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Review Article

MDM4 alternative splicing and implication in MDM4 targeted cancer therapies

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Abstract: The oncogenic MDM4, initially named MDMX, has been identified as a p53-interacting protein and a key upstream negative regulator of the tumor suppressor p53. Accumulating evidence indicates that MDM4 plays critical roles in the initiation and progression of multiple human cancers. MDM4 is frequently amplified and upregulated in human cancers, contributing to overgrowth and apoptosis inhibition by blocking the expression of downstream target genes of p53 pathway. Disruptors for MDM4-p53 interaction have been shown to restore the anti-tumor activity of p53 in cancer cells. MDM4 possesses multiple splicing isoforms whose expressions are driven by the presence of oncogenes in cancer cells. Some of the MDM4 splicing isoforms lack p53 binding domain and may exhibit p53-independent oncogenic functions. These features render MDM4 to be an attractive therapeutic target for cancer therapy. In the present review, we primarily focus on the detailed molecular structure of MDM4 splicing isoforms, candidate regulators for initiating MDM4 splicing, deregulation of MDM4 isoforms in cancer and potential therapy strategies by targeting splicing isoforms of MDM4.

Keywords: MDM4, p53, splicing, cancer therapy

Introduction

The murine MDM4 protein, also known as MDMX, was first discovered through a screen for p53 binding proteins in 1996 [1]. MDM4 shows high similarity with the MDM2 protein, a well-known E3 ubiquitin-protein ligase [2] that promotes proteasomal degradation of p53 [1]. Studies now established that MDM4 and MDM2 both function as important negative regulators of p53 and both are attractive therapeutic targets to reactivate the tumor suppression function of p53 [3, 4]. However, the importance of MDM4 in p53 regulation was not clear for its predicted functional redundancy with MDM2 until revelation in MDM4 knockout mouse studies. *MDM4*$^{-/-}$ homozygous knockout mice died in utero, while concomitant deletion of the *Trp53* gene could avoid the death [5-7], indicating a non-redundant role of MDM4 in regulation of p53. Furthermore, MDM4 was identified to be an MDM2-interacting protein in an unbiased screen [8], which made MDM4 as a potential regulator for MDM2 function in cell. *MDM4* gene amplification or overexpression of MDM4 proteins were observed in many cancer types [4] and targeting MDM4 has been shown to be a valid strategy for p53-based cancer therapy [9, 10]. Enthusiasm for developing MDM4-targeted cancer therapies is on the rise as a complimentary strategy for MDM2-p53 interaction inhibitors since MDM4 overexpression and p53 mutation poses a challenge to MDM2 inhibitor resistance [11, 12]. Importantly, both MDM2 and MDM4 undergo alternative splicing and their splicing isoroms are cancer relevant. While MDM2 splicing has been studied actively and reviewed extensively, MDM4 splicing has drawn attention in recent years. The current review will focus on discussions on the detailed molecular structure of MDM4 splicing isoforms, candidate regulators for initiating MDM4 splicing, deregulation of MDM4 isoforms in cancer, update MDM4-trageting small molecules and implication in MDM4-targeted cancer therapies.

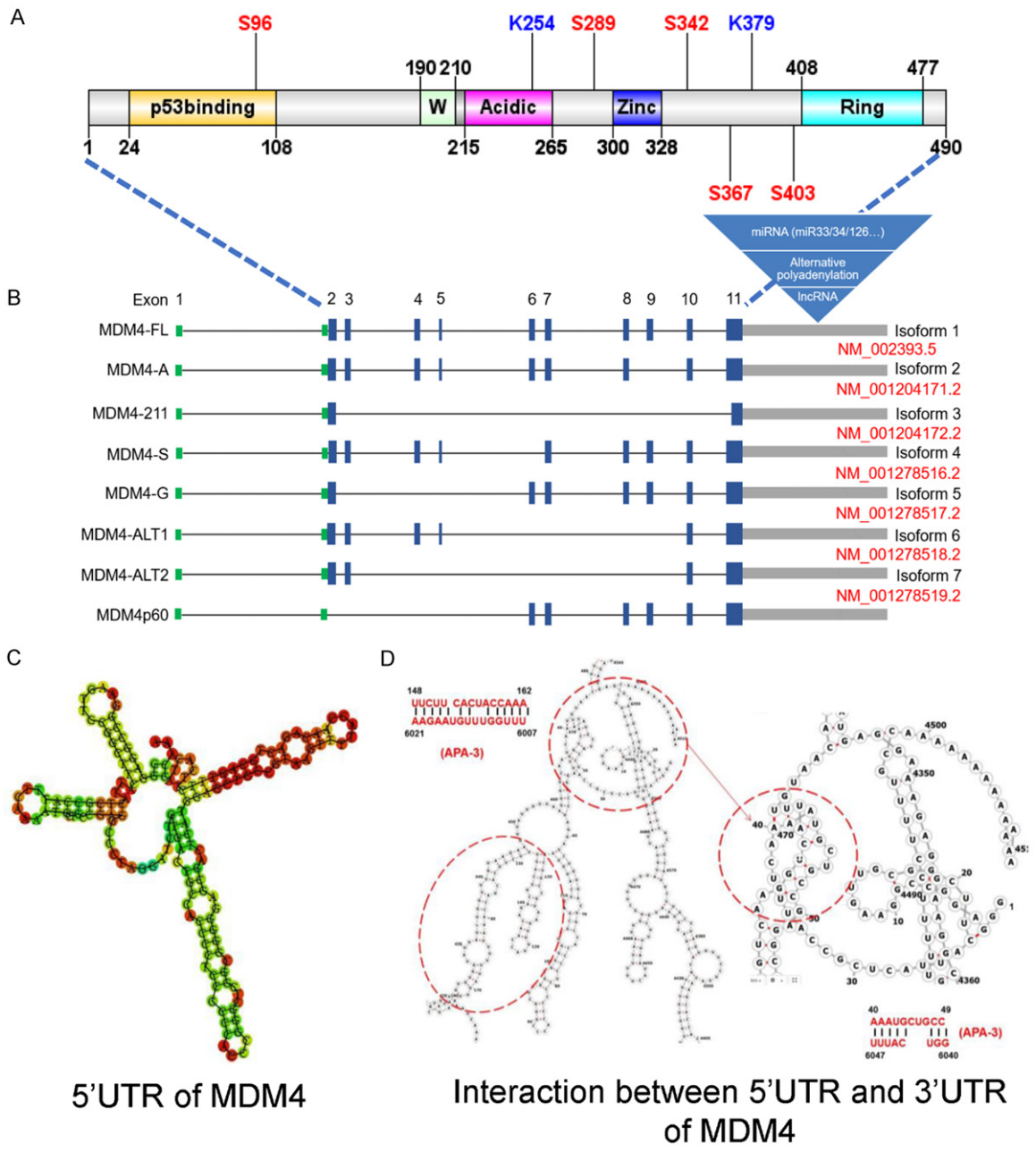

Figure 1. Molecular structures of MDM4 gene and protein. A. A diagram of MDM4 includes known domains and the variants present in this manuscript. B. The 5'UTRs are shown as green bars and the 3'UTRs are shown as grey bars. Separated by introns shown by black lines, exons are indicated by blue solid rectangles. Seven transcripts were listed in NCBI database with indicated accession numbers and one currently identified variant has no accession number. Multiple miRNAs, APA, UPF1 and STAU1 binding motifs were found in 3'UTR region of MDM4 mRNA. C. RNA secondary structure prediction for 5'UTR of MDM4 using online RNA fold program. D. Detailed information about 5' and 3'UTR interacting regions predicted in MDM4 mRNA.

Structure and functions of MDM4

The human MDM4 gene has been mapped to chromosomal locus 1q32 [13]. Currently, about eight isoforms of MDM4 were identified (**Figure 1**). The longest full-length isoform (MDM4 isoform 1, MDM4-FL, NM_002393) contains 11 exons, and encodes a 490-amino acid protein with a predicted molecular weight at 54 kD. The structure for the mRNA and protein of MDM4

are depicted in **Figure 1**. In the following part, we mainly focused on four major conserved domains based on the protein structure: the amino-terminal p53-binding domain, the carboxy-terminal RING domain, the zinc-finger domain and the acidic domain (AD).

The amino-terminal p53-binding domain of MDM4

MDM4 protein includes a p53 binding domain located at N-terminus [14], of which the residues associated with p53 binding are strongly conserved in both MDM4 and MDM2 with 62% similarity [15]. Mutation of either residue of Phe19, Trp23 and Leu26 of p53 will abolish the binding between MDM4 and p53 [15], indicating high similarity of binding pockets of MDM4 and MDM2 for the association with p53 [16]. Nevertheless, a crystal structure analysis revealed the differential binding to p53 between MDM4 and MDM2 [17]. MDM4 has a smaller hydrophobic cleft for binding to the transactivation domain of p53 compared to MDM2, which will impact the binding affinity to the p53-TAD [18]. In addition to the binding pocket difference, full-length MDM4, but not MDM2, comprised a regulatory "WWW element", which could bind to its N-terminal domain and prevent MDM4 from binding to p53 [19]. Some proteins competitively binding to this region may sequester the WWW sequence (aa 190-210, FEEWDVAGLPWWFLGNLRSNY) and unleash the p53-binding power of MDM4. This regulatory mechanism dictates that MDM4 splicing variants lacking the WWW sequence have deregulated high affinity to p53, which explains why they are found to be oncogenic in certain cancer cells [19-21].

The MDM4 RING domain

The second highly conserved motif of MDM4 and MDM2 is RING domain which is located at the carboxy-terminal region of both proteins. The MDM2 RING domain mainly contributes to its ubiquitin ligase activity [22, 23]. However, MDM4 RING domain appears to lack this activity [24]. MDM2 can form homo oligomers via its RING domain in a manner dependent on its extreme C-terminal residues [25, 26] and form heterodimers with MDM4 via RING-RING interaction [8]. In *in vitro* studies, MDM4 RING domain not only stimulates the E3 ligase of MDM2 [27], but in fact, is essential for MDM2-mediated p53 polyubiquitination and degradation [28, 29]. Mouse genetic studies with MDM4 RING domain deletion or structural point mutation established that MDM4 RING domain is essential for p53 regulation *in vivo* during development [30, 31]. Since MDM2 RING domain mutant mice also suffer from p53-dependent lethality, these studies established that RING-RING interface is key for MDM2 and MDM4 to partner to regulate p53 [32]. Interestingly, MDM4 RING domain can compensate the E3 ligase defects of MDM2 CT mutants for ubiquitination [33] and neddylation [34]. MDM4 RING domain contains a potential nuclear localization signal (NLS) (aa 465-469, RRLKK) which is usually hidden by the intramolecular interaction between RING and AD. DNA damage signaling can uncouple this RING-AD interaction by 14-3-3 with phosphor-MDM4 leading to nuclear localization and degradation of MDM4 in MDM3-dependent manner [35-37].

Zinc-finger domain and acidic domain

The function of the zinc-finger domain of MDM4 is largely unknown. Based on limited research, both MDM2 and MDM4 could interact with retinoblastoma protein and regulate its expression, however, the important roles of the zinc-finger domain for mediating these effects were only elucidated for MDM2 [38-40]. The cleavage of MDM4 at a canonical caspase cleavage site, close to the Zinc finger motif, has been proved to affect protein stability [41]. The AD domains mediated differential functions of MDM2 and MDM4. In MDM4, this domain is involved in the intramolecular interactions with the p53-binding domain and the RING domain, while the AD domain of MDM2, whose function couldn't be fulfilled by the analogous domain of MDM4, is essential for p53 ubiquitination [42, 43]. The AD in MDM2 is also involved in the interaction with ribosomal proteins and with p14ARF, but none of which were reported to interact with MDM4 so far. However, these proteins can affect the stability of MDM4 through their interaction with MDM2 [44-46].

Alternative splicing of MDM4

Alternative splicing is one of the fundamental mechanisms to regulate gene expression and

plays important roles in cell biology including cancer cell biology. Through exon retention or skipping, alternative splicing contributes to protein diversity which directly dictates cellular states [47]. Alternative splicing functions as a powerful mechanism for dynamic regulation of protein stoichiometry involved in many pathways by generating spliced transcripts coupled with NMD degradation in the nucleus [48], unstable protein products [49], or stable truncated proteins lacking certain protein-interaction domains. In humans, approximately 95% of multi-exon genes have alternative splicing, and therefore huge effort is needed to garner a full picture of alternative splicing products as well as understanding the functions of different splicing isoforms of a single gene under normal physiological or pathological conditions [50].

Here, based on literature search and sequence analysis for MDM4 transcripts in UCSC Genome Browser and NCBI Nucleotide database, 8 different splicing isoforms of MDM4 were described, compared with the predominant MDM4 isoform 1 (NM_002393) (**Figure 1**).

MDM4 isoform 1 (MDM4-full length, MDM4-FL)

As mentioned above, this transcript variant represents the predominant transcript, and encodes the longest isoform (1) containing all the domains described for MDM4 in the literature and contributing to the most important function of MDM4 in p53 regulation together with MDM4-S.

MDM4 isoform 2 (MDM4-A)

MDM4-A was detected in the cervical cancer cell line C33A cells [51]. MDM4-A was generated by the removal of the exon 9 and annotated as MDM4 isoform 2 (NM_001204171) by National Center for Biotechnology Information (NCBI). This variant lacks an in-frame coding exon 9, resulting in a shorter protein missing most of the acidic region. Also, this alternative deletion of exon9 correlates with the MDM2 degradative activity and potentially controls the stability of MDM4-FL. In the meanwhile, the deletion of acidic domain of MDM4 could release the acidic domain of MDM2 and further promote its degradative function especially toward p53 [51]. From this aspect, MDM4-A possibly performs oncogenic roles through regulating the activity of MDM2 to accelerate the degradation of P53. However, no protein evidence of MDM4-A was provided so far.

MDM4 isoform 3 (MDM4-211)

This isoform was previously identified in the thyroid tumor cell line, ARO [52]. It derives from an aberrant splicing event between the canonical donor site in exon 2 and a cryptic acceptor site in exon 11 of the MDM4-FL mRNA (therefore named as MDM4-211), thus lacking eight consecutive exons from 3 to 10 and part of the last exon 11 (**Figure 1**). The accession number of this isoform in NCBI GenBank was NM_001204172. The open reading frame of this isoform encodes a protein lacking the p53-binding domain, while including the RING-finger domain at the C-terminus. MDM4-211 could only bind and stabilize MDM2 by increasing its half-life [52]. Moreover, the expression of MDM4-211 was limited to cancer types, such as non-small-cell lung cancer [52] and papillary thyroid carcinomas [53], but seldomly observed in normal tissues, indicating that this transcript was generated from an aberrant splicing event occurring only in tumor cells. Even though sharing the similar domains as MDM4-S, the MDM4-211 expression has no relevant to the levels of MDM4-FL transcript suggesting the independency of this splicing event.

MDM4 isoform 4 (MDM4-S)

This isoform is generated by the removal of the internal exon 6 through alternative splicing, which was first reported in rapid growing and transformed cell lines. It was known as MDM4-S, HDMX-S, or HDMX-E previously and annotated as MDM4 isoform 4 (NM_001278516). Since the length of exon 6 is 68 base pairs, this removal produces a shift in the open reading frame (ORF) after codon 114, resulting in the addition of a premature stop codon at amino acid residue 127 [54]. MDM4-S only comprises the p53 binding domain and was proved to be a stronger p53 inhibitor than MDM4-FL [54, 55]. Compared with MDM4-FL, MDM4-S misses an auto-inhibitory sequence and shows more efficient nuclear localization [19]. However, the MDM4-S protein is barely detectable, much lower that MDM4-FL protein, even in a context that the mRNA levels for both isoforms are compatible, indicating potential post-transcriptional mechanisms that negative-

ly regulate MDM4-S translation and/or stability. Besides, accumulating evidences support that the effect of exon 6 skipping is to negatively regulate the expression of MDM4-FL [53, 56, 57].

MDM4 isoform 5 (MDM4-G)

This isoform was detected at the same time as isoform 2 (MDM4-A), and previously known as MDM4-G. It lacks three consecutive exons (3-5) and part of the exon 6. This isoform was annotated as Mdm4 isoform 5 (NM_001278517). MDM4-G contains the same N- and C-termini domains as those of the MDM4-FL, while lacking the integrate p53-binding domain due to the in-frame deletion of aa 27-124 (**Figure 1**). Interestingly, although this variant can not bind to p53, it can still possess a weak inhibitory activity toward p53. This inhibition has been attributed to the stabilization of MDM2 levels and consequently of its oncogenic properties.

MDM4 isoform 6 (MDM4-XALT1)

In a study on MDM4 alternative splicing in responsive to genotoxic stress, two novel alternative transcripts of MDM4, called XALT1 and XALT2 were identified in several human cancer cell lines [58]. XALT1 derives from a splicing event lacking four consecutive exons (6-9) of MDM4-FL mRNA, resulting in a premature stop codon at the exon 10. Currently, XALT1 has been renamed as isoform 6 of MDM4 (NM_001278518). The predicted protein of XALT1 only comprises the p53 binding domain with 24 amino acids longer than the protein encoded by MDM4-S. XALT1 was speculated to suppress p53 transcriptional activity and inhibit the anti-apoptotic function of p53 [59]. The expression of this variant in non-tumor cells might perform different functions [58]. Overall, this isoform is potentially generated through a general splicing mechanism in response to genotoxic stress.

MDM4 isoform 7 (MDM4-ALT2)

XALT2 was identified in the same project as XALT1 [58] and annotated as MDM4 isoform 7 (NM_001278519). This isoform derives from a splicing between exon 3 and exon 10 and lacks six consecutive exons (4-9) as compared with the MDM4-FL (**Figure 1**). The predicted protein of XALT2 transcript lacks the p53 binding domain and retains the COOH-terminal RING finger domain. Thus, this variant may bind MDM4-FL and/or MDM2 splicing isoforms and regulate their function. This isoform has been detected in different tumor cell lines. Its presence was supposed to be another layer of regulation of the p53-MDM2-MDM4 network in response to DNA damage.

MDM4-p60

The MDM4p60 isoform was first reported in 2017, which is generated by alternative splicing between exon 2 and exon 6 to remove most part of the exon 2 and three consecutive exons (3-5). The transcript uses an alternative translation initiation codon to generate a protein lacking the N-terminal p53-binding domain [60]. Previous reports demonstrated that MDM4 could both stabilize and destabilize MDM2 [27, 61, 62]. Besides, with a similarity to the structure of MDM2p76, the MDM4p60 transcript also lacks the N-terminal p53 binding domain and displays a similar function as MDM2p76. MDM4p60 does not have a direct negative effect on p53. On the contrary, it can increase p53 protein levels through blocking the ability of MDM2p90 in mediating p53 degradation. Further investigation will tell whether hMDM4p60 can also play a positive role in the activation of p53 or not.

Candidate regulators for initiating MDM4 splicing

Although eight alternative-spliced isoforms of MDM4 were identified, current studies are mainly focused on two major forms: MDM4-FL (Isoform 1) and MDM4-S (Isoform 4). However, the splicing factors involved in the initiation of MDM4 splicing are still unknown. From a survey of published papers, we summarized candidate regulators involving in the splicing switch from MDM4-FL to MDM4-S isoforms as mechanisms for regulation of MDM4 function (**Table 1**).

It has been reported that the splicing enhancer serine and arginine rich splicing factor 3 (SRSF3) is a necessary, but not sufficient factor for mediating the inclusion of exon 6 in MDM4 mRNA, whereas SRSF7, SRSF9 and SRSF11 facilitate exclusion of exon 6 using MDM4-FL/MDM4-S ratio change as readout [63]. Of note, one study reported that knockdown of the splic-

Table 1. Candidate regulators for initiating MDM4 splicing

Gene	Function	Cell type	Reference
SRSF3	necessary, but not sufficient, to promote inclusion of exon 6 in MDM4 mRNA	Melanoma; neural stem/progenitors; Colon Cancer; cutaneous squamous cell carcinoma	[63-65]
PRMT5	Decreases alternative splicing of MDM4 and activates p53 by inhibiting PRMT5	Melanoma; neural stem/progenitors	[56, 66-68]
PRMT1	Decreases alternative splicing of MDM4 and activates p53 by deletion of PRMT1	Epicardial cells	[69]
RBM11	exogenous RBM11 switches splicing of MDM4 and Cyclin D1 toward the expression of more oncogenic isoforms	Glioblastoma	[70]
Zmat3	Increasing alternative splicing efficiency of MDM4 and inactivate p53 by deletion of Zmat3	lung adenocarcinoma and Liver cancer	[71]
BCAS2	Decreases alternative splicing of MDM4 and activates p53 by deletion of BCAS2	Hematopoietic stem and progenitor cells	[72]

ing factor 3b subunit 1 (SF3B1) induced a dramatically changes for MDM2 splicing patterns, but not for MDM4 [64], whereas another study showed that SF3B1 inhibition, either by RNAi or the SF3B1 inhibitor pladienolide B, promotes the altered mRNA splicing and reduced protein expression of both MDM4 and MDM2 leading to p53 increase in cutaneous squamous cell carcinoma [65]. This discrepancy is probably due to low level of normally spliced MDM4 mRNA in the cells used in this study [64].

In addition to SRSF3, another candidate splicing regulator and transcription cofactor protein arginine methyltransferase 5 (PRMT5) triggers alternative splicing of MDM4 in neural stem/progenitor cells (NPCs) [56] and multiple other cancer types [66]. Depletion of PRMT5 by siRNA or PRMT5 inhibitor GSK3203591 promotes a splicing switch from long isoform to short MDM4 leading to shutdown of the expression of MDM4-FL protein and p53 activation. Besides, as a protein arginine methyltransferase and indirect target of CDK4, PRMT5 also plays important roles in response to the treatment of CDK4/6 inhibitors. Combined treatment of CDK4/6 inhibitor palbociclib and the PRMT5 inhibitor GSK3326595 enhances the efficacy of palbociclib and delays the resistance incidence in melanoma [67, 68]. These studies uncovered a link between CDK4/6 activity and MDM4 expression via PRMT5 and further extended the mechanism of action of CDK4/6 inhibitors far beyond regulation of the cell cycle. Further investigations need to be performed to check the combined treatment of both CDK4/6 and the PRMT5 inhibitors in other cancer cell models such as breast, pancreatic, and esophageal carcinoma.

Arginine methyltransferase member PRMT1 is another regulator of MDM4 splicing for p53 activation during epicardial EMT and invasion [69]. Currently, there are no reports on PRMT1-dependent splicing regulation of MDM4 in cancer cell models, one can speculate that PRMT1 might play similar roles as PRMT5 in regulation of MDM4 splicing in certain types of cancer. RNA-binding proteins can be important regulators of MDM4 splicing machineries. One study has shown that RNA binding motif protein 11 (RBM11) can mediate switches of alternative splicing of MDM4 and Cyclin D1 to express more oncogenic isoforms [70]. Interestingly, another RNA-binding protein (ZMAT3, zinc finger matrin-type 3) was identified to be a key splicing regulator in the p53 tumor suppression program to maintain p53 tumor suppression functions. ZMAT3 is a p53 downstream gene product and can directly modulate exon inclusion of transcripts of diverse pathways including both p53 inhibitors MDM4 and MDM2. SgRNA-guided knockout of ZMAT3 leads to production of full-length MDM2 and MDM4 leading to p53 degradation and suppression. Importantly, ZMAT3 is a key mediator of p53 tumor suppressor function in mouse Kras (G12D)-driven lung, liver cancers and human carcinoma [71]. Finally, breast carcinoma amplified sequence 2 (BCAS2) was first identi-

fied as negative regulator in human cancer cells. Knockout of BCAS2 in zebrafish induces exon-6 skipping with increased production of MDM4-S leading to actvation and p53-mediated apoptosis in hematopoietic stem and progenitor cells [72]. These studies suggest that different MDM4 splicing regulators regulate MDM4 splicing in a tissue-specific manner.

Alternative splicing is a complex process and can be regulated by trans-acting proteins (repressors and activators) and corresponding cis-acting regulatory sites (silencers and enhancers) on the pre-mRNA [73]. Moreover, the secondary structure of the pre-mRNA transcript also plays a role in regulating splicing, such as by bringing together splicing elements or by masking a sequence that would otherwise serve as a binding element for a splicing factor [74, 75]. The super-length 3'UTR (8.4 kb) of MDM4 provides plenty of potential binding sites for splicing factors or splicing enhancers, in addition, it can also form stable secondary structure to facilitate the process of splicing. Although several regulatory molecules in MDM4 splicing have been identified (**Table 1**), more factors are to be uncovered for a better understanding of the underlying splicing mechanisms in the context of disease and cancer types.

Deregulation of MDM4 isoforms in cancer

As a well-known upstream regulator of p53, the functions of MDM4-FL have been intensively investigated. The first report about the aberrant MDM4 expression in malignant gliomas can be tracked back to 1999 that MDM4 gene amplification correlated with high MDM4 expression in 4% of cancer tissues with wild-type p53 [76]. Later, the gene amplification phenomenon of MDM4 was also discovered in other cancer types including breast cancer [77], soft-tissue sarcoma [57], retinoblastomas [78], and cutaneous melanoma [10]. However, no correlation between mRNA expression and protein levels of MDM4 was observed in these cancer samples [79], suggesting that post-transcriptional or post-translational mechanisms might also contribute to the regulation of MDM4 protein levels in tumors.

Although some reports claimed that MDM4-S can be expressed in both normal and tumor cells with higher affinity of p53-binding than full-length MDM4 and the engineered MDM4-S protein primarily showed the nucleus localization [54, 55], there is no conclusive evidence supporting the detection of endogenous MDM4-S protein levels in any normal or cancer cell lines. It was suggested that endogenous MDM4-S might be a very unstable and undetectable protein by regular western blotting, and that the switch from MDM4-FL to MDM4-S is one mechanism that cells undergoing stress use to shut down the expression of MDM4-FL protein, and thereby activate p53 [80]. Alternatively, exon 6 might function as an "nonsense-mediated decay (NMD) switch" exon [56, 81] and could be recognized by the NMD machinery for degradation of MDM4-S transcripts [82]. To sort out these two possibilities warrants further experimentation.

High expression of MDM4-S has been associated with poor prognosis in multiple cancer types including osteosarcoma, soft tissue sarcoma, breast cancer, glioblastoma, melanoma, and chronic lymphocytic leukemia [63, 80, 83-85]. The question is whether MDM4-S is tumorigenic on its own, given its potential in p53 inhibition [19]. To answer this question, Lozano group using mouse models and concluded that MDM4-S overexpression is a consequence of splicing defects in tumor cells rather than a cause of tumor evolution since splicing of MDM4 does not promote tumor development or cooperate with other oncogenic insults to alter tumor latency or aggressiveness, yet it can be used as a possible biomarker [86]. Practically, the MDM4-FL/MDM4-S ratio correlates well with overall levels of full-length MDM4 protein and can be used as a reliable predictor of MDM4 protein abundance in panels of several tumor cell lines, including breast cancer, osteosarcomas, and uveal melanoma [4, 63].

In addition to splicing regulators for regulation of MDM4 expression in trans, whether the untranslated regions of MDM4 mRNA also contributes to its alternative splicing remains unexplored. It is established that the untranslated regions (5'-UTR and a 3'-UTR) in mRNA can play fundamental roles in regulating the stability, function, and localization of mRNA and shaping the multiple diversity of cellular proteome [87]. This layer of regulation also applies to MDM4 since the MDM4's 5'-UTR can inhibit ovalbumin

synthesis when it is connected to the coding sequence of ovalbumin gene [88]. In addition, 5'-UTR of MDM4 has the potential to form a stable secondary structure (**Figure 1C**). Furthermore, miR-885-3p was reported to bind the 5'-UTR of MDM4 mRNA to elevate the MDM4 protein level, instead of inhibiting MDM4 expression by other miRNAs binding to 3'-UTR [89]. Besides, the MDM4 mRNA has an exceptionally long 3'-UTR of about 8.5 kb [90], which provides an opportunity for MDM4 mRNA to harbor many candidate miRNA targets within its 3'-UTR. Around 1,276 potential miRNA binding targets of MDM4 have been reported. However, only a small portion of these candidates can exert functional roles in the regulation of MDM4 translation based on cellular experiments. Up to date, around 24 miRNAs have been validated to regulate the expression of MDM4 in multiple cancer types and most of these miRNAs bind to the 3'-UTR of MDM4 and down-regulate the expression of MDM4 (**Table 2**).

In addition to providing binding sites for miRNAs, the long 3'UTR of MDM4 also contains several alternative polyadenylation (APA) sites with conserved motif sequences (AATAAA). These APA sites do not alter the protein coding frame, while they might affect mRNA stability and translation efficiency [91-94]. Interestingly, the binding motifs of UPF1 and STAU1 can be found in 3'UTR of MDM4 [95]. UPF1 plays essential roles in both Staufen 1 (STAU1)-mediated mRNA decay (SMD) and nonsense-mediated mRNA decay (NMD) [96]. Stau1 functions as a splicing regulator and can regulate the alternative splicing of exon 11 of the human insulin receptor gene (INSR) through binding to Alu elements located in intron 10 [97]. Therefore, it is possible that STAU1 can be a regulator of MDM4/MDM4-S ratio by specifically regulating stability of either isoforms. Another possible mechanism involves the effect of the secondary structure of UTR sequences on mRNA translation. One such example is from p53 studies. The base-pairing interactions between 5- and 3-UTR of p53 positively regulate the translational efficiency of p53 mRNA in a RPL26-dependent manner [98]. Whether MDM4 mRNA uses the similar mechanism is an open question. We tried an online tool named RNA fold (http://www.tbi.univie.ac.at/RNA) to predict the secondary structure of human MDM4 mRNA based on the minimum free energy [99] and got a potential dsRNA structure comprising a complementary sequence located at both 5' and 3'-UTR (**Figure 1D**). Taken together, MDM4 regulation through its long untranslated regions add another layer of complexity of MDM4 splicing and expression.

MDM4, the hub to restore p53 activity

Despite existence of multiple MDM4 isoforms, MDM4-FL is the only established key negative regulator of p53. Mouse models studies further established that MDM4 is a drug target for p53 reactivation therapy [9]. Up to date, several MDM4-targeting strategies have been attempted, including (1) directly blocking p53-MDM4 interaction, (2) degrading MDM4 protein and (3) inhibiting MDM4 expression. Currently, more than 10 inhibitors targeting directly to the MDM4-p53 binding interface have been reported, which could be divided into two main categories, short peptide inhibitors, such as SAH-p53-8 [100], mSF-SAH [101], ATSP-7041 [102] and ALRN-6924 [103]; and small molecular inhibitors, such as Compound B1 [104], CTX1 [105], K-178 [106], Protoporphyrin IX [107], SJ-172550 [108], RO-5963 [109], Pyrrolopyrimidine 3a [110] and WK298 [111] (**Table 3**). Some of these inhibitors show they hit the target leading to p53 activation *in vitro* and some inhibitors displayed promising anticancer efficacy and safety profiles in preclinical models *in vivo* [112]. Currently, only one short peptide inhibitor, ALRN-6924 was advanced to Phase 2 clinical trial study for lymphoma treatment (NCT02264613), Phase 1 for lung cancer (NCT04022876), Phase 1 for advanced or metastatic malignant solid neoplasm (NCT03-725436), and Phase 1 for pediatric Cancer (NCT03654716). These MDM4-p53 disruptor drugs can also be used to combat resistance to MDM2 inhibitors. However, one limitation of targeting the MDM4-p53 interaction strategy is that it will not inhibit the p53-independent oncogenic activity of MDM4.

As an alternative approach, targeting MDM4 protein abundance can be an effective way to overcome such limitation. In this approach, one strategy is to target MDM4 protein for degradation or inhibit MDM4 expression. Since MDM2-MDM4 heterodimers not only plays critical role

Table 2. Candidate miRNAs binding to MDM4 mRNA

Name	Cell Type	Binding region of MDM4	Function	Reference
miR-34a-5p	Non-Small Cell Lung Cancer Cells	4238-4259 of MDM4 3'UTR (CATGCCAGCCTCCACACTGCC)	induces apoptosis of NSCLC cells	[119]
miR-887-3p	Non-small cell lung cancer patients	rs4245739, 32 of MDM4 3'UTR	inhibition of rs4245739 CC genotype on MDM4 expression	[120]
miR-191-5p miR-887-3p	Small cell lung cancer patients	rs4245739, 32 of MDM4 3'UTR	MDM4 rs4245739 SNP contributes to SCLC risk	[121]
miR-191-5p miR-887	Prostate cancer cells	rs4245739, 32 of MDM4 3'UTR	MDM4 rs4245739 SNP A-allele may be associated with an increased risk for prostate cancer	[122]
miR-1307	Breast cancer cell lines MCF-7 and MDA-MB-468	6038-6044 of MDM4 3'UTR (CCGGTCG)	modulating apoptosis by targeting MDM4	[123]
miR-766	Multiple cancer cell lines (MCF7, SBC3 and U2OS)	1405-1412, 2684-2692, 3237-3245, 5519-5524 of MDM4 3'UTR (GGCTGGAG)	induces p53 accumulation and G2/M arrest	[124]
miR-661	Multiple cancer cell lines (MCF7, A549 and H460)	9 target regions in MDM4 3'UTR (CCCAGGC)	downregulates both MDM2 and MDM4 to activate p53	[125]
miR-766	Colon cancer	918-932 of the MDM4 3'UTR (CATCCAAGCTGGAGT)	induces apoptosis of human colon cancer cells	[126]
miR-34a	Colorectal cancer cells	NA	miR-34a negatively regulates MDM4 gene expression to inhibit LOVO cell proliferation	[127]
miR-34a	Multiple cancer cell lines (H1299, MCF7 and U2OS)	6038-1045 of Mdm4 exon 11 (GTCTGATATCACTGCCA)	modulates MDM4 expression via a Target Site in exon 11	[128]
miR-205	Colorectal cancer cells	570-577 of the MDM4 3'UTR (ATGAAGGA)	suppresses cell migration, invasion and EMT of colon cancer	[129]
miR-128	Pancreatic carcinoma cell line MIA PaCa-2	4617-4623 of MDM4 3'UTR (ACUGUG)	induces pancreas cancer cell apoptosis	[130]
miR-191	Ovarian cancer cells	rs4245739, 32 of MDM4 3'UTR	delaying ovarian carcinoma progression and tumor-related death	[131]
miR-191	Oropharyngeal cancer cells	rs4245739, 32 of MDM4 3'UTR	associated with HPV16-positive tumors and survival of oropharyngeal cancer	[132]
miR-10a	Acute myeloid leukemia	511-531 of MDM4 3'UTR (AGGTGTGGGGCGACAGGGT)	associated with MDM4 downregulation in intermediate-risk acute myeloid leukemia	[133]
Let-7	Glioma cells	464-483 of MDM4 exon 7 (CCCCACACTGCCTACCTCA)	let-7 binding to MDM4 is implicated in the DNA damage response	[134]
Let-7	Extravillous trophoblast cell* (htr-8/svneo)	1543-1549 of the MDM4 3'UTR (TTGTACA)	inhibits the migration and invasion of extravillous trophoblast cell	[135]
miR-33a	Renal cell cancer	4436-4442 of the MDM4 3'UTR (CAATGCAA)	inhibits cell growth in renal cancer	[136]
miR-301a-5p	Kidney cell* (HK2)	89-96 of MDM4 3'UTR (GTCAGAGA)	induces kidney cell apoptosis	[137]
miR-885-3p	Squamous cell carcinomas cells	30-54 of MDM4 5'UTR (ACTCGCCATTTCAAAATGCTGCCG)	miR-885-3p might contribute to the regulation of cell viability, apoptosis and/or autophagy in squamous cell carcinoma cells upon cisplatin exposure	[89]
miR-126	Cervical cancer	6389-6396 of MDM4 3'UTR (GTAATAAT)	the upregulation of miR-126 resulted in suppressed proliferation, accompanied by the induced apoptosis of CC cells	[138]
miR-150-5p	Cervical cancer	8180-8186 of MDM4 3'UTR (TTGGGAG)	hsa_circ_0000263 can regulate the expression of MDM4 by affecting miR-150-5p	[139]

* represent non-cancer cell type; NA: Not Available.

Table 3. Inhibitors target for p53-MDMX binding

Inhibitors	Mechanisms of action	References
Short peptide		
SAH-p53-8	Binds to p53-binding pocket of MDMX and blocks the p53-MDMX binding	[100]
mSF-SAH	Covalently binds to p53-binding pocket of MDMX and blocks the p53-MDMX binding	[101]
ATSP-7041	Binds to MDM2 and MDMX and blocks the p53-MDM2/MDMX bindings	[102]
ALRN-6924	Binds to MDM2 and MDMX and blocks the p53-MDM2/MDMX bindings	[103]
Molecular inhibitors		
Compound B1	Binds to p53-binding pocket of MDMX and blocks the p53-MDMX binding	[104]
CTX1	Binds to MDMX, blocks the p53-MDMX binding, and activates p53	[105]
K-178	Inhibits the p53-MDMX binding and activates p53	[106]
Protoporphyrin IX	Blocks the p53-MDM2/MDMX bindings and stabilizes p53 and TAp73	[107]
SJ-172550	Covalently but reversibly binds to p53-binding pocket of MDMX	[108]
RO-5963	dual p53-MDM2 and p53-MDMX inhibitor	[109]
Pyrrolopyrimidine 3a	dual p53-MDMX/p53-MDM2 inhibitor	[110]
WK298	Inhibition the binding of p53-MDMX	[111]

for p53 degradation but also for MDM4 degradation [29]. Some compounds such as camptothecin analog FL118 and HSP90 inhibitor 17AAG can induce MDM2-dependent degradation of MDM4 [113, 114]. Therefore, they can be used to overcome MDM4-mediated oncogenic activities. Another strategy to decrease MDM4 abundance is to manipulate MDM4 alternative splicing: i.e. to induce exon 6-skipping of MDM4 splicing which will introduce a premature stop codon in the mRNA leading to switch from MDM4-FL transcripts to MDM4-S transcripts that is not used for protein translation. This strategy is very effective in shutting down MDM4 expression at splicing level leading to p53 stabilization and reactivation. As mentioned above, several splicing related factors, such as SRSF3 and PRMT5 were reported to regulate MDM4 splicing (**Table 1**). Targeting SRSF3 and PRMT5 will induce MDM4 exon 6-skipping to shut down MDM4 expression. Elegantly, Gerhart et al. have shown that PRMT5 inhibition by a specific inhibitor GSK3203591 induces the alternative splicing of MDM4 and activates the p53 pathway as one of the mechanisms underlying the antitumor activity of PRMT5 inhibitors [66]. Similarly, SF3B1 inhibitor pladienolide B also causes wild-type p53 up-regulation through inefficient mRNA splicing that brought down protein expression of both MDM4 and MDM2, contributing to the antitumor effect of pladienolide B in cutaneous squamous cell carcinoma [65]. Another strategy for splicing manipulation is to use specific antisense oligonucleotides (ASOs). ASO against exon-intron boundaries of MDM4 exon 6 can mimic the *in vivo* splicing event and promotes the exon 6 skipping to decrease the MDM4 abundance (**Figure 2**). Up to date, around 100 different antisense drugs against various targets have been enrolled into various phase II and III clinical trials, including three oral ASOs (fomivirsen, mipomersen and inotersen) [115-117]. Specific ASOs that target MDM4 the exon-intron boundaries of MDM4 exon 6 also demonstrated effectiveness in silencing MDM4 expression in preclinical models [63]. Given the rapid development of ASO-related therapeutics, one can expect a fast advancement of this type of cancer therapeutics to clinic than other chemical entities.

Concluding remarks

Studies in the past 25 years established that up-regulation of MDM4 contributes to p53 inactivation and tumor progression and drug resistance to MDM2 inhibitors. Recent advances highlight the importance of alternative splicing in MDM4 regulation in the context of tumor development via p53 downstream gene ZMAT3 and manipulation of MDM4 splicing for targeted therapies. Although the biological effect of different alternatively spliced MDM4 isoforms is not well understood, MDM4 alternative splicing emerges as an effective regulatory mechanism to shutdown the expression of MDM4-FL mRNA and eliminate MDM4 protein expression. Consequently, MDM4-specific manipulation of splicing with MDM4 exon 6-targted ASO will be an attractive therapeutic strategy for MDM4-

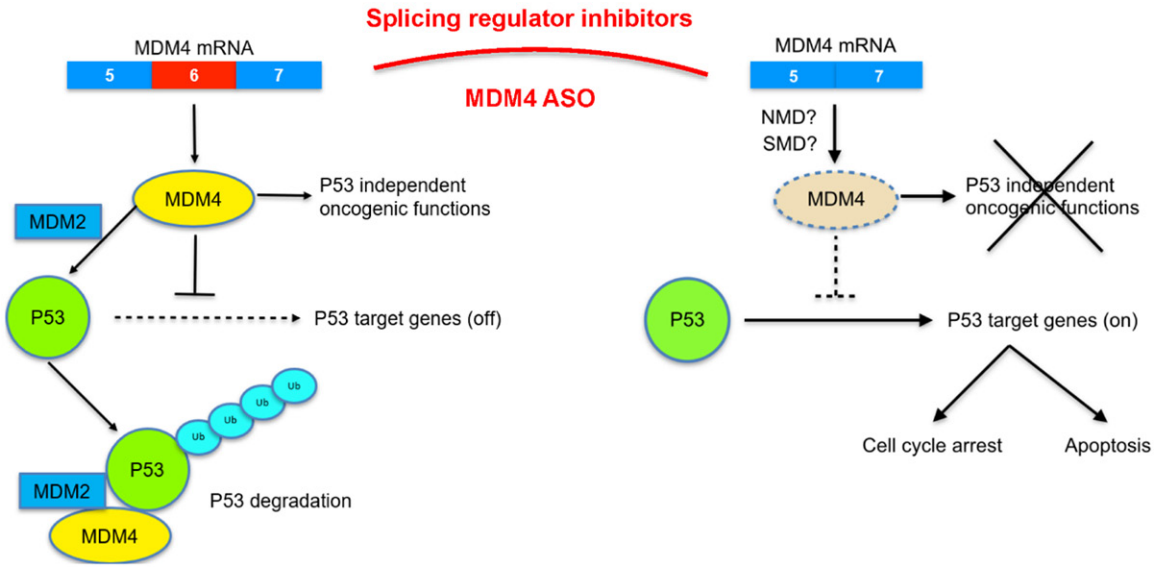

Figure 2. Targeting MDM4 splicing in cancer therapy. In normal adult tissues, exon6 of MDM4 tends to be skipped due to unproductive splicing. This will produce unstable transcript which is prone to be degraded through NMD or SMD pathway and decrease the abundance of MDM4-FL protein, which further transactivates and represses a number of target genes that function in apoptosis, cell cycle, and DNA repair (right part). While MDMX protein is highly expressed in embryonic tissues and in cancers due to the enhanced exon 6 inclusion. MDM4 can work together with MDM2 to mediate p53 degradation and perform both p53-dependent and independent oncogenic functions. Current strategy is that splicing regulator inhibitors (such as pladienoide b or GSK591) or MDM4 ASO can be used to induce MDM4 skipping to decrease the MDM4 abundance, and further inhibit p53-dependent or independent MDM4 oncogenic functions. Either splicing regulator inhibitors or antisense oligonucleotides (ASOs) are very specific, efficient, and clinically compatible approach.

targted p53 reactivation therapy. This strategy also provides advantage over other inhibitors that alter splicing of diverse pathways. Finally, although strategies targeting MDM4 splicing is promising, two issues should be considered. First, the increased p53 activity caused by MDM4-FL inhibition will cause toxicity to normal cells and tissues [50, 118]. Second, cancer cells with reduced MDM4-FL expression due to alternative MDM4 splicing often express a mutant p53 which will not respond to such strategies. Therefore, stratification of cancer patients into MDM4-high and MDM4-low expression groups in clinical trials will help identify patients who will likely benefit from this MDM4-targted therapies.

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Disclosure of conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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