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Mini-Review

Any way you splice it

Mdm2 at the crossroads of tumor surveillance

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***Mdm2* is the most important regulator of p53, the chief responder of various modes of cellular stress, including DNA damage and oncogenic insult. Many alternative and aberrant splice products of the *Mdm2* gene product have been described, but relatively little is known about the origin, function or consequence of these variants. Recently, a novel splice form of mdm2 was discovered which incorporates 108 bp of intronic sequence into the mature *Mdm2* mRNA. The additional sequence encodes in-frame stop codons, resulting in severely truncated mdm2 protein. Most intriguingly, this alternative splice form, termed *Mdm2*$^{+108}$, is acutely induced by the chemotherapeutic agents Adriamycin and Actinomycin D, but not other DNA damaging agents. The effect of *Mdm2*$^{+108}$ induction is a rapid and robust accumulation of p53, arguing that the function of this alternative splice event is to engage the p53 tumor surveillance pathway and restrain proliferation of cells damaged with these potently genotoxic compounds.**

Mdm2 and Cancer

The human gene *HDM2* (often referred to as *Mdm2* for its mouse counterpart) is among the most studied proto-oncogenes in the human genome.[1,2] *Mdm2* was originally discovered on a mouse "double minute" chromosome, and its ability to promote transformation of 3T3 fibroblasts in vitro was subsequently characterized.[3] When the *Mdm2* gene product, mdm2, was next discovered to be associated with p53, the all-important "guardian of the genome," a model for the oncogenic activities of mdm2 quickly emerged.[4] Indeed, all agree that a major cellular function of mdm2 is in the negative regulation of steady-state levels of p53 protein through ubiquitination. In an unstressed cell, mdm2 is charged with targeting p53 for degradation by the 26S proteasome.[5,6] The vital importance of keeping p53 levels in check is illuminated by the embryonic lethality of *Mdm2*$^{-/-}$ mice, an effect that is completely reversed by simultaneous knockout of *Tp53*.[7] Further, the tumor spectrum of transgenic mice that overexpress *Mdm2* overlaps very well with that of *Tp53*$^{-/-}$ mice, confirming p53 as the major target of mdm2 in mice.[1] These observations in mice are confirmed by the rarity of *HDM2* gene amplification co-existent with inactivating mutations of *TP53* in human tumors.[8,9]

However, the story gets murky from there, as researchers report that the spectrum of tumors in *Tp53*$^{-/-}$ mice is not identical to that of *Tp53*$^{-/-}$*Mdm2*$^{-/-}$ DKO mice.[10] Further, mutants of *Mdm2* that are unable to bind p53 retain the ability transform cells in vitro[11] and overexpressed mdm2 can even transform *Tp53*$^{-/-}$ cells.[12] Accordingly, scores of p53-independent functions of mdm2 have been discovered, nearly all of which directly impact cell growth and proliferation.[13,14] The next most important target of mdm2 may be pRb, encoded by *RB1*, the second most commonly mutated tumor suppressor gene (after *TP53*) in the human genome.[15,16] Because pRb is well known to play a direct role in cell cycle progression, differentiation, apoptosis, cellular senescence, and the DNA damage response, it is easy to envision the misregulation of pRb by mdm2 as having dire consequences for tumorigenesis.

Similar to observations in mice, overexpression of mdm2 protein is seen in many types of cancer, fully one-third of sarcomas, and often correlates with poor prognosis and poor response to chemotherapy in a manner similar to that of *TP53* deletion.[8,9,17-19] However, because *Mdm2* is directly transactivated by p53 as part of the autoregulatory feedback loop that regulates p53 levels,[20,21] high levels of mdm2 expression can also indicate intact and active p53. This may partially explain the seemingly paradoxical observation that, depending on tissue tumor type, overexpression of mdm2 can also correlate with *good* prognosis.[8,9,22] Possibly underneath *both* correlations (good vs. bad prognosis) are the p53-independent functions of mdm2 and, possibly even more importantly, additional alternative or aberrant splice variants of *Mdm2* which may or not be detected by the immunohistochemical methods used in the various clinical correlation studies.[22]

Alternative and Aberrant Splicing of *Mdm2*

Those studying mdm2 noticed early on that it could take many splice forms, most notably in cancer-derived established cell lines.[11,23] Over 40 alternate or aberrant splice forms of mdm2 have since been

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reported.[22,24,25] Initially, alternative splice products of Mdm2 were thought to be unique to transformed cells, but they have since been reported in non-transformed cells as well.[26] Alternative splicing is the selective use of seemingly functional exon-intron arrangements into an "intended" gene product, while aberrant splicing refers to errors in splicing, most often due to mutation in donor/acceptor splice sites.[27] Almost always, aberrant splicing results in large truncations of the original gene product either due to the loss of exon(s) in the mature mRNA or due to the inevitable introduction of a nonsense codon as the proper reading frame is lost.[28] The products of aberrant splicing often retain some functional domains and the capacity to participate in protein-protein interactions with binding partners. The result of this is that the mutated gene may not simply serve as a null allele: it may act as a dominant negative, competing with the normal protein for binding to potential effectors, substrates or ligands, or even exhibit gain-of-function properties and novel interactions.[28] In any event, if the alternative splice product confers a growth advantage or disables proper proliferation control, it could potentially promote tumorigenesis.[29,30] Whether aberrant splicing is ever a first-hit initiating event in the pathogenesis cancer is currently an unresolved but important question. That aberrant splice products can exacerbate tumor progression appears fairly certain.[30]

Although it is not known how many of the splice forms of mdm2 are even translated into protein, it is important to keep in mind that full-length mdm2 is almost always retained in the tumors and cell lines that express one or more aberrant versions.[22,24,25,31] This indicates that there is negligible selective pressure for loss of heterozygosity (LOH). In the case of mdm2, one might contrive a simple hypothesis based on plain logic that an effective pro-oncogenic aberrant form of mdm2 would be one that is somehow more aggressive and/or resilient in binding to and destroying p53. However, paradoxically, it happens that the majority of aberrantly spliced mdm2 forms that have been reported to date lack all or most of the p53 binding site, as shown in Table 1 (reviewed in ref. 24).

The function of most of these splice variants remains a mystery, but one common theme in several of the forms that have been studied is that they are capable of binding to the wild-type allele of mdm2.[24,25] This binding can be competitive with p53 and, in the case of an untransformed, unstressed cell, function to keep p53 levels in check and allow the growth-promoting functions of mdm2 to proceed. If cells harboring these kinds of aberrant forms of mdm2 also experience loss or inactivating mutation of p53, the truncated mdm2 proteins could enhance the transformed phenotype if their binding to full-length mdm2 (in the absence of p53 as a competitor) either impairs the p53-independent growth inhibitory functions of mdm2, or enhances the growth promoting functions of mdm2. The only firm conclusion that can be drawn from the relatively few studies of these mdm2 splice variants is that their function and their effect strongly depends on, and varies with, genetic background and cellular context.[22,25] With such a myriad of functions related to cell proliferation, even a slight alteration in mdm2 structure can have a profound impact on cell fate. Nevertheless, transgenic expression of alternative splice forms of mdm2, even those that do not bind p53, has been unequivocally demonstrated to cause transformation of cells in vitro[11] and promotion of tumor growth in vivo.[14]

Just as we know very little about the *function* of aberrant splice forms of mdm2, the *origin* of alternative and aberrant mdm2 splice events has also remained a mystery. Although aberrant splice forms have been reported in untransformed cells, they are more likely to be found in tumor-derived cells and appear to be linked with cellular transformation. The mechanism of how exactly these alternative splice forms come about has yet to be addressed in detail, but sporadic mutation during tumorigenesis is the predominant theory regarding the origins of most mdm2 variants.

Table 1 **Table values indicate the percentage of known/sequenced splice variants of Mdm2 that retain the coding sequence for the indicated protein domains**

	p53-binding	NLS	NES	Acidic domain	lone Zn^{2+} finger	RING finger
> 75% deleted	18%	92%	92%	77%	72%	36%
25–75% deleted	56%	-	-	15%	-	13%
< 25% deleted	15%	-	-	3%	-	10%
intact	10%	8%	8%	5%	28%	41%

Adapted from Bartel et al.[24]

A New Twist—Acute Induction of an Aberrant *Mdm2* Splice Form

Recently, our group stumbled onto a totally unexpected discovery—a previously unreported splice form of mdm2 in mouse cells that, rather than *lacking* parts of the coding region, contains a 108bp insert into the mature *Mdm2* mRNA.[32] While inserting a short region from the middle of the large intron 10, the alternative splicing leaves intact the entire exons 10 and 11 (Fig. 1A). Thus, all the regular splice events occur normally, with the inclusion of additional intronic sequence (Fig. 1B). Because an insertion of 108 bp would maintain the reading frame of the mRNA, our initial speculations were that this sequence could be an alternative exon and that this event was not aberrant splicing, but alternative splicing. However, analysis of the sequence reveals stop codons in all frames, indicating that the translation product of this transcript would be severely truncated (Fig. 1C). Thus, this variant of mdm2 would lack the entire C-terminal region, including the lone zinc finger motif and the RING finger domain, which is known to harbor the E3 ubiquitin ligase activity. However, the regions thought to mediate binding to p53 and pRb would be retained.

An even more striking feature of this novel splice form, which we named $Mdm2^{+108}$, was that it can be acutely induced by two chemotherapeutic agents, Adriamycin (doxorubicin) and Actinomycin D.[32] The appearance of $Mdm2^{+108}$ can be detected within 45 minutes of treatment with either drug, is the dominant form of *Mdm2* within three hours, and by six hours, the normal transcript of *Mdm2* is barely detectable. This rapid alteration in the *Mdm2* mRNA coincides with the switch to the p53-inducble P2 promoter of *Mdm2*.[33,34] However, a myriad of other p53/mdm2-inducing treatments do not induce $Mdm2^{+108}$. Thus, this rapid effect seems to be extremely specific to certain pharmacological agents, not DNA damage in general.

This was the first report of any such acutely inducible change in *Mdm2* splicing and the mechanism leading to this event is a complete mystery.[32] However, the cellular effect of this splicing phenomenon appears relatively straightforward: the aberrant splicing of *Mdm2* to

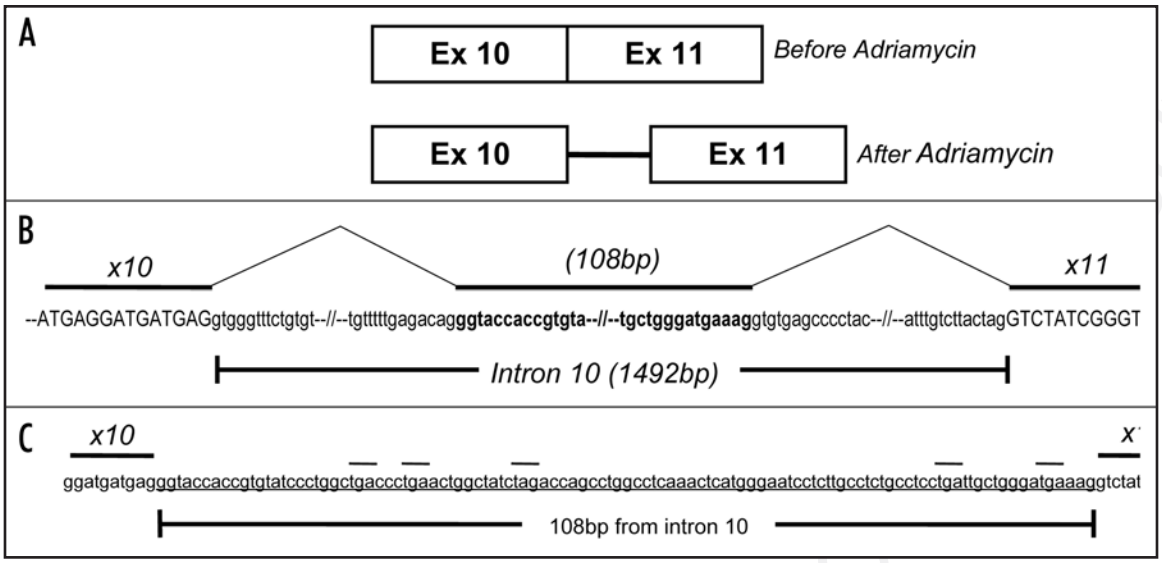

Figure 1. A detailed look at *Mdm2*$^{+108}$. (A) The 108 bp of intronic sequence found in *Mdm2*$^{+108}$ is flanked by wholly intact exons 10 and 11. (B) A closer look at the alternate splice events within intron 10 of the *mdm2* gene. Intronic sequence shown in lower-case letters; the sequence found in *Mdm2*$^{+108}$ shown in bold. Bars above the sequence indicate the apparent mRNA splicing events. (C) Complete sequence of the additional 108 bp found in *Mdm2*$^{+108}$. Bars above indicate putative nonsense codons. Figure adapted from Lents et al.[32]

the *Mdm2*$^{+108}$ form results in a loss of mdm2 function, rapid and robust accumulation of p53, and cell apoptosis or growth arrest.[32] *Mdm2*$^{+108}$, even if translated, would encode an mdm2 mutant that is incapable of ubiquitinating p53. However, following Adriamycin treatment, we were unable to detect this truncated mdm2 using antibodies to the N-terminus of the protein.[32] Instead, we observed only the marked disappearance of full-length mdm2. Released from negative regulation by mdm2, p53 rapidly accumulates in these cells, far above the increase seen following other forms of DNA damage, and the cells are dead within 24 hours.[32]

While the structure-function relationships of most other *Mdm2* splice forms described to date are perplexing and even mysterious, it seems comparatively simple that the physiological relevance of *Mdm2*$^{+108}$ is to add an extra layer of protection against tumorigenesis in cells that are treated with certain potent cellular toxins. By switching to what is essentially a null version of mdm2, those cells can enact an immediate and appropriate p53-mediated response and restrain the further proliferation of the damaged cell. Why cells respond in this fashion to Adriamycin and Actinomycin D, but not other damaging agents such as etoposide, UV irradiation and γ-irradiation, is not clear, but, as discussed in the original report, the common mechanism could lie in the intercalation of these drugs into single-stranded DNA.[32] Adriamycin is known to halt the action of topoisomerase I following breakage of one strand of DNA,[35] and Actinomycin D has been shown to interfere with DNA strand transfer during reverse transcription by HIV-1 reverse transcriptase.[36,37] Thus, it is possible that the trigger for *Mdm2*$^{+108}$ is not DNA damage itself, but the interaction of these drugs with single strands of DNA, such as the bubbles that appear during transcription and DNA replication.

It is likely that the kinetics of transcription and replication would be altered following intercalation of these agents into ssDNA.[38,39] The kinetic model of alternative splicing holds that the timing and kinetic properties of transcriptional elongation by RNA polymerase II influences the fine regulation of alternative splicing for some genes.[40,41] In light of this, a potential (but admittedly speculative) mechanism for the appearance of *Mdm2*$^{+108}$ would go something like this: when Adriamycin (or Actinomycin D) binds to the single-stranded DNA perpetually formed in transcription bubbles, the rate of transcriptional elongation by RNA polymerase is perturbed. Then, alternative splicing results in certain genes, including *Mdm2*, leading to the appearance of *Mdm2*$^{+108}$ and disappearance of full-length of *Mdm2* mRNA. In the context of intact *TP53*, this would initiate the rapid, robust and irreversible accumulation of p53 protein, resulting in apoptosis or cell cycle arrest. This would serve to restrain the growth and proliferation of cells that have suffered the extensive protein and DNA damage characteristic of these very potent and toxic chemotherapeutic agents.

In this way, the 108 bp region within intron 10 of *Mdm2* may indeed be an alternative exon, one whose function is to allow the rapid attenuation of mdm2 function. Thus, the appearance of *Mdm2*$^{+108}$ may be an *alternative* splicing event, rather than an aberrant one, adding yet another mechanism for p53-mediated tumor surveillance. This discovery may contribute a new layer to our understanding of the complex relationship between mdm2 and p53 and open the door for similar discoveries with other genes in other settings. If we've learned nothing else, let this be the lesson: when seemingly incomprehensible data presents itself, we mustn't toss it aside. Discoveries happen at those moments when we get answers that we don't expect.

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