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Functions of the MDM2 oncoprotein

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Abstract. The p53 protein is activated in response to physiological stress resulting in either a G1 arrest of cells or apoptosis. As such, p53 must be tightly regulated, and the MDM2 oncoprotein plays a central role in that regulatory process. The transcription of the *Mdm2* oncogene is induced by the p53 protein after DNA damage, and the MDM2 protein then binds to p53 and blocks its activities as a tumour suppressor and promotes its degradation. These two proteins thus form an autoregulatory feedback loop in which p53 positively regulates MDM2 levels and MDM2 negatively regulates p53 levels and activity. Immediately after ultraviolet (UV) irradiation MDM2 messenger RNA and protein levels fall in a p53-independent fashion, resulting in increased p53 levels. The p53 protein is then activated as a transcription factor by posttranslational modification permitting p53 to initiate its cell-cycle arrest or apoptotic (programmed cell death) functions. At later times, after the repair of DNA, MDM2 levels increase in a p53-dependent fashion. This induction of MDM2 results in the inhibition of p53 transcriptional activity and the degradation of p53 protein. MDM2-p53 complexes in the nucleus are transported to the cytoplasm via signals present in the MDM2 protein, where p53 is degraded in the proteasome. Thus MDM2 acts as a nuclear-cytoplasmic shuttle for the p53 protein. There are many levels at which this process is regulated, and as such there are many places for chemotherapeutic interventions. The amino-terminal domain of the MDM2 protein is all that is required to bind the p53 protein. The MDM2 protein has additional domains and therefore may have additional functions. Any of these MDM2 domains may contribute to MDM2's activities as an oncogene independent of its inhibition of the tumour suppressor functions of p53. Thus MDM2 itself could be a target for cancer therapeutic intervention.

Key words. MDM2; p53; tumour suppressor; oncogene; autoregulatory feedback loop.

Introduction

The p53 tumour suppressor protein is stabilized in response to DNA damage and other stress signals and causes the cell to undergo growth arrest or apoptosis, thus preventing the establishment of mutations in future cellular generations. Mutation or loss of p53 is a very common event in tumour progression; it occurs in about 50% of all tumours analysed, including those of colon, lung, breast and liver [1, 2]. In some tumours with wild-type p53, other changes, such as the amplification of the *Mdm2* gene or overexpression of the MDM2 protein, can block p53 function and promote growth of the tumour [3]. In addition, there are several indications that MDM2 itself may play a role in cell-cycle progression or tumorigenesis independent of its ability to inhibit the tumour suppressor functions of p53.

This article focuses upon the MDM2 protein and its oncogenic functions. It reviews the role of MDM2 in an autoregulatory feedback loop with p53 and the ways in

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which the cell regulates the inhibition of p53 by MDM2. MDM2's oncogenic functions, independent of p53, will also be discussed. Finally, the prospect of targeting MDM2 or the MDM2-p53 interaction as a potential cancer therapy will be reviewed.

MDM2 as an oncogene

MDM2 (murine double minute 2) was first identified as the gene responsible for the spontaneous transformation of an immortalized murine cell line, BALB/c 3T3. The derivative cell line, 3T3-DM, contains 25–30 copies per cell of paired, acentric chromatin bodies called double minutes [4]. These 1–2 megabase (Mb) fragments contain three expressed genes, the second of which (*Mdm2*) has transforming abilities. Cell lines that overexpress MDM2 can form tumours in nude mice, although they do not have the ability to grow in soft agar or form foci [5]. The genomic *Mdm2* clone can also mediate immortalization of rat embryo fibroblasts (REFs) and transformation of REFs (as characterized by focus-forming ability and tumour formation in nude mice) in cooperation with an activated ras gene [6]. Thus MDM2 acts as an oncogene in tissue culture systems.

The *Mdm2* gene can also be classified as an oncogene based on its behaviour in human tumours. Overexpression of MDM2 is found in a wide variety of human tumours, due to one of three different mechanisms: gene amplification [7, 8], increased transcription [9, 10] or enhanced translation [11, 12]. Tumour types include soft tissue sarcomas, osteosarcomas and rhabdomyosarcomas [7, 8, 13–16]; glioblastomas and astrocytomas [17, 18]; B-cell chronic lymphocytic leukaemias, acute myeloid leukaemias, acute lymphoblastic leukaemias, and non-Hodgkin's lymphomas [9, 10]; oral squamous cell and breast carcinomas [19, 20]; and malignant melanomas [21]. In addition, many human-tumour-derived cell lines overexpress MDM2 and are routinely used in laboratories to study the effects of MDM2 overexpression on cells in culture. These cell lines include those derived from osteosarcomas [22], rhabdomyosarcomas [23, 24], neuroblastomas and glioblastoma [25, 26], breast carcinomas [27, 28], and melanomas and choriocarcinomas [11, 12].

An additional connection between MDM2 and cancer is the identification of a cancer-prone Li-Fraumeni family whose normal tissues overexpress MDM2. These individuals contain two wild-type alleles of p53 and do not overexpress p21 (a p53-inducible gene), suggesting that the overexpression of MDM2 is p53-independent [29]. The overexpression of MDM2 in the normal tissues of these individuals suggests that MDM2 may be a direct cause of the high tumour incidence in the family.

Additional evidence that MDM2 functions as an oncogene comes from mice with targeted overexpression of MDM2 in the mammary epithelium during lactation [30]. In the mammary glands themselves, normal development and terminal differentiation are blocked by the high levels of MDM2, and many of the cells become multinucleated and polyploid, which are phenotypes of cells with defective or inactive p53. In the ducts of the breast tissue, hyperplasia occurs as high levels of MDM2 are induced during lactation. In addition to these tumourlike characteristics of duct and gland cells, 16% of the mice develop mammary gland tumours by the time they reach 18 months of age [30], directly demonstrating that overexpression of MDM2 can contribute to tumour formation in the mouse.

MDM2 gene structure and protein domains

In attempt to understand the ability of MDM2 to function as an oncogene, the *Mdm2* gene and its protein products have been intensively studied. This information is summarized below.

Mdm2 gene structure and gene products

The murine *Mdm2* gene is about 25 kilobases (kb) in size and contains at least 12 exons [31]. There are several different isoforms of MDM2 messenger RNA (mRNA) and protein present in various cell lines. For example, in the 3T3-DM cells from which *Mdm2* was originally cloned, there are five MDM2 polypeptides ranging in size from 57 to 90 kilodaltons (kDa) [32]. This observation can at least in part be explained by the presence of multiple *Mdm2* transcripts in most cell lines; at least seven unique transcripts have been described in both mouse and human cells [5, 7]. Many of these result from the use of alternative internal splice sites. In addition, the *Mdm2* gene has two different promoters (see below), leading to transcripts which may initiate translation at different AUG codons [33, 34]. The functions of the multiple forms of MDM2, for example those that lack one or another domain, remain to be elucidated.

MDM2 protein domains

The largest human MDM2 protein consists of 491 amino acids and has several domains that are conserved between species from human to zebrafish (fig. 1) [35]. The first conserved region, in the amino-terminus of MDM2, is the domain that is sufficient for MDM2 to interact with p53 and inhibit its transcriptional activation function [36, 37]. The conserved nuclear localization sequence (NLS) and a conserved nuclear export

signal (NES) sequence of MDM2 mediate its ability to shuttle between the nucleus and the cytoplasm and back [38]. The centre of the MDM2 protein contains a highly acidic region that mediates MDM2's interaction with the ribosomal protein L5 and its associated 5S ribosomal RNA (rRNA) [39]. This acidic domain is immediately followed by a conserved zinc finger domain, and further towards the carboxy-terminus are a conserved caspase 3 cleavage site [40, 41] and three conserved putative DNA-protein kinase (DNA-PK) phosphorylation sites [35]. Finally, MDM2 has two additional conserved zinc fingers in a RING finger conformation [42] which mediate its ability to bind to specific RNA sequences or structures in vitro [43] and may contribute to the regulation of p53 levels [44]. These domains of the MDM2 protein and their interaction with various cellular components may provide pathways by which MDM2 can behave as an oncogene, as discussed in more detail below.

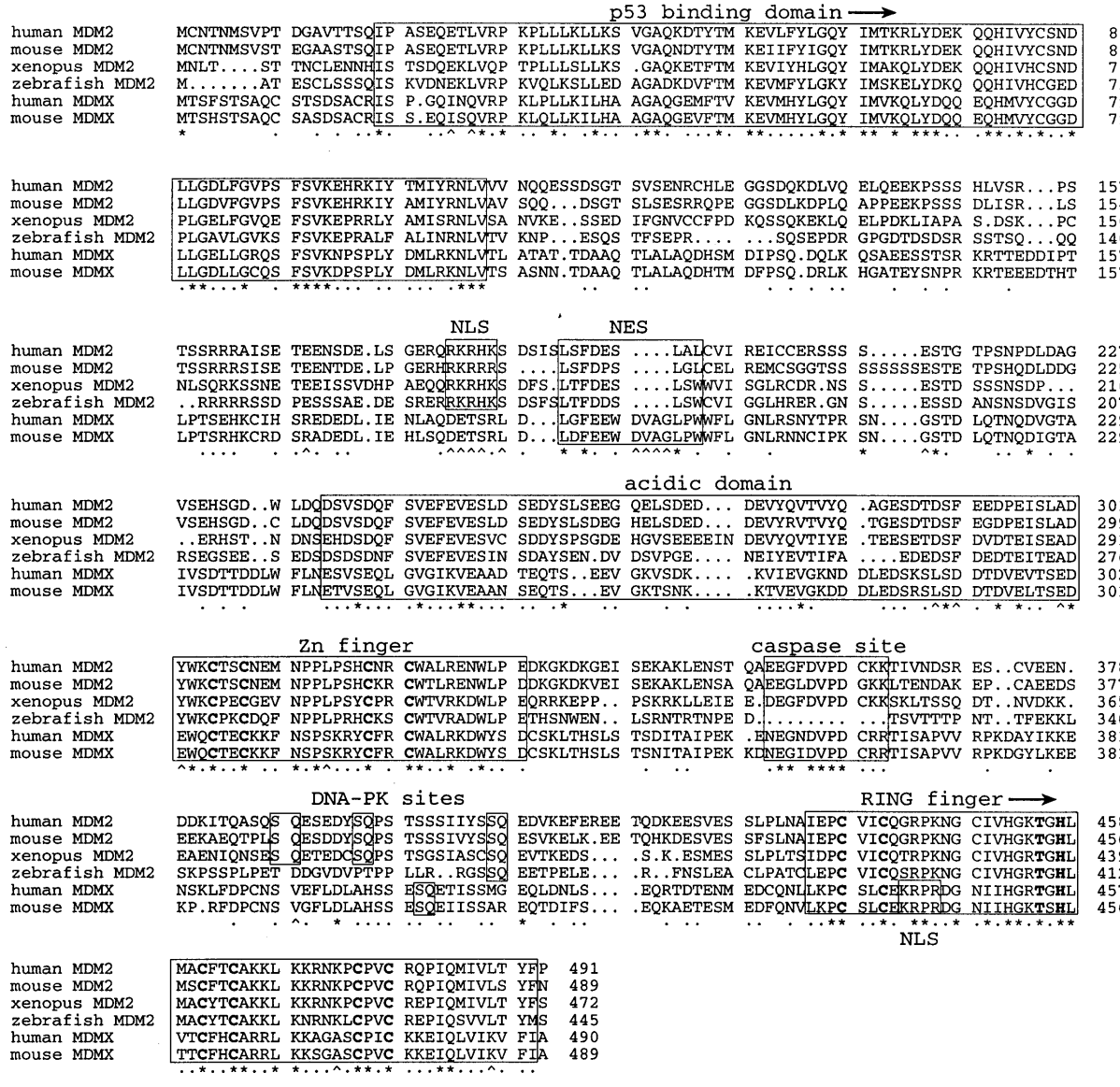

Figure 1. MDM2 protein family sequence alignment. Sequences were aligned using the CLUSTAL W program [75]. GenBank accession numbers for human, mouse and zebrafish MDM2 are M92424, X58876 and AF010255, respectively. Accession numbers for human and mouse MDMX are AF007111 and AF007110, respectively. The sequence for *Xenopus* MDM2 can be found in the paper by Marechal et al. [35].

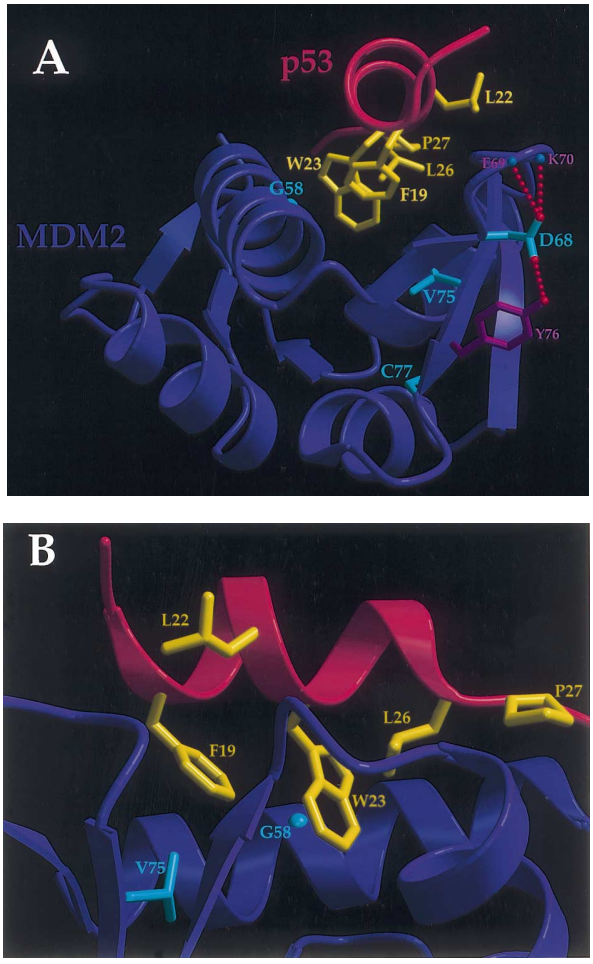

Figure 2. Crystal structure of a complex of MDM2 and p53. (*A*) Amino acids 15–29 of human p53 are shown in pink and form an α helix; amino acids 17–125 of human MDM2 are shown in purple and form a large structure with a deep cleft into which the p53 helix fits. The selected side chains shown in turquoise and yellow are those that when mutated prevent the formation of the complex. Playing structural roles on MDM2 are (in turquoise) residues C77, which is buried in a hydrophobic region of the domain, and D68, which is predicted to form three intramolecular hydrogen bonds as shown by the red dotted lines. Two of the predicted hydrogen bonds are to backbone nitrogens (blue-coloured balls) and one is to the tyrosine side chain of residue 76. These are predicted to stabilize the middle β sheet of the MDM2 domain, the other face of which is in direct contact with p53. (*B*) This close-up view of the p53-MDM2 interaction site is rotated approximately 90° clockwise from the view shown in (*A*). V75 of MDM2 makes van der Waals contacts with F19 of p53, while G58 of MDM2 makes similar contacts with both F19 and W23 of p53.

Interaction with and inhibition of p53

The crystal structure of the complex between the amino-terminal p53-binding domain of MDM2 and a 15-amino acid p53 peptide has been determined (fig. 2) [45]. MDM2 forms a deep cleft, lined with 14 hydrophobic and aromatic amino acids that contact p53 by van der Waals interactions. The p53 peptide forms an amphipathic α helix of two and a half turns, with its hydrophobic surface inserted into the cleft of MDM2. As the p53 peptide alone has no discernible structure by nuclear magnetic resonance spectroscopy (NMR), the helix is presumed to form as a result of an induced-fit with MDM2 [46]. A genetic analysis of the *Mdm2* gene identified two classes of mutations that prevent its interaction with p53: those playing structural roles (at residues D68 and C77) and those directly contacting p53 in the structure (at residues G58 and V75) [47]. A similar mutational analysis with the p53 gene identified residues F19, L22 and W23 of p53 as critical for interaction with MDM2 [48]; these amino acids directly contact MDM2 in the structure [45].

The p53-binding site on MDM2 provides a clue for the functional consequence of the interaction between the two proteins. The amino-terminal domain of MDM2 can block p53's ability to activate transcription in both reporter gene assays in cell culture and in an in vitro transcription assay (see below) [37]. The very same amino acids in p53 (F19, L22 and W23) that contact MDM2 also interact with the human TATA-binding protein (TBP)-associated factors TAFII31 and TAFII70 in TFIID; these interactions are required for p53 to activate transcription [48–50]. These observations provide an attractive model for a mechanism by which MDM2 can block p53's ability to act as a transcription factor, by competing with the transcriptional machinery for p53 interaction. Recent evidence, however, suggests that MDM2 regulates p53 in a second manner. Co-transfection of MDM2 with p53 lowers the steady-state levels and shortens the half-life of p53 [51, 52]. This mechanism of p53 regulation by MDM2 requires both an interaction with p53 as well as another activity of MDM2, that of nuclear-cytoplasmic shuttling, that is described below [38].

Nuclear-cytoplasmic shuttling

In addition to its nuclear localization signal (NLS), MDM2 contains a nuclear export signal (NES) which is both necessary and sufficient to mediate nuclear export of MDM2 [38]. Using a heterokaryon assay where proteins can be observed to shuttle between two nuclei in a cell, it has been shown that MDM2 can shuttle across the nuclear membrane in both directions. This nuclear export function is essential for MDM2's ability to downregulate p53 protein levels, suggesting a model in which MDM2 binds to p53 in the nucleus and transports it to a cytoplasmic proteasome for ubiquitin-mediated degradation [38]. Thus MDM2 has two ways to regulate p53, one by directly blocking

its transcriptional activation function in the nucleus and one by targeting p53 for ubiquitination and degradation in the cytoplasm.

Interaction with ribosomal proteins and RNA

The central acidic domain of MDM2 mediates an efficient interaction with the ribosomal protein L5 and its associated 5S rRNA. MDM2, L5 and 5S rRNA copurify as complexes with or without p53 from various cell lines [39]. In addition, MDM2 binds to specific RNA sequences or structures in vitro via its carboxy-terminal RING finger domain [43]. Specifically, the RING finger domain binds to an RNA structure in the 28S rRNA [38]. Such interactions suggest possible roles for MDM2 in translation control, RNA transport or ribosome biogenesis. The functions of these interactions and potential activities remain to be determined, but it is possible that they contribute to the ability of MDM2 to function as an oncogene.

MDMX

A protein with high homology to MDM2 has recently been cloned from both mouse and human cells, named MDMX [53, 54]. Two murine MDMX transcripts of 7.5 and 10 kb have been seen in all tissues and are much larger and present at higher levels than the one 3.5-kb MDM2 transcript that is present in all mouse tissues in the absence of radiation [5, 53]. Unlike MDM2, transcription of MDMX is not induced by p53 activation after ultraviolet (UV) irradiation [53].

The p53-binding domain is the region most conserved between MDM2 and MDMX, with approximately 50% identity (fig. 1). In fact, after cotransfection with p53, MDMX can be coimmunoprecipitated with p53 and can inhibit p53's ability to activate the transcription of a reporter gene [54]. The carboxy-terminal RING finger is also highly conserved (42% identity), as is the central putative zinc finger. MDMX also contains a putative NLS, although it is at a different location in the protein than in MDM2 [54]. The NES sequence of human MDM2 (**LSFDESLAL**) is altered in human MDMX (**LGF**EEWDVAG**LPW**). Although the hydrophobic residues critical for nuclear export are conserved (fig. 1), it is not clear whether the NES of MDMX is functional. The localization and shuttling abilities of MDMX, as well as its ability to bind to RNA, remain to be determined.

The role of MDMX in cells is not yet known but may prove interesting. It seems likely that MDMX could play a role in the regulation of p53 activity, in the modulation of the MDM2-p53 autoregulatory feedback pathway and/or in the regulation of p53-independent activities of MDM2.

The p53-MDM2 autoregulatory feedback loop

MDM2's induction by p53

Induction of the mouse *Mdm2* gene by p53 was first observed in a system that utilized a temperature-sensitive form of p53, where the expression of MDM2 and its interaction with p53 were enhanced at the permissive temperature for p53 function [55, 56]. This activation is a direct one by p53, as p53 binds to two tandem p53-binding sites in the *Mdm2* gene. The *Mdm2* gene contains two transcriptional promoter elements termed P1 and P2. The upstream P1 promoter is utilized constitutively. When p53 binds to its sites located in the first intron of the *Mdm2* gene, transcription of the P2 promoter, which is located downstream of the p53-binding sites, is induced [33, 55, 57]. Activation of p53 in cells only mildly affects transcription of the upstream P1 promoter if at all, but highly induces P2 to produce a transcript that begins in the second exon. Although these two different *Mdm2* transcripts have identical translational reading frames (the initiating AUG codon is in the third exon), it is possible that they are differentially regulated due to the difference in the 5′ untranslated regions of the transcripts [12]. One reason to suspect that this differential translational regulation may in fact be relevant to MDM2's function or its regulation is that these multiple promoters of the *Mdm2* gene are conserved in the human and mouse *Mdm2* genes [34].

In addition to being observed in temperature-sensitive p53 systems, the induction of MDM2 by p53 has been observed in a variety of cell lines after DNA damage. After UV irradiation of cells, increases in *Mdm2* transcript and MDM2 protein levels are seen that are dependent on the presence of functional p53 protein in the cells [58]. After gamma (γ) irradiation, a similar induction of *Mdm2* occurs, with faster kinetics than after UV irradiation, that is also dependent on the presence of a functional p53 protein [59]. In addition, the induction of MDM2 after γ-irradiation of the whole mouse can be observed and is dependent on the presence of functional p53 gene and protein [60]. These observations directly demonstrate that the p53 tumour suppressor protein induces the expression of *Mdm2* under true physiological settings, in response to DNA damage.

MDM2's inhibition of p53

The MDM2 oncoprotein forms a stable complex with the p53 tumour suppressor protein. The two proteins can be copurified and coimmunoprecipitated from various cell lines [36, 48, 61]. The binding of MDM2 to p53 leads to an inhibition of p53's ability to activate transcription and function as a tumour suppressor as indicated by several independent lines of evidence. First of

all, coexpression of MDM2 with p53 in both mammalian cell systems as well as in yeast leads to a decrease in the level of activation of p53-responsive reporter genes [37, 61, 62]. In addition, MDM2 has been shown to be able to inhibit both the G1 arrest and apoptotic functions of p53 in mammalian cell culture cotransfection experiments [3]. Final evidence of MDM2's ability to inhibit p53 activity comes from studies in mice. Homozygous deletion of the *Mdm2* gene in mice results in very early embryonic lethality, prior to implantation of the embryo. This lethality is completely rescued by the additional homozygous deletion of the *p53* gene, indicating that the lethality is due to the activity of p53 in the absence of regulation by MDM2 [63, 64]. These results clearly demonstrate that the inhibition of p53 activity by MDM2 occurs in a true physiological setting in the very early stages of embryogenesis of the mouse, and that this activity of MDM2 is absolutely essential for life.

MDM2 inhibits p53's ability to function as a tumour suppressor by several different mechanisms. First of all, MDM2 directly blocks the ability of p53 to mediate transcriptional activation. The interaction of MDM2 and p53 maps to the domain that p53 uses to activate transcription [48–50]. These observations lead to a model in which MDM2 competes with the TFIID transcription factor complex for interaction with p53 and therefore directly blocks its ability to activate the transcription of its downstream target genes. Further evidence that this direct blocking mechanism occurs includes the ability of MDM2 (amino acids 1–324) to inhibit the transcriptional activation activity of p53 in an in vitro transcription assay [65].

Recent reports also indicate that MDM2 may have a second mechanism to inhibit p53-dependent transcriptional activation at a promoter. In these experiments, deletion mutants of MDM2 defective in p53 binding are able to repress both p53-activated and basal transcription when brought to a promoter by fusion to a DNA-binding domain [65]. This transcriptional repression domain of MDM2 maps to amino acids 50–222, functions in an in vitro transcription assay as well as by in vivo transfection experiments, and can mediate an in vitro interaction with the 34-kDa protein of TFIIE. These results suggest a model in which p53's recruitment of MDM2 to a p53-responsive promoter both decreases the amount of p53-dependent activation as well as inhibits the basal machinery to dramatically decrease the level of transcription at such a promoter.

In addition to directly blocking p53's ability to activate transcription by this multifaceted mechanism at p53-responsive promoters, MDM2 is able to inhibit p53 activity in another way, by regulating its protein levels. Cotransfection of p53 and MDM2 in a variety of cell lines leads to a lower steady-state level of p53 than when it is transfected alone [51, 52]. MDM2 can also lower the steady-state levels of endogenous p53, both wild-type and mutant [51, 52]. This decrease in steady-state levels of p53 is not due to any effect on the transcription of the p53 gene or on the stability of its message, but is due to the ability of MDM2 to promote the degradation of p53 as evidenced by a decrease in p53 protein half-life with the addition of MDM2 [51, 52]. The ability of MDM2 to cause the degradation of p53 depends not only on its ability to bind to p53, but also on its ability to shuttle from the nucleus to the cytoplasm, leading to a model in which MDM2 binds to p53 in the nucleus and shuttles it to the cytoplasm to deliver it to a cytoplasmic proteasome [38]. MDM2 may in fact act directly as an E3 ubiquitin ligase for p53 in this capacity, utilizing the RING finger at its carboxy-terminus [44].

This latter mechanism, in which MDM2 inhibits p53 via degradation, is a more versatile one than by blocking p53-dependent transcription. For instance, MDM2's ability to inhibit p53-mediated transcriptional activation may not prevent it from performing other activities such as the induction of apoptosis or an enhancement of DNA repair, whereas targeting it for degradation would certainly inhibit all such activities of p53 in addition to that of transcriptional activation.

The p53-MDM2 autoregulatory feedback loop in the cell-cycle checkpoint pathway

In the absence of DNA damage, p53 has a short half-life. This is likely a result of a balance between p53's activation of MDM2 transcription and MDM2's enhancement of the degradation of p53. Thus the autoregulatory feedback loop can control the basal levels of both proteins. After DNA damage, p53 protein levels rise rapidly as the protein becomes stabilized by a posttranslational mechanism (i.e., the phosphorylation of the p53 amino-terminus by DNA-PK [66] or carboxy-terminus by the CDK7-cycH-p36 complex of TFIIH [67]) that causes a dramatic increase in the half-life of p53 [68]. In this event, the autoregulatory feedback loop is broken, allowing the levels of p53 protein to rise and for it to be active as a transcription factor and tumour suppressor. Such an increase in p53 levels could occur by several mechanisms as discussed below, which likely include regulation by the cell of MDM2 levels, its interaction with p53 and/or its subcellular localization. It is also possible that this feedback loop is modulated after DNA damage by signals that affect p53 stability and activity in a manner that is independent of MDM2 (i.e., phosphorylation or acetylation of p53). Whatever the mechanism that blocks the feedback loop, it must be reset after the cell has repaired the damage to its DNA and is ready to reenter

the cell cycle. The now high levels of p53 activate the transcription of the *Mdm2* gene; the resultant high levels of MDM2 protein can now decrease the activity and levels of p53, returning the autoregulatory feedback loop to its basal level and allowing the cell to reenter the cell cycle.

Regulation of the p53-MDM2 autoregulatory feedback loop

At early times after DNA damage, p53 must overcome its inhibition by MDM2 to increase its concentration and perform its growth arrest or apoptotic functions. Interestingly, this can occur in several tumour cell lines that overexpress MDM2. Such cells have an efficient response to DNA damage, as p53 is stabilized and transcriptionally active despite the presence of large amounts of its negative regulator (D. A. Freedman, Y. Jin, A. Cantor and A. J. Levine, unpublished results). These observations indicate that the control of p53 levels and activity by MDM2 may indeed be regulated in and of itself.

Such regulation of the p53-MDM2 autoregulatory feedback loop could occur on several levels. The first of the mechanisms a cell might utilize to overcome inhibition of p53 by MDM2 is blocking complex formation between the two proteins. There is evidence that the interaction between p53 and MDM2 may be blocked in cells after γ-irradiation, by phosphorylation of p53 at human p53 residue S15 [66]. This or a similar mechanism would allow p53 to overcome the potential block by basal levels of MDM2, and the cell could then initiate the appropriate response to the DNA damage. Phosphorylation of MDM2 is another way by which the cell could potentially regulate the formation of the MDM2-p53 complex. For example, disruption of hydrogen-bond formation between Y76 and D68 of MDM2 by mutation of D68 prevents interaction with p53 (see fig. 2) [47]. This tyrosine residue thus presents a potential target for a signalling kinase that can regulate the p53-MDM2 complex after DNA damage. Other posttranslation modifications of either protein or interactions with other proteins remain as additional ways that may function to modulate complex formation under other conditions or as additional layers of control to prevent MDM2 from inhibiting p53 activity when it is needed by the cell.

Another way to control MDM2's regulation of p53 is by changing the levels of MDM2. In fact MDM2 protein and transcript levels decrease rapidly after DNA damage by UV irradiation in a p53-independent manner, before the levels increase due to induction by p53 [69]. The decrease in MDM2 protein levels occurs simultaneously with an increase in p53 levels, suggesting a possible causal relation that would allow p53 to overcome the block by MDM2 after DNA damage. Thus any mechanism the cell utilizes to decrease *Mdm2* transcription or to destabilize the MDM2 protein after DNA damage could contribute to the stabilization of p53 and to the activation of p53-dependent functions under those conditions.

Localization of MDM2 is one final mechanism which may regulate its inhibition of p53. In the nucleus, MDM2 can bind directly to p53 and block its activities as a transcription factor; this mechanism to inhibit p53, however, may not in fact be sufficient in cases where there is an excess of p53 protein as indicated by two independent lines of evidence. The first is that transcriptional activation by a p53 tetramer bound to a dimer of MDM2 can occur in yeast [47], suggesting that the inhibition requires a 4:4 stoichiometric ratio of the two proteins. Second, by mutating the NES sequence of MDM2, MDM2 is retained in the nucleus and cannot target p53 for degradation. In this case, MDM2 is not an efficient inhibitor of large amounts of the p53 protein, although it blocks the activity of p53 when p53 levels are low [38]. In addition, this result suggests that the transport and degradation of p53 by MDM2 does not require a 4:4 stoichiometry. Perhaps one molecule of MDM2 can shuttle a p53 tetramer; it is also likely that MDM2 may function more as a catalyst in this capacity, returning to the nucleus to shuttle out more p53 protein for cytoplasmic degradation. Thus MDM2's ability to target p53 for degradation or at least remove it from the nucleus is likely essential for its ability to block p53's functions as a tumour suppressor, and nuclear-cytoplasmic shuttling by MDM2 is therefore another possible target for regulation of p53 inhibition by MDM2. A cell could potentially block the nuclear export of MDM2 by modification of MDM2 or by altering the nuclear export machinery. The effect of such a block would be the retention of MDM2 in the nucleus, where it would not efficiently inhibit the transcriptional activation activity of the large amounts of p53 protein after DNA damage, allowing p53 to mediate the appropriate cellular response.

The cell thus has several levels at which to regulate the inhibition of p53 by MDM2. One or more of these may function to allow the cell to block the inhibition of p53 by MDM2 in order to fine-tune the level and timing of the p53 response to DNA damage (fig. 3). These same mechanisms of regulation by the cell also represent potential targets for cancer therapies that could lead to activation of p53, cell-cycle arrest or apoptosis and, ultimately, tumour regression. But in addition, MDM2 may have p53-independent oncogenic functions, as described below, which may also provide targets for cancer treatments.

p53-independent oncogenic functions of MDM2

MDM2 binds to the p53 protein and inhibits its functions in tumour suppression. Overexpression of MDM2 contributes to human tumour formation, an activity which is likely due, at least in part, to its ability to block p53 function. Several lines of evidence, however, suggest that MDM2 may have p53-independent activities that can also contribute to tumour formation. First of all, a subset of tumours exist that have both overexpression of MDM2 and mutations in p53 that block its functions as a tumour suppressor; these individuals in fact have a significantly worse prognosis than those individuals whose tumours possess only one of the two alterations [14]. If MDM2's only oncogenic function is the inhibition of p53, having both changes in a tumour would be a redundant mechanism to prevent p53 from performing its tumour suppression functions. These observations suggest that MDM2 may indeed have additional activities that contribute an additional growth advantage to the tumour.

A second line of evidence comes from the transgenic MDM2 mice that overexpress MDM2 in the mammary epithelium during lactation as discussed above [30]. The phenotypes of ductal hyperplasia and decreased gland development, lack of terminal differentiation and polyploidy also occur when the mice are crossed into a p53 null background, although none of these phenotypes are observed in p53 null mice that do not overexpress MDM2 [30]. As MDM2 overexpression confers tumourlike characteristics in the absence of p53, MDM2's gain-of-function activities in this situation do not occur through the inhibition of p53 activity.

A final reason to suspect that MDM2 has a role in cancer independent of its ability to inhibit the function of p53 is the observation that several human tumour cell lines with wild-type p53 have a functional p53 response to various types of DNA damage despite the presence of MDM2 gene amplification or overexpression. This normal p53 response to DNA damage includes the stabilization of p53, the activation of p53-responsive genes, as well as the induction of cell-cycle arrest (D. A. Freedman, Y. Jin, A. Cantor and A. J. Levine, unpublished results). Thus the advantage the high levels of the MDM2 protein impart upon the tumour cells may be from (i) an attenuation, not a complete block, of p53 activity in response to DNA damage, (ii) a p53-independent tumour-promoting activity of MDM2 or (iii) a combination of these possibilities.

There are several activities and domains of MDM2 that could contribute to a p53-independent transforming ability. For example, the roles of RNA binding by the MDM2 RING finger or MDM2's interaction with the L5 ribosomal protein and 5S rRNA (described above) could play a role in MDM2's p53-independent oncogenic functions. In addition, MDM2 has several properties, described below, that may contribute to its oncogenic potential and that do not require any physical or functional interaction between MDM2 and p53.

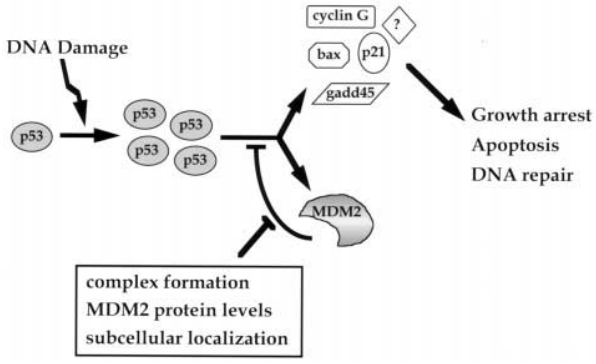

Figure 3. Model of the p53 pathway and the p53-MDM2 autoregulatory feedback loop. DNA damage signals to p53, causing it to become more stable and active as a transcription factor. Some of the genes activated by p53 are shown, and the question mark indicates that not all p53-responsive genes have been identified to date [76–79]. These genes help facilitate the tumour-suppressive outcomes of p53 activation, that of growth arrest or apoptosis and possibly enhanced DNA repair. In addition, p53 activates the transcription of the *Mdm2* oncogene [33], which binds to p53 and blocks its functions as a tumour suppressor. This autoregulatory feedback is in itself regulated by the cell, allowing the level and timing of the p53 response to DNA damage to be fine-tuned.

An interaction with pRB and E2F

The amino-terminal 220 amino acids of MDM2 are sufficient for its in vitro interaction with both the E2F1 and DP1 proteins of the E2F transcription factor complex that mediates progression through S phase [70]. It has also been reported that MDM2 interacts both in vitro and in vivo with the product of the retinoblastoma gene, pRB, which functions to negatively regulate E2F function [71]. Transfection of MDM2 with an E2F reporter gene leads to increased activation by either endogenous or exogenous E2F in several different cell lines, suggesting that MDM2 may play a role in progression through the S phase of the cell cycle via the RB/E2F pathway [70, 71]. This function may represent a p53-independent mechanism by which overexpression of MDM2 contributes to tumour progression.

Inhibition of MyoD activity

The *Mdm2* gene is amplified in a subset of rhabdomyosarcoma cell lines with dominant nondifferentiating phenotypes [24]. In such cells, MyoD-dependent

muscle-specific genes are not expressed, and it was found that MDM2 could block MyoD reporter gene activation by either endogenous or exogenous MyoD. In addition, transfection of antisense *Mdm2* results in the induction of muscle-specific gene expression in the cell lines with amplified *Mdm2*, suggesting that MDM2 is indeed responsible for the lack of muscle-specific gene expression and thus the nondifferentiating phenotype of these tumour cells [24]. The inhibition of the transcriptional activity of MyoD and possibly other cell-type specific transcription factors by MDM2 is likely another mechanism by which overexpression of MDM2 contributes to tumour formation independent of its inhibition of p53.

Targeting MDM2 as cancer therapy

Fifty percent of human tumours have wild-type p53 [1]; blocking MDM2's regulation of p53 in these tumours may enhance p53's activity after treatment with chemotherapeutic DNA-damaging agents and may even activate p53 in the absence of DNA damage. There are multiple levels at which to attempt to block MDM2's inhibition of p53 by drugs. Blocking the interaction between MDM2 and p53, lowering the levels of MDM2 or blocking the nuclear-cytoplasmic shuttling of MDM2 would result in activation of the tumour suppression functions of p53, resulting in apoptosis or cell-cycle arrest.

Several experiments have already begun to show the utility of such approaches. Preventing complex formation between MDM2 and p53 does indeed appear to stabilize the p53 protein, lead to transcriptional activation by p53, and in at least one case result in an arrest of the cell cycle. Microinjection of a monoclonal antibody against the p53-binding domain of MDM2, 3G5 [36], blocks the interaction between MDM2 and p53 and leads to p53-dependent activation of reporter genes in cell lines that have wild-type p53, with either normal or high levels of the MDM2 protein [72, 73]. A similar result is obtained by transfection of a synthetic protein that contains a peptide mimicking the MDM2-binding α helix of p53 which binds in the MDM2 cleft and prevents docking of p53 [72].

Blocking the nucleocytoplasmic shuttling ability of MDM2 is another potential way in which p53 can be stabilized and therefore activated as a cancer therapy. Direct experimental evidence suggests that this is indeed a feasible mechanism to stabilize p53. Blocking the nuclear export of MDM2 by transfection of NLS-rex, an inhibitor of human immunodeficiency virus (HIV) rev protein's nuclear export, also blocks its ability to degrade the p53 protein, the steady-state levels of which subsequently rise in the cell [38]. Also, the addition of leptomycin B, a drug that blocks nuclear export of cellular proteins, leads to an increase in the steady-state levels and half-life of p53 in a variety of cell lines [73a]. This drug, however, is quite toxic to cells regardless of p53 status, as it likely disturbs the activity of many critical cellular regulators. Drugs that specifically block the shuttling ability of MDM2 are thus likely to be efficient stabilizers and activators of p53 and represent another form of potential cancer therapy.

Lowering the levels of MDM2 in cell lines that overexpress it also appears to activate the wild-type p53 protein present in such cells. Using antisense oligonucleotides to the *Mdm2* message in a choriocarcinoma cell line that has wild-type p53 and amplification of the *Mdm2* gene, p53 reporter genes can be activated and the cells induced to undergo apoptosis. The effects of the oligonucleotide treatment are enhanced by the addition of strand-breaking DNA-damaging agents such as topoisomerase I inhibitors, under conditions in which p53 is not activated by the DNA damage alone [74]. This last observation suggests that p53's response to DNA damage in cell lines that overexpress MDM2 may in fact be limited by the presence of large amounts of its negative regulator.

Each of the above ways to block MDM2's inhibition of p53 may have enhanced effects when used in combination with the DNA-damaging agents already used as cancer treatments. Such DNA-damaging drugs are thought to work by activating p53 in the cells of the tumour; one aspect of this activation likely includes blocking its inhibition by MDM2. Presumably other changes, independent of MDM2, also occur that help activate p53, indicating that DNA damage and blocking the inhibition of p53 by MDM2 could act synergistically to induce an enhanced p53-dependent response, such as apoptosis and subsequent tumour regression.

In addition, the drugs that target MDM2 may block other, p53-independent oncogenic activities of MDM2 as described above. By decreasing the cellular levels of MDM2, by altering its subcellular localization, or by blocking its interaction with other cellular proteins, such p53-independent oncogenic activities may be prevented and the advantage they provide to the tumour cells may thus be negated. This therefore suggests that drugs that target MDM2 may even be effective in treating tumours that do not have functional p53 protein. It also provides another reason to suspect that combinations of DNA-damaging drugs to induce p53 and drugs to decrease MDM2 levels or activity would represent very powerful cancer therapies.

Conclusion

The best-characterized activity of the MDM2 oncoprotein is its role in an autoregulatory feedback loop

with the p53 tumour suppressor protein. Its function in this capacity is to regulate the level and timing of the p53 response, allowing the cell to react as efficiently as possible to DNA damage. There are, however, many questions about MDM2 which remain to be answered. For instance, what are the targets of its p53-independent oncogenic activities, and what advantage does MDM2 give tumour cells that overexpress it but still have an efficient p53 response? What are the roles of MDM2-related proteins such as MDMX? Is MDM2 responsible for the low steady-state levels of p53 in the absence of DNA damage? What signals block the inhibition of p53 by MDM2 allowing for p53's response to DNA damage? What activities can regulate the p53-MDM2 interaction? What regulates the p53-independent degradation of MDM2 after UV irradiation? Is the nuclear-cytoplasmic shuttling of MDM2 regulated? What signals control the timing of the p53 response, allowing MDM2 to resume its block of p53 and allow the cell to reenter the cell cycle? Can we take advantage of the knowledge we have accumulated about the interactions of MDM2 and p53 to help develop new drug therapies aimed at killing tumour cells? The answers to these questions will go a long way to understanding the many interesting functions of p53 and MDM2.

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