

REVIEW ARTICLE

Mdm2: Open questions

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Abstract

The Mdm2 oncoprotein and its association with p53 were discovered 30 years ago, and a cornucopia of activities and regulatory pathways have been associated with it. In this review, we will raise questions about Mdm2 and its cousin Mdm4 that we consider worth pursuing in future research, reaching from molecular structures and intracellular activities all the way to development, evolution, and cancer therapy. We anticipate that such research will not only close a few gaps in our knowledge but could add new dimensions to our current view. This compilation of questions contributes to the preparation for the 10th Mdm2 Workshop in Tokyo.

KEYWORDS

Hdm2, Mdm2, Mdm4, MdmX, p53

1 | INTRODUCTION

The Mdm2 oncoprotein and its association with p53 were discovered 30 years ago,¹⁻³ and a cornucopia of activities and regulatory pathways have been associated with it. A number of reviews summarize what we believe to know about Mdm2 and its paralogue and association partner Mdm4.⁴⁻¹⁰ However, open questions remain, similar to the white spots labeled “terra incognita”, Latin for “unknown territory”, on ancient geographical maps or globes. Such unexplored areas were alternatively labeled “hic sunt dracones” (here are the dragons) to indicate the risks and uncertainties for the traveler, and this metaphor could apply to the unexplored features of Mdm2 as well. In this review, we will raise *questions about Mdm2 and Mdm4* that we consider worth pursuing in future research, reaching from molecular structures and intracellular activities all the way to development, evolution, and cancer therapy. We anticipate that such research will not only close a few gaps in our knowledge but could add new dimensions to our current view.

2 | HOW EXACTLY DOES MDM2 ACT ON P53?

The current view emphasizes that Mdm2 forms a complex with p53 and mediates its ubiquitination, followed by proteasomal degradation. However, even this standard summary about Mdm2 leaves open questions such as the following.

What is the exact structure of the complex formed between Mdm2 and a p53 tetramer? The fact that the full-length proteins have never been crystallized, neither alone nor in a complex, makes it difficult to answer this question. However, the advent of cryo-electron microscopy could enable progress in this field. These types of data with the p53-DNA complex (but without Mdm2) were reported already.¹¹

Are the two aminoterminal domains of both proteins representing the only relevant interaction surface? How would the other domains of Mdm2 and p53 fold in relation to each other? What about the dynamics of the complex – does it “breathe” to carry out the transfer to ubiquitin onto various sites on p53? And what is the structural difference between

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the p53-Mdm2 complex and the TAp73-Mdm2 complex? – the latter forming with high efficiency but without detectable destabilization of TAp73.^{12,13}

What is the effect of additional binding partners on the structure of Mdm2 and the complex with p53? The complex of Mdm2 and Mdm4 is held together, at least in part, through the RING finger domains of both proteins,¹⁴ and this association can be separated from the ubiquitin ligase activity of Mdm2,¹⁵ but again, it is subject to ongoing research how the other domains are positioned within the complex of the Mdm2/Mdm4 heterodimer and the Mdm2/Mdm2 homodimer. Additional partners include but are certainly not limited to E2 ligases, p14ARF, and the ribosomal L5/L11/5S-RNA complex. The structures of these complexes remain to be determined, including functional consequences. p53-Bound DNA might well reshape the p53-Mdm2 complex, and the same notion holds for chromatin-associated binding partners of Mdm2, such as members of the polycomb repressor complexes.^{16–18}

Taken together, despite our knowledge on single domains within p53 and Mdm2, we are far from understanding the higher order structures of full-length proteins and their multiple complexes resulting in alternative functions and transcriptional patterns.

3 | IS THERE A MODIFICATION CODE FOR MDM2/MDM4 AND P53, DEPENDING ON THE KIND OF CELLULAR STRESS AND THE DESIRED RESPONSE?

Numerous posttranslational modifications were identified on Mdm2¹⁹ and Mdm4²⁰ as well as p53.²¹ Many (though not all) of these modifications enhance p53 activity and diminish the ability of Mdm2 to bind and degrade p53. The phosphorylations by AKT^{22–24} and by ATM²⁵ (on different residues and with partially opposing effects) are only the most prominent examples.

The function of p53 and Mdm2/Mdm4 is to receive information (largely through the Mdm2 protein) about intrinsic and extrinsic cellular stresses and respond (through the p53 protein) by selective programs of transcriptional activation that either repair the damage produced by the stress and restore homeostasis, or kill the cell, eliminating the consequences of the damage. There are at least 10 stress signals (recognized by stress identifiers and transmitters) that act by inhibiting Mdm2 levels or activity and increase p53 levels, and at least 4 stress signals that act to increase Mdm2 levels or activity and decrease p53 transcriptional functions. Signaling to Mdm2, as well as the p53 responses, are accomplished by either protein modifications, eg phosphorylation, acetylation, methylation, ubiquitination, and sumoylation, protein-protein interactions, or RNA-protein interactions. P53 enhances the fidelity of cellular growth, replication, and division. It not only responds to a stress signal, but when multiple stress signals perturb cell division, the p53 protein modifications and interactions integrate the stresses that are to be responded to and program responses accordingly. As such the Mdm2-p53 node

integrates many diverse functional signal transduction pathways and as such that node is highly connected to a large amount of information that mediates cellular responses.

These considerations at least suggest that there is a code of modifications on both Mdm2/Mdm4 and p53, reflecting the stress input and the biological effects, such as cell cycle arrest, senescence, or cell death of different kinds. Such a code, if it exists, might well depend on the cell type and signaling activities. The future challenge will consist of an integrated understanding of *how combinations of modifications on Mdm2 and Mdm4 are achieved in a stress-specific way, and how this will affect the p53-driven response.*

4 | HOW DOES COMPARTMENTALIZATION OF MDM2 AND MDM4 AFFECT THEIR FUNCTIONS?

Both Mdm2 and Mdm4 can adopt diverse intracellular localizations, and these are subject to dynamic changes. We have reported that Mdm2 shuttles between the nucleus and the cytoplasm, through specific import and export signal sequences.²⁶ Similar findings were also reported for p53,²⁷ and the transport of each binding partner can alter the p53 response.²⁸ Moreover, Mdm2 undergoes relocalization when associating with binding partners. For instance, p14ARF is capable of relocalizing Mdm2 to nucleoli,^{29,30} whereas the acetyl transferase KAT5/Tip60 takes it to promyelocytic leukemia protein (PML) nuclear bodies.³¹ More recently, it turned out that Mdm4 predominantly localizes to the cytoplasm in the absence of Mdm2 but travels to the nucleus in the presence of it.³² Mdm4 localization can further be regulated by phosphorylation through AKT and Chk1, each leading to its association with 14-3-3 proteins.^{33,34} It should be noted, however, that most of these experiments were carried out by overexpressing Mdm2/Mdm4, making it even more important to address the precise compartmentalization of the endogenous proteins, their changes in cellular stress situations, and their impact on p53 activities and cellular responses.

5 | HOW DOES MDM2 AFFECT CELL FATE, INDEPENDENT OF P53?

Although Mdm2 is widely known as a negative regulator of p53, a variety of additional functions have been reported. Most of them were observed by overexpressing Mdm2 from transfected plasmids, raising the uncertainty of their physiological relevance. Still, these activities point out that Mdm2 is at least capable of doing much more than counteracting p53. For instance, overexpressed Mdm2 *hinders* cell cycle progression in the majority of cell types analyzed,³⁵ at least suggesting that it could also have tumor-suppressive properties³⁶ that contribute to p53 activity as an effector.³⁷ Moreover, in a similar setting, Mdm2 binds the MRN complex (in particular Nbs1) and negatively regulates DNA repair.³⁸ On top of this, Mdm2 binds a variety of RNA molecules,^{39–41} including that of p53,⁴² and this

could affect the translation of mRNAs. It remains to be determined whether endogenous Mdm2 also carries out such functions, particularly in the context of p53-induced expression of Mdm2, but also in a setting where p53 is absent. Of note, endogenous Mdm2 can contribute to stemness and chromatin modifications (H2A K119ub1 as well as H3K27me3) in cells that lack p53 altogether.¹⁶ Moreover, and again in the absence of p53, Mdm2 supports the progression of DNA replication forks.⁴³ *A challenging question is whether such activities are enhanced when p53 increases the levels of Mdm2?*

6 | DOES MDM2 PROMOTE OR PREVENT TUMOR DEVELOPMENT?

When p53 is deleted, mice are prone to cancer.⁴⁴ Some p53 target genes were studied in a similar fashion, as they are at least not completely essential for the development of a mouse. In this way, it turned out that none of these p53 target genes, when knocked out, recapitulates the phenotype of p53-null mice, ie the susceptibility to cancer. Even triple knock-outs of *cdkn1a/p21*, *bbc3/puma*, and *pmaip1/noxa* in p53-proficient mice did not induce cancer formation.⁴⁵ This either means that p53 suppresses tumors by transcription-independent mechanisms, or otherwise that a p53 target gene was missing from the reported analyses – and that this missing gene was responsible for tumor suppression. One important gene that could not be analyzed in this way is *mdm2*. Like the other genes mentioned, it is strongly p53-responsive. However, knocking down *mdm2* in p53-proficient mice results in exaggerated p53 activity and embryonic lethality.^{46,47} Thus, it is difficult to determine if Mdm2 (and perhaps Mdm4) are carrying out a tumor-suppressive activity in addition to their p53-regulating function, although this hypothesis has been raised for some time.³⁶

One fact that might support such a scenario is that Mdm2 overexpression, eg by gene amplification, is a rare event in comparison to p53 mutations. Only a subset of sarcomas, as such a relatively rare tumor species, contains such amplifications of Mdm2 on a regular basis (www.cbioportal.org). In contrast, p53 is mutant in roughly 1 out of 2 tumors, including the most common cancer species. Likewise, even when p53 is wildtype, silencing p14ARF represents a far more common way of dampening p53 activity, in comparison to Mdm2 amplifications. *Could tumor-suppressive activities of Mdm2 represent a reason for this failure to observe Mdm2 amplifications in carcinomas? Could this be a reflection of the deleterious effects of Mdm2 overexpression in most cultivated cells?*

7 | ARE THERE ANY TUMOR-PROMOTING MUTATIONS IN MDM2?

If Mdm2 has tumor-suppressing activities, it is expected that these will be difficult to separate from p53 regulation. Otherwise, we would probably find more cancers with a mutation of Mdm2 that preserves p53-binding but silences such tumor-suppressive

activities. However, it remains possible that Mdm2 evolved to comprise p53-regulating as well as tumor-suppressing activities on similar domains, decreasing the likelihood of cancer. Still, if Mdm2 can be activated by posttranslational modifications, *why don't we find activating mutations of Mdm2 in cancer?* Wouldn't it seem “easy” for cancer evolution to enhance its binding to p53 and/or its ubiquitin ligase activity, its stability, or its robustness against phosphorylation and inhibition by ATM?

Another question is whether Mdm2 alterations do in fact exist in tumors, but perhaps not as often as classical missense mutations. Rather, the complex splice pattern of Mdm2^{48,49} might be altered, by dysfunctional splice regulators or even by mutations in Mdm2 introns that would still need to be identified. It remains subject to future research whether some variations in Mdm2 splicing are enhanced in tumors, and if so, whether the resulting Mdm2 variant might antagonize p53 while abolishing additional, cytotoxic activities of Mdm2.

8 | WHAT IS THE ROLE OF MDM2 IN DEVELOPMENT, IF ANY?

When p53 is deleted, this strongly increases the induction of stem cells by the Yamanaka protocol.⁵⁰ Interestingly, even within this background of p53-null cells, the removal of Mdm2 decreases the efficiency of stem cell induction.¹⁶ We have correlated this phenomenon with the ability of Mdm2 to support the activity of polycomb repressor complexes. However, the question remains *whether Mdm2 and p53 affect the pool sizes of stem cells in vivo, or whether they otherwise govern the development of an organism?* At first glance, it appears that the major role of Mdm2 and Mdm4 in development consists in the p53 antagonism. As soon as p53 is removed along with Mdm2 (or Mdm4), mice are born at near-Mendelian ratios (although they are still as cancer prone as the p53 single knockouts).⁵¹ However, these are animals that are kept under very artificial conditions. In nature, even developing organisms are facing stresses that include infectious diseases, malnutrition, and predators. It remains to be determined whether Mdm2 might add robustness to stem cells or other aspects of development under such stresses, and whether this might comprise p53-independent activities as well.

9 | WHAT IS THE ROLE OF MDM2 IN AGING? HOW DOES THE P53-MDM2 AXIS FUNCTION TO AFFECT THE RATE OF AGING?

Cellular senescence occurs in response to intrinsic and extrinsic stresses where the cell withdraws from its cell cycle progression and loses its capacity to replicate. This is commonly irreversible and the cell may lose some of its functions and alter its morphology while it continues to metabolize. Replicative senescence (RS)⁵² is observed when cells duplicate for a certain number of generations, resulting in

telomere shortening. This in turn is recognized as breaks in the DNA by the ATM protein (along with the MRN complex), which phosphorylates and activates checkpoint kinase 2 (Chk2), and the resultant protein modifications weaken the Mdm2 protein binding to p53. P53 is then activated (increased in concentration) as a transcription factor producing p21, PAI-1, PML, and microRNA-34a, which contribute to the inhibition of Cyclin E-Cdk2, partially blocking the release of E2F from its negative regulator, the retinoblastoma protein (Rb). At the same time, increased levels of the p16 tumor suppressor protein inhibit Cyclin D-Cdk4/6 and complete the inhibition of Rb's release of E2F from an Rb-E2F complex blocking entry into S-phase.

A second mediator of cellular senescence results from oncogene-induced senescence (OIS).⁵³ In this case, the mutational activation of *Ras*, *Myc*, or other oncogenes results in the transcription and translation of the ARF protein, which binds to Mdm2 and blocks its ability to ubiquitinate p53. P53 levels increase, resulting in a senescence program similar to the one described above. When this happens in vivo, the p53 transcriptional program includes several cytokines of the innate immune system (interleukin-6, tumor necrosis factor, and macrophage inhibitory cytokine-1) which attract natural killer cells, CD-8 T cells, and monocytes/macrophages, which kill the senescent cells and clean up the debris. This is called the senescence-associated secretory phenotype (SASP).⁵⁴

Over a lifetime, RS, DNA damage repair (DDR) senescence, and OIS produce cells that secrete inflammatory cytokines as part of the SASP. With aging, the efficiencies of the innate immune system and the adaptive immune system decline, and senescent cells remain in vivo resulting in chronic diseases like rheumatoid arthritis, inflammatory disorders, autoimmune diseases, and even cancers. It has been hypothesized that persistence of senescent cells in the body is the (or a) cause of the aging process. Kirkland and his associates have shown that adding isogenic senescent cells to mice results in reduced survival of these mice compared to untreated age-matched mice. This effect was more pronounced in older than in younger mice. There are senolytic drugs, dasatinib and quercetin, that have been shown to preferentially kill and remove senescent cells from a mouse and reduce the levels of SASP in the animals.⁵⁵ When these drugs have been used to treat older mice with high levels of senescent cells, they reduced the physical dysfunctions and extended the life span of these mice by 36% when compared with untreated controls. These drugs also reduced the number of senescent cells and the levels of the SASP in human explants tested in vitro.

These kinds of studies implicate Mdm2 and p53 in playing a central role in aging by initiating senescent cells in response to RS, DDR, and OIS. Is there any evidence to support these ideas? Scoble and her group inserted into the germ line of mice a splice variant of p53, deltaNp53 missing the amino-terminal transcriptional activator. The resultant mice had a much reduced lifespan, aging more rapidly than normal mice.⁵⁶ A mutation at the amino-terminal residue, ser-15 (ser-18 in murine p53), which is phosphorylated by the ATM kinase resulting in p53 activation and senescence, produced a mouse that (unexpectedly) also had accelerated aging.⁵⁷ Extra copies of the p53 gene are lethal. However, up to 4n copies of p53 can be tolerated

with extra copies of p19 ARF, which binds to and regulates Mdm2 activity,⁵⁸ resulting in an increased lifespan and an improved age-related health decline. The conclusions from these observations are that physiological regulation of p53 through Mdm2 delays the aging process, whereas chronic excessive p53 prevents cancer but accelerates the aging process.⁵⁹ In support of these ideas, Lessel and his colleagues⁶⁰ have described a human family where a mutation in the *Mdm2* gene is linked to premature aging. Somewhat similarly, a germline mutation in *Mdm4* leads to shortened telomeres in patients as well as in mice.⁶¹ *The question remains whether the balance between tumor suppression and aging can be manipulated in a favorable manner by therapeutic interventions, to postpone the aging process while still avoiding cancer.*

10 | WHAT IS THE ADVANTAGE OF MDM2 IN EVOLUTION?

The available evidence for the role of Mdm2 in development of the mouse suggests that the only absolutely essential function of Mdm2 is the regulation of p53. This means that Mdm2 forms a genetically inseparable part of the p53 system. It raises the question *why Mdm2 evolved as a separated gene, rather than relying on p53 itself for its functions and its regulation.* Does the evolution of Mdm2 make the p53-Mdm2 system either more efficient in tumor suppression, or more controllable to avoid unnecessary p53-driven cell death? Rather than integrating inputs such as DNA damage signaling on Mdm2 as well as p53, it would have been more efficient to focus all input and output signaling pathways on p53 itself (eliminating Mdm2), eg through posttranslational modifications on p53 that determine its activities and stability.

Nature uses such an alternative regulatory pathway in the case of p63, a p53 paralogue that appears to be the evolutionary precursor in invertebrates of p53 in vertebrates. In vertebrates, p63 has many of the same responses to DNA damage in the germ line that p53 has in the soma. Irradiated oocytes undergo apoptosis depending on p63, not p53.⁶² To activate p63 in this context does not seem to require Mdm2 regulation. Rather, phosphorylation of p63 induces a conformational switch that allows the transcriptionally active isoform(s) of p63 to expose its transactivation domain.^{63,64} It even seems that p63 and its regulation represent the evolutionary older mechanism, in comparison to p53 and Mdm2.⁶⁵ In comparison to p63, p53 has lost much of this C-terminal, regulatory domain.

Why would p53 not use a similar route of regulation, and why do we instead see Mdm2 as its major regulator? What makes the difference in this regard between somatic cells and germ cells? One can speculate that having a separate recipient of stress signaling (Mdm2) could increase the capacities of the system for multiple stress inputs. If signaling pathways end on Mdm2, all they need to do is to *inactivate* its ability to bind and/or ubiquitinate p53. This can be achieved on multiple domains within Mdm2, interfering either with p53 binding, or ubiquitin ligase activity, or just the conformation of Mdm2 that enables the transfer of ubiquitins specifically on p53. In contrast,

if a molecule such as p53 needs to become *more active* in response to a signaling pathway, the opportunities to carry this out are more limited, eg by modifying specific intramolecular switches. Moreover, having a negative regulator between stress signaling and p53 might also lower the chances of inactivating p53 by mutation. If intramolecular switches on p53 would be obligatory for its activation, any mutation within such switchable domains could abolish its tumor-suppressive activity. In contrast, when Mdm2 is carrying out the regulation of an otherwise constitutively active p53 molecule, relatively fewer mutations on p53 can dramatically reduce its activity, and that is what we see in only a handful of hotspot mutations, which represent more than 33% of the p53 mutations observed in cancers.

If some of the above considerations are valid, early evolved versions of Mdm2 should be useful to determine the most well-preserved receptor functions for damage signaling. The discovery of Mdm2 in placozoans (summarized by Lane and Verma⁶⁶) and other ancient organisms should help to clarify the most conserved functions of it.

The p53 pathway has a central node containing p53 and Mdm2 proteins. Feeding into this pathway are 10-15 different types of stress signals that turn on the p53 transcription factor or turn off the p53 transcription factor. Each stress signal has a sensor (ATM for DNA breaks), a transmitter, Chk2 for DNA breaks, and a receptor protein that is modified by the detector and/or transmitter. This receptor is Mdm2 and in turn regulates the level or activity of p53. The p53 transcription factor protein is modified to integrate the stress signals being transmitted and then chooses a transcriptional program that results in either the repair of a stress or damage or decides to kill the cell (by one of several mechanisms) so as to eliminate the damaged cells and prevent cancers from arising. Between the inputs and the outputs of the p53 signal transduction pathway and the information it integrates, ie the stress signals and responses (higher order information), the p53 pathway is the most connected pathway to other cellular functions and the most informed about those functions through one central node. However, the organization or structure of this set of pathways appears vulnerable, not robust at all, because a single mutation in the *p53* gene can disrupt this entire set of multiple pathways in the cell. The fact that the *p53* gene is the single most common mutation in human cancers supports this notion. It is the most vulnerable node in the cell to mutation for cancer development. *Why did evolution choose to construct the p53/Mdm2 pathway in this fashion?*

Almost all of the input signals coming into the central node are received and interpreted by the Mdm2 protein. A p53 mutation does not affect the reception of a stress signal. The insertion of Mdm2 into the p53 pathway, where p53 transcribes the *Mdm2* gene while the Mdm2 protein ubiquitinates p53 and thereby mediates the degradation of the p53 protein, does a number of new things. First, it sets up an autoregulatory loop so that the levels (activities) of these two proteins oscillate 180 degrees out of phase, which does not let either protein get to a very high concentration (like a thermostat) without the loop being broken. Second, a mutation in the *p53* gene has no effect on the ability of Mdm2 to detect a stress signal while

a mutation in a structurally autoregulated p63 would fail to respond to both an input and an output signal. If in addition to p53, Mdm2 has other substrates that respond to stresses, then these other substrates will still function after a stress signal. Thus, it is possible that the input stress signals are more robust, or backed up, than the output signals of p53, because the Mdm2 gene was inserted into the pathway during evolutionary history. If this idea is correct it brings up the question *what other functions does Mdm2 have in response to stress signals, in addition to regulating p53 activity? If the Mdm2 pathway splits into p53 regulation and other responses to a stress, are we missing 50% of what this node does for a cell?*

11 | WHAT IS THE NEED FOR MDM4?

The above considerations apply even more urgently to the evolution of Mdm4. Here, it is even more puzzling why another paralogue of Mdm2 evolved, and what the evolutionary advantage of it might be as opposed to one Mdm2-like molecule. Moreover, like Mdm2, Mdm4 turned out to be essential for murine development, if and only if functional p53 is present. Yet, Mdm2 and Mdm4 do have a few differences.

1. They are regulated differently and their levels and “activity” differ. *What regulators and signaling pathways act on Mdm4 rather than Mdm2, and how do the two proteins divide up their tasks?* Our current knowledge on this was recently summarized.²⁰
2. The Mdm2 KO mouse dies earlier in fetal life than the Mdm4 KO mouse but both are rescued by a p53 KO. This argues that: (i) different efficiencies of the proteins are prevalent during development; (ii) different locations or cell types “prefer” either of the p53 regulators during development; and (iii) each protein is produced at different times in development.⁶⁷ *Where and when is Mdm2 vs. Mdm4 active and required for p53 regulation during development?*
3. While *Mdm2* gene amplifications can occur mainly in sarcomas, *Mdm4* amplifications occur in approximately 10% of glioblastomas and invasive breast carcinomas according to The Cancer Genome Atlas (www.cbioportal.org). This is even more surprising as the two proteins are supposedly most active in a heterodimeric complex. *What is the determinant and mechanism behind this tumor tissue specificity?*

One possibility is that Mdm4 could have been inserted into the p53 pathway because it receives other stress signals not received by Mdm2. *If this is correct then the question is why is there not an Mdm5, 6, 7, 8, and 9 for additional stress signals?*

Mdm2 has a large number of spliced forms.⁴⁸ Perhaps the reason for this is that each spliced form receives a different stress signal than the others. This could represent a way of partitioning Mdm2 into Mdm5, 6, 7, 8, and 9, each protein responding to a diverse stress. A mutation in one Mdm2 spliced form would not necessarily impact another spliced form from signaling to p53 or the alternate Mdm2 substrates. In this way, most mutations in

the Mdm2 protein would have only a small effect on cancer production and not necessarily be selected for, while p53 mutations would be much more common. In addition, any mutation in the *Mdm2* gene that inactivates its function of regulating p53 levels should be a lethal mutation, because too much WT p53 would kill a cell. The mutations in the *Mdm2* gene are most commonly gene amplifications (too much Mdm2 protein, not too little) and, as mentioned above, seem to be tissue restricted to mesenchymal tumors, especially liposarcomas. *Does this suggest that different stem or progenitor cells of different tissues may have different p53-Mdm2 signal transduction pathways, resulting in tissue-specific mutations contributing to cancers?*

Mdm4 is regulated by alternative splicing as well, and the emerging mechanism(s) are defined more sharply than for Mdm2. In particular, exon 6 can be skipped from Mdm4, giving rise to a shorter and less stable isoform, as reviewed recently.²⁰ The inclusion of this exon correlates with tumor-associated deletions of the gene encoding ribosomal protein RPL22,⁶⁸ at least suggesting that such tumors control p53 by giving preference to the synthesis of Mdm4. The splicing pattern can be manipulated by clinically available drugs. For instance, inhibition of cyclin-dependent kinase 4 (CDK4) leads to exon skipping in Mdm4, synergistically with PRMT5 inhibition.⁶⁹ Taken together, *this raises the question whether tumor-specific, full-length Mdm4 can serve as a biomarker or even target in tumor therapy.*

12 | IS THERE A FUTURE OF TARGETING MDM2 AND/OR MDM4 FOR THERAPY?

With the advent of Nutlin⁷⁰ 20 years ago, Mdm2 became druggable, raising the hope that the most successful tumor suppressor could now be activated at will, at least in those 50% of all tumors that retain WT copies of p53. Still, however, no FDA approval has been reached for Mdm2 antagonists, despite multiple attempts to prove their efficacy in the clinics. *Why did this turn out to be so difficult?* Liposarcoma seemed like an ideal tumor entity to be cured by Mdm2 antagonizing drugs, given its 90% frequency of *Mdm2* gene amplifications and the strong in vitro response to Mdm2 antagonists of liposarcoma-derived cells. However, insufficient cell killing, perhaps because of the pharmacokinetic and dynamics, and the occurrence of p53 mutations were hampering clinical successes so far. On top of missing efficacy, toxicities including myelosuppression but also severe nausea and diarrhea (cell death in the gut) were strongly impairing the quality of patients' lives.⁷¹ So is it time to give up?

Alternatively, *are there better ways to target Mdm2 antagonists towards tumors, and to enhance p53-mediated tumor cell death?* Surprisingly, we are still not sure about the determinants that render cells susceptible towards death in response to Mdm2 inhibitors, aside from functional p53. Within cultivated cells with a WT p53 status, there is still a large spectrum of responses, reaching from reversible cell cycle arrest through sustainable senescence all the way to efficient cell death.⁷² If we knew the mechanisms underlying these differential responses, we might have a chance to target

Mdm2 along with such determinants of survival. Then, however, it would still be subject to investigation whether corresponding drug combinations had acceptable toxicities.

Could we improve the efficacy of Mdm2 antagonists by eliminating Mdm2 altogether, rather than "just" blocking its interaction with p53? Recently, proteolysis-targeting chimera (PROTAC)⁷³ drugs to degrade Mdm2 were reported.⁷⁴ If Mdm2 had oncogenic activities on top of its action on p53, such PROTACs should be more capable of interfering with tumor cell proliferation than the classical Mdm2 antagonists. However, as outlined above, Mdm2 also displays a number of activities that are at least capable of hindering proliferation. If such activities are lost, Mdm2-targeting PROTACs might be less efficient. Thus, the biological effects of this exciting class of small compounds still remain to be determined when targeting Mdm2.

What are the advantages of targeting Mdm4, alone or in addition to Mdm2? As Mdm4 represents an essential partner of Mdm2 for many activities, it is at least conceivable to design compounds that bind and inhibit Mdm4. This could have advantages over targeting Mdm2, at least in a subset of malignancies that rely on the enhanced synthesis of full-length Mdm4.⁷⁵ Interestingly, depletion of Mdm4 interferes with the proliferation of breast cancer cells even when p53 is mutant, through activation of the CDK inhibitor p27/CDKN1B.⁷⁶ This further argues that Mdm4 has p53-independent, cancer-promoting activities that might be druggable. Moreover, small compounds can induce the dimerization of Mdm2 and Mdm4 while antagonizing their binding to p53.⁷⁷ Together, these considerations raise the question *which molecular interfaces and activities of Mdm4 would be most helpful to target by small compounds?*

Could Mdm2/Mdm4 antagonists serve different purposes in addition to tumor cell killing? One possibility could be the protection of nontransformed cells and tissues against the toxicities of chemotherapy, in the context of p53-mutant tumors. The idea is to first halt the cell cycle of most normal cells, including tissues that are otherwise rapidly proliferating, eg the bone marrow or the epithelia of the gut and the skin. Mdm2-antagonists would keep them from dividing, whereas they would not halt the proliferation of tumor cells carrying mutant p53. Subsequently, the treatment with chemotherapeutics should selectively affect the tumor, as long as such chemotherapeutics depend on cell proliferation for their efficacy. This applies to nucleoside analogues that are incorporated during S phase – they are essentially of no effect when the cell cycle is arrested.⁷⁸ But similar principles are also applicable to topoisomerase inhibitors (most effective in S phase) or drugs targeting the mitotic spindle, such as taxanes⁷⁹ (most effective during mitosis). This concept, sometimes referred to as "cyclotherapy",^{80,81} has been entertained for two decades but nonetheless has not been clinically established. What would be required to get it to work? Two major obstacles need to be overcome. First, Mdm2 antagonists not only prevent cell cycle progression, but they can also induce cell death, and they are therefore cytotoxic to the bone marrow and the gut on their own. Thus, if possible, new drugs, treatment schedules, and pharmacokinetics would be required to achieve Mdm2 antagonism with as little tissue damage as possible, while still inducing thorough

(but reversible) cell cycle arrest. Second, the regimen applied to the (still cycling) tumor cell would need to be harsh enough to eliminate most, if not all, tumor cells within the relatively short time window during which normal cells can be kept arrested. This would probably require a higher degree of cytotoxicity than what is presently achieved by chemotherapeutics.

Medical applications of Mdm2 antagonists might not be limited to the context of cancer. *So what else could these drugs be used for?* Like some chemotherapeutics, eg methotrexate, Mdm2 antagonists might be useful in immunosuppression. The adaptive immune response is based on the clonal proliferation of few B and T cells with receptors to a given antigen. Halting this proliferation can be very effective to overcome autoimmune diseases such as systemic lupus erythematosus or scleroderma. It remains to be determined whether drugs that disrupt the p53-Mdm2 interaction can be used for such purposes as well.

At present, we are facing a bewildering discrepancy between the unique frequency of p53 mutations in human malignancies and the lack of clinically successful therapeutic approaches that directly target the p53/Mdm2 regulatory system. *How can we change this?*

ACKNOWLEDGMENTS

This work was initiated in preparation for the 10th Mdm2 Workshop in Tokyo, which will now be held in 2022. Our goal is to address a series of questions that both stimulate and focus the field upon the regulatory node of the p53-Mdm2 signal transduction pathway. This pathway is central to the homeostasis, vitality and responses to stress brought about by many diverse pathologies. By publishing this set of challenging questions now, in 2020, we are hoping to hear about some of the answers to these questions at the meeting in 2022. Meetings focused on topics like those discussed in this review are perfect places to advance the field. Everyone, from many diverse fields, is welcome to join this meeting.

CONFLICT OF INTEREST

Arnold J. Levine is on the Board of Directors of PMV Pharma, a company that develops p53 regulators. He is also a member of the board of directors of Pharmabody, a monoclonal antibody company presently involved with hemophilia, complement disorders, and infectious diseases. Matthias Dobbelstein has no conflict of interest.

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How to cite this article: Dobbelstein M, Levine AJ. Mdm2: Open questions. *Cancer Sci*. 2020;111:2203–2211. <https://doi.org/10.1111/cas.14433>