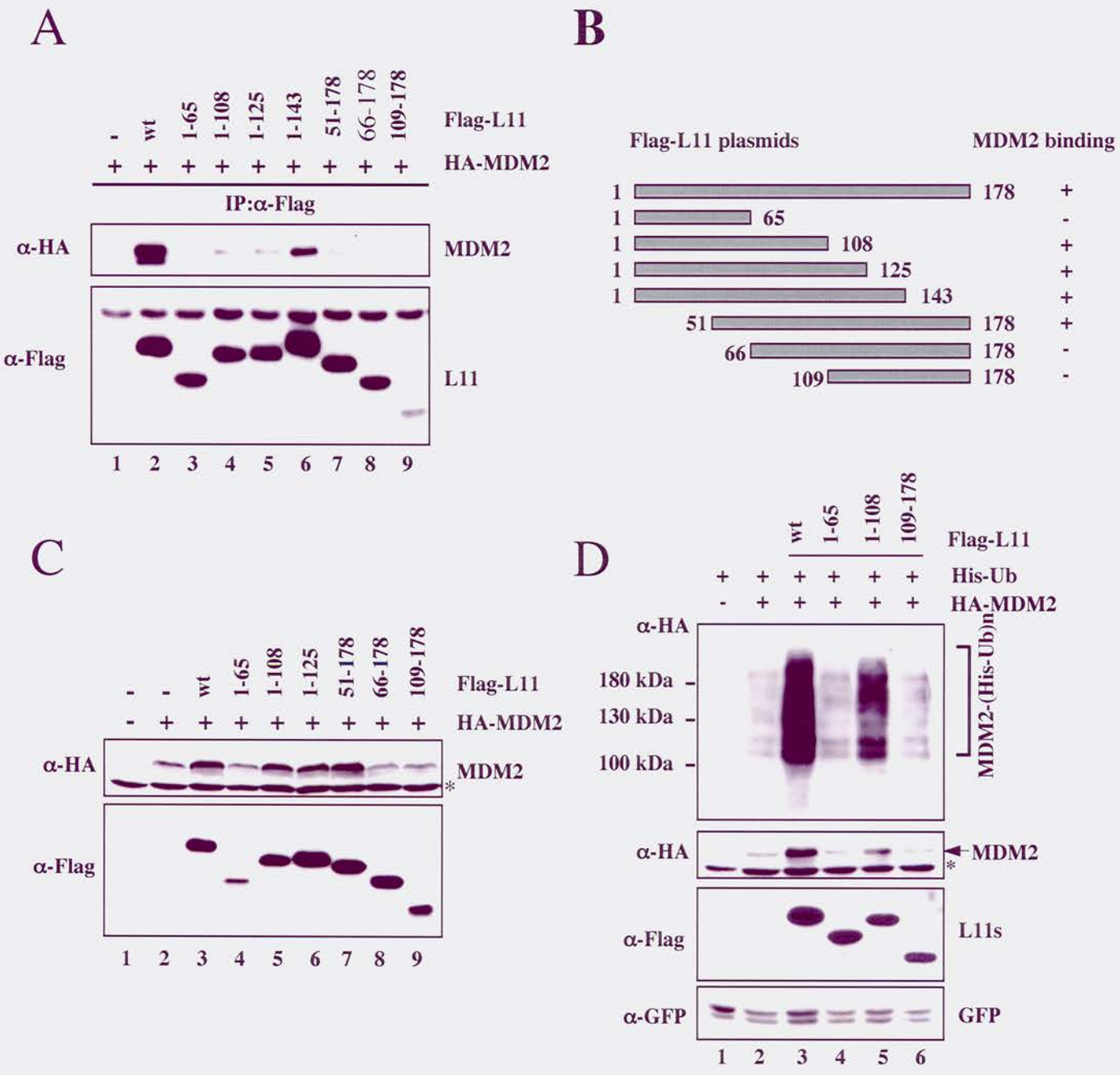
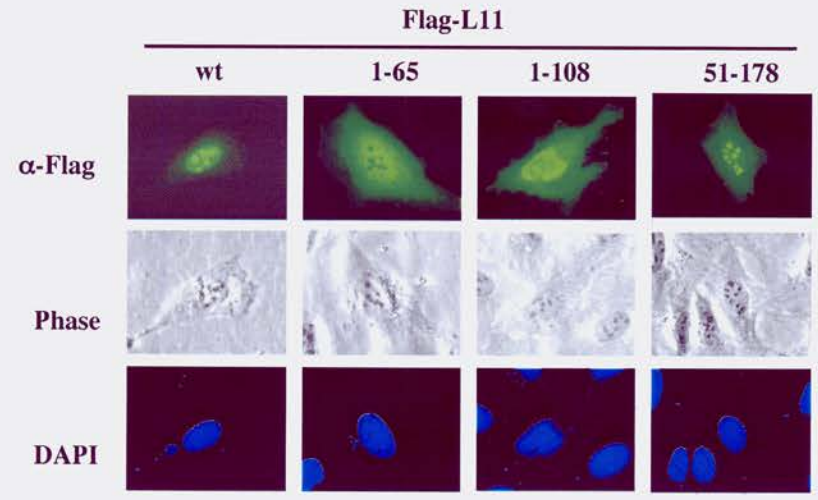

A
- wt 1-65 1-108 1-125 1-143 51-178 66-178 109-178
Flag-L11
+ + + + + + + + +
HA-MDM2
IP:α-Flag
α-HA
MDM2
α-Flag
L11
1 2 3 4 5 6 7 8 9
B
Flag-L11 plasmids
MDM2 binding
1 178 +
1 65 -
1 108 +
1 125 +
1 143 +
51 178 +
66 178 -
109 178 -
C
- - wt 1-65 1-108 1-125 51-178 66-178 109-178
Flag-L11
- + + + + + + + +
HA-MDM2
α-HA
MDM2
*
α-Flag
1 2 3 4 5 6 7 8 9
D
wt 1-65 1-108 109-178
Flag-L11
+ + + + + + His-Ub
- + + + + + HA-MDM2
α-HA
180 kDa
130 kDa
100 kDa
MDM2-(His-Ub)n
α-HA
MDM2
*
α-Flag
L11s
α-GFP
GFP
1 2 3 4 5 6

Fig. 4.7. The nucleolus localization is not required for L11 to activate p53.

(**A**). Localization of wild-type L11 and its deletion mutants in cells. U2OS cells were transfected with wild type (wt) or its deletion mutant (2 μg) plasmids as indicated and immunostained with anti-Flag antibody (green). The cells were also stained with DAPI to show DNA in the nucleus. (**B**). Wild type (wt) L11 and $L11^{1-108}$ mutant, but not other deletion mutants, stabilize and activate endogenous p53 in cells. U2OS cells were transfected with wt L11 or its deletion mutants as indicated. Forty-eight hours posttransfection, cells were harvested and subjected to immunoblot with anti-p53 (DO-1), anti-p21, anti-MDM2 (2A10), or anti-tubulin antibodies as indicated on the left of each panel.


A
Flag-L11
wt
1-65
1-108
51-178
α-Flag
Phase
DAPI

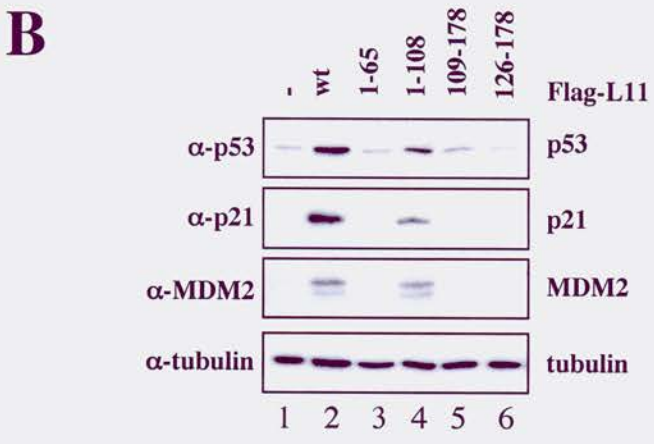
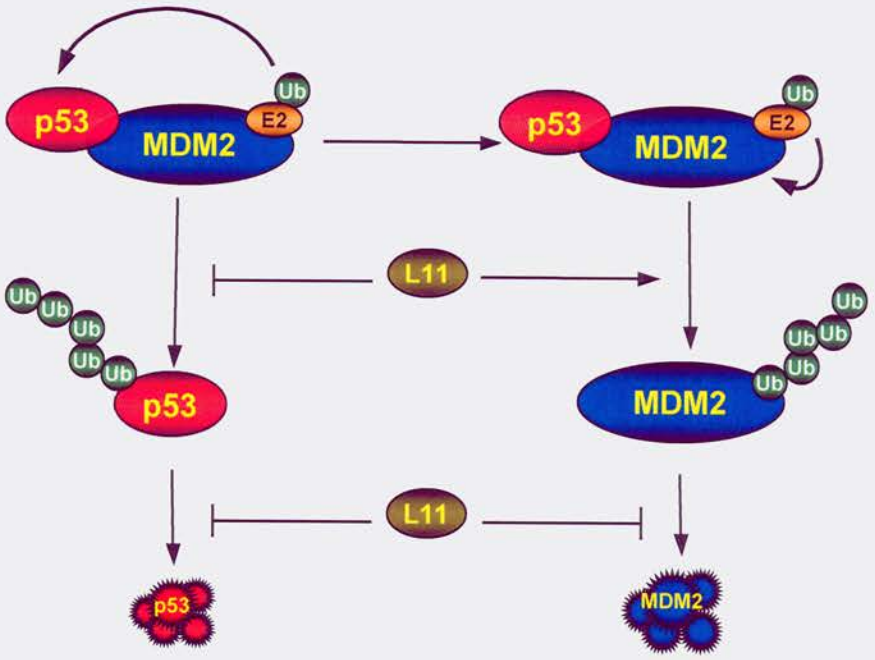

B
-
wt
1-65
1-108
109-178
126-178
Flag-L11
α-p53
p53
α-p21
p21
α-MDM2
MDM2
α-tubulin
tubulin
1 2 3 4 5 6

Fig. 4.8. A proposed model for the differential regulation of MDM2-mediated p53 ubiquitination and MDM2 auto-ubiquitination by L11. Bars indicate inhibition, and arrows indicate activation. Ub represents ubiquitin. See the Discussion for details.


p53
MDM2
E2
Ub
p53
MDM2
E2
Ub
L11
Ub
Ub
Ub
Ub
Ub
p53
MDM2
Ub
Ub
Ub
Ub
Ub
L11
p53
MDM2

CHAPTER FIVE

SUMMARY AND CONCLUSION

The p53 tumor suppressor protein induces expression of MDM2. MDM2, in turn inhibits p53 function by ubiquitinating and targeting p53 for proteasome-mediated proteolysis, thus forming a tight autoregulatory feedback loop. Under physiological conditions, this feedback loop is turned on because of p53-independent residual expression of MDM2 and, as a result, p53 is maintained at a low steady-state level and in an inert form. In response to various stresses, p53 is rapidly stabilized and activated through inhibition of the MDM2-p53 feedback loop. Therefore, tight and precise regulation of this loop is critical for control of the levels of p53 in cells. In principle, there are two models by which the MDM2-p53 feedback loop is inhibited. One involves dissociation of the MDM2-p53 interaction. The other involves direct inhibition of the ubiquitin E3 ligase activity of MDM2 without dissociation of the MDM2-p53 complex.

Ribosomal biogenesis tightly coordinates with cell growth and proliferation. It requires about 80 subunits of ribosomal proteins assembled with rRNAs to form the 80S translation complex. It also requires many nucleolar accessory factors to assist the synthesis, processing, and assembly of rRNAs and subsequent transport of ribosomal subunits to the cytoplasm. Perturbation of ribosomal biogenesis induced by blocking rRNA synthesis, processing, or assembly leads to a ribosomal stress that activates p53 and stalls cell growth. However, the detailed mechanism for how ribosomal stress induces p53 has not been well elucidated until recently when it was shown that, in response to many kinds of stresses, including ribosomal stress, the nucleolus is disrupted

and nucleolar proteins released, indicating that some of the nucleolar proteins might play a role in the regulation of the MDM2-p53 feedback loop.

In this dissertation, it is shown that the ribosomal proteins L5, L11, and L23 inhibit the MDM2-p53 feedback loop and thus activate p53 in response to ribosomal stress induced by treatment with a low dose (5 nM) of actinomycin D, which specifically inhibits RNA polymerase I activity. This inhibition corresponds to the second model in which MDM2-mediated p53 ubiquitnation is directly inhibited by the three L proteins without dissociation of the MDM2-p53 complex. The following data supports the above model. First, ectopic expression of L5, L11, or L23 induces p53 transcriptional activity and p53-dependent G1 cell cycle arrest. Second, treatment of cells with 5 nM of actinomycin D, but not γ irradiation, enhances the MDM2-L protein interactions and leads to p53 stabilization and activation. Third, depletion of L5 and L23 by siRNA inhibits the actinomycin D-induced p53 transcriptional activity and G1 cell cycle arrest.

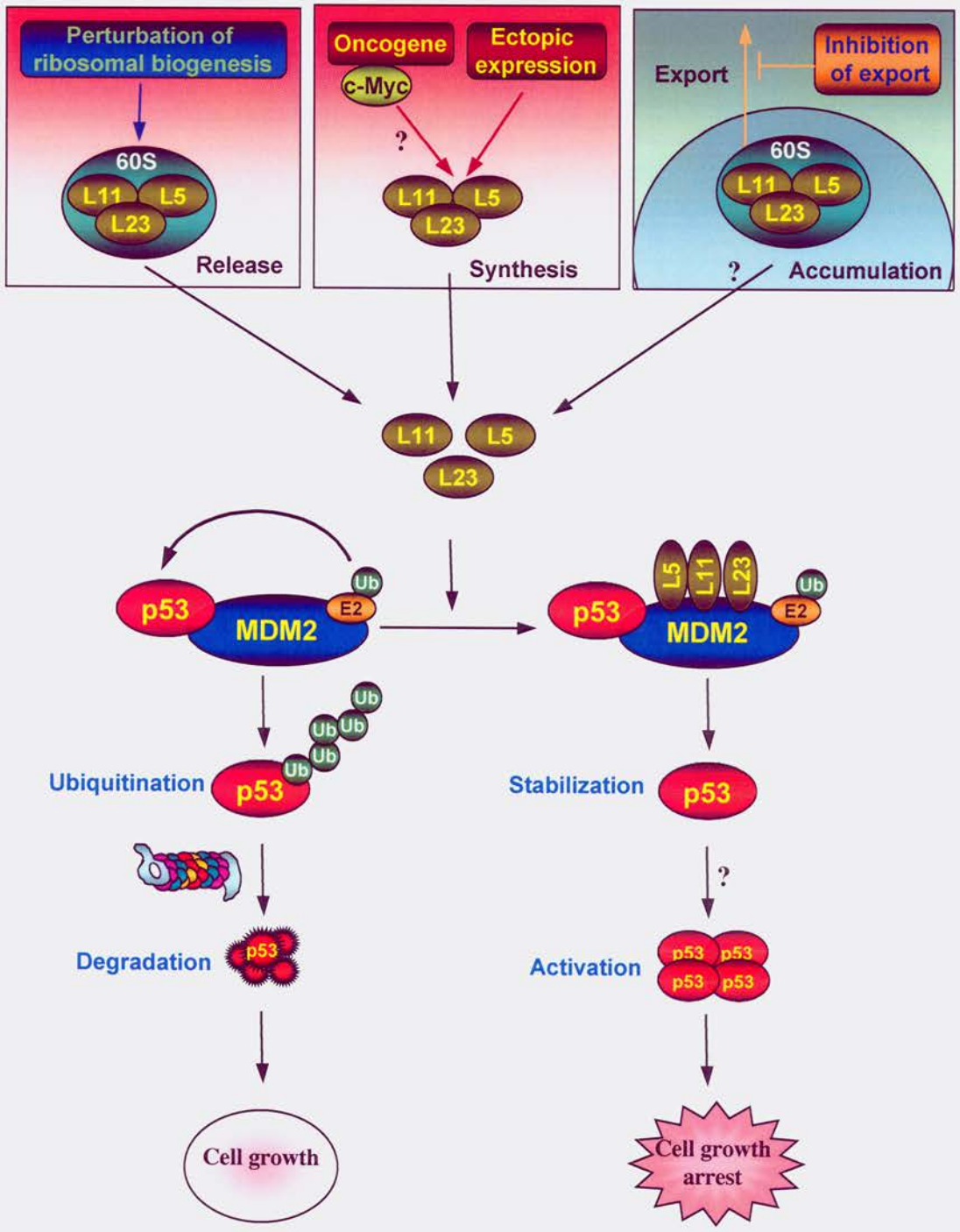
Moreover, this dissertation shows that it is the non-ribosome-associated forms of L5, L11, and L23 that associate with MDM2 and inhibit MDM2-mediated p53 ubiquitination. Therefore, I propose a model for the ribosomal stress-induced p53 activation, as shown in Figure 5.1. There are several possible sources that produce the non-ribosome-associated forms of L5, L11, and L23. First, perturbation of ribosomal biogenesis causes a nucleolar stress that inhibits the assembly of ribosomal subunits, thus releasing the ribosomal proteins. Second, overproduction of the ribosomal proteins by the above ectopic expression or aberrant growth signals such as those induced by c-Myc oncogene could increase the non-ribosome-associated forms of L5, L11, and L23. Third, inhibition of the transport of the 40S and 60S subunits of the ribosome to the cytoplasm,

such as that caused by leptomycin B, could lead to accumulation of the non-ribosome-associated forms of L5, L11, and L23 in the nucleoplasm. Consequently, L5, L11, and L23 form a complex or complexes with MDM2 and inhibit MDM2-mediated p53 ubiquitination. p53 is then stabilized and activated. The mechanism by which p53 is activated upon accumulation induced by L5, L11, and L23 is currently unknown.

In addition, the differential regulation of MDM2-mediated p53 ubiquitination and MDM2 auto-ubiquitination by L5, L11, and L23 has also been pursued in this thesis. L11, like ARF, but not L5 and L23, leads to a dramatic accumulation of the ubiquitinated species and the steady-state level of MDM2, while all three L proteins inhibit MDM2-mediated p53 ubiquitination. Thus we propose a model involving a conformational change of MDM2 and a potential post-ubiquitination mechanism for L11-mediated p53 activation (Fig. 4.8). Binding of L11 to MDM2 could render MDM2 into a conformation that favors its autoubiquitination but not p53 ubiquitination. This conformation might also impair the targeting of the ubiquitinated MDM2 to the 26S proteasome through certain proteasome-interacting proteins such as hHR23, thus blocking the proteasome-mediated proteolysis of both MDM2 and p53. In contrast to L11, L5 and L23 bind to different domains of MDM2 and inhibit the overall ubiquitin E3 ligase activity of MDM2. It would be interesting and important to determine how L11 halts the proteasome-mediated degradation of ubiquitinated MDM2 and p53.

In conclusion, the results presented in this dissertation demonstrate that ribosomal proteins L5, L11, and L23 are important regulators of the MDM2-p53 feedback loop in response to ribosomal stress.

Figure 5.1. A Schematic model for regulation of the MDM2-p53 feedback loop by non-ribosome-associated ribosomal proteins L5, L11, and L23 in response to ribosomal stress. There are several possible sources that produce the non-ribosome-associated forms of L5, L11, and L23. First, nucleolar stress caused by perturbation of ribosomal biogenesis inhibits the assembly of ribosomal subunits, thus releasing the ribosomal proteins. Second, overproduction of the ribosomal proteins by experimentally ectopic expression or aberrant growth signals such as those induced by c-Myc oncogene could increase the non-ribosome-associated forms of L5, L11, and L23. Third, inhibition of the transport of the 40S and 60S subunits of ribosome to the cytoplasm could result in accumulation of the non-ribosome-associated forms of L5, L11, and L23 in the nucleoplasm. Consequently, L5, L11, and L23 form a complex or complexes with MDM2 and inhibit MDM2-mediated p53 ubiquitination and degradation. p53 is then stabilized and activated, and consequently induces cell cycle arrest. Therfore, these L proteins could act as nature inhibitors of the MDM2-p53 feedback loop in cells. By contrast, in rapidly proliferative cells, these L proteins are constantly cooperated into ribosomes and polysomes, thus reducing the non-ribosome-associated forms of L5, L11, and L23 and alleviating their inhibitory effect on the MDM2-p53 feedback loop. Therefore, p53 is constantly degraded by MDM2 and allow cells to growth and proliferation.


Perturbation of ribosomal biogenesis
60S
L11
L5
L23
Release
Oncogene
Ectopic expression
c-Myc
?
L11
L5
L23
Synthesis
Export
Inhibition of export
60S
L11
L5
L23
?
Accumulation
L11
L5
L23
p53
MDM2
Ub
E2
L5
L11
L23
p53
MDM2
Ub
E2
Ubiquitination
p53
Ub
Stabilization
p53
?
Degradation
p53
Activation
p53
p53
p53
p53
Cell growth
Cell growth arrest

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