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p53-Independent Effects of Mdm2

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Abstract

Mdm2 is best known as the primary negative regulator of p53, but a growing body of evidence suggests that Mdm2 also has a number of functions independent of its role in regulating p53. Although these functions are not yet well-characterized, they have been implicated in regulating of a number of cellular processes, including cell-cycle control, apoptosis, differentiation, genome stability, and transcription, among others. It appears that Mdm2 exerts these functions through a surprisingly wide variety of mechanisms. For example, it has been shown that Mdm2 can ubiquitinate alternative targets, can stimulate the activity of transcription factors, and can directly bind to mRNA to regulate its stability. Dysregulation of p53-independent functions could be responsible for the oncogenic properties of Mdm2 seen even in the absence of p53, and may explain why approximately 10 % of human tumors overexpress Mdm2 instead of inactivating p53 through other mechanisms. As the p53-independent functions of Mdm2 present novel targets for potential therapeutic interventions, fully characterizing these cellular and pathogenic roles of Mdm2 will be important in the study of tumor biology and the treatment of cancer.

Keywords

Mdm2; p53; Oncogenesis; Ubiquitination

Mdm2 (murine double minute 2 homolog) is best known for its role as a negative regulator of the tumor-suppressor p53. Due to the ability of p53 to induce cell-cycle arrest and apoptosis, tight regulation of this protein is necessary for normal cellular growth and development. The primary way this regulation is accomplished is through interaction with Mdm2 [26]. Mdm2 is a E3 ubiquitin ligase that ubiquitinates p53 and targets it for proteasomal degradation [15, 17, 49]. Mdm2 is also able to directly bind the N-terminus of p53, inhibiting its activity as a transcription factor for anti-proliferative genes [39]. Mdm2 itself is a transcriptional target of p53, as the *Mdm2* gene possesses a sequence-specific recognition site for p53 within an internal promoter [23, 51]. In this manner, a negative-feedback loop is established, whereby p53 transactivates its own inhibitor. In support of such a regulatory loop, studies of p53 kinetics have shown that elevated levels of p53 following DNA damage decrease in a series of dampened oscillations [29].

In response to various stress signals, the Mdm2-p53 interaction is disrupted. Genotoxic stress causes p53 to undergo phosphorylation and acetylation at a number of residues [1, 54]. However, studies have shown that these modifications are not inherently critical to p53 transactivation function, and are only necessary to counter Mdm2 regulation [13, 58, 61]. This suggests that the primary function of these post-translational modifications are the disruption of the Mdm2-p53 interaction. A number of other mechanisms for regulating this have been characterized. Of note is the inhibition of Mdm2 by either p14^{ARF} in response to oncogenic signaling [53] or several ribosomal proteins in response to ribosomal stress [72].

The importance of the Mdm2-p53 relationship to normal cellular development was dramatically illustrated by the homozygous deletion of *Mdm2* in mice, which results in lethality at the blastocyst stage due to aberrant apoptosis. Strikingly, this phenotype can be completely rescued by the concomitant deletion of p53 [20, 41]. Recently, it has been demonstrated that although p53 transactivation of Mdm2 is necessary for a normal DNA damage response, p53-independent basal expression levels of Mdm2 are sufficient for regulation of p53 in most tissues under homeostatic conditions. This was demonstrated by the use of a transgenic mouse model in which basal levels of Mdm2 are expressed from its p53-independent promoter, but has lost p53-inducible Mdm2 expression [38, 47].

Disruption of the p53 pathway is critical for the development of cancer, as demonstrated by the fact that over 50 % of human tumors contain p53 mutations [16]. Therefore, it is unsurprising that Mdm2 overexpression has been also been implicated in tumorigenesis as an alternative method of inactivating p53 [30]. In one such study, *Mdm2* was found to be overexpressed in 7 % of 3,889 human tumor samples [40]. The highest frequencies of Mdm2 overexpression were found in soft-tissue sarcomas (20 %) and osteosarcomas (16 %), while other tumor types, such as leukemias, lymphomas, and pancreatic carcinomas, showed little Mdm2 overexpression. Importantly, there is a negative association between amplification of Mdm2 and mutation of p53 [45]. This association holds potential clinical implications in the possibility of developing therapeutics that restore p53 function through the inhibition of Mdm2. Indeed, this is the rationale that underlies the investigation of Nutlin-3, a small-molecule inhibitor of the Mdm2/p53 interaction, as a potential therapy in cancers that retain functional p53 [27, 52].

Mdm2 and Cancer

Consistent with its expected role in p53 regulation, ubiquitous overexpression of Mdm2 in transgenic mice predisposes them to spontaneous tumor formation. Surprisingly, however, when Mdm2 is overexpressed in mice in a p53-null background, an increased incidence of sarcomas is observed relative to p53-null mice alone [21]. This suggests that Mdm2 overexpression can also promote tumorigenesis through p53-independent mechanisms. This finding corroborated earlier reports that Mdm2 is able to transform cells *in vitro*, independent of p53 [3]. The increased rate of sarcomas in these mice also parallels the high rate of Mdm2 amplification in human sarcomas. Targeted overexpression of Mdm2 to mammary gland tissue led to the production of polyploid mammary epithelial cells in both p53^{+/+} and p53^{-/-} backgrounds, suggesting a p53-independent role for Mdm2 in the regulation of DNA synthesis and cell-cycle progression [34].

In addition to mutations and changes in expression levels, gene activity can also be regulated through alternative splicing of mRNA. Studies of Mdm2 mRNA splicing showed multiple different-sized transcripts and protein isoforms that vary in their ability to bind to p53 [3, 14]. Analysis of Mdm2 mRNA in human ovarian and bladder cancer samples showed the presence of alternative and aberrant splice variants not found in normal tissue. Interestingly, four out of the five identified alternative transcripts contained partial deletions of the p53-binding domain, and expression of these isoforms *in vitro* confirmed their inability to interact with p53. However, all of the alternative transcripts were able to transform NIH 3 T3 cells, indicating an oncogenic property for these transcripts independent of p53 [55]. Similar transcripts have also been identified in glioblastomas, breast carcinomas, pediatric rhabdomyosarcomas, and oral squamous cell carcinomas [2, 37, 33, 50]. In total, over 40 different alternative splice variants have been identified in human tumors, most of which lack the p53-binding domain. Although the exact function of these splice variants is unknown, the fact that multiple splice variants are associated with specific tumor types, and that most of these splice variants do not interact with p53, suggest that these aberrant isoforms may play a p53-independent role in promoting tumorigenesis.

Although initial studies suggested that overexpression of Mdm2 and mutation of p53 were mutually exclusive in human tumor samples, this was later found to not be the case in certain tumor types. Although the majority of soft-tissue sarcomas contain one or the other, a significant proportion of tumor samples exhibit both an overexpression of Mdm2 and mutation of p53. Interestingly, it was found that there is a significant correlation between tumors containing changes in both proteins and poor patient prognosis [7]. Another confirmatory study showed that the effect on patient survival of Mdm2/p53 cooverexpression was greater than the additive effects of each independently [67]. Additionally, high levels of Mdm2 mRNA expression are correlated with an earlier age of onset of soft-tissue sarcomas [60]. Altogether, these findings point to Mdm2 having p53-independent effects in the process of tumorigenesis.

p53-Independent Effects of Mdm2

How might such p53-independent effects occur? Although the regulation of p53 degradation is by far the most extensively characterized function of Mdm2, a number of other functions have been described.

Since Mdm2 is an E3 ubiquitin ligase, a straightforward hypothesis would be that Mdm2 ubiquitinates other proteins in addition to p53, targeting them for proteasomal degradation as well. Indeed, Mdm2 has been shown to target a number of other proteins involved in cell-cycle regulation and apoptosis.

The Ras/Raf/MEK/ERK pathway couples cell-surface receptor signals to transcription factors that regulate proliferation and differentiation, and mutations or alterations in this pathway are found in many human cancers [10]. When this pathway is activated, ERK phosphorylates Foxo3A, a transcription factor for cell-cycle regulatory proteins. In this phosphorylated form, Foxo3A becomes a target for ubiquitination by MDM2, promoting its degradation. As Foxo3 acts as a tumor suppressor, Mdm2-mediated down-regulation of

Foxo3 could play a role in tumorigenesis in response to oncogenic growth factor signaling [70].

Additionally, Mdm2 has been shown to ubiquitinate the cell-adhesion protein E-cadherin and target it for degradation via the 26S proteasome [69]. E-cadherin has an extensively characterized role in the epithelial-to-mesenchymal transition that occurs when tumors initiate metastasis. Downregulation of E-cadherin causes a loss of cell polarity and cell-cell adhesion, and promotes cell motility and invasiveness; these in turn lead to invasion of the blood stream and metastasis. Interestingly, Mdm2 overexpression occurs more frequently in metastatic and recurrent tumors than it does in primary tumors [9, 28]. Overexpression of Mdm2 is also associated with a poor patient prognosis in a number of cancers [19, 24]. Thus, Mdm2 overexpression could potentially promote tumor invasion and metastasis through the downregulation of E-cadherin. However, whether or not this interaction is important in tumor progression has yet to be confirmed.

Paradoxically, Mdm2 has also been shown to target the transcription factor Slug for degradation. Slug (also known as SNAI1), is a member of the Snail family of transcriptional repressors. It is a key promoter of the epithelial-to-mesenchymal transition, which it stimulates by repressing the transcription of E-cadherin. This was shown to occur through a p53-Mdm2-Slug complex; interestingly, mutant and transcriptionally-inactive p53 inactivates the Mdm2-mediated degradation of Slug [64]. Although Mdm2 is normally thought of as an oncogene, its degradation of an invasion-promoting protein suggests that under certain circumstances, Mdm2 can also act as a tumor suppressor.

Mdm2 has also been shown to interact with the retinoblastoma protein (pRb), a tumor suppressor gene that, like p53, has a major role in cell-cycle inhibition and apoptosis. Unlike the Mdm2-p53 interaction, however, the Mdm2-pRb interaction is not mediated by ubiquitin ligation and degradation. In cells, Mdm2 forms a complex with pRb and disrupts the G1/S checkpoint by preventing pRb from binding to and inactivating certain members of the family of E2F transcription factors [68]. Interestingly, it has also been demonstrated that Mdm2 forms a trimeric pRb-Mdm2-p53 complex, with Mdm2 acting as a linker between these two transcription factors. Binding of pRb to Mdm2 does not inhibit p53 binding; this stands in contrast to other known regulators of the Mdm2-p53 interaction, such as p14^{ARF}, which act by binding to Mdm2 and inhibiting p53 binding. The pRb-Mdm2 interaction is able to inhibit Mdm2-mediated p53 degradation, but does not remove the ability of Mdm2 to inhibit p53 transactivation of its target genes [18]. The finding that pRb impacts the apoptotic ability, but not the transcriptional activity of p53 suggests that the apoptotic effects of p53 may be independent of transactivation [71]. The discovery of cross-talk between the p53 and pRb pathways is particularly exciting, as these are arguably the two most important pathways in the prevention of tumorigenesis.

Mdm2 also impacts the pRb pathway through its interaction with E2F1 and DP1, transcription factors that heterodimerize and activate genes involved in the G1/S-phase transition. E2F1 contains a series of amino acids that are homologous to the activation domain of p53. In contrast to its negative regulation of p53, Mdm2 stimulates the transcriptional activity of E2F1/DP1, promoting progression into S phase [36]. In addition,

Mdm2 increases degradation of the E2F1/DP1 complex, and can prevent p53-null cells from entering E2F-mediated apoptosis [32]. Thus, the Mdm2-E2F interaction both promotes cell growth through increased gene transcription and prevents cell death by inhibiting apoptosis, two activities that are hallmarks of tumorigenesis. However, whether or not the effect of Mdm2 on E2F1 is completely p53-independent is unclear. One study has suggested that this effect is mediated through inhibition of p53-dependent transcription of p21, the resultant increase in cyclin-dependent kinase activity causing phosphorylation/inactivation of pRb, which in turn stimulates E2F1 activity [12, 66]. These effects are not necessarily mutually exclusive; it is possible that this occurs through both p53-dependent and -independent mechanisms. The nature of the Mdm2-E2F1 relationship and its effect on cellular growth and tumorigenesis warrants further investigation.

The RING finger domain of Mdm2, which is responsible for its E3 ubiquitin ligase activity, also serves as a binding site for the closely related protein MdmX [59]. Mice lacking MdmX have similarity to Mdm2-knockout mice, in that both have an embryonic-lethal phenotype, although MdmX-null lethality occurs at a later stage of development and is associated with cell cycle arrest. Both phenotypes can be rescued by the loss of p53 [35, 48]. MdmX, despite possessing homology to the RING finger domain of Mdm2, does not possess E3-ubiquitin ligase activity. MdmX also represses p53, but does not do so through p53 degradation [25]. It has been suggested that Mdm2 homo-oligomers have different functions than Mdm2-MdmX heterodimers and can modify each other's function and regulation. The growing evidence surrounding the Mdm2-MdmX-p53 triumvirate suggests a complex relationship between these proteins that modifies their functions, modifications, and stabilities [62]. The interaction between Mdm2 and MdmX, and what role it plays in the regulation of p53-dependent and -independent effects of Mdm2, remains an active area of investigation.

Mdm2 and Genome Instability

Recently, increasing evidence has been found that suggests that Mdm2 overexpression can cause genome instability in a p53-independent manner. As already noted, targeted expression of Mdm2 to the mammary gland in transgenic mice gave the surprising phenotype of mammary epithelial cells that were hypertrophic and polyploid, indicating that these cells had undergone multiple rounds of S phase without mitosis. This effect was found to be p53-independent, as the same phenotype was observed in both wild-type and p53-knockout backgrounds [34].

The Eischen laboratory has reported an interesting interaction between Mdm2 and Nbs1 (also called Nibrin or NBN), a member of the Mre11/Rad50/Nbs1 complex (more commonly referred to as MRN) that functions in initiating DNA double-strand break repair and activating cell-cycle checkpoints. Nbs1 is thought to localize the complex to double-stranded DNA breaks and play a role in activation of ATM signaling. Mdm2 overexpression in p53-null cells induced chromosome breakage and delayed DNA double-stranded break repair, but not in cells with a mutated form of Nbs1. Through mutational analysis of their respective binding domains, it was demonstrated that Mdm2 directly interacts with Nbs1 and inhibits its function, leading to a delayed DNA-damage response, possibly through reduction

in ATM signaling. When this Mdm2-Nbs1 interaction is disrupted, the rate of DNA damage repair is restored [4].

Genomic instability is a common characteristic of many cancers [43]. Previous studies of Mdm2 overexpression had revealed that elevated Mdm2 levels led to increased chromosome/chromatid breaks, centrosome hyperamplification, and aneuploidy [6, 63]. At that time, however, it was assumed these effects were mediated by Mdm2 repression of p53, as loss of p53 can also promote genome instability [8, 11, 31]. However, recent evidence, such as the demonstration of Mdm2 inhibition of Nbs1, argues that Mdm2 increases genetic instability directly and in a manner independent from the instability caused through inhibition of p53. This novel function of Mdm2 could have implications for cancer treatment, as it presents a new target interaction for chemotherapeutic drugs, as well as the appropriate clinical use of Mdm2 inhibitors such as Nutlin [4].

Mdm2, Transcription, and Translation

Although its primary role is involved the degradation of proteins, one unexpected function of Mdm2 that has been described is Mdm2-dependent regulation of translation. Mdm2 is known to regulate the translation of p53 via two different mechanisms. First, Mdm2 has been shown to directly interact with p53-encoding mRNA and impact its translation. Binding of p53-mRNA to the RING finger domain of Mdm2 stimulates translation of p53, while simultaneously inhibiting the E3 ligase ability of Mdm2 [42]. Fascinatingly, this suggests that while Mdm2 negatively regulates p53 on the protein level, it is a positive regulator of p53 on the mRNA level. In agreement with this, it has also been found that “silent” mutations of p53 – that is, nucleotide changes of the gene encoding p53 that do not change its amino acid sequence – have an impact on levels of p53 activity. Presumably, a silent mutation could alter the secondary structure of mRNA, thus altering its regulation by mRNA-binding proteins. This suggests that Mdm2-mRNA binding is an alternative method through which Mdm2 regulates p53 activity [5].

Secondly, Mdm2 has been shown to ubiquitinate and target the ribosomal protein RPL26 for degradation. RPL26 plays a critical role in the translation of p53-encoding mRNA following DNA damage [57]. Under normal conditions, Mdm2 targets RPL26 for degradation, secondarily prohibiting translation of p53. In response to DNA damage, the Mdm2 inhibition of RPL26 is attenuated, causing a rise in p53 translation [44].

Both of these mechanisms have been described as alternative methods by which Mdm2 regulates the activity of p53. In addition, they suggest additional mechanisms through which Mdm2 could regulate other cellular processes as well. Indeed, it has been recently been shown that Mdm2 binds to and stabilizes the mRNA encoding Slug. In p53-null cells, Mdm2 stabilization of Slug mRNA caused an increase in the amount of Slug protein and induced Slug-dependent effects, such as repression of E-cadherin and increased invasiveness [22]. The direct binding of Mdm2 to mRNA and altering its transcription is a novel mechanism by which Mdm2 could have tumorigenic effects independent of p53.

Future Directions

In conclusion, Mdm2 has been shown to have a number of different functions independent of its role as a regulator of p53. Through the demonstration of alternate ubiquitination targets, its effects on genome stability, and interactions with mRNA and ribosomal proteins, it is becoming increasingly clear that Mdm2 regulates cellular processes on a number of different levels. Although these p53-independent functions are not fully understood, they may contribute to the role of Mdm2 in oncogenesis. As Mdm2 is implicated in a significant portion of human tumors, these functions present novel targets for potential clinical therapies. In order to provide a clinical benefit, however, the exact nature of these p53-independent functions will need to be further characterized.

Based on current knowledge, several avenues of research need to be pursued. For one, over 40 different splice variants of the Mdm2 mRNA have been isolated from normal and tumor cells [3]. Studying the potentially distinct biological roles of these isoforms is likely to reveal more about the various functions of Mdm2. The finding that some human tumors express Mdm2 splice variants that lack the p53-binding domain suggests that these splice variants may contribute to oncogenesis through p53-independent effects [55]. One interesting area of further study would be to identify the effects of expressing these distinct splice variants in targeted tissues to determine what role they have in tumor development.

Additionally, given the variety of splice variants of Mdm2, a more rigorous analysis of Mdm2 overexpression in human cancers is warranted. Simple measurement of gene amplification or mRNA expression levels may not be truly reflective of the actual protein levels of Mdm2 and its variant forms in human tumors. A more detailed analysis that takes into account the existence of distinct isoforms will need to be performed in order to determine what isoforms are expressed in specific tumor types. Mdm2 overexpression seems to be associated with better prognosis in some tissue types, and worse prognosis in others [46]. However, these analyses have, for the most part, used methods that do not distinguish between splice variants. Comparing the expression of distinct isoforms in tumor samples with clinical outcome data could reveal whether or not Mdm2 isoforms have an effect on patient prognosis and potentially explain this paradoxical finding.

Nutlin-3 has been investigated for use as an agent that disrupts the Mdm2-p53 interaction. However, as a small-molecule inhibitor of Mdm2, it also has the potential to inhibit or alter p53-independent effects of Mdm2 as well. It has been shown that Nutlin-3 can increase cell toxicity following DNA damage in p53-null prostate cancer cells, where it acts as a radiosensitizer [56]. Recently, Nutlin-3 has also been shown to inhibit the epithelial-to-mesenchymal transition in p53-null cells by interfering with the TGF- β 1-SmadSnail/Slug axis [65]. The exact mechanisms by which Nutlin-3 exerts p53-independent effects remain unclear. Such findings have important clinical relevance: traditional thinking would indicate that drugs such as Nutlin would only be clinically useful in cancers that retain wild-type p53. However, if Mdm2 inhibitors are found to interfere with the tumor-promoting, p53-independent effects of Mdm2, they could potentially be useful as chemotherapeutic agents in a much wider spectrum of tumors.

Given the central role of p53 in tumor suppression, it is unsurprising that most research on Mdm2 has been focused on its regulation of p53. Nevertheless, a growing body of evidence suggests that Mdm2 has a number of other p53-independent functions, both in normal cellular biology and tumorigenesis. The exact mechanism of these functions, under which conditions they occur, and their significance to oncogenesis have yet to be determined. As Mdm2 emerges as an important player in tumor development in its own right, fully characterizing its p53-independent functions will certainly be important in the study of tumor biology and the development of new therapies for the treatment of cancer.

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