MDM2 and MDMX Regulators of p53 Activity

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MDM2 and MDMX Regulators of p53 Activity

Jamil Momand, Paul Joseph Aspuria, and Saori Furuta

SUMMARY

MDM2 possesses three activities that, together, effectively inhibit the p53 tumor suppressor. First, it binds to p53 and sterically blocks p53 interaction with TATA box protein accessory factors thereby shutting down its transcriptional transactivation function. Second, MDM2 shuttles p53 from its site of action within the nucleus into the cytoplasm. Third, MDM2 is an E3 ligase that transfers ubiquitin onto lysine residues of p53. Ubiquitinated p53 is rapidly degraded by the 26S proteosome. Because the MDM2 oncoprotein mediates three progressive stages of inhibition, it is the principal regulator of p53 activity. The *MDM2* gene is located on chromosome 12q14.3-q15 and is amplified in several types of neoplasms, most of which are of mesenchymal tissue origin. MDM2 binding to p53 can be inhibited by phosphorylation of either MDM2 or p53. The kinases responsible for this phosphorylation are activated by cell stressors in general (hypoxia, nitric oxide, hydrogen peroxide) and DNA damaging agents in particular (ionizing radiation, UV-light). MDM2 can be inhibited by RAS or MYC oncoproteins. RAS and MYC activate the tumor suppressor protein $p19^{Arf}$ which sequesters MDM2 into the nucleolus and, in doing so, allows p53 levels to rise. The *MDM2* gene is activated by p53, which means that, in effect, p53 inhibits itself through MDM2. The cell requires a fine balance of MDM2 and p53 to maintain cell growth

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and a rapid response to stressors. MDMX is a paralog of MDM2 that has retained the ability to inhibit p53 binding to TATA box protein accessory factors. MDMX does not possess other p53 inhibitory activities. There has been recent progress in the development of small molecules that block MDM2 from binding to p53. Although these molecules are in the early stages of development, it is hoped that they will contribute to the war on cancer. This chapter summarizes the key studies that have increased our understanding of the interplay between p53, MDM2, and MDMX.

7.1. INTRODUCTION

Every sophisticated engineering system allows for regulation via feedback. Feedback mechanisms ensure that systems do not spiral out of control. In the area of cell growth one can consider tumor suppression as a system in which p53 is the central operator and that MDM2 and MDMX are essential proteins in the feedback mechanism that controls p53. The *mdm2* gene was initially characterized as an amplified oncogene by Dr. Donna George at the University of Pennsylvania (Cahilly-Snyder et al., 1987; Fakharzadeh et al., 1991). Its protein product, MDM2 (sometimes named HDM2 when referring to the human protein), downregulates p53 in distinct stages. First, p53 forms a complex with MDM2 and, through this interaction, is prevented from increasing the transcription of its effector genes. Second, MDM2 shuttles p53 away from the genome out to the cytoplasm. Finally, MDM2 marks p53 for degradation. MDMX (also known as MDM4) was later discovered because its sequence is similar to MDM2 (Shvarts et al., 1996). MDMX does not promote p53 degradation nor transport it from the nucleus to the cytoplasm. However, like MDM2, MDMX inhibits p53-mediated transactivation by masking the transactivation domain. Both oncoproteins have been shown to be abnormally upregulated in human tumors. In this chapter, we will discuss the molecules that regulate the ability of MDM2 and MDMX to control p53. We will explore avenues that may lead to anticancer therapies based on MDM2/p53 interactions and highlight areas that will likely be investigated in the near future.

7.2. MDM2 AND MDMX STRUCTURE/FUNCTION RELATIONSHIPS

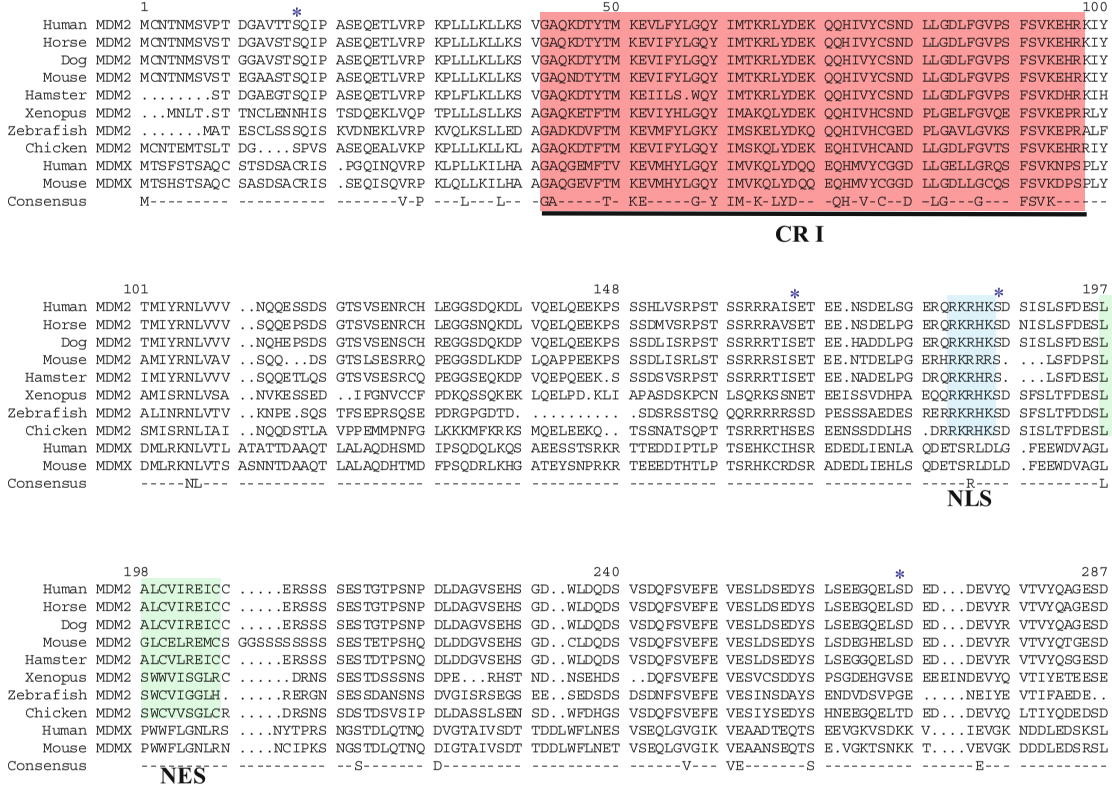
From its mRNA sequence, MDM2 is predicted to be composed of 491 amino acid residues and its gene is located on human chromosome 12q13-14 (Oliner et al., 1992). Note that we will use the term MDM2 to refer to the human ortholog unless otherwise noted. MDM2 is highly phosphorylated (Momand et al., 1992) and is quickly turned over with a half-life of approximately 20 minutes (Hinds et al., 1990; Olson et al., 1993). A common method used to identify important functional regions of proteins is sequence alignment and previous studies that compared MDM2 and MDMX amino acid sequences pinpointed three regions of high similarity (Momand

et al., 2000; Piette et al., 1997). Figure 7.1A shows an alignment of 8 MDM2 and 2 MDMX sequences using the Multialign algorithm (Barton and Sternberg, 1987) where these three similar regions are denoted with the abbreviations CR1, CR2, and CR3 ("CR" is an abbreviation for conserved region). Figure 7.1A also shows the sites where MDM2 is posttranslationally modified and the regions that control its subcellular localization. Analysis of CR1, CR2, and CR3 predicts that MDM2 binds p53, partakes in regulating the transport of molecules in and out of the nucleus, and transfers ubiquitin onto protein substrates.

The functions of the three conserved regions accurately describe the biochemical activities of MDM2. CR1 (amino acid residues 27–94) is the portion of MDM2 that binds to p53. Most of the binding takes place through van der Waals forces (Kussie et al., 1996). CR2 (residues 301–329) contains a sequence that is similar to Ran-1, a protein that controls nuclear export via a zinc-binding sequence (Yaseen and Blobel, 1999). MDM2 shuttles between the nucleus and the cytoplasm and controls the export of p53 from nucleus, but CR2 has not been identified as a necessary domain for this process. CR3 (residues 437–483) binds zinc (Lai et al., 1998) and contains a critical cysteine residue at position 464 that is necessary for transferring ubiquitin onto p53 (Honda et al., 1997). Ubiquitination of several lysine residues near the p53 carboxyl terminus is required for its degradation by the 26S proteosome (Kubbutat et al., 1999; Lai et al., 2001). In some cases, Cys 464 is also necessary to transport p53 from the nucleus to the cytoplasm (Boyd et al., 2000; Geyer et al., 2000). Interestingly, MDMX, which also contains a conserved cysteine residue within CR3, fails to transfer ubiquitin onto p53 making it likely that regions outside of CR3 are necessary for MDM2 ubiquitin ligase activity. Figure 7.1B shows a schematic diagram of the linear sequence of MDM2 and some of the key regions that control its activity. Structural analysis of MDM2 and MDMX should help to further elucidate their functions.

At the moment, we know the structure of the interface between CR1 of MDM2 and p53. X-ray diffraction studies performed by Dr. Nikola Pavletich's group at Columbia University followed by nuclear magnetic resonance spectroscopy studies showed that CR1 creates a hydrophobic cleft for p53 (Kussie et al., 1996; Stoll et al., 2001). Analysis of the interface region has provided insight into the design of molecules that bind MDM2 and release p53 for the purpose of preventing the spread of cancer. Figure 7.2A shows a portion of MDM2 bound to p53 depicted as filled van der Waals surfaces. Electron-rich regions are red, electron-poor regions are blue, and neutral regions are white. The p53 peptide is presented as a red α-helix with five side chains in the shapes of balls and sticks. The peptide bears the sequence Glu^{17}-Thr-Phe-Ser-Asp-Leu-Trp-Lys-Leu-Leu-Pro-Glu-Asn^{29}, but only side chains of Phe19, Leu22, Trp23, Leu25, and Leu26 are shown for clarity. Phe19, Trp23, and Leu26 side chains lie on one face of the alpha-helix and direct their hydrophobic atoms into the pocket of MDM2. On the opposite face lies two other hydrophobic side chains, Leu22 and Leu25. It appears that at least one of these, Leu25, may interact with His73 of MDM2. Thus, MDM2 interacts with residues on opposite faces of the p53 alpha-helix that constitutes the transactivation domain.

A


1
50
100
Human MDM2 MCNTNMSVPT DGAVTTSQIP ASEQETLVRP KPLLLKLLKS VGAQKDTYTM KEVLFYLGQY IMTKRLYDEK QQHIVYCSND LLGDLFGVPS FSVKEHRKIY
Horse MDM2 MCNTNMSVST DGAVSTSQIP ASEQETLVRP KPLLLKLLKS VGAQKDTYTM KEVIFYLGQY IMTKRLYDEK QQHIVYCSND LLGDLFGVPS FSVKEHRKIY
Dog MDM2 MCNTNMSVST GGAVSTSQIP ASEQETLVRP KPLLLKLLKS VGAQKDTYTM KEVIFYLGQY IMTKRLYDEK QQHIVYCSND LLGDLFGVPS FSVKEHRKIY
Mouse MDM2 MCNTNMSVST EGAASTSQIP ASEQETLVRP KPLLLKLLKS VGAQNDTYTM KEVIFYLGQY IMTKRLYDEK QQHIVYCSND LLGDLFGVPS FSVKEHRKIY
Hamster MDM2 ....ST DGAEGTSQIP ASEQETLVRP KPLFLKLLKS VGAQKDTYTM KEIILS.WQY IMTKRLYDEK QQHIVYCSND LLGDLFGVPS FSVKDHRKIH
Xenopus MDM2 ...MNLT.ST TNCLENNHIS TSDQEKLVQP TPLLLSLLKS AGAQKETFTM KEVIYHLGQY IMAKQLYDEK QQHIVHCSND PLGELFGVQE FSVKEPRRLY
Zebrafish MDM2 ....MAT ESCLSSSQIS KVDNEKLVRP KVQLKSLLED AGADKDVFTM KEVMFYLGKY IMSKELYDKQ QQHIVHCGED PLGAVLGVKS FSVKEPRALF
Chicken MDM2 MCNTEMTSLT DG....SPVS ASEQEALVKP KPLLLKLLKL AGAQKDTFTM KEVIFYLGQY IMSKQLYDEK EQHIVHCAND LLGDLFGVTS FSVKEHRRIY
Human MDMX MTSFSTSAQC STSDSACRIS .PGQINQVRP KLPLLKILHA AGAQGEMFTV KEVMHYLGQY IMVKQLYDQQ EQHMVYCGGD LLGELLGRQS FSVKNPSPLY
Mouse MDMX MTSHSTSAQC SASDSACRIS .SEQISQVRP KLQLLKILHA AGAQGEVFTM KEVMHYLGQY IMVKQLYDQQ EQHMVYCGGD LLGDLLGCQS FSVKDPSPLY
Consensus M--------- ---------- -------V-P ---L---L-- -GA-----T- KE-----G-Y IM-K-LYD-- -QH-V-C--D -LG---G--- FSVK-------
CR I
101
148
197
Human MDM2 TMIYRNLVVV ..NQQESSDS GTSVSENRCH LEGGSDQKDL VQELQEEKPS SSHLVSRPST SSRRRAISET EE.NSDELSG ERQRKRHKSD SISLSFDESL
Horse MDM2 TMIYRNLVVV ..SQQEPSDS GTSVSENRCH LEGGSNQKDL VQELQEEKPS SSDMVSRPST SSRRRAVSET EE.NSDELPG ERQRKRHKSD NISLSFDESL
Dog MDM2 TMIYRNLVVV ..NQHEPSDS GTSVSENSCH REGGSDQKDP VQELQEEKPS SSDLISRPST SSRRRTISET EE.HADDLPG ERQRKRHKSD SISLSFDESL
Mouse MDM2 AMIYRNLVAV ..SQQ...DS GTSLSESRRQ PEGGSDLKDP LQAPPEEKPS SSDLISRLST SSRRRSISET EE.NTDELPG ERHRKRRRS. ...LSFDPSL
Hamster MDM2 IMIYRNLVVV ..SQQETLQS GTSVSESRCQ PEGGSEQKDP VQEPQEEK.S SSDSVSRPST SSRRRTISET EE.NADELPG DRQRKRHRS. ...LSFDESL
Xenopus MDM2 AMISRNLVSA ..NVKESSED ..IFGNVCCF PDKQSSQKEK LQELPD.KLI APASDSKPCN LSQRKSSNET EEISSVDHPA EQQRKRHKSD SFSLTFDESL
Zebrafish MDM2 ALINRNLVTV ..KNPE.SQS TFSEPRSQSE PDRGPGDTD. .... .SDSRSSTSQ QQRRRRRSSD PESSSAEDES RERRKRHKSD SFSLTFDDSL
Chicken MDM2 SMISRNLIAI ..NQQDSTLA VPPEMMPNFG LKKKMFKRKS MQELEEKQ.. TSSNATSQPT TSRRRTHSES EENSSDDLHS .DRRKRHKSD SISLTFDESL
Human MDMX DMLRKNLVTL ATATTDAAQT LALAQDHSMD IPSQDQLKQS AEESSTSRKR TTEDDIPTLP TSEHKCIHSR EDEDLIENLA QDETSRLDLG .FEEWDVAGL
Mouse MDMX DMLRKNLVTS ASNNTDAAQT LALAQDHTMD FPSQDRLKHG ATEYSNPRKR TEEEDTHTLP TSRHKCRDSR ADEDLIEHLS QDETSRLDLD .FEEWDVAGL
Consensus -----NL--- ---------- ---------- ---------- ---------- ---------- ---------- -----R---- ---------L
NLS
198
240
287
Human MDM2 ALCVIREICC .....ERSSS SESTGTPSNP DLDAGVSEHS GD..WLDQDS VSDQFSVEFE VESLDSEDYS LSEEGQELSD ED...DEVYQ VTVYQAGESD
Horse MDM2 ALCVIREICC .....ERSSS SESTGTPSNP DLDAGVSEHS GD..WLDQDS VSDQFSVEFE VESLDSEDYS LSEEGQELSD ED...DEVYR VTVYQAGESD
Dog MDM2 ALCVIREICC .....ERSSS SESTGTPSNP DLDAGVSEHS GD..WLDQDS VSDQFSVEFE VESLDSEDYS LSEEGQELSD ED...DEVYR VTVYQAGESD
Mouse MDM2 GLCELREMCS GGSSSSSSSS SESTETPSHQ DLDDGVSEHS GD..CLDQDS VSDQFSVEFE VESLDSEDYS LSDEGHELSD ED...DEVYR VTVYQTGESD
Hamster MDM2 ALCVLREICC .....ERSSS SESTDTPSNQ DLDDGVSEHS GD..WLDQDS VSDQFSVEFE VESLDSEDYS LSEGGQELSD ED...DEVYR VTVYQSGESD
Xenopus MDM2 SWWVISGLRC ......DRNS SESTDSSSNS DPE...RHST ND..NSEHDS ..DQFSVEFE VESVCSDDYS PSGDEHGVSE EEEINDEVYQ VTIYETEESE
Zebrafish MDM2 SWCVIGGLH. .....RERGN SESSDANSNS DVGISRSEGS EE..SEDSDS DSDNFSVEFE VESINSDAYS ENDVDSVPGE .....NEIYE VTIFAEDE..
Chicken MDM2 SWCVVSGLCR .....DRSNS SDSTDSVSIP DLDASSLSEN SD..WFDHGS VSDQFSVEFE VESIYSEDYS HNEEGQELTD ED...DEVYQ LTIYQDEDSD
Human MDMX PWWFLGNLRS ....NYTPRS NGSTDLQTNQ DVGTAIVSDT TDDLWFLNES VSEQLGVGIK VEAADTEQTS EEVGKVSDKK V....IEVGK NDDLEDSKSL
Mouse MDMX PWWFLGNLRN ....NCIPKS NGSTDLQTNQ DIGTAIVSDT TDDLWFLNET VSEQLGVGIK VEAANSEQTS E.VGKTSNKK T....VEVGK DDDLEDSRSL
Consensus ---------- ---------- --S------- D--------- ---------- ------V--- VE-------S ---------- ------E--- ----------
NES

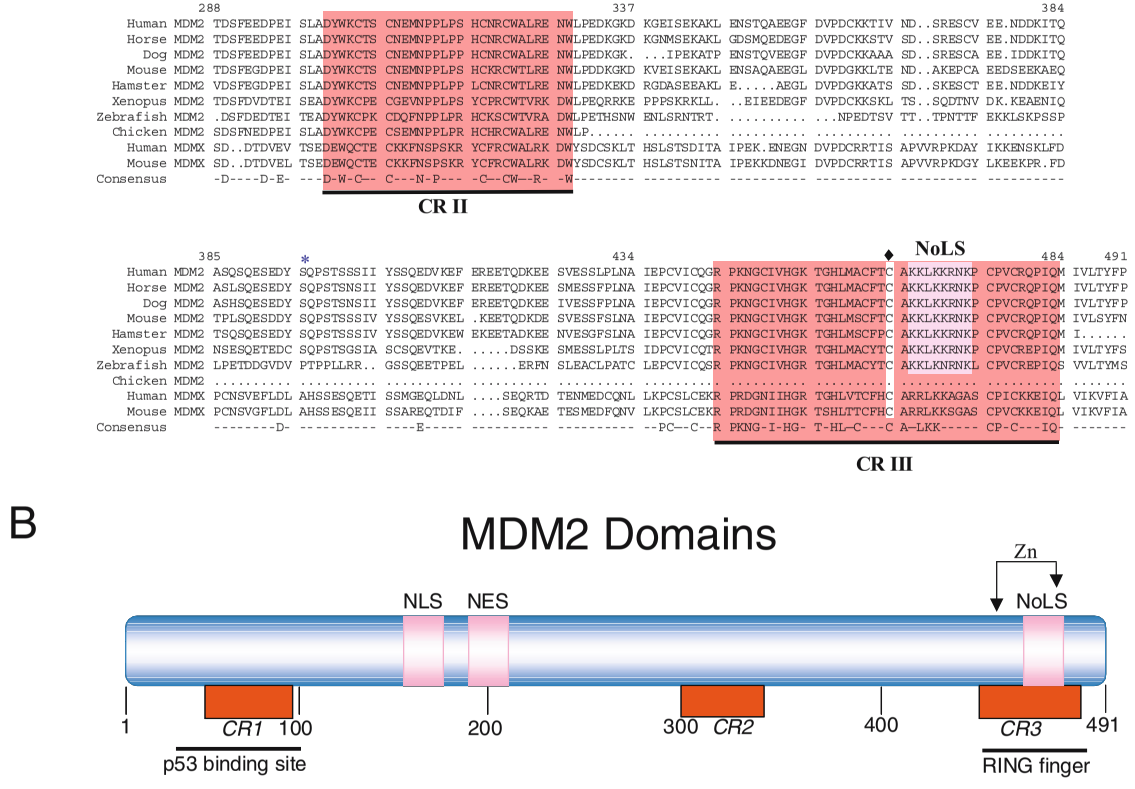


Figure 7.1. MDM2 and MDMX protein domains. **A:** Sequence alignment of MDM2 and MDMX protein sequences. CR1, conserved region 1; CR2, conserved region 2; CR3, conserved region 3; NLS, nuclear localization sequence; NES, nuclear export sequence; NoLS, nucleolus localization sequence; black diamond, cysteine required for ubiquitin transfer to p53; blue asterisk, phosphorylation site. Sequences were aligned with the MultiAlign program. **B:** Schematic diagram of MDM2 protein domains (see text for details). Known Zn binding sites and the RING finger domain are indicated.

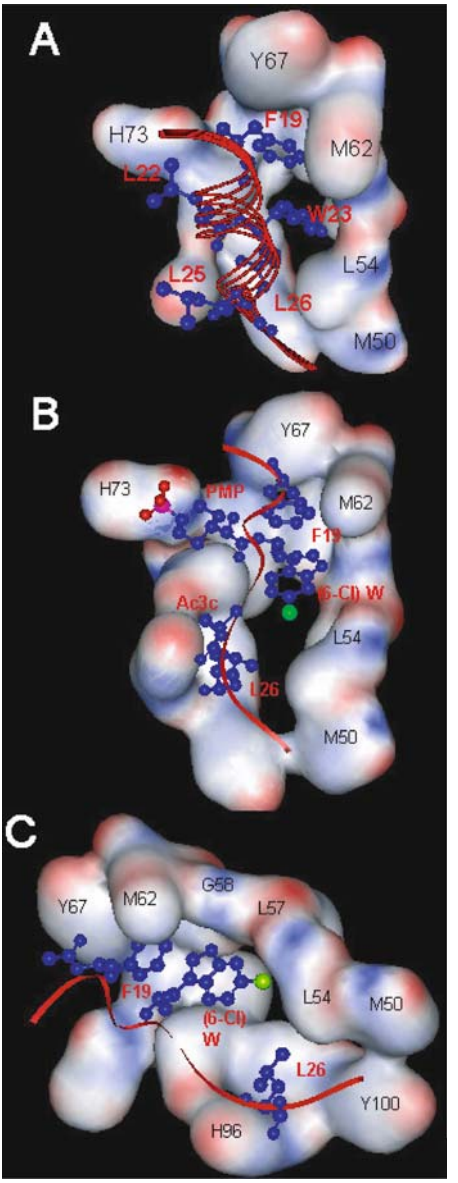


Figure 7.2. Peptide inhibitors bound to MDM2 interactions. **A:** p53 peptide bound to MDM2. The red ribbon represents the backbone peptide alpha-helix of p53. The three p53 residues (Phe 19, Trp 23, Leu 26) on one face of the alpha-helix form van der Waals interactions with the MDM2. Two other p53 residues (Leu 22 and Leu 25) on the opposite face of the alpha-helix also appear to interact with MDM2. MDM2 residues are depicted as filled van der Waals surfaces, with red indicating high electron density and blue indicating low electron density. The MDM2 residues involved in binding p53 are labeled with black font. **B:** Model of CGP 84700 bound to MDM2. The CGP 84700 peptide model was created by substituting the side chains of CGP 84700 for the p53 peptide side chains. **C:** Different orientation of CGP 84700 bound to MDM2. All models were generated using WebLab ViewerPro©. Coordinates for MDM2 and p53 residues were obtained from data deposited in the Protein Data Bank (Kussie et al., 1996). Peptide atom color code: blue, carbon; pink, phosphorous; red, oxygen; green, chlorine.

Aside from knowing the shape of p53 when it is bound to MDM2 it is also important to understand how its conformation changes upon binding. A synthetic p53 peptide containing residues 14–28 has the propensity to form a two-β-turn structure stabilized by Phe19, Leu22, Trp23, and Leu25 (Botuyan et al., 1997). The conformation of this two β-turn structure is similar to the structure of the p53 peptide when it is bound to MDM2. This scenario opens the possibility that this portion of p53 is "primed" for MDM2 binding. The structure of the MDM2-p53 complex has paved the way for the design of potent inhibitors of binding.

7.2.1. Artificial Modulation of MDM2-p53 Complex Formation

Dr. Bert Vogelstein and his colleagues at Johns Hopkins University presented the first evidence that MDM2 inactivates p53 in human tumors (Oliner et al., 1992). They demonstrated that MDM2 is overexpressed in a large percentage of sarcomas. This result gave the impetus for scientists to design inhibitors of MDM2 as a possible therapeutic to reestablish p53 tumor suppressor activity in these tumors. In one general approach, small molecules have been synthesized (or isolated) that can release p53 from MDM2. In a second approach, oligonucleotides have been designed to bind and destroy MDM2 mRNA. Table 7.1 shows patents and patent-pending applications related to MDM2. The majority of these patents cover approaches to inhibit MDM2 activity, and are discussed below.

To create small molecules that prevent MDM2 from binding p53, it was necessary to accurately map the regions of interaction. To map these sites, truncated MDM2 transcripts were translated in the presence of p53 and complexes were captured by coimmunoprecipitation (Chen et al., 1993). Using this method, it was discovered that the two proteins interact through regions near their amino termini. Confirmation that these two domains interact came from yeast two-hybrid assays (Oliner et al., 1993). Next, peptide libraries were used to narrow the binding region within p53 to residues 18–23 (^{18}TFSDLW^{23}) (Picksley et al., 1994). Amino acid replacements at Leu22 and Trp23 effectively prevented its ability to bind MDM2 (Lin et al., 1994). This "double mutant" p53 was also incapable of activating genes driven by a p53-responsive promoter, indicating that the MDM2-binding domain and the domain responsible for transactivation overlap.

7.2.2. High Affinity Molecules that Dissociate MDM2 and p53

With the sites required for interaction accurately mapped, reagents were developed to inhibit MDM2-p53 complex formation. Five of these inhibitors and their respective IC_{50} values are presented in Table 7.2. IC_{50} values represent the amount of inhibitor necessary to reduce the binding of p53 to MDM2 by 50%. A compound developed by Novartis, named CGP 84700, has the exceptionally low IC_{50} of 5 nM, and is sufficiently hydrophobic to penetrate cultured tumor cells and induce accumulation of p53 (Chene et al., 2000). Treatment of cancer cells with CGP 84700 leads to p53-mediated transactivation and promotes cell suicide (apoptosis), indicating its potential as a therapeutic. Two views of a model of CGP 84700 bound to MDM2

Table 7.1. List of patents and patent-pending applications related to MDM2.

Assignee or inventor	Year granted or applied	Patent or application number	Title
University of Johns Hopkins	1995	US5,411,860; WO9320238A3	Amplification of human MDM2 gene in human tumors
University of Johns Hopkins	1997	US5,618,921	Antibodies for detection of human MDM2 protein
University of Dundee	1998	US5,770,377	Interruption of binding of MDM2 and p53 protein and therapeutic application thereof
Ludwig Institute for Cancer Research	1998 2001	WO9813064A1; US6,204,253	Factors which interact with oncoproteins
Cancer research campaign technology limited	1998	WO9801467A2;	Inhibitors of the interaction between p53 and MDM2
University of Dundee	1998	WO9847525A1	Materials and methods relating to inhibiting the interaction of p53 and MDM2
Ruiwen Zhang	1999	WO9910486A3	MDM2-specific antisense oligonucleotides
Zeneca Limited	2000	WO0015657	Piperizine-4-phenyl derivatives as inhibitors of the interaction between MDM2 and p53
David Lane et al.	2001	App. No. 20010018511 (US)	Inhibitors of the interaction between p53 and MDM2
Yijia Bao et al.	2001	App. No. 20010018183	Simultaneous measurement of gene expression and genomic abnormalities using nucleic acid microarrays
Loren J. Miraglia et al.	2001	App. No. 20010016575	Antisense modulation of human MDM2 expression

Table 7.2. Small molecule inhibitors of p53-MDM2 interaction.

Common Name	Molecular structure	IC_{50} μM	Reference
p53 peptide	Ac-Thr^{18}-Phe-Ser-Asp-Leu-Trp^{26}-NH_2	286–1000	Picksley et al. (1994)
CGP 84700	Ac-Phe^{19}-Met-Aib-Pmp-(6-Cl) Trp-Glu-Ac_3c-Leu^{26}-NH_2[a]	0.005	Chene et al. (2000)
Chalcone	1,3-diphenyl-2-propen-1-one, compound B-1	117	Stoll et al. (2001)
Chlorofusin (B-1)	$C_{66}H_{99}O_{19}N_{12}Cl$	4.6	Duncan et al. (2001)
Nutlin-3	$C_{34}H_{29}O_4N_4Cl_2$	0.09	Vassilev et al. (2004)

[a]Where Aib is α-aminoisobutyric acid, Pmp is phosphonophenylalanine, (6-Cl)Trp is 6-chorotryptophan, Ac_3c is 1-amino-cyclopropane carboxylic acid.

are shown in Figures 7.2B and C. The model was created by using the structure of the p53 peptide in Figure 7.2A as a scaffold to build the compound in an alpha helical form with minimal alteration of the MDM2 cleft. Chalcones, a class of molecules derived from 1,3-diphenyl-2-propen-1-one, also show promise as inhibitors of MDM2-p53 complex formation (Stoll et al., 2001). These molecules were originally isolated from plants, and have a wide variety of anticancer effects. Of the chalcones tested, the most effective at inhibiting MDM2-p53 complex formation is derivative B-1, which binds to the region of MDM2 that normally binds Trp 23 of p53. Another molecule that releases MDM2 from p53 is chlorofusin, a circular peptide isolated from the fungus *Fusarum* (Duncan et al., 2001). This nine amino acid peptide was isolated by screening over 53,000 compounds that could potentially inhibit MDM2-p53 interaction. It will be exciting to see if any of these inhibitors can be further developed as an anticancer therapeutic with minimal toxic side effects. Scientists at Hoffman-La Roche and Pharma developed a series of *cis*-imidazoline analogs named Nutlin-1, -2, and -3 (Vassilev et al., 2004). These small organic compounds dissociated recombinant p53 from MDM2 with the median IC_{50} in the 100 to 300 nM range. The compounds inhibit cell cycle progression and promote apoptosis in a p53-dependent manner. Initial studies of Nutlin-3 on a human osteosarcoma cell-line xenograft in nude mice demonstrated that it was as effective as doxorubicin in reducing tumor volume although the effective dose of Nutlin-3 was 20-fold higher.

7.2.3. Antisense Therapy

A second potential therapeutic route to inhibit MDM2 function is to use antisense oligodeoxyribonucleotides (ODNs). In this approach, the ODN binds the transcript and forms a localized duplex, which then becomes a target for degradation by the endogenous nuclease RNAse H. Two research groups have reported that ODNs reduce the levels of MDM2 transcript and MDM2 in cultured cells (Chen et al., 1998; Teoh et al., 1997). Using multiple myeloma cells, Teoh et al (1997) showed that the ODN 5′-dGACATGTTGGTATTGCACAT-3′ (complements nucleotides 1–20

of the coding sequence within the transcript) reduces MDM2 expression, increases p53 protein levels, and inhibits DNA synthesis. A second group created the ODN 5′-dGATCACTCCCACCTTCAAGG-3′, which is complementary to nucleotides 714–733 of MDM2 transcript (Chen et al., 1998). This ODN contains a phosphoramidite backbone that is designed to decrease its degradation by nucleases, and appears to be very effective in destroying MDM2 transcripts.

Strategies to treat cancers with MDM2 targeting agents are in the initial stages of exploration. To proceed with their development, one should be cognizant of their potential uses and limitations. Generally, such agents are proposed to release p53 and thus cause cancers cells to undergo apoptosis. From the perspective of the p53 autoregulatory loop, there are four molecular scenarios that could benefit from anti-MDM2 therapy: (1) The *mdm2* gene is amplified and MDM2 is overexpressed; (2) MDM2 mRNA is overexpressed in the absence of gene amplification; (3) The *p*53 gene is wild type but MDM2 is not overexpressed; and (4) p53 is not properly phosphorylated for activation. The third and fourth scenarios may not be obvious candidates for anti-MDM2 therapy. In the case of the third scenario, one must consider a cancer cell where the signaling pathway that *inactivates* MDM2 is defective. An example of this scenario is the ARF signaling pathway (see ARF-MDM2 complex formation below). The ARF protein normally activates p53 by inhibiting MDM2. ARF is inactivated in a large number of cancers allowing MDM2 to constitutively inhibit p53. In such cancers anti-MDM2 therapy may be able to activate p53. The fourth scenario that may benefit from anti-MDM2 therapy is one where p53 is not properly modified by kinases and acetylases—modifications known to increase p53 activity (Giaccia and Kastan, 1998). Overexpression of unmodified p53 by anti-MDM2 therapy may suppress tumors because the high level will be sufficient to activate target genes. While most of the benefit of anti-MDM2 therapy to cancers will likely be derived from p53 activation, such therapy may also benefit cancers with low Retinoblastoma tumor suppressor activity. MDM2 has been shown to inhibit RB's ability to prevent tumor cell growth (Xiao et al., 1995). A major challenge currently facing scientists is to develop an efficient anti-MDM2 therapeutic delivery vehicle that spares potential toxic side effects.

7.2.4. SUMO-1 Modification

In the past few years there has been some progress in our understanding of how MDM2 is regulated by posttranslational modifications. A list of these modifications and their functional consequences is presented in Table 7.3. One of the major modifications is the covalent attachment of a polypeptide to a lysine residue of MDM2. Based on the number of amino acids encoded by the *mdm2* gene, MDM2 should have a molecular size of 54 kDal. However, MDM2 isolated from mammalian cells has a relative molecular size of 90 kDal as determined by denaturing polyacrylamide gel electrophoresis (Barak and Oren, 1992; Momand et al., 1992). This paradox of varying molecular sizes was partially solved when it was discovered that the majority of MDM2 is covalently bound to the small-ubiquitin-like modifier protein (SUMO-1),

Table 7.3. Modifiers of MDM2 protein and putative outcomes.

MDM2 modification (molecule/site)	Modifier of MDM2	General characteristics of modifier	Stressor/effect on modification	Effect on MDM2	Effect on p53	References
Phosphoryl group/ser17	DNA-dependent protein kinase	Phosphorylates proteins when bound to double stranded DNA molecules-often as a result of DNA damage	Data not available	Decrease binding to p53	Increase	Mayo et al. (1997)
Phosphoryl group/Ser166	Data not available	Data not available	Mitogen activation of PI3-kinase and Akt-PKB serine-threonine kinase/ increase phosphorylation	Promotes translocation to nucleus	Lowers levels of p53	Mayo and Donner, (2001)
Phosphoryl group/Ser186	Data not available	Data not available	Mitogen activation of PI3-kinase and Akt/PKB serine-threonine kinase/increase phosphorylation	Promotes translocation to nucleus	Lowers levels of p53	Mayo and Donner (2001)
Phosphoryl group/ser269	Creatine Kinase 2	Associates with general transcription factors	Data not available	Data not available	Data not available	Gotz et al. (1999)

Table 7.3. (Continued)

MDM2 modification (molecule/site)	Modifier of MDM2	General characteristics of modifier	Stressor/effect on modification	Effect on MDM2	Effect on p53	References
Phosphoryl group/ser395	Ataxia Telangiectasia a Mutated protein	DNA-damage responsive kinase; mutated in AT patients leading to radiosensitivity	IR/increase phosphorylation of MDM2	Data not available	Correlates with increase in p53 protein levels	Khosravi et al. (1999); Maya et al. (2001)
SUMO-1/unknown	E1 activating complex (Aos1-Uba2); E2 (Ubc9); E3 (unknown)	Modifier of lysine residues on proteins	UV/decrease level IR/decrease level	Increase protein level	Lower protein level	Buschmann et al. (2000); Melchior and Hengst (2000); Buschmann et al. (2001)
Ubiquitin/lys446	E1 activating complex (?); E2 (?); E3 (MDM2)	Modifier of lysine residues on proteins	Data not available	Decrease protein level	Increase protein level	Buschmann et al. (2000)
Ubiquitin/cys464	E2 (Ubc9)	Transfer ubiquitin from E2 to E3	Data not available	Part of transfer reaction to ubiquitinate p53	Transfer of Ub to Lys residues of p53	Honda et al. (1997)

which consists of 101 amino acid residues (Buschmann et al., 2000). SUMO-1 attachment increases the size of MDM2 and enhances the ubiquitin ligase activity, which likely hastens p53 proteolysis.

A hallmark of p53 activation after DNA damage is stabilization through decreased degradation. Because MDM2 is responsible for p53 degradation, one would predict that DNA damage would reduce its ability to ligate ubiquitin onto p53. In line with this prediction, MDM2 modification by SUMO-1 is inhibited in response to DNA damage and correlates with higher p53 levels (Buschmann et al., 2000). The mechanism controlling SUMO-1 modification in response to DNA damage will likely be another major research front in the future.

7.2.5. Nuclear-Cytoplasmic Translocation

Localization of MDM2 within the cell is tightly regulated, as is suggested by the high number of subcellular localization signals it contains. Within its primary amino acid sequence are regions responsible for nuclear import and export (Roth et al., 1998), and nucleolar import (Lohrum et al., 2000) (see Fig. 7.1). These sequences help MDM2 control the transport of itself and the transport of p53. First, one must place MDM2's control of p53 transport within the context of what we know about p53 subcellular trafficking. After p53 synthesis in the cytoplasm it is transported to the nucleus. Under nonstressed conditions p53 is quickly exported from the nucleus whereupon it meets its destruction by the 26S proteosome in the cytoplasm. This cycle of nuclear import, nuclear export, and destruction is broken when the cell is stressed. Upon stressor treatment, p53 export from the nucleus is blocked and it accumulates. Once the concentration reaches a threshold level it binds target genes that execute its tumor suppressor function. Work in Dr. Arnold Levine's laboratory at Princeton University showed that MDM2 is the transporter that carries p53 from the nucleus to the cytoplasm (Roth et al., 1998; Tao and Levine, 1999). Interestingly, a few clinical cancer cases have shown that high levels of p53 reside in the cytoplasm but not in the nucleus. This phenomenon, termed nuclear exclusion, suggests that there is defect in the p53 accumulation process in the nucleus of these cancer cells. Cancers showing this phenotype include inflammatory breast carcinomas, retinoblastomas, neuroblastomas, and colorectal carcinomas (Moll et al., 1992, 1995; Domagala et al., 1993; Schlamp et al., 1997). Recent studies suggest that MDM2 may play a role in p53 nuclear exclusion. For example, experimental overexpression of MDM2 in cells expressing wild-type p53 can, in some cases, lead to p53 nuclear exclusion (Rodriguez-Lopez et al., 2001). Furthermore, some tumor cells displaying nuclear exclusion require MDM2 to maintain p53 in the cytoplasm (Lu et al., 2000). It is possible that these cells express a highly active MDM2 that exports p53 and a defect in p53 degradation mechanism. Therefore, tumor cells displaying p53 nuclear exclusion may be good candidates for anti-MDM2 therapy.

Exactly how MDM2 shuttles p53 out of the nucleus has been the subject of several recent studies. MDM2-mediated export of p53 requires CRM1, a mammalian export receptor that recognizes a leucine-rich nuclear export signal (Freedman and

Levine, 1998). Research has shown that Cys 464 within the RING finger domain of MDM2 is required for p53 nuclear export (Boyd et al., 2000; Geyer et al., 2000). This cysteine residue is also essential for ubiquitin ligase activity but it is not clear if p53 is ubiquitinated prior to export. There appear to be subtle nuances in the control of p53 nuclear export. Complicating the export issue is the fact that both p53 and MDM2 contain nuclear export sequences. Two studies have shown that p53 nuclear export requires a nuclear export sequence located within residues 340–351 but does not require the MDM2 nuclear export sequence (Boyd et al., 2000; Geyer et al., 2000). Another study has shown that export of p53 requires nuclear export sequences from both p53 and MDM2 (Tao and Levine, 1999). A third study has shown that p53 is capable of nuclear export in the absence of MDM2 (Stommel et al., 1999). How can we account for these apparent differences? Perhaps the mechanism of p53 nuclear export depends on other parameters that include species type, cell type, MDM2/p53 expression levels, and cell growth state.

An interesting twist on MDM2 control of p53 nuclear export was recently discovered (Zhang and Xiong, 2001). The first p53 nuclear export sequence discovered was located near its carboxyl terminus (Roth et al., 1998) far away from the MDM2 binding domain. However, Dr. Yue Xiong's laboratory at the University of North Carolina uncovered a second p53 nuclear export sequence located near the N-terminus, where MDM2 binds. Under nonstressed circumstances the N-terminal p53 export sequence is functional; p53 forms a complex with MDM2 and is shuttled out of the nucleus. Phosphorylation of p53 within this sequence, however, prevents MDM2 binding and, in addition, blocks its export. In this instance, kinases activated by genome damage can release p53 from MDM2 and at the same time retain p53 within the nucleus. In sum, p53 nuclear export is strongly influenced by MDM2.

7.3. STRESSOR INDUCED REGULATION OF MDM2–p53 INTERACTION

7.3.1. Phosphorylation of p53

Activation of p53 is characterized by an increase in protein level, nuclear accumulation, and the attainment of posttranslational modifications that enhance its ability to transactivate effector genes. Two well-characterized stressors that lead to p53 activation are ionizing radiation and inappropriate oncogene activation. Each stressor inhibits MDM2 activity but in unique ways. In 1991, Dr. Michael Kastan and his colleagues at Johns Hopkins University showed that ionizing radiation promotes upregulation of p53 activity and halts cellular proliferation (Kastan et al., 1991). Since this seminal discovery, several investigators have showed that p53 upregulation in response to DNA damage correlates with certain specific posttranslational modifications (see Giaccia and Kastan, 1998) for review). Phosphorylation of p53 occurs at Ser 15, Thr 18, and Ser 20 and prevents its ability to bind to MDM2 either by direct interference (Thr 18, Ser 20) or by alteration of its local conformation

(Ser 15) (Shieh et al., 1997; Unger et al., 1999a, b; Bean and Stark, 2001, 2002; Hirao et al., 2000; Sakaguchi et al., 2000). Recently, DNA damage has also been shown to result in MDM2 phosphorylation (Khosravi et al., 1999; Maya et al., 2001).

7.3.2. Phosphorylation of MDM2

One kinase that phosphorylates MDM2 is the *Ataxia Telangiectasia Mutated* gene product, ATM (Khosravi et al., 1999; Maya et al., 2001), the same one that modifies p53 in response to some forms of DNA damage. ATM appears to be responsible for phosphorylating MDM2 at Ser 395. Although Ser 395 does not reside in a known subcellular localization motif, its phosphorylation does appear to inhibit MDM2 export and assist in upregulating p53 activity. Another kinase that phosphorylates MDM2 is DNA-dependent protein kinase (Mayo et al., 1997). DNA-dependent kinase is activated by genome damage but the site of phosphorylation on MDM2 is not yet clear. It is likely that, with the development of phosphopeptide-specific antibodies, MDM2 phosphorylation regulation will be explored extensively in the next few years.

7.3.3. ARF–MDM2 Complex Formation

Classical yeast genetic studies showed that DNA damage promotes cell cycle arrest (reviewed in Hartwell and Weinert, 1989). Later, mammalian cell culture studies demonstrated that DNA damage could also lead to apoptosis (Clarke et al., 1993; Lowe et al., 1993). More recently, oncogene activation has been shown to lead to these cellular outcomes as well (reviewed in Sherr, 2001). Activation of oncogenes can be deleterious to the organism by signaling cells to divide at inappropriate times. If the cell receives a signal from an oncogene to divide when DNA is damaged, it risks the chance of sustaining a mutation. When this cell stress pathway was uncovered it did not take long to show that oncogene activation triggered a p53 response. Early studies demonstrated that *myc* oncogene activation led to apoptosis and that this process required p53 (Ramqvist et al., 1993; Wang et al., 1993). A key discovery was that the *Ink4a*/*ARF* tumor suppressor gene was required for oncogene signaling to p53 (de Stanchina et al., 1998; Zindy et al., 1998). The *Ink4a*/*ARF* gene produces two transcripts, each possessing tumor suppressor activity. In humans the first transcript produces $p16^{Ink4a}$, which inhibits cyclin D1/Cdk4 complex, a negative inhibitor of Rb. The second transcript of this gene produces ARF (in humans this is sometimes known as $p14^{Arf}$ and in mice as $p19^{Arf}$), which binds and inhibits MDM2 activity. ARF also binds and prevents MDMX from interacting with p53 (Jackson et al., 2001). The current view of the mechanism of MDM2 and MDMX inactivation by ARF is presented in Figure 7.3. In the absence of oncogene activation p53 is removed from the nucleus by MDM2 and degraded by the 26S proteasome. A separate p53 subpopulation is directly bound to MDMX in the nucleus and is prevented from activating its effector genes. Upon oncogene activation ARF levels increase and bind MDM2 and MDMX, releasing p53 to transactivate its appropriate target genes. ARF then sequesters both MDM2 and MDMX into the nucleolus. Oncoproteins that activate

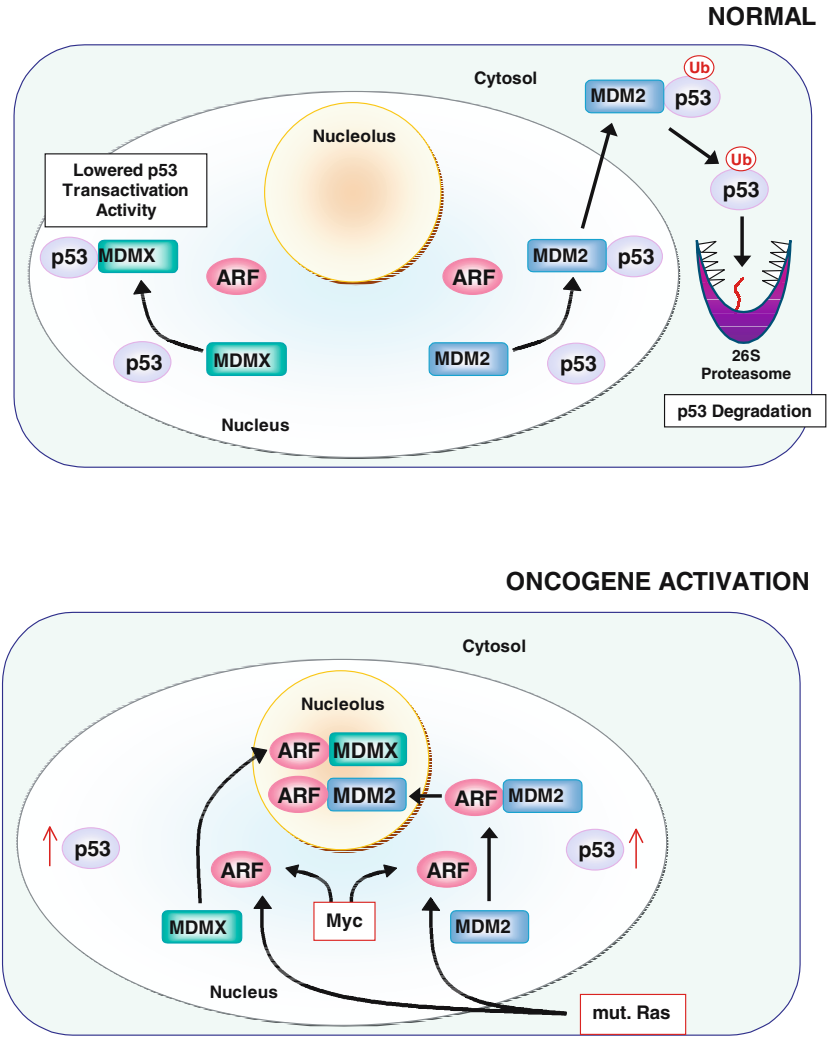


Figure 7.3. Oncogene activation of MDM2 and MDMX. Under normal conditions p53 is bound to MDMX in an inactive complex or shuttled to the cytoplasm by MDM2 where it is degraded by the 26S proteasome. When the Myc or mutant Ras oncoprotein is abnormally activated, ARF levels increase. Upon binding ARF, MDMX is released from p53 and shuttled into the nucleolus. When MDM2 binds ARF it fails to ubiquitinate p53 and is shuttled to the nucleolus. The released p53 transactivates genes that arrests cell proliferation or induces apoptosis.

p53 through the ARF pathway include myc (Pomerantz et al., 1998; Zindy et al., 1998), polyoma virus middle T-antigen (Lomax and Fried, 2001), E2F1 (Bates et al., 1998), viral oncoprotein E1A (de Stanchina et al., 1998), and mutant Ras (Ries et al., 2000). The list of mitogenic molecules that activate p53 through this pathway is likely to expand in the near future.

Recent studies suggest that ARF actually downregulates MDM2 activity by two mechanisms. First, MDM2 bound to ARF is inhibited in its ability to ubiquitinate p53 (Honda and Yasuda, 1999; Xirodimas et al., 2001). Second, ARF sequesters MDM2 into the nucleolus. These would seem to be redundant inhibitory mechanisms but redundancy appears to be the rule when dealing with MDM2 biochemical activities, not the exception. It is likely that the combination of the two mechanisms of MDM2 inactivation results in a more rapid and a more efficient response than a single one. Perhaps in the absence of nucleolar sequestration, upon initial binding to ARF, MDM2 is inactivated but remains in the vicinity of p53. Because binding is reversible, some MDM2 can dissociate from ARF and bind to p53 again. To be a more effective inhibitor ARF removes MDM2 to the nucleolus and allows newly synthesized ARF to bind and inhibit other MDM2 molecules. In sum, the two major upstream signals that activate p53 do so by distinct mechanisms. DNA damage modulates phosphorylation of MDM2 and p53. Oncogene activation controls ARF binding to MDM2. A fertile area of future research will be the delineation of more mechanisms that control MDM2. Other stressors known to control p53 activity include hypoxia, hyperoxia, and chemical carcinogens. These stressors may modulate MDM2 and MDMX through either of the routes listed above or through other posttranslational mechanisms such as oxidation, reduction, oligomerization, acetylation, SUMO-1 modification, and dephosphorylation.

7.3.4. Oligomerization and Acetylation

Aside from phosphorylation and ARF binding, other posttranslational events control MDM2–p53 complex formation. For example, p53 must form a dimer for efficient MDM2 binding (Maki, 1999) and p53 is incapable of being acetylated when it is bound to MDM2 (Ito et al., 2001; Kobet et al., 2000). Acetylation helps recruit transcriptional coactivators to p53 to help it increase its ability to transactivate genes (Barlev et al., 2001). Lysine residues near the C-terminus of p53 are substrates for acetylation. It is likely that the RING finger domain of MDM2 is involved in blocking acetylation because this domain is necessary for ubiquitinating lysine residues near the C-terminus of p53. If p53 deacetylation is inhibited it becomes more stable (Ito et al., 2001) suggesting that acetylation protects lysines from ubiquitination.

7.4. GENETICS OF MDM2 AND MDMX

Mouse genetics can be a powerful tool to test hypotheses derived from experiments performed with cultured mammalian cells. Prior to the mouse genetic studies, experiments using cultured cells led to the prediction that a mouse lacking *mdm2*

would overexpress p53. Overexpression of p53 should lead to cell death or cell cycle arrest. This hypothesis was dramatically confirmed when it was shown that *mdm2* –/– mouse embryos fail to survive after day 6.5–7.5 postgestation (Jones et al., 1995; Montes de Oca Luna et al., 1995). Tissues recovered from aborted embryos showed signs of having undergone p53-mediated programmed cell death (Montes de Oca Luna et al., 1995). Interestingly, the *mdm2* –/– mouse was rescued when placed in a *p*53 null genetic background. The double knockout mouse exhibits a phenotype almost identical to a mouse that expresses no p53. Both mice are born with a normal phenotype but tumors arise within the same timeframe and the type of tumors that develop are similar. Thus, *mdm2* appears to mainly function to maintain p53 protein in check.

To determine the physiological significance of MDMX an *mdmx* knockout mouse was created (Parant et al., 2001). Like the *mdm2* –/– mouse, the *mdmx* –/– mouse was not viable and aborted at approximately 6.5 days postgestation. However, death was not due to excessive programmed cell death. Instead, cells in the aborted embryo simply failed to proliferate. It is possible that p53 was unchecked and that the cell cycle arrest genes it normally regulates were overexpressed. To partially test this hypothesis, a mouse with a knockout in both *mdmx* and *p*53 genes was created. Surprisingly, the double knockout mouse survived and appeared normal. The oldest mouse at the time of the publication was four months and failed to show any signs of abnormality (It is predicted that the mouse will develop tumors similar to the p53 knockout mouse.) The mouse genetic studies indicate three important points: (1) MDM2 is a negative regulator of p53-mediated apoptosis activity; (2) MDMX is a negative regulator of p53-mediated cell cycle arrest activity; (3) MDM2 cannot substitute for MDMX and vice versa during mouse development.

It is intriguing that MDM2 and MDMX cannot substitute for one another given that both molecules bind to the same region within p53. It is possible that there may be cell-specific or time-specific expression of each p53 inhibitor, both being independently critical to embryo survival. It is also possible that control of p53's ability to transactivate its many effector genes is divided between MDM2 and MDMX. In the developing embryo, MDM2 may control proapoptotic genes while MDMX may control growth arrest genes.

7.5. THE AUTOREGULATORY LOOP

Soon after the discovery of MDM2 it was observed that its level was increased when p53 was experimentally overexpressed (Barak et al., 1993). This increase was due to a higher level of MDM2 transcript and was mediated by the binding of p53 to two specific sequences within intron 1 (Juven et al., 1993; Wu et al., 1993). DNA damage also leads to p53-mediated upregulation of the *mdm2* gene (Chen et al., 1994). These studies gave rise to the notion that p53 could regulate its own inhibition through an autoregulatory loop (reviewed in Zambetti and Levine, 1993). In this loop, it is thought that p53 activates its own destruction by transcribing *mdm2*, thus maintaining itself at a low level. Stressors activate signaling pathways that prevent p53 from

binding MDM2, thus allowing its level to increase and transactivate effector genes that promote apoptosis and cell cycle arrest. When cell cycle arrest is the outcome, cells may eventually resume proliferation once the genome is repaired. Once p53 activates *mdm2*, its levels are lowered to a point where cells can proliferate again. Interestingly, p53 does not regulate *mdmx*. Presented in Figure 7.4 are the major discoveries of the molecules involved in the p53 and MDM2 pathways and the autoregulatory loop. Each arrow that connects two molecules in the pathway corresponds to a discovery made by scientists. The width of each arrow is proportional to the number of citations each discovery received per unit time (see Appendix 7.1 for details on how the width of each arrow was calculated). The black arrows correspond to the molecular events that are active in the autoregulatory loop that controls p53 activity. The yellow arrows show the molecular events that occur when the cell genome is damaged. The pink arrows show the molecular events that are activated by oncogene stimulation. The

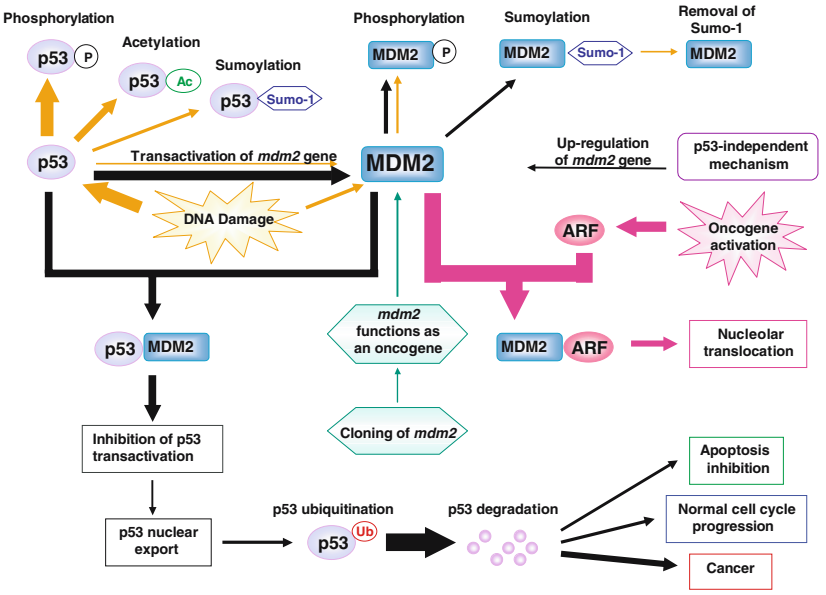


Figure 7.4. Major milestones of p53 autoregulation. The p53 protein activates *mdm2* resulting in an increase in MDM2 protein levels. In the absence of DNA damage (depicted with black arrows) p53 transactivates the *mdm2* gene. MDM2 binds p53 and ultimately assists in its degradation. In doing so, p53 is prevented from promoting apoptosis or inhibiting the cell cycle. Excess production of MDM2 lowers the level of p53 and can ultimately lead to cancer. DNA damage activates p53 (depicted in yellow arrows) by promoting p53 phosphorylation, p53 acetylation, SUMO-1 conjugation to p53. Subsequently, the *mdm2* gene is activated. DNA damage also promotes MDM2 phosphorylation and removal of SUMO-1 from MDM2. A second route to MDM2 inhibition is via oncogene activation (depicted in pink arrows). Oncogene activation increases the level of ARF, which in turn sequesters MDM2 into the nucleolus. Green arrows depict the discovery of the *mdm2* oncogene. The thickness of the arrows correlates with the number of citations received for each part of the pathway. (See Appendix 7.1 for details of calculations of arrow thicknesses.)

green arrows depict the major steps made in the discovery of the *mdm2* gene. This mode of p53 activation allows it to act quickly after application of the stressor.

7.6. *mdm2* GENE STRUCTURE AND TRANSCRIPTION

Murine *mdm2* consists of 12 exons and spans approximately 25 kb (Montes de Oca Luna et al., 1996; Jones et al., 1995). We now know that regulation of *mdm2* takes place at the transcriptional level, posttranscriptional level, and the posttranslational level. In this section we describe how differential promoter usage and alternative splicing regulate the MDM2 transcript. The first two exons of the *mdm2* gene do not code for protein, and two physically distinct promoters within *mdm2*, named P1 and P2, have been identified in both humans and mice (Barak et al., 1994; Landers et al., 1997; Zauberman et al., 1995). The P1 promoter is located 5′ to exon 1 and the P2 promoter is located just 5′ to exon 2. The P2 promoter contains two p53-responsive elements but elements that control the P1 promoter have not been identified. Studies on *mdm2* transcriptional regulation have yielded a complex pattern of mRNA production.

In adult murine tissues MDM2 transcripts initiated from P1 are approximately five times more abundant than transcripts initiated from P2 (Mendrysa and Perry, 2000). As expected, when a mouse is exposed to ionizing radiation, P2 initiated transcripts increase in a p53-dependent fashion. In the *p*53 knockout mouse, P2-initiated transcripts, named S-MDM2 transcripts, are present at low levels, but fail to increase after ionizing radiation treatment. The fact that S-MDM2 transcripts are detected in the *p*53 knockout mouse indicates that transcription factors other than p53 can express the S-MDM2 transcript. Interestingly, P2 becomes active upon removing cells from normal mice embryos and placing them in culture. This suggests that P2 becomes p53 dependent only in a state of constant stress. The process of culturing promotes that stress and implies that perhaps all cultured cells contain an active p53 autoregulatory loop. The corollary to this hypothesis is p53 is not downregulated by MDM2 under nonstressed conditions. This has implications for anti-MDM2 therapy. It was previously thought that a major impediment to anti-MDM2 therapy could be the inappropriate activation of p53 in noncancer tissue. This would elicit a cell death response in normal cells. If, however, the autoregulatory loop is active only after a stress event then anti-MDM2 therapy becomes attractive because there may be no p53-elicited cell death in nonstressed normal tissues. The transcription factors that maintain basal levels of MDM2 mRNA in nonstressed tissues are not known.

Transcription factors other than p53 can increase MDM2 mRNA. Dr. Moshe Oren and his colleagues at the Weizmann Institute in Rehovot have shown that DNA sequences corresponding to AP-1, EtsA, and EtsB transcription factor binding sites are observed within intron 1 (Ries et al., 2000). Ras oncoprotein activates a signaling pathway that leads to upregulation of AP-1, EtsA, and EtsB and, ultimately, cell proliferation. The existence of this pathway indicates that Ras can mediate its oncogenic function by MDM2 upregulation. The factors linking Ras and the DNA binding proteins are Raf, MEK, and MAPK. Combined with other studies previously discussed in this chapter, this observation indicates that Ras can activate two opposing pathways

culminating with either upregulation or downregulation of MDM2. In one pathway, Ras activates ARF, which inhibits MDM2 through complex formation. In the other pathway, Ras activation increases transcription of *mdm2*. How can we explain these two seemingly contradictory outcomes? It is known that p53 levels are extremely low in proliferating cells and it may be that in such cells the Ras pathway that upregulates *mdm2* may be active, leading to low p53 levels. In this cell growth phase, Ras fails to activate ARF. However, if Ras is activated at an inappropriate time, it triggers ARF activation and upregulates p53. The switch that modulates these pathways is unknown. In cells that have lost the ability to properly express the *ARF* gene the repercussions of the *mdm2* activation by Ras becomes magnified. In such cells, Ras is predicted to decrease the level of p53 protein and drive them toward cancer formation.

An alternatively spliced form of MDM2 transcript affects its ability to inhibit p53. Truncated versions of MDM2 have been demonstrated to exist for almost 10 years (Olson et al., 1993; Haines et al., 1994). But, until recently, little was known as to how such truncated forms could control p53 activity. A murine *mdm2* transcript lacking exon 3 has been observed in cell lines (Saucedo et al., 1999). This transcript codes for a truncated version of MDM2, named $p76^{MDM2}$, which is translated beginning at codon 50. Interestingly, $p76^{MDM2}$ increases the stability of full-length MDM2 and enhances p53 transactivation capacity (Perry et al., 2000). One hypothesis as to the mechanism of $p76^{MDM2}$-mediated upregulation of p53 is that it may compete for factors that normally bind or modify full-length MDM2 (perhaps E2 of the ubiquitin activating pathway). Binding these factors prevents MDM2 from properly inhibiting p53. The ratio of full-length MDM2 to $p76^{MDM2}$ is not uniform amongst tissues and, when the ratio of $p76^{MDM2}$ to full-length MDM2 is high, it is predicted that the p53 protein will be able to quickly stabilize after DNA damage.

Like its murine counterpart, the human *mdm2* gene produces two transcripts named L-MDM2 and S-MDM2 (Landers et al., 1997; Zauberman et al., 1995). Figure 7.5 delineates how these transcripts are generated. The L-MDM2 transcript is the product of the constitutive promoter, P1. Exon 2 encoded sequences are removed from the mature L-MDM2 transcript resulting in an RNA sequence that consists of exon 1 encoded sequence juxtaposed to exon 3 encoded sequence. The S-MDM2 transcript is the product of the p53 inducible promoter, P2. The S-MDM2 transcript is translated at eightfold higher efficiency than the L-MDM2 transcript, resulting in high expression levels of its protein product (Landers et al., 1997). Both S-MDM2 and L-MDM2 transcripts encode the full-length MDM2 protein.

7.7. MDM2 AND MDMX INVOLVEMENT IN CANCERS

Gene amplification is one of several mechanisms by which cancer cells can overexpress oncogenes. In fact, the *mdm2* gene was originally observed to be amplified in a mouse cell line that had spontaneously become tumorigenic (Cahilly-Snyder et al., 1987; Fakharzadeh et al., 1991). *mdm2* gene amplification has been detected in 14 types of tissues displaying abnormal growth patterns (Momand et al., 1998). The overall frequency of *mdm2* amplification in these tissues is 7%. Based on the

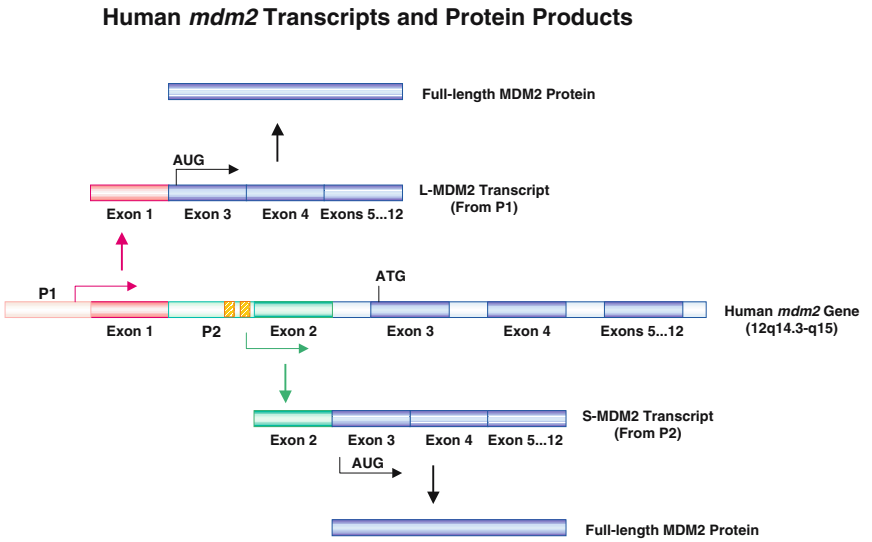


Figure 7.5. Alternative splice forms of human MDM2 transcripts. Human *mdm2* consists of 12 exons. The first two exons do not code for protein. Promoter P1 is located upstream of exon 1 and P2 is located within intron 1. Two yellow hatched regions represent the two p53-response elements within P2. L-MDM2 is transcribed beginning at exon 1 but splices out exon 2 to form the mature transcript. S-MDM2 is transcribed starting at exon 2. Full-length MDM2 is produced from both transcripts.

phenotype of the *mdmx* knockout mouse one would predict that the human *mdmx* gene might be upregulated in some cancers. This prediction was recently confirmed when in a study of 208 gliomas it was discovered that *mdmx* was amplified in five tumors (Riemenschneider et al., 1999). Because MDM2 and MDMX control p53 one would expect that mutations in the *p53* gene would be an infrequent event in cancers with *mdm2* and *mdmx* gene amplification. In all cases of cancers with *mdm2 or mdmx* amplification there was virtually no evidence for *p53* gene mutations, indicating that genetic alterations in *mdm2*/*mdmx* and *p53* rarely occur in the same tissue (Momand et al., 1998; Riemenschneider et al., 1999). Because ARF lies directly upstream of MDM2 within the oncoprotein activation pathway one would predict that the *ARF* gene would be mutated in high percentage of tumors; studies now attest to this (Gardie et al., 1998; Gazzeri et al., 1998). Taken together, we now know that the chance of observing an inactivating mutation in *ARF*, an activating mutation in *mdm2*/*mdmx*, or an inactivating mutation in *p53* is very high in human cancers.

7.8. CONCLUDING REMARKS

A striking take-home lesson presented by the p53 autoregulatory loop is that the ability of MDM2 to inhibit the tumor suppressor activity of p53 is biochemically redundant. MDM2 appears to target p53 for destruction in three progressive

stages—targeting, removal, and destruction. In the targeting stage, the specific domain of p53 required for transcription is bound by MDM2 allowing for immediate cessation of p53 activity. Fulfillment of this stage allows p53 to bind MDM2 while it is bound to DNA, immediately shutting down transcription. In the removal stage, MDM2 continuously escorts p53 away from the nucleus; thus, maintaining a low concentration of p53 in the vicinity of its target genes. In the destruction stage, the initial step of p53 degradation is MDM2-mediated ubiquitination. In the absence of any of these stages MDM2 becomes a less efficient inhibitor and, therefore, may allow p53 to be active at inopportune times. The last two stages of MDM2 inhibition harkens back to the thermodyanamic principles that control product formation in chemical reactions. The change in free energy of the reaction depends, in large part, on efficient removal of products which drives the reaction forward. This biochemical redundancy is necessary for efficient responses to cellular needs. It is likely that redundant forms of inhibition are waiting to be found in other biochemical systems.

ACKNOWLEDGEMENTS

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APPENDIX 7.1. CALCULATIONS AND REFERENCES FOR CITATIONS USED TO SET ARROW THICKNESS IN FIGURE 7.4.

Original communications were identified and placed under separate subject headings. The number of citations for each communication until November 2001 was obtained from the ISI Web of Science ® internet site. The number of citations for each communication was divided by the number of months elapsed since publication to obtain the citation rate. The citation rate was added to other citation rates within the same category and listed as "Total". This Total number divided by 2 was set as the point width of each arrow used in Figure 7.4.

p53 phosphorylation:

Phosphorylation at S15 upon DNA damage (Siliciano et al., 1997) **Dec (47 Months); # Cited: 227; Correction = 227/47 = 4.83**

Phosphorylation at S20 upon DNA damage

(Chehab et al., 1999) **Nov (24 Months); # Cited: 67; Correction = 67/24 = 2.79**

(Unger et al., 1999) **Apr (31 Months); # Cited: 88; Correction = 88/31 = 2.84**

Phosphorylation at S37 upon DNA damage

(Shieh et al., 1997) **Oct (49 Months); # Cited: 388; Correction = 388/49 = 7.92**

Phosphorylation at T18 upon DNA damage

(Sakaguchi et al., 2000) **Mar (20 Months); # Cited: 26; Correction = 26/20 = 1.3**

Total = 19.68

p53 acetylation:

(Gu and Roeder, 1997) **Aug (51 Months); # Cited: 532; Correction = 532/51 = 10.43**

Total = 10.43

p53 Sumoylation:

(Rodriguez et al., 1999) **Nov (24 Months); # Cited: 63; Correction = 2.63**

(Gostissa et al., 1999) **Nov (24 Months); # Cited: 55; Correction = 55/24 = 2.29**

Total = 4.92

MDM2 phosphorylation:

S17 by DNA-PK (Mayo et al., 1997) **Nov (48 Months); # Cited: 91; Correction = 91/48 = 1.90**

S395 by ATM (Khosravi et al., 1999) **Dec (23 Months); # Cited: 61; Correction = 61/23 = 2.65**

(Maya et al., 2001) **May (6 Months); # Cited: 4; Correction = 4/6 = 0.67**

S269 by CK2 (Gotz et al., 1999) **Dec (23 Months); # Cited: 5; Correction = 5/23 = 0.22**

S166 by PI3K/Akt(PKB) (Mayo and Donner, 2001) **Sep (2 Months); # Cited: 1; Correction = ½ = 0.50**

S186 by PI3K/Akt(PKB) (Mayo and Donner, 2001) **(2 Months); # Cited: 1; Correction = ½ = 0.50**

Total = 6.44

MDM2 Sumoylation:

(Buschmann et al., 2000) **Jun (17 Months); # Cited: 37; Correction = 37/17 = 2.18**

(Melchior and Hengst, 2000) **Sep (14 Months); # Cited: 1; Correction = 1/14 = 0.07**

Total = 2.25

Cloning of MDM2:

(Cahilly-Snyder et al., 1987) **May (174 Months); # Cited = 153; Correction = 153/174 = 0.88**

Total = 0.88

Identification of MDM2 as an oncogene:

(Fakharzadeh et al., 1991) **Jun (125 Months); # Cited = 336; Correction = 336/125 = 2.69**

Total = 2.69

p53 up-regulation by DNA damage:

(Kastan et al., 1991) **Dec (119 Months); # Cited = 2266; Correction = 2266/119 = 19.09**

Total = 19.09

Transactivation of MDM2 gene by p53:

(Wu et al., 1993) **Jul (100 Months): # Cited: 728; Correction = 738/100 = 7.38**

(Barak et al., 1993) **Feb (105 Months): # Cited: 558; Correction = 558/105 = 5.31**

(Juven et al., 1993) **Dec (95 Months): # Cited: 216; Correction = 216/95 = 2.27**

Total = 14.96

Transcriptional up-regulation of MDM2 independent of p53:
(Perry et al., 2000) **May (18 Months); # Cited: 8; Correction = 8/18 = 0.44**
(Barak et al., 1994) **1994 Aug (51 Months); # Cited 129; Correction = 129/51 = 2.53**
Total = 2.97

MDM2/p53 complex formation:
(Momand et al., 1992) **Jun (113 Months); # Cited: 1358; Correction = 1358/113 = 12.02**
Total = 12.02

p53 transactivation inhibition by MDM2:
(Momand et al., 1992) **Jun (113 Months); # Cited: 1358; Correction = 1358/113 = 12.02**
Total = 12.02

p53 export by MDM2:
(Tao and Levine, 1999) **Jun (29 Months); # Cited: 81; Correction = 81/29 = 2.79**
Total = 2.79

Ubiquitin ligase activity of MDM2:
(Honda et al., 1997) **Dec (47 Months); # Cited: 216; Correction = 216/47 = 4.60**
Total = 4.60

p53 degradation by MDM2:
(Haupt et al., 1997) **May (54 Months); # Cited: 705; Correction = 705/54 = 13.06**
(Kubbutat et al., 1997) **May (54 Months); # Cited: 629; Correction = 629/54 = 11.65**
(Midgley and Lane, 1997) **Sep (50 Months); # Cited: 106; Correction = 106/50 = 2.12**
Total = 26.83

Cancer progression upon p53 inhibition by MDM2:
(Oliner et al., 1992) **Jul (112 Months); # Cited: 1053; Correction = 1053/112 = 9.40**
Total = 9.40

Normal cell cycle progression upon p53 degradation by MDM2:
(Chen et al., 1994) **March (92 Months); # Cited: 226; Correction = 226/92 = 2.46**
Total = 2.46

DNA damage induced upregulation of MDM2 gene:
(Chen et al., 1994) **March (92 Months); # Cited: 226; Correction = 226/92 = 2.46**
Total = 2.46

Apoptosis inhibition upon p53 degradation by MDM2:
(Haupt et al., 1996) **Feb (69 Months); # Cited: 150; Correction = 150/69 = 2.17**
(Chen et al., 1996) **May (66 Months); # Cited: 145; Correction = 145/66 = 2.20**
Total = 4.37

Oncogene Activation of ARF:

(Kamijo et al., 1998) **Jul (40 Months); # Cited : 198; Correction = 198/40 = 4.95**

(de Stanchina et al., 1998) **Aug (39 Months); # Cited: 188; Correction = 188/39 = 4.82**

(Zindy et al., 1998) **Aug (39 Months); # Cited: 238; Correction = 238/39 = 6.10**

Total = 15.87

MDM2/ARF complex formation:

(Pomerantz et al., 1998) **Mar (44 Months); # Cited: 755; Correction = 755/44 = 17.16**

(Zhang et al., 1998) **Mar (44 Months); # Cited: 395; Correction = 395/44 = 8.98**

Total = 26.14

MDM2/ARF complex nucleolus translocation:

(Weber et al., 1999) **May (29 Months); # Cited: 140; Correction = 140/29 = 4.83**

(Tao and Levine, 1999) **June (27 Months); # Cited: 145; Correction = 145/29 = 5.00**

Total = 9.83

DNA damage induced MDM2 Phosphorylation changes:

S395 by ATM (Khosravi et al., 1999) **Dec (23 Months); # Cited: 61; Correction = 61/23 = 2.65**

(Maya et al., 2001) **May (6 Months); # Cited: 4; Correction = 4/6 = 0.67**

Total = 3.32

DNA damage induced MDM2 SUMO-1 removal:

(Buschmann et al., 2000) **Jun (17 Months); # Cited: 37; Correction = 37/17 = 2.18**

Total = 2.18

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