Review Article
Functions of MDMX in the Modulation of the p53-Response

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The MDM family proteins MDM2 and MDMX are two critical regulators of the p53 tumor suppressor protein. Expression of both proteins is necessary for allowing the embryonal development by keeping the activity of p53 in check. Upon stresses that need to activate p53 to perform its function as guardian of the genome, p53 has to be liberated from these two inhibitors. In this review, we will discuss the various mechanisms by which MDMX protein levels are downregulated upon various types of stress, including posttranslational modifications of the MDMX protein and the regulation of \textit{mdmx} mRNA expression, including alternative splicing. In addition, the putative function(s) of the described MDMX splice variants, particularly in tumor development, will be discussed. Lastly, in contrast to common belief, we have recently shown the existence of a p53-MDMX feedback loop, which is important for dampening the p53-response at later phases after genotoxic stress.

1. Introduction
The tumor suppressor protein p53 is maintaining genome integrity following stress signals by either inducing a cell cycle arrest, allowing a complete checkup of the genome before resuming DNA replication, inducing senescence, or by stimulating apoptosis when the cell is beyond rescue [1]. In addition, p53 has been shown to directly affect DNA repair by modulating expression of number of DNA repair genes [2]. During tumor development, inhibition of angiogenesis is part of the tumor suppressor function of p53 [3]. In recent years, it has been shown that p53 also affects the metabolic switch which occurs in many tumors, in which energy is obtained by glycolysis rather than via oxidative phosphorylation [4]. p53 is activated by a plethora of stimuli, including DNA damage, hypoxia, and oncogene activation, the final outcome of the activation being very dependent on the strength and type of the stimulus and the type of targeted cell.

Whatever stimulus used to activate p53, it has become clear that for full, but controlled p53 activation, a tight control of activity and levels of the main p53 inhibitors, MDM2 and MDMX, is necessary [8, 9]. Various mouse models provide the best evidence regarding the importance of MDM2 and MDMX in controlling p53 activity under normal conditions. Loss of either MDM2 or MDMX expression leads to embryonal lethality, which is rescued by simultaneous loss of p53. Tissue-specific deletion of either the \textit{mdm2} gene or the \textit{mdmx} gene showed differences between cell types for their dependency on MDM2 and MDMX to keep p53 in check. These mouse models have recently been discussed in two excellent reviews [9, 10].

The MDMX and MDM2 proteins have a similar structural organization (Figure 1).

MDM2 and MDMX bind via their N-terminal hydrophobic region to a short alpha-helical stretch within the p53 N-terminus [11]; this N-terminal region is best conserved between the two proteins. Other conserved structures include the C-terminal RING finger domain, a Zn-finger and a central acidic region. The RING finger domains of MDMX and MDM2 are involved in their homo- and hetero-multimerization [12]. MDM2 has convincingly been shown to form both homo-oligomers and hetero-oligomers with MDMX. In contrast, no significant homo-oligomerization of MDMX could be found. This deficiency could have strong implications for the function of MDMX (see below).

MDM2 is an E3 ubiquitin ligase that targets p53 for ubiquitination and degradation. Essential for p53 ubiquitination are both the MDM2 C-terminal region including
Figure 1: Schematic representation of MDMX protein structure in comparison with MDM2. Most indicated protein modification sites and protein-protein interactions shown are discussed in the text. Modifications of MDMX by sumoylation (K254, K379) have not yet shown to affect function or regulation of MDMX [5]. Phosphorylation of S298 stimulates the association of MDMX to p53 [6], and phosphorylation of S96 has been reported to affect the regulation of subcellular localization of MDM2 [7].

The RING finger and the acidic domain. Corresponding regions of MDMX cannot fulfill this function [13, 14]. MDM2-mediated monoubiquitination of p53 leads to nuclear export, thereby inhibiting the p53’s function as a transcription activator. Moreover, it has become evident that Mdm2-dependent mono-ubiquitination of p53 promotes the localization of p53 to the mitochondria, where stress-induced HAUSP-p53 complexes result in nonubiquitinated, apoptotically active p53 [15]. Low levels of Mdm2 favor mono-ubiquitination of p53, leading to this mitochondrial transcription-independent induction of apoptosis upon stress. In contrast, high levels of Mdm2 and/or in combination with p300/CBP, promote polyubiquitination, which results in proteasome-dependent degradation of p53 [16, 17]. As mentioned, MDMX has no detectable ubiquitin-ligase itself, but has been proposed to inhibit p53 in a dual way. First, MDMX binds tightly to the N-terminal transcription activation domain of p53, in that way inhibiting its function as transcription activator [18]. Binding of MDMX to this part of p53 prevents the interaction of p300, which results in reduced acetylation of p53 which is involved in activation of p53 [19, 20]. Interestingly, p300 acetylates several lysines at the C-terminal region of p53 that are also targeted by Mdm2-mediated ubiquitination [17]. This might implicate that MDMX indirectly stimulates the MDM2-mediated ubiquitination by reducing the acetylation of those lysines. It should be noted that the importance of modifications of the C-terminal lysines for correct regulation of p53 stability and activity in vivo appears to be limited. Krummel et al. [21] showed that a knock-in mutant mouse in which the C-terminal lysines were replaced by arginines developed normally. Several assays showed no difference in regulation of wild-type p53 and p53-7KR. Only a minor increased activation of p53 in the thymus upon ionizing radiation was found, and, interestingly, p53-7KR MEFs could not escape from senescence, in contrast to normal MEFs, indicating an increased activity of p53-7KR. The difficulty with interpretation of the results is that one cannot distinguish between the functions of the various possible modifications on the lysines (ubiquitination, sumoylation, neddylation, methylation, and acetylation). It could well be that a certain modification on one lysine counteracts the effect of a different modification on a nearby lysine. In Mdmx-deficient MEFs, we have seen increased acetylation of the one analyzed lysine in p53, K379. A full proteomic/mass spec approach would be interesting to analyze the ratio of various lysine modifications at the p53 C-terminus and the effect of different MDMX expression levels on that ratio.

What has become quite clear is that the ratio between the levels of MDM2/MDMX strongly determines the outcome on p53 stability. A relative high level of MDMX might compete p53 away from MDM2, leading to p53 stabilization [22]. In addition, as mentioned above, MDM2 and MDMX interact via their respective C-terminal RING finger domains, and it has been proposed that under nonstress conditions, the MDM2/MDMX heterodimer functions as an active ubiquitin ligase via providing an extended interaction motif with the E2 protein [23–25]. One might argue that only a dimer/oligomer of MDM2/MDMX RING fingers can bind the E2; since MDMX cannot homodimerize, MDMX on its own is defective as a ubiquitin ligase. This might certainly be true, but is not the whole explanation since it has been shown that just replacing the MDMX RING with an MDM2 RING cannot rescue the ubiquitin-ligase activity of MDMX [13, 14].

In this review, we will focus on the regulation of MDMX expression upon various forms of stress. In most cases,
a downregulation of MDMX protein is observed, which is needed for full p53 activation. On the other hand, under certain lethal conditions, a positive role for MDMX in p53-induced apoptosis has been found. These various and apparently contradictory results will be discussed. This includes the recently discovered p53-MDMX feedback loop, which affects the outcome of genotoxic insults in cells.

2. MDMX Regulation upon Genotoxic Stress

2.1. Inactivation of MDMX at the Protein Level. Interestingly, initial reports indicated that MDMX levels were not affected by DNA damage or differentiation or during cell growth \[18, 26\]. These wrong conclusions were caused by the absence of high-affinity antibodies and by our limited insight into the ways MDMX mRNA levels were regulated. The first to notice that DNA damage influences MDMX were Li and coworkers. They observed that DNA damage induces the nuclear translocation of exogenously expressed MDMX, but an effect on MDMX protein levels was not discovered \[27\]. Although it had been reported that MDMX could be degraded by a proteasome-dependent mechanism, which is enhanced by p53- and Adriamycin-induced caspase-dependent cleavage of MDMX \[28\], only a year later it was recognized that MDMX is a target of the ubiquitin-ligase activity of MDM2 and that endogenous MDMX protein is degraded upon DNA damage, in an MDM2-dependent fashion \[29–31\]. Importantly, overexpression of MDMX prevented p53 stabilization and activation \[31\]. Notably, in these studies, no effect on the total amount of MDMX mRNA by ionizing radiation (10 Gy) was observed. Others and we subsequently showed that the MDM2-mediated degradation of MDMX is strongly stimulated upon ATM-dependent phosphorylations on three serine residues, S342, S367, and S403 \[32–34\]. These phosphorylations have multiple effects, including the temporal nuclear accumulation of MDMX in a 14-3-3-dependent manner \[34–36\].

Increased association of MDM2 with MDMX upon phosphorylation of the latter has been suggested \[32\] as an explanation for the increased ubiquitination and degradation of MDMX. Alternatively, we have shown that upon ATM-dependent MDMX phosphorylation, the interaction with the deubiquitinase HAUSP is strongly decreased and that HAUSP interaction-expression is needed to maintain endogenous MDMX levels \[35, 37\]. Whether the observed increased interaction with 14-3-3 proteins and the decreased interaction with HAUSP are causally related has not been proven. Interestingly, recently the interaction motifs for HAUSP on human MDMX have been mapped. It was found that both an N-terminal motif (\(8^{N}AQC8^{11}\)) and more C-terminal motif (\(398^{N}AHSS401\)) in MDMX can interact with HAUSP \[38\]. The latter is very close to the S403, which is directly phosphorylated by ATM. Although not tested for this particular motif, it was found that phosphorylation of a serine residue two positions C-terminal of the core motif could strongly inhibit the interaction with HAUSP as assayed by peptide arrays. So, possibly both the direct phosphorylation event on S403, and the interaction of 14-3-3 around phosphorylated S342 and S367 are involved in decreasing the HAUSP/MDMX interaction.

Although one of the HAUSP interaction motifs in MDM2 is also close to the phosphorylation targets Y394 and S395, these phosphorylation events do not appear to affect the interaction with HAUSP \[38\]. Even though MDM2 lacks classic consensus sites for interaction, 14-3-3 proteins have also been shown to bind MDM2. Two sites have been mapped; one within the middle domain of MDM2, dependent on phosphorylation by Pim1-kinase on S166 and S186 \[39\]. However, this event appears to stabilize MDM2. On the other hand, 14-3-3[σ] was shown to interact with the RING finger domain of MDM2, in a phosphorylation-dependent manner, and this interaction enhanced MDM2 ubiquitination and degradation \[40\]. Unfortunately, the involved phosphorylation events (target sites, signaling cascade) have not been investigated, but the 14-3-3[σ] interaction domain was found rather far downstream of S395, which is needed for the temporarily destabilization of MDM2 upon ionizing radiation \[41\]; so, a causal relation is not obvious. In addition, whether the observed effects on MDM2 stability are dependent on this specific isoform of 14-3-3 has not been extensively investigated.

The above-reported regulation of MDMX protein levels via ATM/Chk2 phosphorylations and 14-3-3/HAUSP interactions were all upon treatment of cells with IR or IR-mimetic NCS (neocarzinostatin). Surprisingly, UV-C-induced DNA damage also increases phosphorylation of MDMX/Ser367 in a Chk1-dependent manner, but in this case, the increased interaction with 14-3-3 results in cytoplasmic sequestering of MDMX \[42\]. This manner of MDMX inactivation also turned out to be important for a full p53-stabilization upon DNA damage. Unfortunately, the authors did not investigate total levels of endogenous MDMX after longer time-points after UV-C irradiation; it is not excluded that the observed change in localization, seen three hours after UV-C, is a first step in the inactivation of the MDMX protein, followed by a total disappearance of full-length MDMX protein either via proteasomal degradation or via alternative splicing of the MDMX pre-mRNA (see below). The physiological relevance of the UV-C mediated responses is questionable, since humans are not exposed significantly to UV-C. The use of UV-A or UV-B irradiation would have been better in that respect.

A recent elegant study with the use of a mouse knock-in model has shown the importance of the S342, S367, and S403 MDMX phosphorylations, not only for a DNA damage response, but also in the regulation of tumor suppression by p53 \[43\]. As could be extrapolated from the cell culture studies, thymocytes or MEFs derived from the 3SA mutant mice show decreased degradation of MDMX upon ionizing radiation or neocarzinostatin treatment, and a concomitant reduced p53 stabilization and activation. More striking is the observation that crossing the 3SA mutant mice with Eμ-Myc knock-in transgenic mice strongly decreased the latency period for tumor formation. In the developing tumors, no selection for p53 mutation was found in the 3SA mice, indicating that the expression of the mutant, degradation resistant MDMX is sufficient to inhibit p53’s
tumor suppressor function. Although the involvement of other mechanisms cannot be excluded, it was found that the overexpression of the Myc transgene did result in activation of the double-strand DNA break DNA damage response, which could explain the observation that prevention of DNA damage-associated phosphorylations of MDMX impact on the tumor development [43]. It should be noted that it is not excluded that other, yet undefined, kinases are involved in the phosphorylation of MDMX which can be activated by Myc overexpression.

In addition to the serine phosphorylations mentioned above, also tyrosine phosphorylation of MDMX upon imposing double-strand DNA breaks has been reported [44]. Tyrosine 55 and tyrosine 99 were identified as targets of the c-Abl tyrosine kinase. Phosphorylations on these sites increased upon DNA damage. Interestingly, Y99 phosphorylation was found to inhibit the p53/MDMX interaction, which was supported by structural modeling. Function of Y55 phosphorylation is not yet known, but appeared to be dependent on Y99 phosphorylation, and the authors suggest a possible role for Y55 phosphorylation in the recovery phase of p53 activation. These results indicate that double-strand DNA breaks not only targets MDMX to activate p53 via the ATM/Chk2 pathway to reduce MDMX levels, but also via a very quick pathway, c-Abl mediated phosphorylation that inhibits the MDMX/p53 interaction.

3. Inactivation of MDMX at the Posttranscriptional mRNA Level

Extensive alternative mRNA splicing of mdm2 has been observed, especially in tumor cells [45]. In addition, existence of some splicing variants of mdmx has also been reported, mainly in tumors/tumor-derived cell lines (reviewed recently by [46]). The so far identified mdmx mRNA variants obtained by alternative splicing are depicted in Figure 2. Chandler and colleagues investigated whether genotoxic stresses would also affect the splicing pattern of mdm2 and mdmx [47]. It was observed that mdm2 showed alternative splicing upon treatment with rather high doses of UV-C or cisplatin, but IR did not affect the splicing pattern. Similarly, mdmx alternative splicing was observed upon 30 or 50 J/m² UV-C, but not with 10 J/m². Unfortunately, the effect of cisplatin treatment on mdmx mRNA splicing was not tested in this study. What is missing from these studies is a clear quantification of the levels of the alternative spliced mRNAs compared to the normal spliced mRNA. Even better would have been to investigate whether the putatively produced alternative protein products are indeed synthesized endogenously. For MDMX that might be difficult, because only few high-quality antibodies are available, but for MDM2 it could have been performed. Possible functions of the protein products of splicing variants have been investigated almost only by strong overexpression, which might not reflect the physiologically relevant situation. Therefore, no conclusions can be drawn regarding a physiological function of the alternative splicing after these high levels of DNA damage. The authors note that they also detect low levels of another mdmx splicing variant, mdmx-S [47]. This splicing variant, a result of exon 6 skipping, was first described by Rallapalli and colleagues [48]. The alternative splicing was mainly found in some cancer cell lines and could be increased in nontransformed cells upon serum-stimulation. The mdmx-S mRNA encodes essentially only the p53 binding domain, and a number of unique C-terminal amino acids (Figure 2). In overexpression studies the MDMX-S product was found to be a stronger inhibitor of p53 activity, most likely because this short
protein localized into the nucleus much more efficiently than full-length MDMX [49].

Importantly, in recent experiments, we found that after exposure of normal human fibroblasts or MCF-7 cells to relatively low levels of UV-C (10 J/m²), splicing of mdmx mRNA is changed, but that preferentially the mdmx-S variant is expressed (Figure 3). This is a relative quick event in the human skin fibroblasts and occurs somewhat slower in the MCF-7 cells. Under these conditions, the X-Alt1 and X-Alt2 splice variants as described by Chandler et al. [47] could not be detected. In addition, we found that cisplatin (30 μM) strongly induced the switch in ratio between full-length mdmx- and mdmx-S mRNAs in the breast carcinoma MPE600 cell line (Figure 3). Similar results were obtained in other tumor cell lines, such as U2OS, upon treatment with comparable concentrations cisplatin or with 20 μM doxorubicin (data not shown).

At the moment it is not known via which mechanism alternative splicing of mdnx is induced upon genotoxic stress. From the literature, a few mechanisms can be proposed. First, it has been shown that upon stress the subcellular localization of proteins involved in splice site selection is affected, like the hSlu7 protein (3′ site selection) and the hnRNP A1 proteins (5′ site selection). Treatment of cells with osmotic shock or UV-C leads to cytoplasmic localization of hnRNP A1, which affects the choice of 5′ splice site [50]. Since with mdnx the change is the choice of the 3′ splice site, involvement of hSlu7 is more likely [51]. However, in that publication, it is mentioned that cisplatin treatment does not affect hSlu7 subcellular localization, so regulation of hSlu7 is most likely not the single mechanism by which the splicing of mdnx mRNA is altered. More recently, it was reported that alternative splicing upon UV-C treatment is coregulated with the inactivation of RNA polymerase II by inducing the hypophosphorylation of the CTD region [52]. Although such a mechanism cannot be excluded at the moment in the alternative splicing of mdnx mRNA, changes in subcellular localization of certain factors involved in splicing are likely to play a role in the alternative splicing of mdnx mRNA. We have found that the treatment of several types of cells with Leptomycin B, which blocks nuclear export of proteins containing a Rev-like nuclear export signal, strongly increases alternative splicing of mdnx, leading to increased levels of mdnx-S mRNA and reduced mdnx full-length mRNA (Figure 3), accompanied with a reduced MDMX protein level (not shown).

Even so, it is unclear what the biological importance of this alternative splicing is. It can be a means to reduce the amount of full-length MDMX protein (which indeed is found after sublethal doses of UV-C [31,53]), needed for full activation of p53. In this case, alternative splicing would lead to a loss of function. On the other side, it could be a mechanism to produce an MDMX protein form that is resistant to MDM2-mediated degradation, which could have a function in attenuating the p53 response at later time-points after the damage. In that case, it would be a gain of function.

Putative biological functions of protein products of the mdnx splicing variants will be discussed later.

Markey and Berberich [54] have recently uncovered another mechanism leading to reduction of MDMX protein levels upon genotoxic agents. They found that exposing cells to doxorubicin (0.5 μg/mL; which is ~0.85 μM) leads to a strong reduction in the total mdnx mRNA levels; this effect was found to be fully p53-independent. Similarly, cisplatin reduces mdnx mRNA levels in doses varying from 25 to 100 μM, on various cell lines, independent of their p53 status. In addition, the authors show that the decrease of MDMX protein levels after doxorubicin treatment could not efficiently be rescued by proteasome inhibition or knockdown of p53 and MDM2, when measured 24 hrs after start of treatment. It would have been nice if the authors would
have shown a time-course of doxorubicin treatment and analyzed both MDMX protein and mRNA. It could well be that initially degradation of the MDMX protein occurs upon the activation of the DNA damage signaling cascade, and that the reduced mRNA level is a later effect. These authors also investigated the alternative splicing of *mdmx* mRNA upon doxorubicin treatment. The remarkable observation here is that for this experiment they use a 100 μM concentration of doxorubicin, which is more than 100-fold higher than used in their initial experiment; so, it cannot be compared to the earlier experiment. It could be, but that is not commented upon, that at the lower concentration of doxorubicin no significant alternative splicing can be found. Anyway, the authors do find some increase in X-Alt2 levels, but actually a small decrease in X-Alt1. Again, total levels of *mdmx* mRNA strongly decrease, indicating that X-Alt2 only represents a very small fraction of the total *mdmx* mRNA. Interestingly, the decrease in *mdmx* mRNA levels is caused by decreased stability of the *mdmx* mRNA. The authors suggest a putative involvement of microRNA miR-34a in this regulation of *mdmx* mRNA stability. This is interesting in the view of the earlier observation that transcription of the miR34 family members is upregulated upon p53 activation, which has been shown to be important for a full p53 response upon DNA damage [55, 56]. However, the regulation of miR-34a by p53 does not explain the p53 independency of the *mdmx* mRNA destabilization upon DNA damage. Unfortunately, it was not addressed in this study whether the decrease in *mdmx* mRNA after doxorubicin or cisplatin treatment is important for the biological outcome of the treatment. So, the physiological importance of the observed *mdmx* mRNA reduction still has to be investigated.

4. A Proapoptotic Function of MDMX upon Lethal Doses of Stress

In all the experiments presented above, the idea was that a decrease in MDMX expression is needed to get a full-blown p53-response upon DNA damage. And indeed, in most cases, a decrease in MDMX levels is observed and some experiments have shown that prevention of that MDMX degradation is inhibiting the p53 response [31, 35]. However, these experiments were all done with a sublethal dose of IR or treatment with IR-mimetic agents like NCS.

The group of Fabiola Moretti has obtained results that appear to be in conflict with what has been discussed above. In an initial report, it was presented that in an (inducible) overexpression system high levels of MDMX actually stimulate doxorubicin (adriamycin)-induced apoptosis [57]. This correlated with an increased induction of Bax transcription, while p21WAF1 was induced similarly in control- and MDMX-overexpressing cells, suggesting that high MDMX expression facilitated p53-mediated induction of proapoptotic genes specifically. These experiments were all performed with the use of nontransformed mouse or human cells, with doxorubicin as the genotoxic agent. The main drawback of this study is that a pro-apoptotic function of endogenous levels of MDMX protein was not investigated. Furthermore, it could be that MDMX has a different function in untransformed versus tumor cells upon adriamycin treatment. In this respect, it is of interest to note that in the colorectal tumor cell line HCT116 an active p53 seems to inhibit adriamycin-induced apoptosis [58], so a pro- or antiapoptotic function of MDMX could very well be strongly cell type specific.

In a follow-up study, the authors made the very intriguing observation that a fraction of MDMX is localized to the mitochondria and that upon lethal stress MDMX could function as an anchor for S46-phosphorylated p53, leading to transcription-independent apoptosis [53]. Unfortunately, the genotoxic agents used were now UV-C and cisplatin, which makes comparison with the earlier publication difficult. Strikingly, it was found that low levels of UV-C irradiation did reduce MDMX protein levels in MCF-7 cells, but that high, lethal doses (40 J/m²) of UV-C did not or much less. At these high doses, reduced levels of MDM2 were found, fitting with earlier reports that lethal doses of genotoxic agents can inhibit mdm2 transcription [59, 60], partly by activation of HIPK2 [61, 62]. The results also lead to two tentative conclusions. First, the observed alternative splicing of *mdmx* mRNA after high UV-C doses in MCF-7 cells [47] would not lead to a reduction in full length MDMX protein. Second, under these conditions the earlier reported downregulation of *mdmx* mRNA levels upon genotoxic stress [54], admittedly a different type, is not occurring.

Most strikingly in this study is the observation that reducing MDMX levels by siRNA did decrease the induction of apoptosis upon UV-C irradiation. A similar result was obtained upon treatment of control- and MDMX-knockdown MCF-7 cells with a-aminitin, which blocks polymerase II transcription but is reported to induce a p53-dependent apoptosis strictly by stimulating translocation of p53 to the mitochondria.

Similarly, reducing MDMX levels in A2780 cells partly prevents cisplatin-induced apoptosis (20 μM cisplatin). This concentration of cisplatin did not induce significant alternative splicing of *mdm2* mRNA in MCF-7 or U2OS cells [47]. The effects on *mdmx* mRNA were not investigated in this study, but a similar dose of cisplatin (25 μM) caused a small upregulation of *mdmx* mRNA in MCF-7, but already a downregulation in some other cell lines [54]. We found recently in a few osteosarcoma cell lines that 20 μM cisplatin reduces the total levels of *mdmx* mRNA and increases the *mdmx*-S: *mdmx*-FL ratio, independent of the p53 status (Lenos et al., manuscript in preparation).

It is difficult to reconcile the data from Moretti and colleagues with the earlier presented data. However, there are a number of points to keep in mind. The data obtained with ionizing radiation or IR-mimetic usually did not induce a high level of apoptosis, but mainly a cell cycle arrest. When UV-C was used, it was at relative low doses (5 or 10 J/m²), which might give some apoptosis induction, but not very high. The alternative splices as described by Chandler et al. (2006) [47] were only seen at rather high doses of UV-C or cisplatin; these are in the same range as Moretti and colleagues used, but Chandler et al. did not investigate effects on protein levels. What can be noticed from that study
is that while the dose of UV-C resulted in almost complete alternative splicing of mdm2 mRNA (full length is hardly detectable anymore), the main mdmx mRNA expressed upon treatment with 50 J/m² UV-C is still the full-length mRNA. So, it is possible that under these ruthless conditions MDMX protein is still expressed.

What we do find somewhat surprising, though, is that apparently the induction of apoptosis by 40 J/m² is largely dependent upon the targeting of p53 into the mitochondria. In several cellular systems, it has been reported that UV-C (and cisplatin) can induce apoptosis in p53-null or p53-mutant cells [52, 63–65], indicating that wild-type p53 status is not a universal essential factor for UV-C or cisplatin-induced apoptosis. It will be interesting to see whether modulation of MDMX levels in p53-deficient cells also affects the UV-C or cisplatin-induced apoptosis.

In an attempt to understand and reconcile the various and sometimes contradicting results, a very well-structured study should be performed in which one or two wild-type p53 expressing cell lines, if possible with their p53-deficient counterparts, were treated with various types of stress-inducing agents, mainly genotoxic agents like doxorubicin, cisplatin, IR, and UV-C irradiation, but also with a Polymerase-II transcription inhibitor like α-amanitin. Cells should be extensively analyzed at early and late time-points for expression of relevant genes (p53, MDM2, MDMX, p53-targets) at protein and mRNA level (including analyses of splicing variants) and for the biological outcome of the treatment. The latter can be done primarily by FACS analyses to determine DNA profile (PI staining), apoptosis (Annexin V staining), and DNA replication (BrDU incorporation). The importance of MDMX regulation can be determined by using MDMX knockdown derivatives of the used cell lines. Such a study should be able to provide the answers to several questions regarding the regulation of MDMX by the various agents, and the importance of that for the biological response.

5. Role of MDMX in the Ribosomal Stress Response: Essential Part of the DNA Damage Response?

In the last years, a strong link between nucleolar- and ribosomal stress and p53 activation has been established. Very importantly, Rubbi and Milner showed that disturbance of nucleolar integrity is important for the DNA damage response [66]. As such, they already showed that the DNA damage response and ribosomal stress are causally linked. Several groups subsequently showed that upon nucleolar disruption, ribosomal proteins are released, and several of those interact with MDM2 and prevent the MDM2-mediated degradation of p53 (recently reviewed by [67, 68]). Very striking, none of the ribosomal proteins shown to interact with MDM2 interact with MDMX, suggesting a selective targeting by ribosomal proteins [69]. However, that does not imply that MDMX is not important for the ribosomal stress response. Indeed, these researchers show that upon treatment of cells with Actinomycin D or 5-FU, at concentrations that are reported not to induce a significant DNA damage response but only to give ribosomal stress, MDMX levels are downregulated. Importantly, that downregulation is needed for full p53 activation. The MDMX degradation is MDM2-dependent, but is not associated with increased phosphorylation of MDMX-Serine367, so is distinct from the ATM/Chk2-stimulated MDMX degradation. Interestingly, the treatment with these compounds leads to release of L11 protein from the nucleoli, which binds MDM2 and inhibits MDM2-mediated degradation of p53, but stimulates the MDMX ubiquitination by MDM2 [69]. MDMX over-expressing cells showed an increased resistance to apoptosis induction by 5-FU, and indeed MDMX also inhibited the growth-suppressing effects of 5-FU on xenografts of HCT116 cells. Also in this case words of caution are needed. In the model tested, the HCT116 cells, the 5-FU effects are largely dependent on wild-type p53 function, as shown earlier [38]. However, other studies indicate that the effects of 5-FU on colorectal tumor cells are only very limited dependent on p53 status, and then only at really low concentrations [70]. Furthermore, in a small panel of 4 breast cancer cell lines, all expressing wild-type p53 and sensitive to Nutlin-3, only two showed a partial p53-dependence on growth inhibition by 5-FU, while one cell line actually is sensitized for 5-FU by reduced p53 levels [71]. The differential effects can possibly in part be explained by a difference in pRB status, which seems to dictate the dependence of 5-FU responsiveness on wild-type p53 status [72]. Therefore, it would be of importance to investigate whether in other settings (e.g., p53 proficient, but pRB-deficient) MDMX levels also have an impact on 5-FU sensitivity.

A recent study from the Prives’ lab showed another cross-talk between ribosomal proteins and MDM2/MDMX regulation of p53 [73]. They identified the small ribosomal subunit protein S7 as an MDM2-interacting protein, which can inhibit the MDM2-mediated degradation of p53. Interestingly, they found that S7 could only perform its function sufficiently in the presence of MDMX. A putative involvement of S7 in MDM2-mediated degradation of MDMX was not investigated. A striking observation of these investigators was also that knockdown of either the ribosomal protein L11 or S7 attenuated the p53 activation not only by agents inducing ribosomal stress, but also DNA damaging agents like doxorubicin and neocarzinostatin. These data confirm the earlier mentioned observations by Rubbi and Milner, who already showed that DNA damage only affects p53 stability when it is causing nucleolar disruption [66].

6. Alternative Splicing of MDMX mRNA: What Is Its Use?

At the moment, 6 different splicing variants of MDMX have been described, which are schematically depicted in Figure 2. As mentioned before, the first reported splicing variant is the mdmx-S mRNA, expression of which seemed to correlate with transformed cells and with normal cells stimulated to grow after serum-stimulation [48]. This was attributed to the fact that MDMX-S protein, which essentially comprises
only the p53-binding domain (amino acids 1–114), and 26 unique C-terminal amino acids, is a stronger inhibitor of p53 as assayed by overexpression studies [49]. What is important to note is that the authors denote a protein of about 32 kDa in COS cells as the endogenous MDMX-S protein, which is significantly larger than the predicted molecular weight of approximately 16 kDa. The authors propose that this shift is caused by post-translational modification of the MDMX-S protein, but the type of modification was not determined. Strikingly, in vitro translation of the mdmx-S mRNA yielded also the 32 kDa protein, and a protein of about 17 kDa, which could represent the “real” MDMX-S protein. We have recently obtained evidence that the 32 kDa protein is most likely not representing the true MDMX-S protein.

Transfection of an MDMX-S expression vector, encompassing the total MDMX coding region but just lacking exon 6, with an N-terminal HA-tag, results in expression of a protein around 24 kDa detected with anti-HA antibody (Figure 4). Expression level of this protein is much lower than that of full-length HA-MDMX protein upon transfection of the same amount of plasmid DNA. In addition, smaller proteins around 18 kDa and 14 kDa are found, which are even lower expressed. However, we also made an expression vector in which we truncated the mdmx cDNA at an XbaI restriction site, just downstream of the predicted stop codon of MDMX-S protein in exon 7. So, this construct theoretically has the same coding potential. When this construct is expressed, only the smaller proteins of
about 18 and 14 kDa can be detected with anti-HA, again at low levels compared to full-length HA-MDMX. Importantly, an antibody raised against the C-terminal unique amino acids of MDMX-S (Frank Bartel, personal communication) recognizes only the 18 kDa protein, identifying that as the real MDMX-S protein. The anti-MDMX antibody MX-82, with its epitope around amino acid 100 of MDMX, recognizes both the 18 kDa and 14 kDa protein, but not the 24 kDa protein. Two conclusions can be drawn from these results. First, the 24 kDa protein, possibly reflecting the 28 kDa protein mentioned by Rallapalli et al. [48], is most likely an artifact of the overexpression of the mdmx-S cDNA, because it appears to lack the MX-82 epitope which should be included in the MDMX-S protein. Secondly, the smaller protein of about 14 kDa lacks a C-terminal part of MDMX-S; again most likely some artifact of the overexpression. Importantly, when we use the same expression plasmids for in vitro translation, we only detect the 18 kDa protein, either with an anti-MDMX antibody (1328; raised against bacterially produced MDMX-S protein) or with anti-MDMX-S specific antibody (Figure 4). In addition, His6-tagged bacterially produced MDMX-S protein also has an apparent molecular weight of about 18 kDa.

As mentioned, we find that the MDMX-S protein is much lower expressed compared to full-length MDMX, in contrast to previous reports, but confirming our previous finding [20]. This is not a result of transfection efficiency, as can be seen by the expression of the transfection control (Figure 4), and similar results were obtained after transduction of cells with lentiviral expression vectors for MDMX-S (not shown). Furthermore, real-time Q-PCR analysis showed that mdmx-S mRNA was even higher expressed than full-length mdmx in these transfections (Figure 4(b)). Notably, treatment of transfected or transduced cells with proteasome inhibitor MG132 strongly increased the MDMX-S protein level, suggesting that the protein is very unstable. The levels of full length MDMX protein are only mildly increased upon MG132 treatment, while no effect is seen on the 24 kDa protein. Although we have no evidence at the moment, the rapid degradation of MDMX-S is possibly caused by the unfolded protein response (UPR); it is possible that this truncated MDMX protein is not properly folded, and as such is targeted via UPR for degradation.

In addition, we have put much effort in detecting the endogenous MDMX-S protein, which we expected not to be such a problem because the recently developed monoclonal antibody, MX-82, has its epitope within the MDMX-S region. However, even in cell lines that express more mdmx-S mRNA compared to FL-mdmx mRNA, only full-length MDMX protein can be detected (data not shown). Therefore, we propose that the induction of mdmx-S mRNA is a means of reducing full-length MDMX levels, and not so much the generation of a new MDMX Figure 3, where it can be seen that full-length mdmx mRNA decreases as mdmx-S mRNA levels increase upon various stress conditions, whereas MDMX-S protein levels are still undetectable (not shown).

The MDMX-A and MDMX-G variants were cloned from C33A cells, a somewhat exceptional cell line in that it expresses high levels of mutant p53 but also high levels of MDMX [30]. The corresponding MDMX proteins could not unequivocally be identified, no biological function has been determined, and whether this particular splicing event is affected by any event, like stress conditions, is unknown. Therefore, these splicing variants will not be discussed here any further.

As mentioned above, UV-C irradiation was found to lead to alternative splicing of MDMX, resulting in X-Alt1 and X-Alt2. Again, no functional studies have been performed, synthesis of predicted alternative MDMX proteins has not been investigated, and the ratio between the full-length MDMX and the alternative splicing variants is not known. Therefore, a biological relevance of these splicing variants is still hypothetical.

Giglio and colleagues are the only group to report functional experiments on an endogenously expressed MDMX splice variant [74]. They identified an mdmx mRNA variant, which they called mdmx211, in a thyroid tumor cell line (ARO), which has structural similarity to the X-Alt2 variant (see Figure 2). The protein products produced by these variants both lack the major part of the p53-binding domain, but still contain the C-terminal domain including the RING finger. A difference is that the X-Alt2 variant contains all exon 11 encoded amino acids, including the Zn-finger, while the mdmx211 variant lacks the N-terminal part of the exon 11. Overexpression experiments indicate that MDMX211 protein can stabilize MDM2 and prevents the degradation of p53 by MDM2, reflecting what has been published about full-length MDMX protein [75]. Overexpression of MDMX211 increased colony formation of both p53-wt and p53-negative cells, indicating a p53-independent oncogenic role of MDMX211. Physiologically more relevant is the specific knockdown of this MDMX variant in ARO cells. Reducing levels of mdmx211 mRNA about 50% reduces the viability of this cell line, suggesting that both full-length MDMX and the alternative MDMX211 form are contributing to the proliferation of these cells. However, the effects of specific knockdown of full-length MDMX was not investigated in parallel, nor were relative levels of MDMX and MDMX211 proteins compared. Knockdown of MDMX211 did reduce MDM2 levels, but p53 levels or activity was not investigated, probably because these cells harbor a mutant p53. Again, these results are indicating a p53-independent oncogenic function of MDMX211. It is unfortunate that the authors have not studied the effect of specific knockdown of MDMX211 and full-length MDMX in the FRO cell line, which appears to express similar levels of MDMX211, but contains a wild-type p53 gene [76]. The comparison between MDMX211 and FL-MDMX in this cell line would especially have been interesting in the experiment investigating the sensitivity to adriamycin treatment. As mentioned earlier, Mancini and colleagues published that overexpression of full length MDMX could increase the sensitivity to adriamycin [57]; so, one would predict that knockdown of MDMX would increase the number of viable cells after adriamycin treatment. This would then indeed be opposite from the effects of knockdown of MDMX211 in ARO cells, which increases the sensitivity to adriamycin in
these thyroid tumor cells [74]. All in all, these results indicate that MDMX-211 has oncogenic potential, but whether that is increased compared to full-length MDMX is not known at the moment.

A number of studies investigated the expression levels of mdmx mRNA and of mdmx splicing variants in a panel of tumors. An analysis of malignant gliomas showed an amplified mdmx gene in a subset of tumors, none of which had p53 mutation. Interestingly, ratio between mdmx-S/mdmx-FL mRNA was found to be significantly higher in grade IV glioblastomas compared to grade III anaplastic gliomas or grade II gliomas [77]. Bartel and colleagues studied 66 soft tissue sarcomas for mdnx gene amplification and mdmx-FL/mdmx-S mRNA expression [78]. They also found an association between tumor grade and overexpression of the mdnx-S splice form. Furthermore, from their analyses they concluded that the mdnx-S is a prognostic factor that predicts a poor outcome and a significantly shortened survival time for STS patients. No correlation between p53 status and mdnx-S expression was evident. Keeping in mind that our analyses so far suggest that increase in the ratio of mdnx-S mRNA compared to mdnx-FL mRNA is more a means to decrease full-length mdnx, it could imply a tumor suppressor function for the MDMX protein, as is also suggested from the study of Moretti and colleagues [79]. They found that in 57 papillary thyroid tumors, which all contain a wild-type p53 gene, a decrease of full length MDMX is associated with tumor stage (stage 1 versus others), indicating downregulation of MDMX protein levels during tumor progression. An increased ratio mdnx-S/mdnx-FL (ratio ≥1.5) mRNA is found in a significant percentage of these thyroid tumors, but is not associated with any clinical data. Expression of the mdnx211 mRNA is also found in a subset of tumors, again not correlating with any patient data. Together, these data on the expression of mdnx-FL and mdnx mRNA splice variants in combination with other analyses of mdnx gene amplification, mdnx mRNA expression and MDMX protein analyses in tumors [80–83] strongly indicate that the function of MDMX in tumorigenesis is strongly dependent on the tumor type. Although in several tumor types MDMX appears to have an oncogene function, in others it seems to be antioncogenic. So far, the study by Moretti and colleagues has been the only indicating a tumor suppressor role of MDMX in wild type p53 expressing human tumors; unfortunately, the authors do not show any data on the levels of p53 in these tumors. Possibly, the p53 gene is still wild type but the expression of the p53 mRNA is silenced yielding an essential p53-null tumor. This is important in the light of other publications in mouse models indicating an antiproliferative function of MDMX [84, 85]. In the first study a growth suppressive function of mouse MDMX in a p21/WAF1-deficient background was observed [84]. In addition, in a p53-null background loss of MDMX increased the growth of mouse embryo fibroblasts and endogenous levels of MDMX suppresses tumorigenicity in p53-null mice [85]. In p53-null cells, MDMX prevents the chromosome loss in hyperploid cells by inhibiting spindle multipolarity, in that way stimulating genome stability. Thus, under certain conditions MDMX indeed can function as an antioncogene. It would be interesting to investigate whether the reduced MDMX expression in later stage thyroid tumors [79] is correlated with low expression of p21/WAF1 and/or aneuploidy. Recently, two mouse models have been published that overexpress an mdmx transgene. Unfortunately, the mice show different phenotypes, which cannot easily be explained at the moment. In the first model, the generally overexpressed MDMX protein induces spontaneous tumor formation, mostly sarcomas, indicating an oncogene function of MDMX [86]. MDMX overexpression also strongly accelerated the tumor development of p53 heterozygous mice. Unfortunately, the effect of MDMX overexpression was not investigated in a p53-null background. In the light of the study of Jones and colleagues [85], showing that loss of MDMX in p53-null mice accelerates tumor formation, one might predict that overexpression of MDMX should reduce the rate of spontaneous tumor formation in the p53-null mice. Surprisingly, the second mdmx transgenic mouse model, in which a Myc-tagged MDMX is overexpressed, does not accelerate tumor formation, neither the spontaneous nor the Epi-Myc induced tumorigenesis [87]. Interestingly, only a heterozygous myc-mdmx transgenic mouse is viable; the homozygous transgenic mice die during embryogenesis in a p53-independent manner, due to massive vascular maturation defects. An in-depth side-by-side comparison between the two mdmx transgenic mouse models could possibly explain the observed differences in phenotypes.

In conclusion, most available data suggest that the human mdmx gene functions as an oncogene when overexpressed in tumors still expressing wild-type p53, which is supported by most mouse models. However, the question whether expression of MDMX or alternative MDMX proteins produced by alternative splicing has an oncoprotein or a tumor suppressor protein function in tumors lacking p53 (or p21) or expressing a mutant p53 has still to be answered.

7. Transcriptional Regulation of MDMX Expression

Up till recently, it was only Gilkes and colleagues who investigated regulation of transcription initiation of the mdmx gene [88]. They found that, like the mdm2 P2 promotor, mitogenic signaling stimulates transcription of the mdmx promotor. Especially two Ets-family binding sites are important in that respect, and the MEK/ERK signaling pathway was found to be involved in the induction of MDMX promotor activity upon growth factor stimulation. Expression of oncogenic K-Ras also activated the MDMX promotor. Importantly, in a panel of colon tumors they find an increasing MDMX expression with tumor stage, and this increased expression of MDMX does correlate to some extent with increased activity of the MAPK pathway as measured by phospho-ERK staining. It is intriguing to note that around 50% of later stage colon tumors stain positive for MDMX in this study and that approximately 50% of colon tumors contain p53 mutations. It would have been interesting had the authors determined...
the p53 status of the samples they used for MDMX staining to investigate a putative-negative-correlation.

These results indicate that enhanced mitogenic signaling in tumor cells which have lost p14ARF expression might inhibit the tumor suppressor activity of p53 by enhancing both mdmx and mdm2 gene transcription [88, 89].

More recently, a new twist to the p53/MDM2/MDMX interactions has been added. Wei and colleagues had reported that the intron 1 of the human mdmx gene contains a p53RE, as identified in a global p53 ChIP experiment [90]. Li et al. were the first to publish that this p53RE indeed mediates transcriptional activation of the mdmx gene upon activation of p53 by Nutlin-3 [91]. Furthermore, they provide evidence that reducing MDMX levels in the TGCT (testicular germ cell tumor) cell line NT2/D1 induces low levels of apoptosis, but strongly sensitizes these cells for growth inhibition by Nutlin-3. However, this effect was not observed in other TGCT cell lines tested. The authors do suggest that in the NT2/D1 cells MDMX is involved in a negative feedback loop, similar to MDM2. These studies do not distinguish between the importance of the constitutive mdmx transcription and the p53-induced transcription. In collaboration with the groups of Jeremy Blaydes and Frank Bartel, we also recently reported that the previous identified p53RE in the first intron of mdmx is directing p53-induced transcription of the mdmx gene [92]. In addition to the earlier publication, we showed that the p53RE initiates transcription from an alternative promoter (mdmx P2), which includes an alternative first exon (named exon 1β) in the mdmx mRNA. This exon contains an in-frame upstream ATG relative to the canonical first ATG, which results in the synthesis of 18 extra-N-terminal amino acids. It was found that the mdmx P2 promoter can be induced in almost all cells tested that express wild-type p53 (only a few primary human cell cultures showed a very low or undetectable expression). Furthermore, every type of p53 activation (Nutlin-3, DNA damage, ribosomal stress, Leptomycin B, activation of p14ARF expression) increased transcription from the mdmx P2 promoter. Most importantly, the inclusion in the mdmx P2 mRNA of a unique exon provided us with the opportunity to investigate specifically the contribution of the p53-induced mdmx mRNA expression in the p53 response. Indeed, shRNA targeting sequences in the unique exon 1β increased the p53 response upon treatment of cells with Nutlin-3 or DNA damage (etoposide) and enhanced the p53-induced growth inhibition. These results together strongly establish mdmx as a p53 target gene, involved in a negative feedback loop to attenuate the p53 response.

8. Conclusions and Future Perspectives

When the identification of the mdmx gene was presented in 1996 [18], the p53 field was not immediately impressed. The added value of MDMX to MDM2 could not be recognized. MDM2 was expected to be sufficient to keep p53 in check and to regulate the p53 response. However, the p53-dependent embryonal lethality of mdmx knockout mice established MDMX as an essential regulator of p53 activity. In more recent years, it has become clear that during development MDM2 is the more general regulator of p53 activity. However, the intricate regulation of MDMX has been shown to be essential for a controlled p53 response upon various forms of stress, as much as MDM2 regulation. Although great insight into regulation of MDMX expression and activity has been obtained, much has still to be learned. How is MDMX degraded upon ribosomal stress, since DNA-damaged induced phosphorylations are not involved? How is the alternative splicing of MDMX upon certain types of genotoxic stress regulated? Do the various identified mdmx mRNAs encode proteins with important biological functions in the coordination of stress response or in tumorigenesis? Under which circumstances is MDMX acting as an oncogene, and when is it stimulating the p53 tumor suppressor response? The answers to these questions will be important for understanding the role of MDMX in tumorigenesis, and for obtaining more insight into the possibility to regard MDMX as a target for therapeutic intervention in cancer.

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