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Extra Views

A New Twist in the Feedback Loop

Stress-Activated MDM2 Destabilization is Required for p53 Activation

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ABSTRACT

The p53 tumor suppressor is a transcription factor that is activated by diverse genotoxic and cytotoxic stresses. Upon activation, p53 prevents the proliferation of genetically unstable cells by regulating the expression of genes that initiate cell cycle arrest, apoptosis, and DNA repair. Consequently, p53 must be kept inactive in unstressed cells as its inappropriate activation can cause premature senescence and death. p53 inhibition occurs primarily through the E3 ubiquitin ligase, MDM2. Because MDM2 is also a p53 target gene, stresses paradoxically activate p53 while simultaneously increasing MDM2 expression. Therefore, a challenge has been to explain how the abundant MDM2 is prevented from inhibiting p53, thus ensuring that p53 can execute an appropriate stress response. Here we discuss a new mechanism for p53 activation involving DNA damage-induced auto-degradation of MDM2. Our data reveal that DNA damage leads to the destabilization of MDM2, which correlates with p53 stabilization and target gene induction. Conversely, p53 levels and activity decrease when MDM2 returns to a more stable state later in the stress response. The destabilization of MDM2 is required for p53 activation, as blocking MDM2 degradation via proteasome inhibition prevents p53 transactivation in DNA-damaged cells by enabling MDM2 to bind and inhibit p53. MDM2 destabilization is controlled by DNA damage-activated post-translational modifications and by its own RING domain, implying a possible role for the RING domain-interacting protein, MDMX, in regulating MDM2 stability. We propose that accelerated degradation of MDM2 limits its binding to p53 during a stress response and enables p53 to accumulate and remain active, even as p53 transcriptionally activates more *MDM2*. Thus, the induction of *MDM2* RNA by activated p53 may create a reserve of MDM2 that can inactivate p53 once the DNA damage stimulus has abated and MDM2 is restabilized. As many tumors inactivate wild type p53 through MDM2 overexpression, exploiting the pathways that trigger MDM2 auto-degradation may be an important new strategy for chemotherapeutic intervention.

MDM2 KEEPS p53 INACTIVE IN UNSTRESSED CELLS

Upon activation by various genotoxic or cytotoxic stresses, the p53 tumor suppressor is activated to direct a transcriptional program that prevents the proliferation of genetically unstable cells. Consequently, the results of inappropriate regulation of p53 are dire: the loss of p53 function through mutation, deletion, or constitutive degradation predisposes cells to tumorigenesis, while errant p53 activation can lead to premature senescence or apoptosis. Thus, the appropriate positive and negative regulation of p53 is a life-or-death matter for the cell.

The primary means of negatively regulating p53 is through MDM2. This oncogene was initially discovered in a locus amplified on double minute chromosomes in a tumorigenic mouse cell line.[1] MDM2 overexpression enables primary human fibroblasts expressing E1A and activated ras to form tumors in nude mice, thus MDM2 behaves as a bona fide oncogene.[2] MDM2 plays an important role in the etiology of human cancer as it is amplified or overexpressed in a subset of human tumors expressing wild type p53.[3,4] The importance of MDM2 in the control of p53 is evidenced by the embryonic lethality of *MDM2* knockout mice, which presumably occurs due to rampant p53-dependent apoptosis and consequently can be suppressed by concurrent deletion of *p53*.[5,6]

MDM2 prevents p53-dependent gene expression through diverse mechanisms. It inhibits p53 transactivation by binding and occluding the p53 N-terminal transactivation domain, preventing the interaction of p53 with the basal transcription machinery.[7-9] Various stresses result in the acetylation of p53 by the histone acetyl transferases PCAF and

p300/CBP; however, this too can be blocked by the association of p53 with MDM2.[10-14] In addition to these direct mechanisms of transcriptional inhibition, MDM2 can indirectly inhibit p53-dependent gene expression by ubiquitinating and degrading p53.[15,16] MDM2 is a RING domain-containing E3 ubiquitin ligase, and as such, associates with an E2 ubiquitin conjugating enzyme to facilitate the catalysis of ubiquitin chains on both p53 and itself.[17-19] Once poly-ubiquitinated, p53 and MDM2 are subject to proteasome-dependent degradation.[20,21] An interesting recent report demonstrates that the ubiquitination activity of MDM2 is not just for degradation: it might also inhibit p53 target gene activation by ubiquitinating histones in p53-responsive promoters.[22] It is unclear whether all of the above mechanisms of p53 inhibition are utilized by MDM2 universally, or whether there might be specific contexts in which some of these mechanisms are preferred. For example, p53 activity is increased in mouse thymocytes expressing decreased levels of MDM2, despite the fact that p53 protein levels are the same as those observed in wild type mice.[23] This suggests that the mechanisms by which MDM2 inhibits p53 may be context-dependent.

Because p53 is a transcription factor, nuclear localization is critical for its activity.[24-26] Thus, it comes as no surprise that some human tumors arise that exclude wild type p53 from the nucleus.[26-29] MDM2 is purported to additionally negatively regulate p53 by inducing its nuclear export, either by binding a nuclear export receptor with its own intrinsic nuclear export signal and escorting p53 through the nuclear pore[30] or by unmasking the nuclear export signal in p53 via a ubiquitination-dependent change in p53 conformation.[31-33] The ensuing change in the subcellular localization of p53 is proposed to enable its degradation by cytoplasmic proteasomes.[34] However, p53 can also be ubiquitinated and degraded in the nucleus.[35-38] Because both p53 and MDM2 are predominantly nuclear in unstressed cells,[38] and because the half-life of p53 is significantly shorter than its rate of nuclear export,[31,38,39] it can be inferred that a significant proportion of p53 degradation occurs in the nucleus.

MDM2 INHIBITION BY ARF BINDING AND p53 PHOSPHORYLATION IS UNLIKELY TO BE UNIVERSAL

In a stress, p53 not only transcriptionally activates genes involved in cell cycle arrest or apoptosis, but also its own negative regulator, *MDM2*. Thus, MDM2 and p53 participate in an auto-regulatory feedback loop.[40,41] The *MDM2* gene has two promoters—one that is p53-independent and transcribed at constitutively low levels in unstressed cells and a second that p53 activates under most conditions of stress.[42,43] Consequently, while it has been suggested that some stresses such as transcriptional inhibition activate p53 through the downregulation of MDM2 transcription,[44,45] most stresses result in the significant accumulation of both p53 and MDM2 in the nucleus. Therefore, in order for p53 to induce the appropriate transcriptional response, stressed cells must have mechanisms to mitigate the inhibitory activity of MDM2.

One way by which p53 might be activated despite the presence of high levels of MDM2 is through ARF, the "alternative reading frame" product of the INK4a/ARF locus.[46] ARF overexpression results in the inhibition of MDM2 and consequently the stabilization of p53,[47-49] though the precise mechanisms by which this occurs remain controversial.[50] The role of ARF in inhibiting MDM2 is compelling because *ARF* expression is commonly lost in cell lines[48] and in Eμ-*myc*-driven lymphomas[51] that retain wild type p53, and because accentuated p53-dependent apoptosis in Eμ-*myc MDM2*$^{+/-}$ B cells is prevented by the loss of one allele of *ARF*.[52] However, the tumor spectrum of *ARF*-null mice is not the same as that observed in mice that are *p53*-null, indicating that *ARF* loss is not an equivalent substitute for loss of *p53*.[53] In addition, the repertoire of stresses that activate ARF is limited: it is induced by oncogenes such as myc,[54] ras,[55] E2F1,[56] and E1A,[57] and by senescence in part via the alleviation of negative regulation by Bmi-1,[58-61] but ARF is only partially required for the activation of p53 after DNA damage.[62] Moreover, ARF is not required for p53 activation in all tissues, as p53 activity is uncompromised in brain epithelium of *ARF*-null mice.[63] Together, these observations raise questions about the generality of ARF-dependent mechanisms for alleviating the inhibition of p53 by MDM2.

p53 phosphorylation is a second mechanism by which the inhibition of p53 by MDM2 might be alleviated in a stress response. Multiple stresses result in the phosphorylation of p53 on multiple sites in the N-terminus adjacent to and overlapping with the MDM2 binding domain.[64,65] Early studies speculated that these modifications might stabilize and activate p53 by preventing MDM2 binding. However, contradictions between in vitro and in vivo studies as well as the observation that p53 does not have to be phosphorylated to be activated have made the biologically relevant effects of these modifications challenging to discern. In vitro assays of MDM2 association with phosphorylated p53 peptides have come to widely disparate conclusions. For example, various studies show that MDM2 has a reduced affinity for p53 peptides phosphorylated at serines 15,[66,67] 20,[67,68] or 37,[66] or threonine 18.[67,69-71] Other studies show exactly the opposite: that MDM2 can bind p53 peptides phosphorylated at serine 15,[68-74] 20,[69-71,74] or 37,[70,73,74] or threonine 18.[68] Still other studies indicate that combinations of the above modifications are required to inhibit MDM2 binding.[70,72] In contrast with the above reports, full length p53 constructs mutated at multiple phosphorylation sites, either singly or in combination, have no defects in MDM2-dependent degradation or accumulation after stress in transiently transfected cells.[75,76] These widely contrasting findings suggest that in vivo analyses of p53 phosphorylation might provide a more accurate picture of the role of these modifications in mitigating the effects of MDM2 inhibition.

Surprisingly, in vivo studies of p53 phosphorylation site mutants hint at a less profound role for these modifications in p53 activation, as mice expressing endogenous p53 mutated at the murine equivalents of serine 15 or 20 have only partial defects in p53 activity and stability. For example, serine 15 mutant mice have partially attenuated apoptosis in the retina[77] and in thymocytes[78,79] upon exposure to γ-irradiation, though MEFs have no significant defects in cell cycle arrest.[79] The protein levels of endogenous p53 mutated at this site seem to be regulated normally by MDM2: they are low but increase after stress similarly to wild type p53,[78-80] in spite of the fact that this mutation also prevents subsequent phosphorylation at the murine equivalent of threonine 18.[80] Importantly, these mutant mice do not get tumors,[79] an observation contrary to what one might predict if serine 15 and threonine 18 phosphorylation prevented the negative regulation of p53 by MDM2. p53 activity is more seriously perturbed in mice mutated at the murine equivalent of serine 20, though as in the serine 15 mutant mice, the impact of this alteration seems to be tissue-dependent.[81] p53 protein levels in serine 20 mutant MEFs are indistinguishable from wild type, but markedly decreased in thymocytes and the cerebellum.[81] In addition, these mice are tumor-prone but the distribution of these tumors is more limited in these mice than those that are *p53*-null, consistent with a

role for serine 20 phosphorylation in a subset of tissues.[81] These in vivo studies suggest that while N-terminal phosphorylation partially prevents the inhibition of p53 by MDM2 in some tissues, additional mechanisms must exist to enable a full p53 response in all tissues.

Studies showing that p53 is not phosphorylated at canonical sites after diverse genotoxic and cytotoxic stresses raise additional questions about the role of these modifications in stabilizing and activating p53. For example, actinomycin D,[82] taxol,[83] nocodazole,[83] and leptomycin B[38] activate p53-dependent gene expression, though none of these treatments lead to p53 phosphorylation at serine 15. In addition, neither actinomycin D nor deferoxamine treatment leads to serine 20 phosphorylation,[82] and threonine 18 phosphorylation is not observed in normal human lymphoblasts treated with multiple stresses.[84] While many other agents do lead to phosphorylation at these sites, the consequences of these modifications is not always clear: we found that p53 is unstable and transcriptionally inactive at early and late times after DNA damage, despite phosphorylation at serine 15.[38] In addition, serine 15 phosphorylated p53 binds MDM2 in DNA-damaged cells as long as they are pretreated with proteasome inhibitors to stabilize MDM2 (see ref. 38 and below). Together, these data suggest that p53 N-terminal phosphorylation might be neither necessary nor sufficient to prevent MDM2 from binding p53 in stressed cells, though they do not rule out a role for these modifications in fine-tuning p53 function, for example, by enabling p53 to bind histone acetyltransferases[73,78,84,85] or by determining p53 promoter choice.[78,79]

A NEW MECHANISM OF MDM2 INHIBITION: MDM2 AUTO-DEGRADATION

The ubiquitin ligase activity of MDM2 is selective, and therefore has the potential to be subject to differential regulation. MDM2 does not promiscuously degrade all its binding partners, as it can bind ARF,[86] p73,[87,88] E2F,[89] and PML,[90-92] but there is no evidence thus far that any of these serve as substrates for MDM2 ubiquitination. Moreover, auto-ubiquitination of MDM2 is likely to be regulated through mechanisms distinct from p53 ubiquitination, as MDM2 poly-ubiquitinates itself but only mono-ubiquitinates p53 in vitro.[93] Because only proteins with ubiquitin chains consisting of at least four ubiquitin moieties are recognized as substrates by the proteasome,[94] an E4 (such as p300[95]) might be necessary to extend ubiquitin chains on p53, but not MDM2, prior to degradation. However, recent evidence indicates that when expressed at high enough levels, MDM2 can poly-ubiquitinate p53 without the assistance of an E4.[96] Nonetheless, there is evidence that the choice of auto-ubiquitination versus substrate ubiquitination can be context dependent. In an elegant experiment performed by Fang et al.,[18] the RING domain of MDM2 was shown to play an important role in substrate selection: substituting this domain for that of an unrelated protein (Praja1) prevented MDM2 from ubiquitinating p53 but did not prevent it from ubiquitinating itself. These observations indicate that the selective auto-ubiquitination of MDM2 might be an important means by which the cell can activate p53.

We recently found that regulated MDM2 auto-degradation is an important mechanism by which p53 is activated in cells treated with DNA damage.[38] The half-life of MDM2 protein decreases in normal human fibroblasts treated with the DNA damaging agents neocarzinostatin (NCS), UV irradiation, and BCNU. We also found that MDM2 destabilization is required for p53 activation. The timing of the DNA damage-dependent decrease in MDM2 half-life coincides with the peak of p53 stability and transcriptional activity, suggesting that although p53 induces the expression of high levels of *MDM2* RNA in a stress response, the resulting protein might be incapable of associating with and inhibiting p53 because of its rapid rate of turnover. Indeed, when we blocked MDM2 destabilization with proteasome inhibitors, p53 was incapable of transcriptional activation in DNA-damaged cells. This inhibition of p53 activity is most likely due to its increased association with stable MDM2, as p53 target gene induction was restored by concurrent treatment with nutlin, a small molecule that prevents the association of MDM2 with p53.[38,97] Interestingly, the destabilization of MDM2 by DNA damage was reversible: at later times in the DNA damage response, the half-life of MDM2 returned to that in unstressed cells and p53 again became unstable and inactive. This suggests a possible role for p53-dependent transcription of MDM2 in stressed cells: the increase in *MDM2* RNA enables the production of a reserve of MDM2 with the potential to inhibit p53 later when the stress is alleviated and p53 is no longer needed, thus ensuring the long-term viability of the cell.

Our findings are consistent with previous reports that indicate that subtle changes in MDM2 levels are likely to significantly affect p53 function. For example, MDM2 haploinsufficiency in mice expressing Eμ-myc is sufficient to activate p53, leading to increased apoptosis in spleen and a decrease in lymphomas.[98] In addition, a partial reduction of MDM2 levels in vivo leads to increased p53 transcriptional activity, decreased proliferation of MEFs in culture, and increased apoptosis in lymphatic and epithelial tissues in the absence of a stress.[23] More recently, a single nucleotide polymorphism was found in the *MDM2* promoter that enhances its transcription.[99] The increased MDM2 protein generated by this allele is sufficient to decrease the functionality of the p53 pathway, resulting in an acceleration of the onset of tumor formation and an increase in the tumor burden in carriers of this allele.[99] Interestingly, peptides, antibodies, and small molecule inhibitors that prevent MDM2 binding are sufficient to activate p53 dependent gene expression and apoptotic programs in the absence of any stress signals and their associated post-translational modifications.[97,100-104] These findings suggest that the most critical requirement for p53 activation is the abrogation of inhibition by MDM2.

THE REGULATION OF MDM2 DESTABILIZATION

How might the switch from p53 ubiquitination to MDM2 auto-ubiquitination be controlled? Though more than 1000 publications have been devoted to the study of the 14 phosphorylation sites on p53, MDM2 has at least 19 phosphorylation sites of its own,[105] most of which are of unknown functional consequence. MDM2 is phosphorylated by the DNA-damage activated kinases ATM,[106,107] DNA-PK,[108] ATR,[109] and c-Abl,[110] and it is de-phosphorylated at multiple sites after γ-irradiation.[111] Because NCS activates ATM,[112] which in turn can phosphorylate MDM2,[106,107] we asked whether ATM controls MDM2 destabilization. We observed that mutating the ATM phosphorylation site only partially prevents the destabilization of MDM2 in NCS-treated transfected cells,[38] and the half-life of MDM2 only partly decreases in NCS-treated *ATM* mutant fibroblasts (J. Stommel, unpublished observation). Because MDM2 destabilization is completely inhibited by wortmannin,[38] we conclude that this process is likely to be controlled by phosphorylation at multiple sites and by multiple DNA damage-activated kinases of the PI 3-kinase family, such as ATM or ATR.[113] MDM2

phosphorylation is likely to play a significant role in determining the activity of p53 through the control of MDM2 stability.[38]

In addition to phosphorylation, we found that MDM2 destabilization requires its intrinsic ubiquitin ligase activity, as a construct with a dysfunctional RING domain fails to become unstable after DNA damage.[38] This is an especially intriguing finding in light of a prior observation that the MDM2 RING domain plays an important role in ubiquitination substrate choice.[18] The switch from auto- to p53 ubiquitination by MDM2 might involve post-translational modification of the RING domain. For example, acetylation of this domain appears to inhibit the ubiquitin ligase activity of MDM2, though this seems to effect the ubiquitination of both p53 and MDM2.[114] The RING domain also binds ATP, though the functional consequences of this are uncertain as some MDM2 mutants that cannot bind ATP block p53 degradation and MDM2 ubiquitination, while others enhance both.[115]

The RING domain also binds accessory proteins that might contribute to the regulation of ubiquitination. MDMX might be the most interesting candidate for this role. MDMX was initially discovered as a p53-binding protein with significant homology to MDM2, though unlike its namesake, the *MDMX* gene is not transcriptionally activated by p53 in stressed cells.[116] MDMX binds p53 through a domain similar to that of MDM2,[69,117,118] and it has a RING domain through which it binds MDM2.[119,120] However, in contrast with MDM2, MDMX is missing a critical cysteine in its RING domain, which precludes it from acting as a ubiquitin ligase.[121-125] Early reports concluded that MDMX opposes MDM2 by binding and stabilizing p53,[117,119,126] which seemed very reasonable in light of the inactive RING domain of MDMX. Consequently, it came as a surprise that like MDM2, MDMX knockout mice die as embryos, and this lethality can be rescued by concurrent p53 deletion.[127-129] Together, the genetic data were more consistent with a role for MDMX as a negative regulator of p53. Later molecular evidence supported the genetics, showing that MDMX binds MDM2 and enhances its ability to ubiquitinate and degrade p53.[122,130] Moreover, siRNA to MDMX results in increased p53 protein abundance and activity.[122,124] Thus, MDM2 and MDMX behave in a manner similar to the ubiquitin ligase pair, BRCA1 and BARD1: BRCA1 alone has weak ubiquitin ligase activity and BARD1 has none, but as a RING-RING heterodimer, the two are more potent.[131,132]

The apparent inconsistencies between the earlier work showing that p53 is stabilized by MDMX and the later work showing that MDMX enhances MDM2 ubiquitin ligase activity are clarified by the discovery of two limitations of the in vitro systems used to study MDMX. First, many studies used C-terminally tagged MDMX, but this construct does not behave like the untagged counterpart.[133] Second, Gu et al. performed a careful titration of MDMX levels in cotransfections with MDM2 and found that at low levels MDMX cooperates with MDM2 in degrading p53, but at high levels it stabilizes p53.[122] Because MDMX homodimers can bind the p53 transactivation domain, it is likely that at supraphysiological levels MDMX homodimers are formed that have no ubiquitin ligase activity. These MDMX homodimers should compete with the more active MDM2 homodimers or MDM2-MDMX heterodimers for p53 binding, thereby stabilizing p53. Conversely, at physiological MDMX levels, MDMX-MDM2 heterodimers might be the predominant species, resulting in accentuated p53 degradation. Therefore, studies employing overexpression protocols are likely to give conflicting (and possibly artifactual) results depending on the extent of MDMX overexpression.

Together, these data raise the intriguing possibility that MDMX could switch the ubiquitin ligase activity of MDM2 away from MDM2 and toward p53. Perhaps MDM2 is stabilized by binding a partner with no ubiquitin ligase activity, and consequently has enough time to bind and inhibit p53. Interestingly, DNA damage and ARF overexpression lead to the degradation of MDMX by MDM2.[124,133] It is tempting to speculate that by decreasing MDMX levels in a stress, the abundance of MDM2 homodimers is increased, and that this species has more intrinsic ubiquitin ligase activity toward itself than to p53, leading to its enhanced degradation and resultant p53 degradation. Further experiments are required to test this possibility.

A number of recent reports suggest that the choice of whether MDM2 or p53 is degraded might also occur downstream of ubiquitination, perhaps through selective de-ubiquitination or by regulating access to the proteasome. For example, the ubiquitin hydrolase HAUSP has been implicated in regulating the stability of both p53[134] and MDM2[135,136] by de-ubiquitinating each of these proteins under different experimental conditions. The acidic domain of MDM2 might also be involved in determining whether ubiquitinated p53 is degraded, as deleting parts of this domain prevents the degradation of ubiquitinated p53.[137-140] It may be that this deletion prevents the extension of ubiquitin chains that enable efficient recognition of substrates by the proteasome, as this domain binds p300,[137] a protein that can act as a p53 E4 ubiquitin ligase in vitro.[95] Conversely, transfected p300 stabilizes MDM2,[141] which suggests that p300 might play an important role in switching the ubiquitin ligase activity of MDM2 away from itself and toward p53. The MDM2 acidic domain deletion mutants might also be incapable of targeting ubiquitinated p53 to the proteasome, perhaps due to a defect in binding hHR23A, the human homologue of *S. cerevisiae* Rad23.[142] This protein, known for its role in DNA repair, can act as a bridge between the proteasome and a ubiquitinated substrate.[143,144] When bound to MDM2, hHR23A enhances the degradation of ubiquitinated p53, though its impact on MDM2 degradation is unclear.[142,145] Interestingly, hHR23A binds in a region of MDM2 that is dephosphorylated in cells treated with γ-irradiation,[111] so it is tempting to speculate that DNA damage stabilizes p53, in part, through preventing the interaction of MDM2 with hHR23A.

A NEW MODEL FOR THE REGULATION OF p53 THROUGH MDM2 AUTO-DEGRADATION

In conclusion, the control of MDM2 auto-degradation, both by its mitigation in unstressed cells and its augmentation in stressed cells, is likely to play a critical role in the appropriate regulation of p53 activity. By rapidly degrading MDM2, the cell can ensure that p53 can be active despite the high level of newly synthesized nuclear MDM2 that is induced by p53 in a stress. In addition, the differential control of MDM2 stability along with the stress-dependent increase in *MDM2* transcription enables the creation of a reserve of MDM2 protein that, once restabilized, can rid the cell of the high levels of p53 that accumulate during the stress response. Many types of human tumors inactivate p53 by overexpressing MDM2, including 50% of pediatric acute lymphoblastic leukemias, one-third of sarcomas, 20% of nonHodgkin's lymphomas, and 10% of malignant gliomas.[146] MDM2 overexpression in these tumors correlates with poor prognosis and lethality. Proteasome inhibitors have shown promise as chemotherapeutic agents,[147] and while it is tempting to speculate that these might work well in tumors that overexpress

MDM2, our data show that the stabilization of MDM2 should hinder the efficacy of these drugs in this subset of tumors.[38] Therefore, targeting the switch between MDM2 auto-ubiquitination and p53 ubiquitination might represent an important new point of exploration for novel chemotherapeutic agents.

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