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MDM2 and MDM4 splicing: an integral part of the cancer spliceome

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1. ABSTRACT

MDM2 and MDM4, the murine double minute proteins, are oncogenes that function as important regulators of various proteins. One fundamental role for these proteins is regulation of the tumor suppressor, p53. Precise regulation of p53 is vital for coordinated malignant suppression and cell survival. Alternative splice forms of MDM2 as well as MDM4 have been associated with various cancers. Indeed, UV irradiation triggers alternative splicing of both MDM2 and MDM4. Coordinated alternative splicing in response to cellular stress or in cancerous cells regulates the posttranscriptional expression of these two genes and likely others. This concert of stress responsive mRNAs comprises the cancer spliceome and provides a fingerprint of coordinated alternative splicing in these aberrant cells. Although various transcripts have been described for both proteins, here we provide a precise catalog of the alternatively spliced transcripts of both genes and the cancers with which they are associated.

2. INTRODUCTION

The MDM family of genes function to regulate expression of the p53 tumor suppressor gene. The *Mdm2* gene was originally isolated as an amplified gene in transformed murine 3T3 cell lines (1). In 1996, MDM4, also known as MDMX, was identified as a p53 binding protein that was related to MDM2 (2). Both proteins function to regulate p53 by direct binding and either blocking transactivation or subsequently targeting it for degradation (2-6). Stringent control of the p53 pathway is regulated by both MDM2 and MDM4. Due to its powerful growth suppressive activities, p53 is activated in response to damage. Frequently, human tumors are linked to the mis-regulation of this pathway.

MDM2 protein consists of a p53 binding domain, nuclear localization and export signals, an ARF binding domain and a RING domain (7-9). MDM2 binds p53, targeting it for degradation and thereby blocking its ability

to act as a transcriptional regulator (3, 10, 11). The role of MDM2 in the p53 pathway is underscored by the rescue of embryonic lethality in *Mdm2* knockout mice by generation in a p53 null background (12, 13). Similarly, *Mdm4* knockout mice, which are embryonically lethal, also develop normally with double knockout of p53 (14). Although both knockouts are lethal, they appear to work through different mechanisms. Whereas, *Mdm2* knockout embryos die due to massive apoptosis at the blastula stage; *Mdm4* embryos have a loss of cellular proliferation and die within day 7-11, suggesting divergent pathways in their regulation of p53.

The first alternatively spliced isoform of *MDM2* was described in 1996 (15). For both *MDM2* and *MDM4*, alternatively spliced transcripts have been documented in various tumors, as well as in response to certain cellular stress. *MDM2* alternatively spliced forms are expressed in many cancers including pediatric high grade gliomas, astrocytomas, rhabdomyosarcomas, liposarcomas and in adult lymphomas and cancers of the breast, ovary and lung (15-22). Although not as well studied, *MDM4* alternative splice forms have been associated with soft tissue sarcomas and papillary thyroid carcinomas (23-25). The function of these alternative forms have yet to be fully defined, yet it appears as though they may be part of the normal regulation of p53 in response to stress. Indeed, in response to UV stress and in some tumors, certain MDM2 forms can bind full length MDM2, re-localize it to the cytoplasm and therefore allow transcriptional regulation by p53 (21, 26-28). Here we catalog the known alternatively spliced forms of MDM2 and MDM4 with their cancer association.

3. ALTERNATIVE SPLICE FORMS OF MDM2

Variation in mRNA transcripts can occur by various mechanisms. Alternative promoters can generate transcripts of various sizes, as in the case of *MDM2* where two independent promoters, P1 and P2, generate transcripts lacking exon one or exon two, respectively (29). For other genes, the existence of alternate polyadenylation sites modifies the length of subsequent transcripts. However, for *MDM2* the greatest diversity in transcripts is generated through alternative splicing.

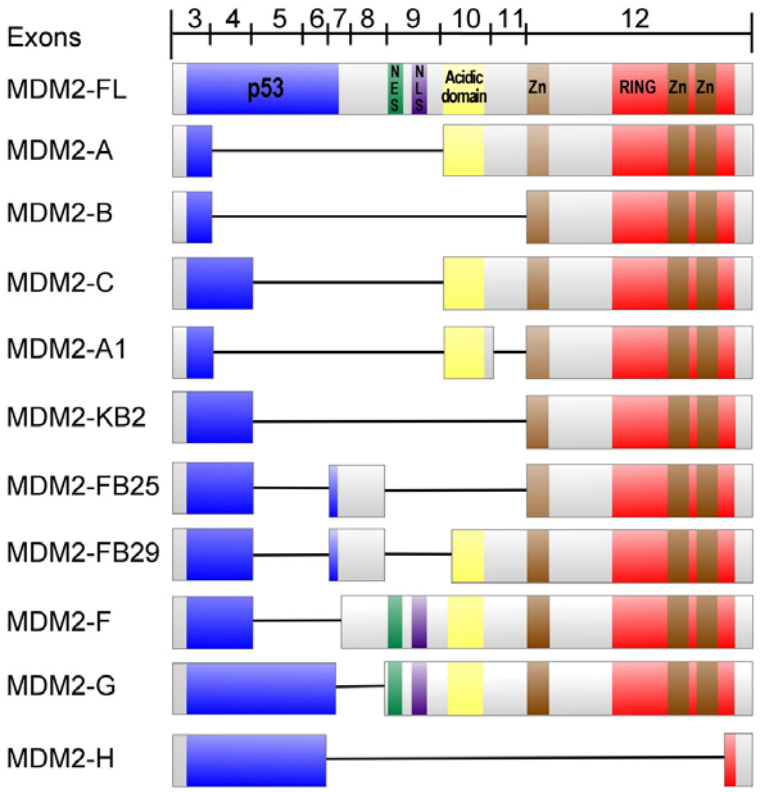
Alternative splicing allows for increased diversity from a single pre-mRNA transcript. Greater than sixty percent of genes are affected by alternative splicing which occurs when exclusion or inclusion of exons generate varying mRNA transcripts from a single gene (30). Alterations in alternative splicing due to mutations in cis-acting splicing elements, as well as changes in trans-acting regulatory proteins can result in the alteration of certain transcripts that ultimately affect tumor development and progression. Cis-acting mutations have been found in LKB1, KIT, and BRCA1 (31-33). Changes in trans-acting regulatory proteins have been associated with regulation of Ron, RAC1 and CD44 (34-36). Certainly these alterations in splicing can result in many variant mRNAs that are altered from normal expression and therefore comprise a cancer spliceome of alternatively spliced mRNAs in any given cancer.

In the early 1990s, reports arose of various transcripts generated from the *MDM2* gene. Not only was there characterization of multiple MDM2 protein isoforms (37), but also, sequencing from clones of both human (1) and murine (10) cDNA libraries revealed the presence of multiple transcripts. Further analysis confirmed the existence of these transcripts and identified the resulting proteins in NIH3T3 cells (38). With the determination of the organization and structure of the *MDM2* gene (39) the two previously described transcripts of *MDM2* were demonstrated to be products of alternatively spliced *MDM2* pre-mRNA. Following these studies, multiple murine *Mdm2* transcripts were described in Eu-Myc transgenic mice (40) as well as murine mammary tumor models (41). These mRNAs were determined to be lacking the p53 binding domain (27) or c-terminal ring domain, respectively.

MDM2 protein isoforms were described in human breast carcinomas as well as in human leukemia bone marrow samples (42, 43). It was in 1996 that Sigalas *et al.* described six distinct *MDM2* transcripts, from a nested PCR strategy on ovarian and bladder tumors as well as leukemia cell lines. These same alternative splice forms were also present in glioblastomas (16). These transcripts included the full-length transcript as well as alternative forms of which three represent alternatively spliced transcripts and two by unknown processes, based on sequence analysis of consensus splice sites (Figure 1). All of these three transcripts were found to lack portions of the p53-binding domain. In addition, these transcripts lack the nuclear localization signal, nuclear export signal and a portion of the acidic domain.

In soft tissue sarcomas not only were the previously described forms, *MDM2-A* and *MDM2-B*, observed but also another spliced form *MDM2-KB2* which lacks a portion of the p53 binding domain as well as the nuclear localization signal, nuclear export signal and acidic domain observed in the full length transcript (44). In both rhabdomyosarcoma (RMS) tumors and cell lines, six bonafide alternatively spliced transcripts were observed including *MDM2-A, MDM2-B, MDM2-C*. In addition, three other forms, *MDM2-A1, MDM2-fb25, and MDM2-fb29,* were observed which lack portions of the p53 binding domain, NLS and NES apparent in *MDM2-FL* (44). Further, study of the *MDM2-B* transcript identified its expression in response to genotoxic stress suggesting a physiological role for these transcripts (45). This study further determined that this is an evolutionarily conserved response in both mouse and human cell models that activates the p53 tumor suppressor pathway.

Three novel transcripts were identified in a panel of liposarcoma, *MDM2-F*, *MDM2-G* and *MDM2-H* (46). In contrast to the previously described variants, *MDM2-G* and *MDM2-H,* both contain intact p53 binding domains. Also *MDM2-F* and *MDM2-G* retain their NLS, NES and acidic domains. Indeed, these forms appear to retain the ability to bind p53, however may not be able to signal its degradation (Figure 2). In contrast, the isoforms lacking the p53-binding domain may function similar to MDM2-B,



Exon	Alternative names	Tumor Types
3-12	-	19 tumor types, see Momand et. al. (51)
3,10-12	ALT-2	Ova, STS, HL, Bl, GM, RMS, LS, LC, BC
3,12	ALT-1	Ova, STS, HL, Bl, GM, RMS, LS, LC, BC
3,4,10-12	ALT-3	Ova, STS, HL, Bl, GM, RMS, LS, LC, BC
3,10,12		Rhabdomyosarcomas (RMS)
3,4,12	ALT-4	Rhabdomyosarcomas (RMS)
3,7,8,10-12		Rhabdomyosarcomas (RMS)
3,4,7,8,12		Rhabdomyosarcomas (RMS)
3,4, part of 7-12		Liposarcomas (LS)
3-6,9-12		Liposarcomas (LS)
3,4, part 12		Liposarcomas (LS)

Figure 1. Summary of MDM2 mRNA splicing products, their alternative names, the exons they retain and tumor associations. The exons are depicted in a line graph above the above the protein isoforms, which are depicted as boxes (for the portions included) and as lines (for the portions removed by splicing). The various domains are shown by color: the p53 binding domain, blue; nuclear export signal (NES), green; nuclear localization signal (NLS), yellow; Zinc finger domains (Zn), tan; RING domain, red. Abbreviations: Ovarian (Ova), Soft Tissue Sarcoma (STS), Hodgkin's Lymphoma (HL), Bladder (Bl), Glioblastomas (GM), Rhabdomysosarcomas (RMS), liposarcomas (LS), lung carcinoma (LC), breast cancer (BC).

which can bind full length MDM2 and inhibit its ability to bind p53 and therefore limit its ability to target p53 for degradation (21).

Several other forms of *MDM2* transcripts have been described. However, these aberrant forms do not contain consensus splice sites and therefore do not appear to be the product of alternative splicing. Indeed, there is some question as to whether some of these forms may be due to splicing-like events or experimental artifact (Figure 3a). This phenomenon can occur during the reverse transcription reaction when template switching happens due to the presence of repetitive sequences. The switching events result in cDNAs with sequences deleted from the endogenous mRNA (47, 48). Repetitive sequences even as short as 8 base pairs when coupled with a less thermostable RT can produce such false transcripts. Indeed, when *MDM2* transcripts were observed in response to UV irradiation, the use of two different reverse transcriptase reagents generated different panels of transcripts (Figure 3b). Sequencing confirmed the presence of *MDM2-B*, but also identified artifactual transcripts. *MDM2* exon 4 and 5 sequences contain the repeat 5'-gaaagag-3'. This same sequence is also found in exon 12. Template switching from the exon 12 repeat to a more 5' copy of the sequence can produce a false transcript of 433 or 562 bp (Figure 3c). These products were observed both before and following genotoxic stress. Sequence analysis of these and other products with repetitive sequences suggests a role for template switching in the identification of a portion of *MDM2* aberrant transcripts.

4. ALTERNATIVE SPLICE FORMS OF MDM4

Although less studied than *MDM2*, there have already been five described alternative transcripts of *MDM4*. *MDM4-S* (Figure 4) was observed in several human and murine cell lines, particularly those that were proliferating or oncogenic (25). This transcript contains a deletion of exon 6 that causes the translation of a truncated protein. This "short" form of *MDM4* contains only the p53-binding domain and was determined to also alter p53 transcriptional activity. Recently, over-expression of this transcript was found in soft tissue sarcoma tumors (24). Thyroid tumor cell lines unveiled the existence of the *MDM4-221* isoform (49). This transcript lacks nine internal exons resulting also in a protein without the p53-binding domain. Not only does this isoform appear to stabilize inactive p53, but also inhibits MDM2 degradation of p53 while stabilizing MDM2 protein levels. Consistent

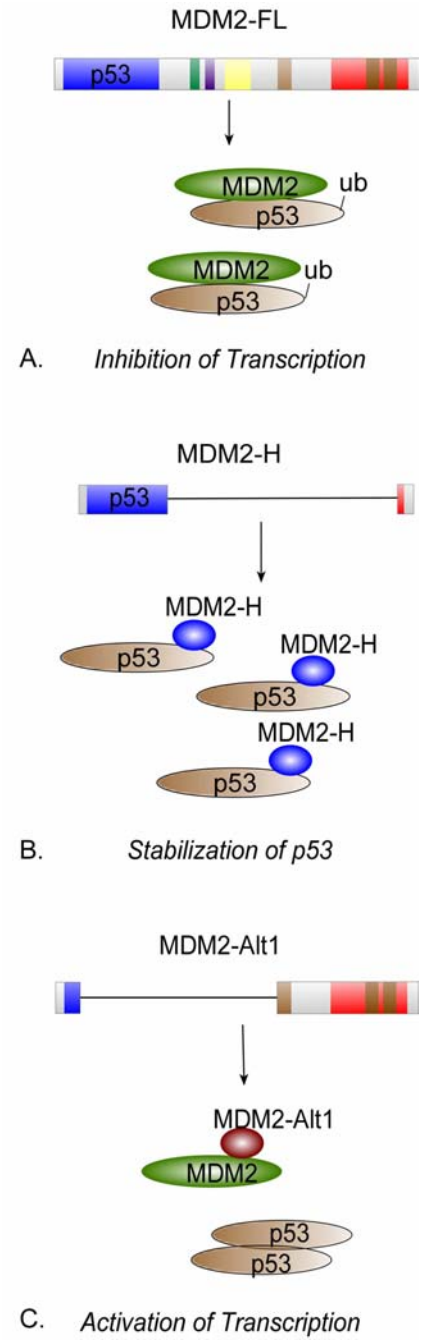


Figure 2. Models representing the interaction of MDM2 splice variants with p53. Under normal conditions MDM2-FL binds p53 and targeting it for degradation. Transcripts retaining the p53 binding domain can bind and stabilize p53. In contrast, for those lacking the p53 domain such as MDM2-Alt1, binding to full length MDM2 allows for the activation of p53 tumor suppressive activity.

with this role, this isoform was found in human lung tumors that express high levels of MDM2. More recently, both *MDM4-221* and *MDM4-S* were found expressed in papillary thyroid carcinomas while full length *MDM4* levels decrease (23).

Two other alternative splice variants, *MDM4-A* and *MDM4-G,* were isolated from C233A cells (50). *MDM4-A* lacks exon nine which results in a 50 amino acid deletion in the predicted protein therefore resulting in a loss of the acidic domain. In contrast, *MDM4-G* lacks exons 3-5 and a portion of 6, which contains a cryptic splice site. This deletion results in a loss of the p53-binding domain. Although these splice forms appear to alter p53 and stabilize MDM2, their specific physiological role is still unclear.

As reported in *MDM2* alternative splicing, two forms of *MDM4* are responsive to genotoxic stress. *MDM4-Alt1* and *MDM4-Alt2* appear in human cell lines in response to UV treatment (28). MDM4-Alt1 retains only the p53-binding domain similar to MDM4-S, which has been shown to bind p53 more strongly, and therefore maintains a p53 suppressive activity (Figure 5) (25, 50). In contrast, MDM4-Alt2 lacks the p53-binding domain similar to MDM2-B, which is also UV responsive. Potentially this isoform functions in an analogous manner to MDM4-221, which can stabilize p53 by inhibiting its degradation by MDM2. *MDM4* alternative splice forms appear to follow similar patterns as seen in *MDM2*. Interestingly, both respond to genotoxic stress with the expression of specific isoforms. Potentially these splice forms are generated through a general stress responsive splicing mechanism. Regardless, both *MDM2* and *MDM4* alternatively spliced isoforms appear to be tumor specific and potentially prognostic.

5. CANCER IMPLICATIONS OF ALTERNATIVE SPLICE FORMS

MDM2 has long been described as an oncogene. Over-expression of full-length MDM2 leads to transformation (1). Indeed, amplification of MDM2 has been identified in 19 different tumor types with the highest frequency observed in soft tissue tumors, osteosarcomas and esophageal carcinomas (51). Although overexpression of full-length MDM2 is well described as oncogenic, the emergence of the various alternative splice forms widens the scope of this gene's involvement in cancer.

All ten described alternative forms of *MDM2* have been observed in various tumor samples. *MDM2-A, -B, -C, -A1, -KB2, -FB25, and -FB29* have all been observed in RMS tumors and cell lines with high frequency (52). *MDM2-A, -B* and *C* have been observed in ovarian, as well as, bladder cancers (15). *MDM2-A, -B*, and *-C* have also been verified in Hodgkin's Lymphoma cell and primary tumors (53). *MDM2-B, -C, -F, -G* and *-H* were described in liposarcomas (46). Although there have been some reports that *MDM2* alternative splice forms correlate with poor prognosis (16, 44), other studies have found no correlation with these alternatively spliced transcripts to outcome (22). Further understanding of the mechanism by which these isoforms are regulated and how they regulate downstream events will certainly shed light on the role they play in tumorigenesis and what role, if any, they play in prognosis.

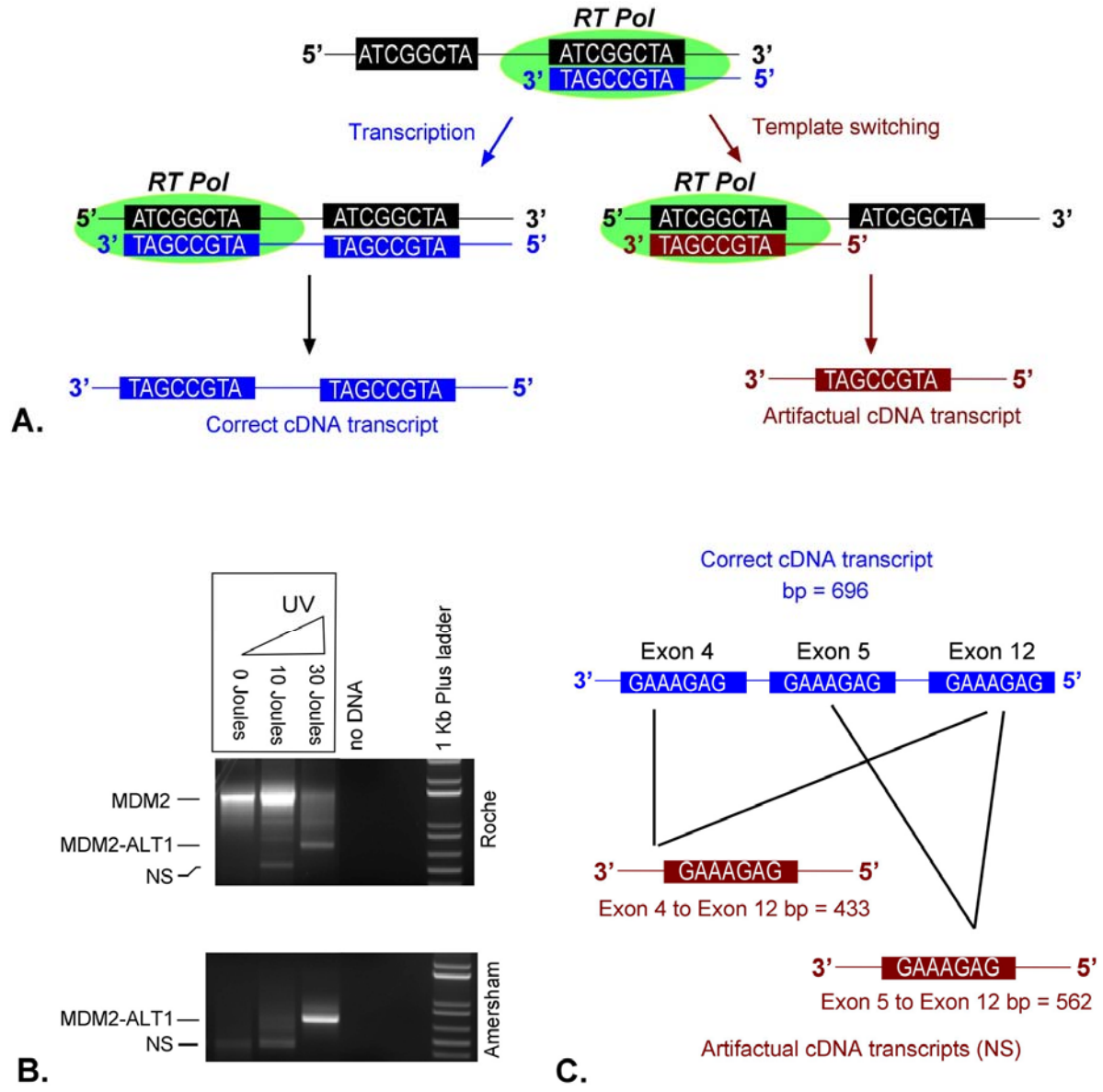
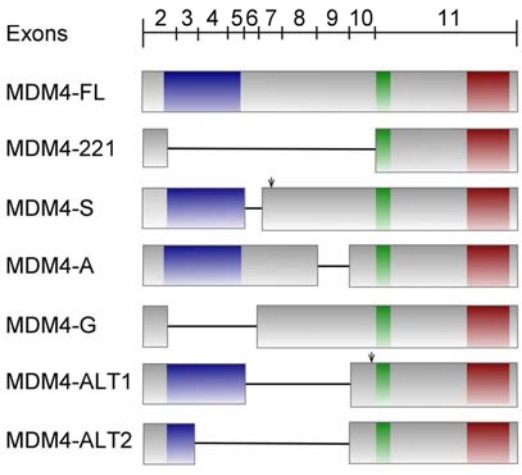


Figure 3. Artifactual reverse transcription products. A. Schematic of reverse transcription generating correct products and those generated by template switching. Duplication of the ATCGGCTA octamer results in skipping of internal sequences by reverse transcriptase and generation of a shorter aberrant transcript. B. Human breast carcinoma MCF-7 cells were subject to increasing amounts of UV irradation, 0, 10 and 30 J/m^2. RNA was harvested 24 hours after treatment using the RNAeasy kit, Quiagen (Valencia, CA) and used for subsequent nested RT-PCR reaction using RT polymerase from either Roche (Indianapolis, IN) (*top panel*) or Amersham (Piscataway, NJ) (*bottom panel*). Use of different enzymes resulted in varying panels of resulting transcripts. The full-length RNA is depicted as MDM2, the MDM2-B transcript is depicted as ALT1, and the nonspecific transcripts resulting from template switching are depicted as NS. C. Schematic of resulting transcripts, due to repeat sequences in Exon 5 and Exon 12. There are aberrant short transcripts of 562 bp and 433 bp. Amersham RT, the less thermostable enzyme, produces the aberrant transcript and is unable to reliably detect full-length RNA.

MDM4 alternative transcripts have been identified in various tumors as well. As with *MDM2*, there are reports of *MDM4* alternatively spliced transcripts in certain tumors. *MDM4-S* was described in soft tissue sarcomas and *MDM4-221* has been identified in lung carcinomas, both of these variants have been observed in papillary thyroid carcinomas. As with *MDM2* the role of alternative splice forms in prognosis is still under examination. However, *MDM4-S* expression in soft tissue sarcomas appears to be associated with poor prognosis. Further investigation as to the splice forms expressed in various tumor types will certainly unveil the role of this less studied family member in tumorigenesis.



Exon	Alternative names	Tumor Types
2-11	-	-
2,11	HDMX221	Non-Small Cell Lung Tumor
2-5, 7-11	HDMX-E HMDX-S	Soft Tissue Sarcomas (STS)
2-8, 10, 11	HDMX-A	-
2, 7-11	HDMX-G	-
2-5, 10, 11	HDMX-ALT1	-
2,3,10,11	HDMX-ALT2	Rhabdomyosarcomas (RMS)

Figure 4. Summary of MDM4 mRNA splicing products, their alternative names, the exons they retain and tumor associations. The exons are depicted in a line graph above the above the protein isoforms, which are depicted as boxes (for the portions included) and as lines (for the portions removed by splicing). The various domains are shown by color: the p53 binding domain, blue; Zinc finger domains (Zn), green; RING domain, red. Arrows represent stop codon and termination of translation.

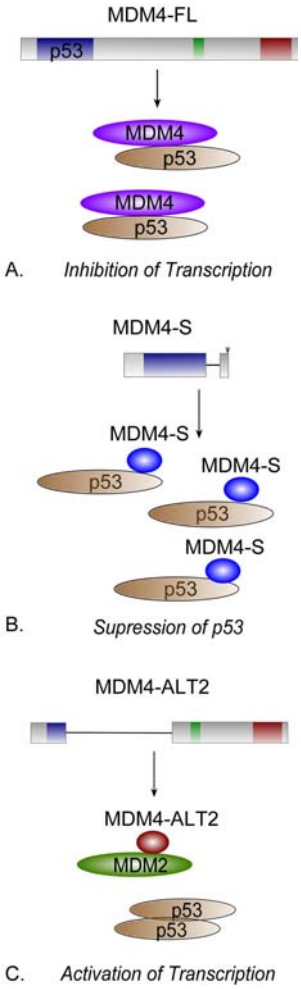


Figure 5. A representation of the MDM4 alternatively spliced transcripts interaction with p53. Full-length MDM4 binds to p53 to suppress its transctivation activity. Transcripts such as MDM4-S, which retain the p53 binding domain, have been shown to bind and suppress p53. For transcript that lack the p53 binding domain, interaction with MDM2 allow for the activation of p53 tumor suppressive activity.

6. SUMMARY AND PERSPECTIVES

Both MDM2 and MDM4 have been described as modulators of the tumor suppressor p53. The discovery of various splice forms of both these genes has complicated what appeared to be a very straightforward method of regulation. Whereas these alternatively spliced transcripts were originally discovered in various tumor samples, specific isoforms of both genes appear to be UV responsive and are alternatively spliced in response to damage. This provides the true biological role of these splice forms as protection from genotoxic stress. Initial studies suggest that the *MDM2* alternatively spliced forms regulate full length by binding through the ring domain and inhibiting its regulation of p53. However, the question still remains as to what is the mechanism by which this protective role may be modulated to a transforming phenotype and subsequently tumorigenesis. It is counterintuitive that if these alternative forms allow for p53 regulated apoptosis and cellular senescence how they lead to unregulated cell growth. Indeed, recent reports hint to the ability of MDM2 to inhibit Nbs1 and inhibit DNA break repair and therefore provides evidence that MDM2 may lead to transformation independent of p53 (54).

Whether by p53 dependant or independent pathways, the mechanism by which alternative splicing of *MDM2* and *MDM4* are regulated by the cellular response to DNA damage and the potential role the resulting transcripts have in tumorigenesis provide an interesting avenue for understanding cancer progression. Further, the role alternative splicing plays not only in the cellular response to DNA damage, but also in determining the cancer spliceome requires further elucidation. Determining the global mechanism by which alternative splicing alters the mRNA concert within tumorigenic cells should not only open the door to novel regulatory mechanisms for the cell, but may provide targets for novel therapeutic interventions.

7. ACKNOWLEDGEMENTS

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