# Dual function of MDM2 and MDMX toward the tumor suppressors p53 and RB

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## ABSTRACT

The orchestrated crosstalk between the retinoblastoma (RB) and p53 pathways contributes to preserving proper homeostasis within the cell. The deregulation of one or both pathways is a common factor in the development of most types of human cancer. The proto-oncoproteins MDMX and MDM2 are the main regulators of the well-known tumor suppressor p53 protein. Under normal conditions, MDM2 and MDMX inhibit p53, either via repression of its transcriptional activity by protein-protein interaction, or via polyubiquitination as a result of MDM2-E3 ubiquitin ligase activity, for which MDM2 needs to dimerize with MDMX. Under genotoxic stress conditions, both become positive regulators of p53. The ATM-dependent phosphorylation of MDM2 and MDMX allow them to bind *p53* mRNA, these interactions promote p53 translation. MDM2 and MDMX are also being revealed as effective regulators of the RB protein. MDM2 is able to degrade RB by two different mechanisms, that is, by ubiquitin dependent and independent pathways. MDMX enhances the ability of MDM2 to bind and degrade RB protein. However, MDMX also seems to stabilize RB through interaction and competition with MDM2. Here, we will contextualize the findings that suggest that the MDM2 and MDMX proteins have a dual function on both p53 and RB.

## INTRODUCTION

The p53 and retinoblastoma (RB) proteins are two key tumor suppressors. Mutations in one or both are found in all human cancer tumors and both have been extensively studied as potential therapeutic targets in drug development programs. p53 is a transcription factor in which converge many cellular stress pathways such as oncogene activation, hypoxia, DNA damage, and endoplasmic reticulum stress, to induce different biological cell responses such as cell cycle arrest in G1 or G2, DNA repair, senescence, or even apoptosis [1]. As a result of these features, p53 is named "the guardian of the genome". In a normal healthy cell, p53 remains at a very low concentration, but after cellular stress, its level increases. Due to the importance of p53, it has to be very tightly controlled; MDM2 and MDMX are known as the main regulators of this tumor suppressor protein and are altered in many human cancers [2-7]. Their negative influences toward p53 have been extensively characterized. Nevertheless, both are also positive

regulators of p53 expression; the cellular conditions are the key determinant in whether p53 is up- or down-regulated [8-11].

Conversely, RB is implicated in many cellular processes such as cell cycle regulation, differentiation, chromatin remodeling, and mitochondria-mediated apoptosis [12-14]. Loss of RB function can occur through mutation in the *RB* gene itself, by hypermethylation of the *RB* promoter, by binding of viral proteins such as E7 of the human papillomavirus or E1A of the adenovirus, or through post-translational modifications with tumor-associated kinase activity [15-17]. Given the importance of the functions of RB in the cell, its activity and levels are also tightly regulated. Phosphorylation is the most well-characterized post-translational modification of RB, particularly phosphorylation of Cdk/cyclin complexes, which plays a role in RB inhibition during cell cycle control [18]. However, only a few E3 ubiquitin ligase proteins have been reported as regulators of RB: during virus infection, cullin 2 is able to target RB for degradation via the human papillomavirus protein E7; SCF[SKP2] is

**Table 1: Effects of MDM2 and MDMX on the tumor suppressors p53 and RB**

| MDM2 | Positive effect | Negative effect | Binding site |
|---|---|---|---|
| Effect on p53 | [8, 9, 11] Under DNA damage: after ATM phosphorylation, MDM2 enhances p53 translation. | [27, 89] Under normal conditions: p53 degradation via ubiquitination and inhibition of transactivity of p53. | [25, 26, 90] p53 hydrophobic pocket residues 18 to 23 (N-terminal region); MDM2 p53 binding domain (N-terminal region), a secondary interaction site between DNA binding domain of p53 and the acidic domain of MDM2. |
| Effect on RB | NR* | [32] Under MDM2 overexpression: RB is degraded via ubiquitination.<br><br>[35] Under non-stress conditions: RB is degraded independent of ubiquitination.<br><br>[43] Under DNA damage: degradation after p38-dependent phosphorylation of RB. | [30] MDM2 residues 254 to 264 (ac. domain); RB residues 785 to 803 (the C-terminal pocket) [32]. |
| **MDMX** | | | |
| Effect on p53 | [10] Under DNA damage: after ATM phosphorylation, MDMX is an RNA chaperone to enhance p53 translation. | [50, 91] Under normal conditions: inhibition of transactivity of p53 and ubiquitination and degradation via MDM2. | [47, 92] p53 hydrophobic pocket (N-terminal region); MDMX p53 binding domain (N-terminal region), a secondary interaction site between DNA binding domain of p53 and the acidic and RING domains of MDMX. |
| Effect on RB | [54] Under normal conditions: stabilization of RB by competition with MDM2. | [55] Under normal conditions: degradation in an MDM2-dependent manner. | [55] MDMX residues 432 to 481 (C-terminal RING domain); RB C-terminal pocket.<br><br>[54] RB C-terminal pocket; MDMX ΔC-terminal. |

*NR: not reported

also able to target RB via the Epstein-Barr EBNA3C protein; and under non-viral infection conditions, RB is ubiquitinated by NRBE3 and MDM2 [19-23]. The regulatory activity of MDM2 and MDMX proteins on the tumor suppressor RB is becoming the subject of focus. We yet know that MDM2 is able to degrade RB through two different mechanisms, while MDMX, on the one hand, helps MDM2 to degrade RB, and on the other, it is also able to avoid the MDM2-dependent degradation of RB. However, the conditions under which each event occurs have not yet been clarified.

To illustrate the relevance of the interplay between MDM2/MDMX and RB and p53 in human cancers, we will give an overview of progress in the field, the similarities and differences in the regulation of these important tumor suppressors through MDMX and MDM2, and finally analyze the crosstalk between the p16[Ink4a]/RB/E2F and the p14[ARF]/p53/MDM2/MDMX pathways.

## MDM2 in the regulation of RB and p53

p53 and MDM2 interact directly through the N-terminal regions of both proteins (Figure 1). It has been proposed that this first interaction in the N-terminal promotes a second contact that involves the acidic domain of MDM2 and the DNA-binding domain of p53 [24-26]. The interaction between p53 and MDM2 induces proteasomal degradation of p53 via polyubiquitination [27, 28], hence the reputation of MDM2 as the main negative regulator of p53. The effect described above is responsible for keeping p53 at very low levels, under normal cellular conditions. Less well-known is the fact that MDM2 can also act as a positive regulator of p53. Under genotoxic stress conditions, ATM phosphorylates MDM2 at Ser395, near to its RING domain, allowing the protein to expose a site for the *p53* mRNA interaction that enhances p53 translation [9, 11]. Using either doxorubicin or etoposide to induce DNA damage in the H1299 cell line, it is possible to see an MDM2-dependent enhancement of transfected p53 levels (Table 1), a phenomenon that helps to explain the fact that MDM2 is one of the first p53-transcribed genes. Thus, MDM2 has a dual role toward p53, and the key to allow it to switch from negative to positive p53 regulator is the cellular environmental condition.

RB and MDM2 also interact directly; in this case, the acidic domain (residues 254 to 264) of MDM2 is responsible for binding with the RB C-terminal region (residues 785 to 803) (Figure 1). The same region of RB is involved in the interaction with the E2F1 transcription factor [29-31]. An important implication of the interaction

between RB and MDM2 is the reduction of RB levels. In 2005, two different reports showed that MDM2 promotes RB degradation. On the one hand, Uchida et al. found that MDM2 promotes RB ubiquitin-dependent degradation *in vivo* in HEK293, NIH3T3, HCT116, MEF, SRB1, and U2OS cell lines [32]. This effect was suppressed by the presence of p14[ARF], as has previously been shown for p53 [33, 34]. They also observed that this effect is selective toward RB, since neither p107 nor p130 underwent MDM2-dependent ubiquitination even when they interacted with MDM2 [32]. In the same year, Sdek et al. determined that MDM2 promotes proteasomal degradation of RB, but in an ubiquitin-independent manner [35]. Using the U2OS, C33A, H1299, SJSA-1, SAOS, and ts20 cell lines, they carried out a series of experiments where they detected ubiquitinated forms of p53 but not of RB. They confirmed these results using the ubiquitin mutants K47R and K48R, which both block polyubiquitination, and

even in the presence of these mutants they were able to observe the RB degradation. Finally, they observed the formation of a triple complex between MDM2, RB, and C8, a subunit of the 20S proteasome. RB is able to interact directly with C8, but the presence of MDM2 enhances this interaction, promoting RB proteasomal degradation independent of ubiquitination [35]. Likewise, other proteins such as p53 and p21 are degraded by ubiquitin-dependent and independent mechanisms [36-41]. The above-described facts seem to confirm that MDM2 is a key negative regulator of RB (Table 1). However, the cellular conditions and the signals that control one or other degradation pathway could be different, and are not yet known.

More than 10 years ago, it was observed that MDM2 preferentially interacts with the hypophosphorylated form of RB [29, 42]. In 2011, the team of Harbour showed that indeed, MDM2 interacts with a p38MAPK-dependent
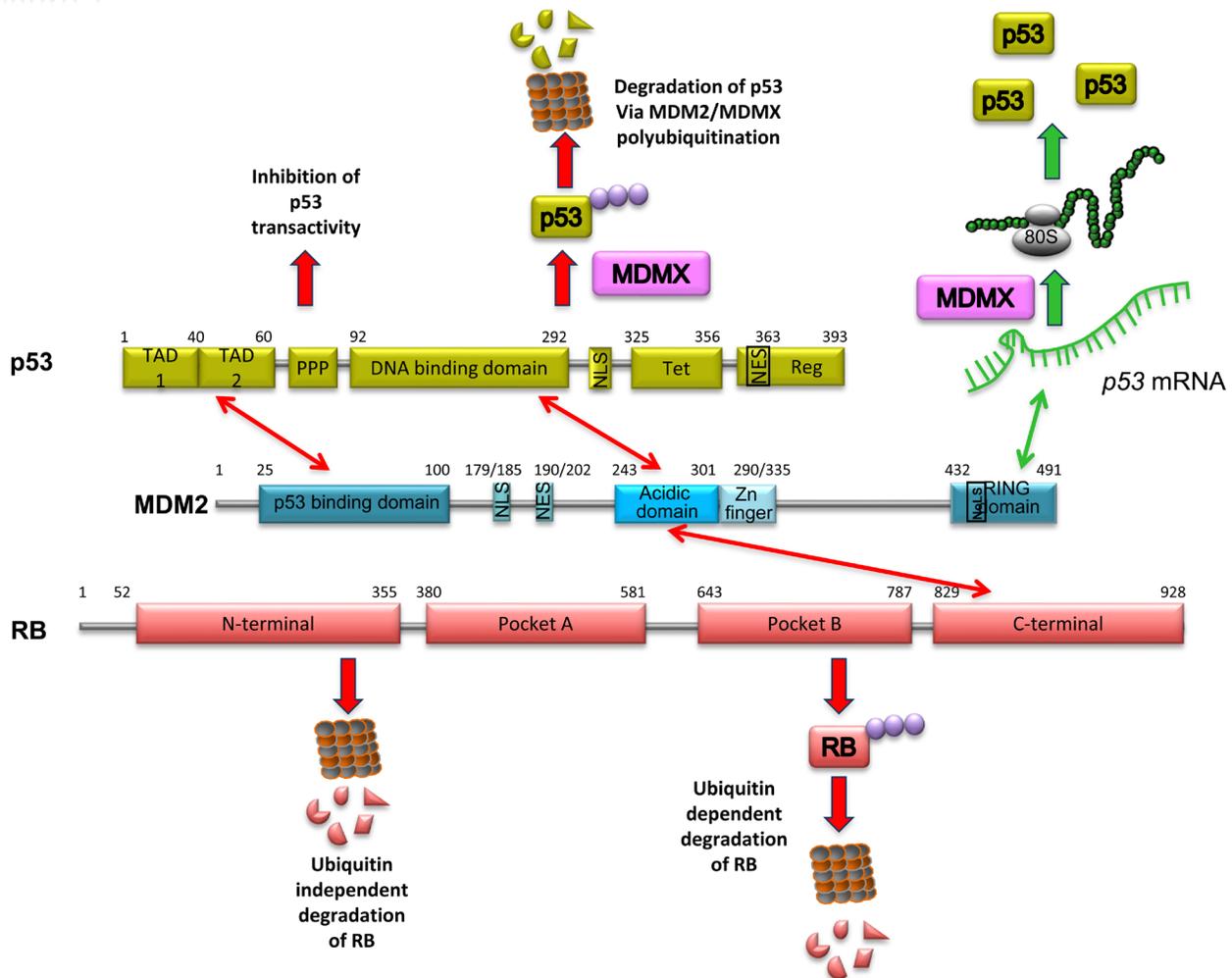


**Figure 1: Schematic representation of MDM2, p53 and RB.** The different domains of each proteins are indicated, the sites of interaction between the proteins and the physiological consequences of each interaction.

phosphorylated RB protein in Ser567 that is independent of Cdk phosphorylated RB sites [43]. p38MAPK is activated in response to different cellular stresses, such as DNA damage, osmotic shock, inflammatory response, and heat shock. After DNA damage, p38MAPK is activated in an ATM-dependent manner via Serine/threonine-protein kinase TAO involved in cell cycle regulation [44]. Under these conditions it seems that the p38α isoform is able to induce Ser567-RB phosphorylation. This phosphorylation promotes the interaction of RB with MDM2 and is involved in p53-dependent apoptosis in the Mel202 cell line. Using RNAi, it was also shown that p53 is not involved in Ser567-RB phosphorylation, or even in its degradation, but the absence of p53 under this condition inhibits cell death [43].

MDM2 is thus able to interact with the two tumor suppressor proteins; the site of interaction is different and the MDM2-RB complex is indeed able to bind p53. The formation of the triple complex RB-MDM2-p53 is stronger under DNA damage conditions, when p53 is stabilized [42]. However, RB and p53 are not able to interact directly, suggesting that MDM2 is a bridge between RB and p53 [42, 45].

## MDMX in the regulation of RB and p53

MDMX is a protein paralogous to the MDM2, they share a high similarity in their RING domains. However MDMX does not have detectable E3 ubiquitin ligase activity [46-48]. The MDMX and p53 interaction has also been very well described. The N-terminal domain of MDMX interacts with the hydrophobic pocket of the N-terminal of p53 [47]. The interaction represses the transactivity of p53; moreover, even when there is no detectable p53 ubiquitination mediated by MDMX itself, its presence stabilizes MDM2 via RING-RING interaction, and is indeed essential to promoting MDM2-mediated polyubiquitination of p53 under normal cellular conditions [49, 50]. In the same way as MDM2, under genotoxic stress conditions, MDMX switches from being a negative to a positive regulator of p53. The Ser403 near
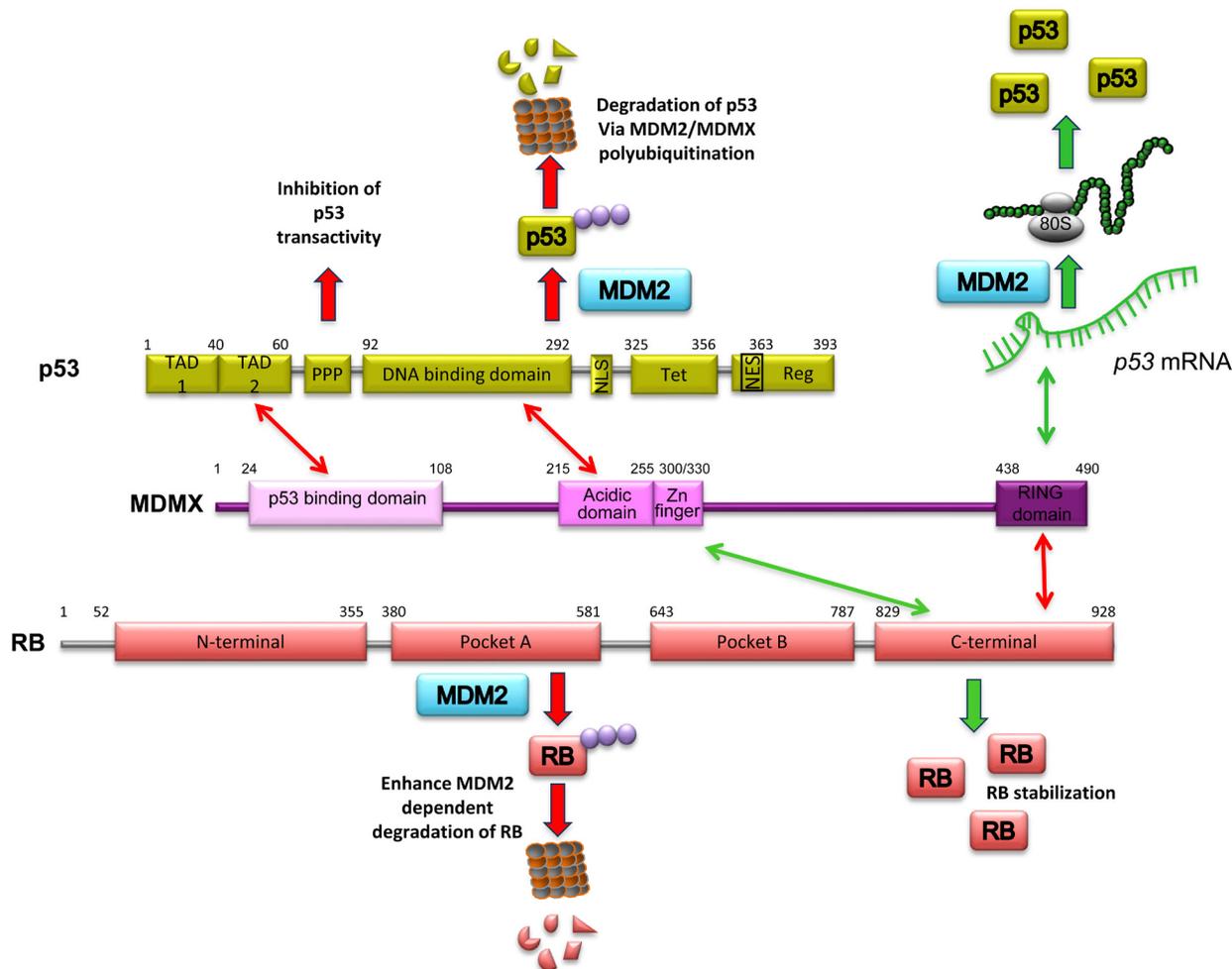


**Figure 2: Schematic representation of MDMX, p53 and RB.** The different domains of each proteins are indicated, the sites of interaction between the proteins and the physiological consequences of each interaction. Note that depending on the region where RB binds to MDMX, the outcome may be MDM2-dependent RB degradation or RB stabilization.

to the RING domain of MDMX is ATM-dependently phosphorylated following DNA damage; this event promotes the binding of MDMX to *p53* mRNA and acts as an RNA chaperone to properly fold the *p53* mRNA, optimizing the correct recognition for MDM2; together the two proteins enhance p53 translation ensure proper cellular response [10]. Evidence supports the idea that MDM2 and MDMX are partners and work in collaboration through the formation of a heterodimer to down-regulate p53 under normal conditions [51, 52], but also to up-regulate p53 after DNA damage [53] (Table 1).

Less is known about the MDMX and RB interplay; nonetheless, MDMX directly interacts with the RB protein. Due to the high level of identity between MDM2 and MDMX, it is not surprising that the C-terminal region in RB responsible for the recognition of MDM2 is also involved in the binding with MDMX [54]. In 2006, using the U2OS, HCT116, HEK293, and MEF cell lines, Kitagawa and colleagues, observed that the ectopic expression of MDMX inhibited RB degradation via MDM2 and stabilized RB in cells. The suggested mechanism to explain this observation is that MDMX blocks or interferes with the RB-MDM2 binding by competition with MDM2 for the C-terminal region of

RB. Interestingly, a construct of MDMX that lacks the C-terminal RING domain was still able to bind RB, suggesting that the site of interaction in MDMX is apart from its C-terminal domain (Figure 2).

Using the U2OS and MEF cell lines, the direct interaction between MDMX and the C-terminal region of RB was recently corroborated. Surprisingly, in this case, the C-terminal RING domain of MDMX is involved in the binding with the C-terminal region of RB [55]. More remarkable is the fact that they observed that MDMX induces RB degradation in an MDM2-dependent manner. This effect required an MDMX-MDM2 interaction (Figure 2), whereas MDM2-mediated degradation of RB does not require the presence of MDMX [55, 56]. Accordingly, MDMX is both a negative and a positive regulator of RB, although the conditions under which each event occurs have not been described (Table 1).

**Crosstalk between RB/E2F and p53/MDM2/ MDMX pathways**

Inactivation of pathways p16[Ink4a]/RB/E2F and p14[ARF]/p53/MDM2/MDMX are important mechanisms



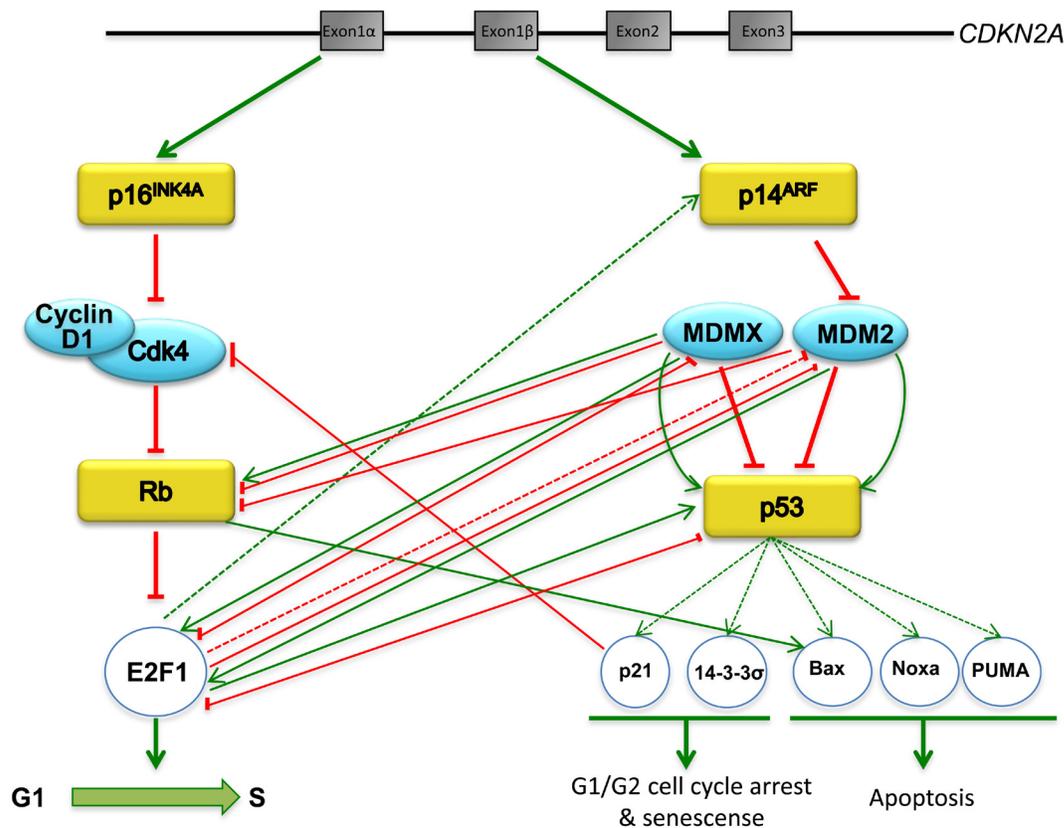**Figure 3: Schematic representation of the p16[Ink4a]/RB/E2F1 and p14[ARF]/p53/MDM2/MDMX pathways.** Tumor suppressor proteins are shown in yellow squares, proto-oncogenes are shown in blue ovals. Red lines represent negative regulation, green arrows represent positive regulation, dotted lines represent regulation at a transcriptional level.

in the development of human cancer [57]. A correlation has been shown between the concomitant inactivation of both pathways and chemotherapy [58], such as in the case of some ovarian cancers [59, 60], uveal melanoma [61], malignant rhabdoid tumors [62], retinoblastoma [63], and breast cancer [58, 64].

A deficiency in the p16$^{Ink4a}$/RB/E2F1 pathway could arise either from the loss of function of one of the two tumor suppressors RB or p16$^{INK4}$, or from an event that allows overexpression of cyclin D1 and/or Cdk4 [65, 66] (Figure 3). Meanwhile, the p14$^{ARF}$/p53/MDM2/MDMX pathway can be inactivated by loss of function of p53 or p14$^{ARF}$, or by overexpression of either MDM2 or MDMX. This is exemplified in retina cancer, where 65% of tumors have an extra copy of MDMX and 10% have an extra copy of MDM2 when p53 is wild type [67-70]. The tumors with loss of RB correlate with high levels of p14$^{ARF}$ or p53, and the explanation of this phenomenon is that in the absence of RB, E2F1 is liberated and triggers p14$^{ARF}$ that in turn will sequester MDM2, allowing p53 to accumulate (Figure 3). However, in a scenario with overexpression of MDM2, there is no p53 accumulation, stimulating tumor growth.

Both p16$^{INK4}$ and p14$^{ARF}$ tumor suppressor proteins are encoded by the same locus on chromosome 9p21, through the use of two independent promoters, and encompass a unique first exon (E1α for *INK4A* and E1β for *ARF*) followed by two common exons, E2 and E3 (Figure 3). These exons are translated using different open reading frames [34, 71]. p16$^{INK4}$ inhibits Cdk4/cyclin D1 activity and promotes RB to block cell cycle progression through the interaction with E2F1. Meanwhile, p14$^{ARF}$ is able to bind to MDM2, preventing p53 polyubiquitination and degradation. The activation of p53 triggers p21 which causes cell cycle arrest in the G1/S phase due to suppression of Cdk/cyclin complexes. p53 could also mediate the induction of 14-3-3-σ that results in G2/M arrest [72, 73]. Through the induction of genes such as *Noxa, PUMA,* or *Bax*, p53 promotes apoptosis [1, 74]. Recently it has been shown that RB binds directly to the Bax protein at the outer membrane of the mitochondria, inducing apoptosis [14].

As mentioned above, MDM2 can also down-regulate RB, promoting cell cycle progression, or by interaction with the E2F1 transcription factor. The region of MDM2 that interacts with E2F1 is within the conserved p53-binding domain involving the amino acids 1 to 220 [75]. Given that MDM2 is an E3 ubiquitin ligase, one might expect that the principal function of this protein would be the ubiquitination of its substrates. In this particular case, MDM2 acts to prevent the ubiquitination of E2F1 by competing with the E2F1 E3 ubiquitin ligase SCF$^{SKP2}$ [76]. In turn, E2F1 inhibits the expression of MDM2 in a p53-dependent manner [77].

In 2003, Strachan et al. described a direct interaction between E2F1 and MDMX that inhibits the function of E2F1 as a transcription factor [78]. This interaction takes place in the central portion of MDMX and near to the E2F1 DNA binding domain, between residues 117 to 241 of E2F1, with the interaction impairing the ability of E2F1 to bind DNA [78]. Thus, MDMX can affect the expression of proteins such as p53 or p14$^{ARF}$ through the inhibition of E2F1. Moreover, an elevated expression of MDMX results in E2F1 delocalization into the cytoplasm [79]. However, it is important to note that MDMX is also able to enhance the E2F1 transactivation function through the RB degradation that results in release of E2F1 and therefore cell cycle progression [55]. Taken together, MDMX is able to enhance E2F1 function in an RB-dependent manner; but is also able to repress E2F1 activity through two different mechanisms; directly by blocking the DNA binding capacity of E2F1, and indirectly through delocalization of the transcription factor. Again, the physiological conditions that trigger one or the other are not yet known. E2F1, in turn, is able to target both MDM2 and MDMX for proteolytic degradation, in a mechanism independent of the proteasome that does not need the transcriptional activity of E2F1 [80]. Using a battery of protease inhibitors, the authors suggest that the MDMX and MDM2 degradation induced by E2F1 may be via cathepsin-like proteases, and this mechanism could also have a role in E2F1-mediated apoptosis.

Finally, we would like to discuss the direct interaction of the two transcription factors in these pathways, E2F1 and p53. In 1995, O'Connor et al. demonstrated, *in vitro* and *in vivo*, a physical interaction between the two proteins: through this interaction, p53 can suppress E2F1 transcriptional activity independently of RB [81]. Likewise, E2F1 and its partner DP1 were able to repress p53 transactivity [81, 82]. More recently, it has been shown that the cyclin A interaction domain of E2F1 is essential for the p53 binding that results in p53 stabilization and enhanced apoptosis p53-dependent under DNA damage conditions [83]. In these examples, it is clear that the same interaction can affect the function of the proteins, depending on the cellular conditions.

## CONCLUSIONS AND PERSPECTIVES

Since its discovery more than 30 years ago, the tumor suppressor protein RB has been extensively studied in a variety of cellular processes that implicate its interaction with many different partners. The best-characterized is its regulation of E2F1 and the Cdk/cyclin complexes in cell cycle progression. However, some conflicting findings have flooded the studies of the regulation of MDM2 and MDMX on RB and other members of this pathway. Until a few years ago, MDM2 and MDMX were considered the main negative regulators of p53 [49, 84-88], but recent evidence supports the idea that they can switch to being positive regulators of p53 depending on the cellular conditions [8-11]. Taken together, these characteristics may help

to explain the different and sometimes contradictory effects that have been described about the regulation of these two oncogenes on the RB pathway members. What is clear is that more knowledge is required to elucidate the mechanisms controlling the relationship of the two pathways, which may be helpful in developing programs for anticancer therapies.

## ACKNOWLEDGMENTS

## CONFLICTS OF INTEREST

There is no conflict of interest.

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