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Regulation of MDM4

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1. ABSTRACT

Mouse double minute 4 (MDM4), also known as MDMX or HDMX (human MDMX), is a critical negative regulator of the tumor suppressor p53. Under normal growth conditions, MDM4 contributes to the repression of p53 activity. Upon DNA damage, it becomes important to down-regulate MDM4 to allow a full p53 response. Here, the mechanisms by which MDM4 activity is controlled are reviewed and discussed, starting with alterations in copy number, then control of transcription, mRNA stability, translation, and finally post-translational interactions, modifications, localization, and targeting by recently developed drugs.

2. INTRODUCTION

Mouse Double Minute 4 (MDM4, MDMX, HDMX) was originally cloned in 1996 (from mouse (1)) and 1997 (from human (2)) in a screen to find proteins that bind the tumor suppressor p53. Since then, it has been recognized as a kind of counterpart to the related protein MDM2. Both are able to bind p53 and inhibit transactivation of p53 target genes. MDM2 is able to act as an E3 ubiquitin ligase to target p53 for degradation, but despite their similarities, MDM4 does not definitively have this E3 activity. Nevertheless, MDM4 has become recognized as a critical regulator of p53. This is underscored by the fact that knockout of MDM4 is

embryonic lethal, but can be rescued by loss of p53 (3-5), similar to the MDM2 knockout. MDM2 and MDM4 play non-overlapping roles in regulation of p53, as knockout of MDM4 in the heart does not prevent normal development (6), and knockout in the central nervous system causes cell death by apoptosis for MDM2 and cell cycle arrest for MDM4 (7). Further knockouts of these genes demonstrated that MDM2 mainly functions to control p53 stability, while MDM4 controls p53 activity (8, 9). MDM4 must be repressed in order to fully activate p53. MDM4 also influences p53 activity through other mechanisms, and has biological significance apart from p53, evidenced by promotion of bipolar mitosis by MDM4 in the absence of p53 (10).

Despite the importance of MDM4, it has been less well studied than MDM2 and relatively less is known of its regulation. There have been a number of good reviews in recent years on individual aspects of MDM4 regulation and functions, such as its role in apoptosis (11), splicing of MDM4 (12), and the importance of pharmacological targeting of MDM4 (13-15), but little specifically dealing with regulation of MDM4. Therefore, here I will present a review of what we know about how MDM4 is regulated, from amplification of gene copy number to post-translational interactions and modifications.

3. COPY NUMBER

MDM4, located at in humans at 1q32, is amplified in a significant percentage of cancers, many of which retain wild-type p53. Amplification of MDM4 was first identified in a small fraction of malignant gliomas. Riemenschneider *et al.* (16) observed MDM4 5- to 25-fold amplification in 2.4% (5 of 208) of gliomas, all of which carried wild-type p53 and did not show amplification of MDM2. Initially there was some controversy over whether it was MDM4 amplification that was favored in the development of malignant gliomas or the neighboring gene, contactin 2 (CNTN2, (17)). A follow up study of 17 genes near MDM4 in 8 malignant gliomas with amplification of 1q32 showed that amplification of MDM4 was the only event common to the tumors, with the other genes representing coamplification ((18)).

In a study of over 500 primary tumors, Danovi *et al.* (19) observed MDM4 overexpression in a variety of tumors. 18.5% of 27 colon cancers, 18.2% of 88 lung cancers, 42.9% of 14 stomach cancers, 27.3% of 11 testicular cancers, 23.1% of 13 cancers of the larynx, 15.4% of 13 uterine cancers, and 14.3% of melanomas showed overexpression of MDM4. None of the 25 prostate cancers screened showed MDM4 amplification. 18.8% of breast cancer samples showed at least 3-fold overexpression of MDM4. By FISH, 5% of breast cancers were shown to carry over 6 copies of MDM4, and an additional 33% had lower gains of 4-6 copies. Importantly, there was mutual exclusivity between gain of MDM4 or MDM2 and p53 mutation. None of the breast tumors with amplification of MDM4 carried mutations in p53 or amplification of MDM2. Looking at human tumor cell lines from a variety of tissues, Ramos *et al.* found MDM4 overexpression in 13 of 31 lines (41.9%) (20). Similarly, MDM4 overexpression has been observed in almost 50% (39/99) of colon tumors (21). These may or may not have been due to increased copy numbers, but it underscores the significant frequency of increased MDM4 expression.

A fourth study of gliomas was carried out by Arjona *et al.* (22). Eighty-six samples were analyzed for amplification of genes at 1q32. Although 65% of the samples showed amplification of at least one gene, MDM4 was only the second most common of the four genes examined, present in 27.9% of samples. LRNN5 (leucine-rich repeat protein, neuronal, 5), in comparison, was amplified in 51%. Interestingly, MDM4 amplification was already present in 28.6% of low-grade astrocytic tumors, showing that MDM4 amplification may represent an early event in carcinogenesis. In contrast to other studies, 52% of glioma samples with MDM4 amplification also had MDM2 amplification or mutations in p53. In another study from the same lab (23), only 4 of 40 oligodendrogliomas displayed amplified MDM4, whereas 33% showed amplified LRNN5.

66 soft tissue sarcomas (STS) were examined by Bartel *et al.* (24) for a splice variant of HDMX termed HDMX-S (discussed later) and for MDM4 amplification. 17% of STS samples had up to 9-fold amplification of the MDM4 gene, which correlated to a tumor's staging but not grading, and was associated with poor prognosis. MDM4 appeared to be the target of amplification, as none of 5 tumor samples without MDM4 amplification showed amplification of adjacent genes.

Retinoblastoma, a tumor type originally hypothesized to arise from intrinsically death-resistant cells of the retina upon loss of both copies of the retinoblastoma tumor suppressor (RB), has long been associated with gain at 1q. In a review of retinoblastoma cytogenetics, Potluri *et al.* found that 44% of retinoblastoma tumors had additional copies of chromosome 1q (25). Meta-analysis of retinoblastoma CGH studies by Corson and Gallie (26) showed 53% of these tumors carry gains of 1q. Retinoblastoma was found to have frequent amplification of MDM4 (27). Of 49 retinoblastoma samples examined, an astonishing 65% has additional copies of MDM4. As observed for breast cancers (19), there was a strong inverse correlation between amplification of MDM4 and amplification of MDM2 or mutation of p53. Countering the importance of MDM4 in retinoblastoma, Dimaras *et al.* (28) studied copy number gains during progression from retina to retinoma to retinoblastoma in 4 patients. In each case, KIF14 (at 1q31) was gained. MDM4 was only significantly gained (5 copies) in one case, where it was co-amplified with the nearby KIF14.

Another tumor type with high expression of MDM4 is head and neck squamous carcinoma (HNSC, (29)). Although copy number changes were not examined, high MDM4 protein levels were detected in 50% of 56 HNSC samples by IHC. Many of these also showed high levels of MDM2, and 67.9% of 28 tumors positive for MDM4 simultaneously carried high levels of wild-type

p53. Again, overexpression of MDM4 or MDM2 appears to be mutually exclusive of p53 mutation.

Schlaeger *et al.* (30) used array comparative genomic hybridization (aCGH) to examine 67 samples of hepatocellular carcinomas (HCC) of varying etiologies. One sample was found to have low level amplification of MDM4. However, significantly increased MDM4 expression was observed in a pool of 20 HCC and 4 HCC cell lines compared to normal liver tissue. 11 of 24 samples examined by western blot showed increased MDM4 protein expression. Amplification and/or overexpression of MDM4 was independent of etiology.

In urothelial cell carcinoma (UCC), MDM4 is often gained (31). 16.1% of 109 samples had gains of MDM4 (1.2% had extrachromosomal amplification), and this correlated with high expression of MDM4 (2-3 fold), and invasiveness. Again, gains of MDM4 were mutually exclusive of p53 mutation.

The frequency of MDM4 amplification independent of p53 mutation or gains of MDM2 emphasizes the importance of MDM4 in control of p53 activity. Interestingly, there is tissue specificity in the frequency of MDM4 amplification in human cancer, with very frequent gains in retina, but apparently little selective advantage provided to prostate cells, for example. Further cytogenetic studies will continue to fill out our picture of MDM4 across additional tumor types, and follow-up studies at the molecular level may elucidate the causes of tissue specificity.

4. TRANSCRIPTION

Even when it was first cloned, MDM4 was observed to not be induced in response to UV irradiation (1), immediately differentiating its transcriptional control from that of MDM2. MDM4 promoter activity has been generally thought to remain relatively stable throughout cell growth and arrest, but there have been a few reports to the contrary. A study of the interaction of ribosomal proteins with MDM4 uncovered a 20% reduction in MDM4 mRNA and 30% reduction in promoter activity in response to ribosomal stress (through low-level acinomycin D treatment), but not to DNA damage (32). I observed no consistent decrease in MDM4 promoter activity following DNA damage, but did observe a decrease in full length MDM4 (flMDM4) mRNA (discussed in *5.1 pre-mRNA splicing*) (33).

There has to date been one published study that looked exclusively at regulation of the MDM4 promoter (21). Because MDM4 overexpression apparently occurs more frequently than MDM4 gene amplification, Gilkes *et al.* examined the promoter of MDM4 for transcription factor binding sites that could regulate its expression. Four conserved DNA binding sites for the c-Ets-1, Elk-1, Aml-1, and Cdxa transcription factors, were located within the 120 base pairs upstream of the transcription start site. Mutation of these sites led to decreasing MDM4 promoter activity and decreased MDM4 protein expression. Loss of the Aml-1, c-Ets-1, and Elk sites in particular was able to reduce promoter activity to nearly the level of the promoterless vector. Overexpression of Ets-1 was able to induce the MDM4 promoter, and conversely, knockdown of Ets-1 and Elk-1 decreased promoter activity. Gilkes *et al.* were able to show that MDM4 expression is induced by mitogenic signaling through Ras/Raf/MEK/ERK signaling pathway, which increased binding of Ets-1 and Elk-1 to the MDM4 promoter. Stimulation of the MAPK pathway by IGF-1 was shown to increase MDM4 expression. Colon cancer samples positive for phospho-ERK (indicating active MAPK signaling) were twice as likely to have MDM4 overexpression. The importance of transcription in control of MDM4 levels was underscored by the fact that MDM4 protein levels correlated with mRNA levels in a panel of cell lines, *but did not correlate with MDM2 expression* in unstressed cells, nor was MDM4 protein half-life increased by MG132 in unstressed cells. It appears, therefore, that under normal growth conditions it is transcription, rather than proteosomal degradation brought on by MDM2, which is primarily responsible for control of MDM4 expression.

5. POST-TRANSCRIPTION

5.1. pre-mRNA splicing

Similar to MDM2, several alternatively spliced forms of MDM4 have been identified and characterized. An excellent graphic depicting these isoforms is given in Jeyaraj *et al.* (12). The earliest, MDM4-S (MDMX-S) is the result of a 68 base pair internal deletion in exon 6, which shifts the reading frame to create a stop codon in exon 7 (34). Therefore, the encoded protein consists of the p53 binding domain (amino acids 1-100, (1) followed by 13 unique amino acids. This unique tail appears necessary for the enhanced stability of the protein (35). Strangely, it is also required for optimal binding to p53, despite being outside the p53 binding domain. MDM4-S was originally identified in murine NIH3T3 cells, and then in human fibroblasts and human tumor cell lines (34, 36). MDM4-S was more easily detected in growing cells. When *in vitro* transcribed and translated, the MDM4-S protein is 17 kDa in size, but when expressed *in vivo* it is much larger at 27 kDa, presumably due to unknown post-translational modifications. When overexpressed, MDM4-S was able to strongly repress p53-mediated transcription, reducing apoptosis. MDM4-S showed greater binding to p53 than does full length MDM4 (flMDM4), which could account for the stronger repression of p53. In view of p53's activity in shuttling MDM4 into the nucleus (37), this could also be the reason MDM4-S was partly present in the nucleus (35). Clinically, MDM4-S was the major MDM4 gene product in 14% of soft tissue sarcomas and correlated with decreased survival and increased risk of tumor-related death (24, 38). MDM4-S was also found to be overexpressed in 29 of 83 papillary thyroid carcinoma samples with wild type p53, and present in matched normal tissue (39). One would expect from these data that knockdown of MDM4-S would potentially reactivate p53. This remains to be demonstrated, as does the efficacy of MDM4-targetted drugs against this highly truncated protein.

MDM4-A (Mdmx-A) and MDM4-G (Hdmx-G) were identified by de Graaf *et al.* (36) from human C33A

cervical carcinoma cells. MDM4-A is the result of a small deletion 50 amino acids in the center of MDM4, removing the acidic domain while remaining in frame. MDM4-G is lacking 97 amino acids, mostly within the p53 binding domain. Accordingly, MDM4-A was able to bind p53 while MDM4-G was not. MDM4-A inhibits p53, and weakly increased the levels of MDM2 protein. MDM4-G strongly increased MDM2. Conversely, both were reduced by the ubiquitin ligase activity of MDM2, independent of caspase activity. Interestingly, MDM4-A is especially susceptible to MDM2-mediated degradation. This is complicated by the presence of a second, slightly larger MDM4-A protein band that is more stable. Modifications of MDM4-A may therefore stabilize the protein, but these modifications remain to be identified. Expression of these variants outside of C33A cells has not been observed, and indeed, expression of endogenous MDM4-A or MDM4-G protein has not been observed even in C33A.

MDM4-211 (HDMX211) was cloned from the human thyroid tumor line ARO (40). It was so named because the protein consists only of amino acids encoded by exons 2 and 11 including a portion of the p53 binding domain (insuffienct to bind p53), the caspase signal, and the ring finger. Therefore MDM4-211 is able to bind MDM2 but not p53, similar to MDM4-G. Binding to MDM2 inhibits its E3 ubiquitin ligase activity, stabilizing p53 and MDM2 itself. Again, this is reminiscent of the increase of MDM2 by MDM4-G expression (36) or by full length MDM4 (41, 42). Similar to other splice variants, there was no correlation between MDM4 expression and MDM4-211 expression. Interestingly, the increase in p53 elicited by MDM4-211 does not result in increased p53 activity, as p53 remains associated with the (now E3 incompetent) MDM2. MDM4-211 expression was able to increase colony formation, and knockdown decreased viability. It was found to be expressed in 2 of 5 non-small cell lung tumors which also expressed high MDM2 protein (and zero of 16 normal samples) . It seems therefore that this variant promotes tumorigenesis in the presence of MDM2 and p53. In the light of MDM4-G and MDM4-211, it will be interesting to see if all MDM4 variants deficient in p53 binding have oncogenic effects in the presence of stable MDM2 and p53. MDM4-211 was later found to be overexpressed in 15 of 83 papillary thyroid carcinoma samples with wild type p53, but not in matched normal tissue (39). Tumors overexpressing MDM4-211 also showed increased MDM2 protein expression, consistent with the ability of MDM4-211 to inhibit MDM2 ubiquitination and auto-ubiquitination.

MDM4-ALT1 (XALT1) and MDM4-ALT2 (XALT2) were identified by Chandler *et al.* in MCF7 and H1299 cells exposed to UV (43). The MDM4-ALT1 transcript consists of the 5' end, including the p53 binding domain, followed by a portion of exon 10. The exons encoding the zinc finger, caspase signal, and Ring domain are lacking (or deleted). The MDM4-ALT2 transcript includes sequence encoding a portion of the p53-binding domain along with all of exons 10 and 11, which encode the zinc finger, caspase signal, and p53-binding domain. These transcripts have not yet been shown to express a protein. If expressed, an MDM4-ALT1 protein is predicated to function similarly to MDM4-S, which also contains the p53 BD but not the Ring domain, to repress p53 (34, 35). An MDM4-ALT2 protein would possibly resemble MDM4-G (36) and MDM4-211 (40) in binding and stabilizing MDM2.

In response to DNA damaging agents cisplatin and doxorubicin, we recently showed that the levels of full length MDM4 mRNA are decreased in cancer cell lines and normal fibroblasts (33). This decrease did not appear to be due to changes in promoter activity, but did correspond to increases in the damage-induced splice variant MDM4-ALT2. Interestingly, MDM4-ALT1 levels were unchanged. Additionally, there are potential binding sites for the microRNA miR34a in the 3' UTR of MDM4, and miR34a has been shown to be upregulated by DNA damage (44-49). This is discussed further in *5.2 mRNA stability and microRNAs*.

5.2. mRNA stability and microRNAs

The decrease in flMDM4 in response to DNA damage does not appear to be entirely due to the diversion of MDM4 pre-mRNA toward the damage-induced transcripts MDM4-ALT1 and MDM4-ALT2 (33). In absolute terms, the number of MDM4-ALT2 transcripts increased only about $1/100^{th}$ of the amount by which flMDM4 transcripts decreased. Obviously there could be other splice variants of MDM4 that remain to be identified. However, a decrease in the half-life of flMDM4 mRNA was also observed following DNA damage, from almost 6 hours in undamaged MCF7 cells down to 4 hours with doxorubicin treatment. This suggests active regulation at the level of mRNA stability.

One mechanism by which this could be achieved is the interaction of a damage-induced microRNA, targeting MDM4 mRNA for degradation. A possible culprit is miR-34a, which has been shown to be upregulated by DNA damage (44-49) and which has been reported to decrease MDM4 mRNA levels in HCT116 when overexpressed (45). The MDM4 3' UTR is of extraordinary length (over 9400 base pairs in humans) and contains a cluster of potential miR-34a recognition sites (33). Furthermore, introduction of anti-miR-34a increased MDM4 expression in undamaged MCF7 cells. Whether this is a direct effect on the 3' UTR of MDM4 mRNA is currently under investigation. Interestingly, the MDM4 3' UTR also contains five K-box sequences, which are similar to the recognition sequence for miR-23a. Overexpression of miR-23a, however, did not affect MDM4 levels in preliminary experiments (data not shown).

6. TRANSLATION

There are to date no reports of specific regulation MDM4 protein levels by modulation of mRNA translation. However, MDM4 likely plays an indirect role in the control of global cap-dependent translation through its inhibition of p53. The eukaryotic initiation factor 4E (eIF4E), which is critical for cap-dependent mRNA translation, is itself

regulated by p53. p53 binds to c-myc, preventing c-myc from increasing transcription of eIF4E. Inactivation of p53 by MDM2 reciprocally increases eIF4E promoter activity (50), and it is reasonable that inhibition of p53 by MDM4 would have the same effect. Additionally, active p53 is able to cause accumulation and dephosphorylation of 4E-BP1 to inhibit protein translation (51-53).

7. POST-TRANSLATION

7.1. Protein-protein interactions and modifications

There have been myriad proteins reported to interact with the full-length MDM4 protein. Many of these interactions impinge upon the ability of MDM4 to repress transactivation by p53, but there are many other functions attributed to MDM4. Another recent review detailed several binding partners of MDM4 (54). Here, I will briefly enumerate and summarize the protein-protein interactions and modifications of MDM4, which are illustrated in Figure 1.

As mentioned previously MDM4 is able to bind p53, thereby inhibiting its ability to transactivate target genes that promote cell cycle arrest or apoptosis (1, 2, 55, 56). Besides direct binding, MDM4 is able to stabilize MDM2 (41, 42) and promote the ubiquitin ligase activity of MDM2 through heterodimerization to reduce p53 protein levels (57). Overexpression of MDM4 relative to MDM2 can have the opposite effect, stabilizing p53 (56). MDM4 also binds ASPP1 and ASPP2, preventing them from activating p53 (58). Interaction of MDM4 with p300/CBP prevents activating acetylation of p53 (59, 60).

Downstream of p53, MDM4 has been shown to act in several ways on the RB tumor suppressor. MDM4 can promote degradation of $p21^{Ink4a}$ (an inhibitor of cyclin dependent kinases which inactivate RB) possibly through bringing it together with the 26S proteosome (61-63). MDM4 has also been shown to interact directly with and inhibit the activity of E2F1 (64, 65). Conversely, E2F1 is able to indirectly cause the degradation of MDM4, probably through activation of a cathepsin-like protease (66). Furthermore, MDM4 can inhibit the ubiquitin ligase activity of MDM2 toward RB itself (67, 68), which would allow accumulation of RB and inhibition of cell cycle progression. Through interaction with the receptor SMADs SMAD2 and SMAD3, MDM4 can prevent propagation of TGF-β signaling (59, 69), primarily allowing cell cycle progression but presumably other effects due to the pleiotropic nature of TGF-β.

Many signals converge on MDM4. Immediately upstream is MDM2, which ubiquitinates MDM4, targeting it for degradation (36, 70, 71). This activity of MDM2 is promoted by interaction with the ribosomal protein L11 in response to ribosomal stress but not DNA damage (32). Binding of 14-3-3 encourages MDM2 ubiquitination of MDM4 by colocalizing MDM4 to the nucleus following DNA damage (72-74). There is some controversy over the effect of 14-3-3 interaction, as others have reported that this stabilizes MDM4 to inhibit p53 (72) or causes cytoplasmic localization of MDM4 (75). Import of MDM4 can alternatively be carried out by importin α, although this has been viewed as potentiating the interaction of MDM4 and p53, rather than encouraging degradation by MDM2 (76). The ubiquitin ligase COP1 also inhibits ubiquitination by MDM2, including MDM2 auto-ubiquitination (77), resulting in increased MDM4 protein levels. Similarly, the ubiquitin ligase PIRH2 increases MDM4 levels (ibid), but a mechanism remains to be demonstrated. Ubiquitination of MDM4 is promoted by the ARF tumor suppressor (71). ARF has also been reported to bind and inhibit MDM4 (78), and to encourage sumoylation of MDM4, although this latter effect has been shown not to affect the activity or stability of MDM4 (79, 80). Competition by MDM4 does prevent ARF-mediated sumoylation of MDM2, resulting instead in MDM2 auto-ubiquitination (79). Ubiquitination of MDM4 is counteracted by the activity of the de-ubiquitinases HAUSP (81) and USP2a (82). MDM4 can also be degraded through cleavage by caspase 3 (83).

MDM4 is regulated by phosphorylation. In response to DNA damage, MDM4 is phosphorylated by both ATM (at S403, (84)) and the ATM-target Chk2 (at S342 and S367, (85)). This encourages ubiquitination by MDM2 (73), inhibits deubiquitination by HAUSP (86, 87), and allows 14-3-3 binding & nuclear accumulation, leading to degradation (74). Casein Kinase 1 α (CK1α) phosphorylates MDM4 at S289, and this appears to be required for the interaction of MDM4 with p53 (88). C-Abl also phosphorylates MDM4 in response to damage, which inhibits the interaction of MDM4 with p53 (89). It has also been reported that phosphorylation at S367 can be mediated by AKT, and that this stabilizes MDM4 and promotes binding of 14-3-3 (72). Phosphorylation by CDK1 (CDC2p34) led to nuclear export of MDM4 (90).

7.2. Localization

MDM4 inhibits p53 by dual mechanisms: by binding p53 and inhibiting its ability to transactivate genes, and by affecting the localization of p53. MDM4, unlike MDM2, does not contain a canonical nuclear localization signal (NLS) and wild type MDM4 is primarily expressed in the cytoplasm (37, 91). A potential NLS in the Ring finger domain was suggested (1), and a mutant of this region prevents binding of a MDM4 to importin α3 *in vitro* and translocation of truncated MDM4 into the nucleus after DNA damage (76). The role of this cryptic NLS and importin α3 remains to be clarified, but there is clearly a role for MDM2 and p53 in control of MDM4 localization.

Upon DNA damage, MDM4 is translocated to the nucleus in p53-positive cells, but not p53-null cells (37). Interestingly, MDM2 is also capable of shuttling MDM4 to the nucleus independent of p53, dependent on its ability to bind MDM4 and its intact NLS. Although much MDM2 localizes to the nucleolus upon overexpression, this was not the case with MDM4 brought to the nucleus by MDM2. U2OS cells in which p53 is inactivated by binding HPV16 E6 protein, and which express no MDM2, nevertheless showed some nuclear MDM4, indicating that there may exist other proteins capable of bringing MDM4 into the nucleus (ibid).

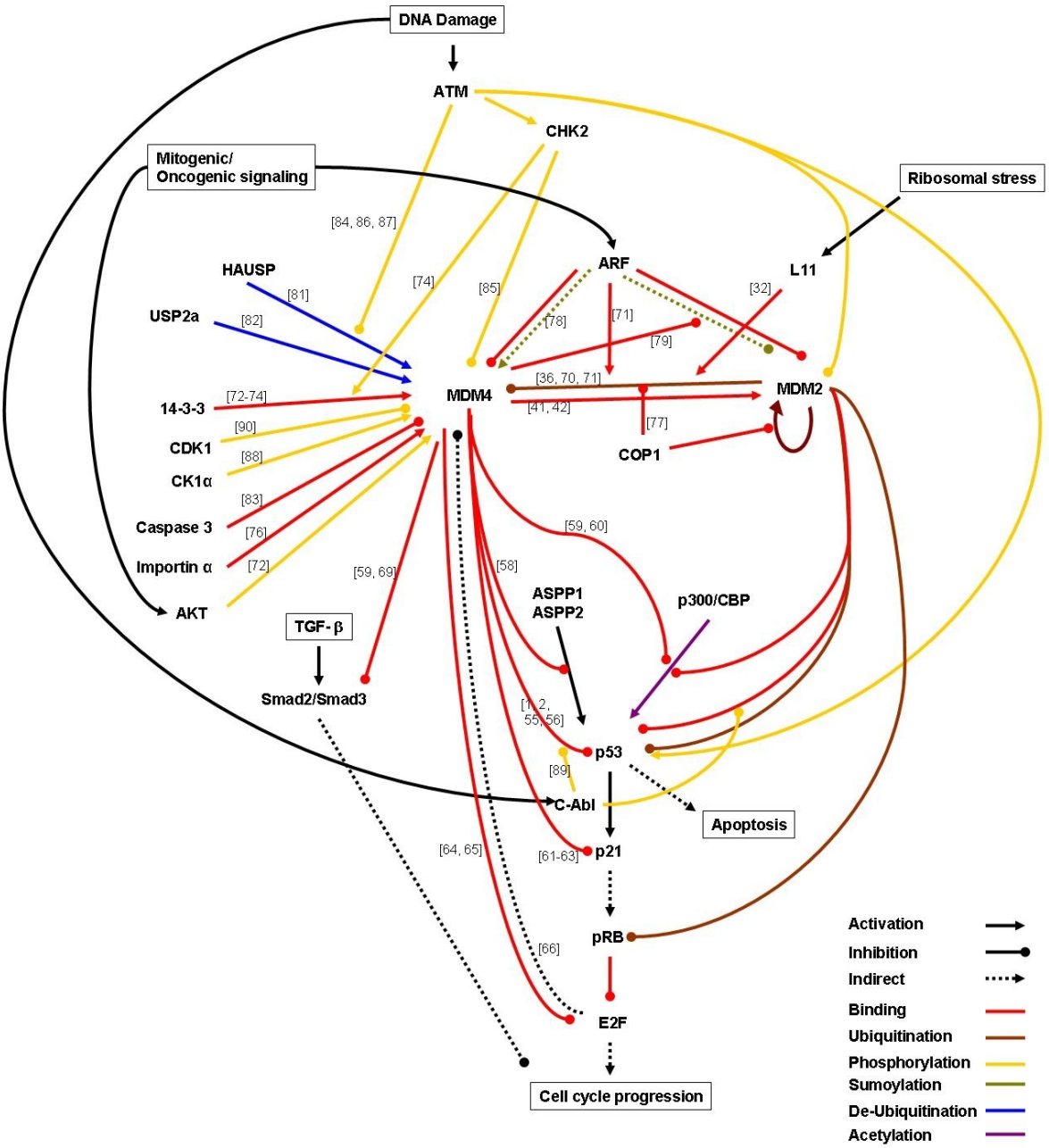

Figure 1. Interaction network of MDM4. Type of interaction is indicated by the color of connections. Direct interactions are solid lines, indirect interactions are dashed. Positive or negative regulation is indicated by arrow head type. Intersections of lines on other lines indicate promotion or inhibition of an interaction (e.g., MDM2 and MDM4 block acetylation of p53 by p300/CBP). Bracketed numbers correspond to references. For readability, some interactions mentioned in the text are omitted: MDM4 is able to stabilize MDM2 (41, 42) and promote the ubiquitin ligase activity of MDM2 through heterodimerization to reduce p53 protein levels (57). Overexpression of MDM4 relative to MDM2 can have the opposite effect, stabilizing p53 (56). MDM4 inhibits the ubiquitin ligase activity of MDM2 towards p53 and itself (67, 68).

Ohtsubo *et al.* elaborated on the localization of MDM4 in the cytoplasm versus the nucleus (92). When not phosphorylated by DNA damage pathways, MDM4 localizes to the cytoplasm. In fact, mutant MDM4 lacking phosphorylation sites for ATM, Chk1 and Chk2 is able to inhibit p53 by binding and sequestering it in the cytoplasm, but only when MDM2 is also present (this requirement being attributed to enhanced monouibuitination of p53 by the MDM2/4 complex, encouraging to nuclear export). Controlled localization of MDM4 to the cytoplasm represents another mechanism to modulate the ability of MDM4 to inhibit p53. However, the picture is complicated

by the ability of cytoplasmic MDM4 to promote the pro-apoptotic function of p53.

Recently Mancini *et al.* (11, 93) showed that a portion (10-29% in the cell lines tested) of MDM4 protein outside the nucleus is located inside the mitochondria. This localization is apparently dependent on the COOH terminus of MDM4, and the level of mitochondrial MDM4 does not change after induction of apoptosis. However, during apoptosis p53 (especially p53Ser46^{P}) is recruited to the mitochondria and indeed anchored there by binding to MDM4. MDM4 acts as a scaffold to bring together p53 and BCL2 at the mitochondria, associated with release of cytochrome C and induction of the p53 intrinsic apoptotic pathway. The mitochondrial binding between MDM4 and BCL2 appears even under normal growth conditions, and it is the recruitment of p53Ser46^{P} which correlates with apoptosis. This study elucidates a fascinating pro-apoptotic role for MDM4 following lethal genotoxic stress, in contrast with its more canonical pro-growth role under undamaged conditions, and provides a potential mechanism for the switching of the p53 response from growth arrest to apoptosis.

7.3. Drug targeting

Because wild type p53 is retained in approximately half of all human cancers (94), it has long been appreciated that inhibitors of p53 present attractive drug targets. The identification of nutlins showed that reactivation of p53 could be achieved by inhibiting MDM2, resulting in p53 accumulation, cell cycle arrest, and apoptosis of p53-positive tumor cells (95). There is evidence that the increased MDM2 levels following nutlin treatment can decrease MDM4 protein levels in certain tumor cell lines (96). Because the major activity of MDM4 is to bind p53 and repress its ability to transactivate target genes, inhibition of MDM2 alone is not expected to result in efficient activation of p53. In fact, MDM4 expression is a significant predictor of non-response to nutlin (97). Therefore, there have been several recent efforts to design inhibitors of MDM4.

Several small inhibitory peptides have been described. The IC_{50} for the earliest MDM2 peptide inhibitor to be described, 12/1 (55, 98), was determined to be 150 nM for MDM2 and 1.25 μM for MDM4 (99). Through phage display, the peptide pDI (peptide dual inhibitor) was identified as binding to the p53-binding domains of both MDM2 and MDM4 (ibid). The IC_{50} for pDI was 10 nM for MDM2 and 100 nM for MDM4. Adenovirus expressing a thioredoxin scaffold displaying the pDI peptide was used to express pDI in cells, where it bound MDM2 and MDM4 and disrupted binding to p53 without inducing p53 Ser^{15} phosphorylation. pDI expression activated p53 and induced apoptosis in MDM4-positive tumor cell lines. Importantly, pDI was able to inhibit the growth of tumor xenografts that depended on MDM4 to suppress p53. As with other inhibitory peptides, pDI is limited to tumor cells accessible to virus. This same lab later examined mutants derived from pDI (100). The most efficient of these mutants was pDIQ, a quadruple mutant with an IC_{50} of 110 nM for MDM4 (8.0 nM for MDM2, making it the strongest MDM2 inhibitor yet described). Large conformational changes in the inhibitors were apparent when bound to MDM4 versus MDM2.

It was hypothesized that the differences in pDI binding to MDM2 versus MDM4 could be due to stronger binding of MDM4 to p53 (ibid). However, the crystal structure of the p53 binding domain of MDM4 showed that the there are a few significant differences between the p53-binding clefts of MDM2 and MDM4; therefore, any inhibitor designed to block the binding of MDM2 to p53 would necessarily be less efficient at binding MDM4 (101, 102). This study showed that the affinities of MDM2 and MDM4 for a p53 peptide are, in fact, similar and range from 0.1-2 μM depending on the p53 peptide. Despite the similarity between the p53-binding regions of MDM2 and MDM4, nutlin-3 was unable to disrupt more than 20% of MDM4 binding, even at a 50:1 molar ratio. The small differences in the shape of the hydrophobic pocket on MDM4 are enough to prevent a strong interaction with nutlin. The one reported success of using nutlin to activate MDM4 in retinoblastoma (27) was likely an indirect effect, or an effective intraocular concentration so high as to be "rancid".

In contrast, Kallen *et al.* described the crystal structure of the N-terminus of MDM4 bound to a chlorine-substituted eight amino acid p53 peptide (Ac-Phe-Met-Aib-Pmp-6-Cl-Trp-Glu-Ac_3c-Leu-NH_2 , or "peptide 2"), which induced surprising changes in the MDM4 bind cleft to allow binding to MDM4 and MDM2 (103). For MDM4 the K_d was 36 nM, and 7 nM for MDM2.

Another peptide inhibitor of MDM4 was built on the β^3 3_{14} helix. These are made up of beta amino acids, in which the amino group is joined to the beta carbon rather than the alpha carbon used in most natural amino acids. The proteolysis-resistant 3_{14} helix has a periodicity such that side chains can line up along one face of the helix to mimic protein binding sites. Harker *et al.* (104) described a β^3 peptide, βp53-12, which bound MDM4 (K_d = 518 ± 41.3 nM). An IC_{50} was not determined, but appears to exceed 100 μM. This same peptide was able to bind much better to MDM2 (K_d = 28.2 ± 4.79, IC_{50} = 6.32 ± 0.316), as would be expected from the work of Popowicz *et al.* (102). Additionally, cell permeability remains an obstacle to practical use of β^3 peptides.

Another screen of a phage display library identified a stronger inhibitor of MDM2 and MDM4 binding to p53, termed pMI (105). In contrast to the expectation that MDM2 inhibitors will bind significantly less well to MDM4, a surface plasmon resonance-based competition assay found PMI to have low nanomolar affinities for both MDM2 and MDM4 (K_d = 3.4 nM and 4.2 nM, respectively). IC_{50} values for MDM2 and MDM4 were later determined by Phan *et al.* (100) as 20nM and 40 nM, respectively. Unfortunately, pMI showed poor killing of p53+/+ cells compared to Nutlin-3, likely due to the usual problems with peptide inhibitors: inefficient uptake, degradation, and endosomal sequestration (105).

Another exception to the trend of MDM2-binding molecules binding less well to MDM4 is L-NAPA 25. Hayashi *et al.* (106) built this duel inhibitor of MDM2 and MDM4 on an artificial N-acrylpolyamine (NAPA) scaffold. L-NAPA 25 was able to inhibit binding of p53 to MDM2 and MDM4 equally (IC_{50} = 2.6 ± 0.2 μM and 2.7 ± 0.1 μM, respectively). Cell killing potential remains to be tested, and the difficulties of synthesizing the inhibitor remains a challenge.

Most recently, the first small-molecule inhibitor of MDM4 has been described (107). Reed *et al.* screened a chemical library of nearly 300,000 compounds and ultimately identified SJ-172550. It reversibly bound MDM4 to efficiently kill MDM4-overexpressing retinoblastoma cells (cell lines Weri1 and RB355) and other lines with high MDM4 (HCT116 and SJSA-X). It showed favorable stability, solubility, low redox potential, and a low micromolar binding constant (EC_{50} = 4.3 μM). Permeability, however, was relatively low. Cells exposed to SJ-172550 did not accumulate p53, but induced p53-dependent apoptosis. Because of this, it seems likely that SJ-172500 binds to MDM4 to inhibit binding to p53, removing the ability of MDM4 to repress p53 transcriptional activity. This represents a powerful tool in the study of the MDM4:p53 interaction and an important step toward the design of clinically effective inhibitors of MDM4.

8. PERSPECTIVE

The history of our knowledge of MDM4 shows that the more we learn, the more complicated the picture becomes. What we once perceived as steady-stay mRNA expression and translation countered by MDM2-dependent ubiquitination and degradation is now better understood as a careful balance of mitogen-dependent transcription, damage-induced splicing, microRNA-mediated message half life, and other regulatory mechanisms. Inhibition of the transactivation ability of p53 by MDM4 has been complicated by p53-independent functions (10), regulation of localization, and a multitude of protein-protein interactions.

The regulation of MDM4 mRNA remains one area of increasing interest. The role of microRNA in regulation of MDM4 remains to be elucidated. Additionally, alternative splicing of MDM4 represents a way to regulate the activity of the MDM4 protein, not just by lowering full length MDM4 (flMDM4) levels, but by creating alternative proteins. Various truncated MDM4 proteins have been shown to act more strongly to repress p53 and associate with tumor progression, or to conversely promote p53 stability by binding and inhibiting MDM2. A biological role for several of these splice variants remains to be demonstrated.

The growing appreciation of the importance of MDM4 in the life of a cancer cell has made it a priority target for the development of new therapeutics. Already we have seen the potential clinical payoff of targeting MDM4 using even a non-specific inhibitor in retinoblastoma (27). The announcement of the specific small molecule inhibitor SJ-172550 (107) represents an exciting development that will doubtlessly spur further design of clinically useful MDM4 inhibitors. It will be interesting to see the effectiveness of this new class of inhibitor in models of cancers where MDM4 is overexpressed, and what synergy they have with inhibitors of MDM2 and traditional chemotherapeutics.

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10. REFERENCES:

1. Shvarts, A., W.T. Steegenga, N. Riteco, T. van Laar, P. Dekker, M. Bazuine, R.C. van Ham, W. van der Houven van Oordt, G. Hateboer, A.J. van der Eb, and A.G. Jochemsen, MDMX: a novel p53-binding protein with some functional properties of MDM2. *Embo J* 15(19), 5349-57 (1996)

2. Shvarts, A., M. Bazuine, P. Dekker, Y.F. Ramos, W.T. Steegenga, G. Merckx, R.C. van Ham, W. van der Houven van Oordt, A.J. van der Eb, and A.G. Jochemsen, Isolation and identification of the human homolog of a new p53-binding protein, Mdmx. *Genomics* 43(1), 34-42 (1997)

3. Finch, R.A., D.B. Donoviel, D. Potter, M. Shi, A. Fan, D.D. Freed, C.Y. Wang, B.P. Zambrowicz, R. Ramirez-Solis, A.T. Sands, and N. Zhang, mdmx is a negative regulator of p53 activity in vivo. *Cancer Res* 62(11), 3221-5 (2002)

4. Migliorini, D., E. Lazzerini Denchi, D. Danovi, A. Jochemsen, M. Capillo, A. Gobbi, K. Helin, P.G. Pelicci, and J.C. Marine, Mdm4 (Mdmx) regulates p53-induced growth arrest and neuronal cell death during early embryonic mouse development. Mol *Cell Biol* 22(15), 5527-38 (2002)

5. Parant, J., A. Chavez-Reyes, N.A. Little, W. Yan, V. Reinke, A.G. Jochemsen, and G. Lozano, Rescue of embryonic lethality in Mdm4-null mice by loss of Trp53 suggests a nonoverlapping pathway with MDM2 to regulate p53. *Nat Genet* 29(1), 92-5 (2001)

6. Grier, J.D., S. Xiong, A.C. Elizondo-Fraire, J.M. Parant, and G. Lozano, Tissue-specific differences of p53 inhibition by Mdm2 and Mdm4. *Mol Cell Biol* 26(1), 192-8 (2006)

7. Xiong, S., C.S. Van Pelt, A.C. Elizondo-Fraire, G. Liu, and G. Lozano, Synergistic roles of Mdm2 and Mdm4 for p53 inhibition in central nervous system development. *Proc Natl Acad Sci U S A* 103(9), 3226-31 (2006)

8. Barboza, J.A., T. Iwakuma, T. Terzian, A.K. El-Naggar, and G. Lozano, Mdm2 and Mdm4 loss regulates distinct p53 activities. *Mol Cancer Res* 6(6), 947-54 (2008)

9. Toledo, F., K.A. Krummel, C.J. Lee, C.W. Liu, L.W. Rodewald, M. Tang, and G.M. Wahl, A mouse p53 mutant lacking the proline-rich domain rescues Mdm4 deficiency and provides insight into the Mdm2-Mdm4-p53 regulatory network. *Cancer Cell* 9(4), 273-85 (2006)

10. Matijasevic, Z., H.A. Steinman, K. Hoover, and S.N. Jones, MdmX promotes bipolar mitosis to suppress transformation and tumorigenesis in p53-deficient cells and mice. *Mol Cell Biol* 28(4), 1265-73 (2008)

11. Mancini, F. and F. Moretti, Mitochondrial MDM4 (MDMX): an unpredicted role in the p53-mediated intrinsic apoptotic pathway. *Cell Cycle* 8(23), 3854-9 (2009)

12. Jeyaraj, S., D.M. O'Brien, and D.S. Chandler, MDM2 and MDM4 splicing: an integral part of the cancer spliceome. *Front Biosci* 14 2647-56 (2009)

13. Marine, J.C., M.A. Dyer, and A.G. Jochemsen, MDMX: from bench to bedside. *J Cell Sci* 120(Pt 3), 371-8 (2007)

14. Toledo, F. and G.M. Wahl, MDM2 and MDM4: p53 regulators as targets in anticancer therapy. *Int J Biochem Cell Biol* 39(7-8), 1476-82 (2007)

15. Wade, M. and G.M. Wahl, Targeting Mdm2 and Mdmx in cancer therapy: better living through medicinal chemistry? *Mol Cancer Res* 7(1), 1-11 (2009)

16. Riemenschneider, M.J., R. Buschges, M. Wolter, J. Reifenberger, J. Bostrom, J.A. Kraus, U. Schlegel, and G. Reifenberger, Amplification and overexpression of the MDM4 (MDMX) gene from 1q32 in a subset of malignant gliomas without TP53 mutation or MDM2 amplification. *Cancer Res* 59(24), 6091-6 (1999)

17. Rickman, D.S., M.P. Bobek, D.E. Misek, R. Kuick, M. Blaivas, D.M. Kurnit, J. Taylor, and S.M. Hanash, Distinctive molecular profiles of high-grade and low-grade gliomas based on oligonucleotide microarray analysis. *Cancer Res* 61(18), 6885-91 (2001)

18. Riemenschneider, M.J., C.B. Knobbe, and G. Reifenberger, Refined mapping of 1q32 amplicons in malignant gliomas confirms MDM4 as the main amplification target. *Int J Cancer* 104(6), 752-7 (2003)

19. Danovi, D., E. Meulmeester, D. Pasini, D. Migliorini, M. Capra, R. Frenk, P. de Graaf, S. Francoz, P. Gasparini, A. Gobbi, K. Helin, P.G. Pelicci, A.G. Jochemsen, and J.C. Marine, Amplification of Mdmx (or Mdm4) directly contributes to tumor formation by inhibiting p53 tumor suppressor activity. *Mol Cell Biol* 24(13), 5835-43 (2004)

20. Ramos, Y.F., R. Stad, J. Attema, L.T. Peltenburg, A.J. van der Eb, and A.G. Jochemsen, Aberrant expression of HDMX proteins in tumor cells correlates with wild-type p53. *Cancer Res* 61(5), 1839-42 (2001)

21. Gilkes, D.M., Y. Pan, D. Coppola, T. Yeatman, G.W. Reuther, and J. Chen, Regulation of MDMX expression by mitogenic signaling. *Mol Cell Biol* 28(6), 1999-2010 (2008)

22. Arjona, D., M.J. Bello, M.E. Alonso, A. Isla, J.M. De Campos, J. Vaquero, J.L. Sarasa, M. Gutierrez, and J.A. Rey, Real-time quantitative PCR analysis of regions involved in gene amplification reveals gene overdose in low-grade astrocytic gliomas. *Diagn Mol Pathol* 14(4), 224-9 (2005)

23. Alonso, M.E., M.J. Bello, D. Arjona, V. Martinez-Glez, J.M. de Campos, A. Isla, E. Kusak, J. Vaquero, M. Gutierrez, J.L. Sarasa, and J.A. Rey, Real-time quantitative PCR analysis of gene dosages reveals gene amplification in low-grade oligodendrogliomas. *Am J Clin Pathol* 123(6), 900-6 (2005)

24. Bartel, F., J. Schulz, A. Bohnke, K. Blumke, M. Kappler, M. Bache, H. Schmidt, P. Wurl, H. Taubert, and S. Hauptmann, Significance of HDMX-S (or MDM4) mRNA splice variant overexpression and HDMX gene amplification on primary soft tissue sarcoma prognosis. *Int J Cancer* 117(3), 469-75 (2005)

25. Potluri, V.R., L. Helson, R.M. Ellsworth, T. Reid, and F. Gilbert, Chromosomal abnormalities in human retinoblastoma. A review. *Cancer* 58(3), 663-71 (1986)

26. Corson, T.W. and B.L. Gallie, One hit, two hits, three hits, more? Genomic changes in the development of retinoblastoma. *Genes Chromosomes Cancer* 46(7), 617-34 (2007)

27. Laurie, N.A., S.L. Donovan, C.S. Shih, J. Zhang, N. Mills, C. Fuller, A. Teunisse, S. Lam, Y. Ramos, A. Mohan, D. Johnson, M. Wilson, C. Rodriguez-Galindo, M. Quarto, S. Francoz, S.M. Mendrysa, R.K. Guy, J.C. Marine, A.G. Jochemsen, and M.A. Dyer, Inactivation of the p53 pathway in retinoblastoma. *Nature* 444(7115), 61-6 (2006)

28. Dimaras, H., V. Khetan, W. Halliday, M. Orlic, N.L. Prigoda, B. Piovesan, P. Marrano, T.W. Corson, R.C. Eagle, Jr., J.A. Squire, and B.L. Gallie, Loss of RB1 induces non-proliferative retinoma: increasing genomic instability correlates with progression to retinoblastoma. *Hum Mol Genet* 17(10), 1363-72 (2008)

29. Valentin-Vega, Y.A., J.A. Barboza, G.P. Chau, A.K. El-Naggar, and G. Lozano, High levels of the p53 inhibitor MDM4 in head and neck squamous carcinomas. *Hum Pathol* 38(10), 1553-62 (2007)

30. Schlaeger, C., T. Longerich, C. Schiller, P. Bewerunge, A. Mehrabi, G. Toedt, J. Kleeff, V. Ehemann, R. Eils, P. Lichter, P. Schirmacher, and B. Radlwimmer, Etiology-dependent molecular mechanisms in human hepatocarcinogenesis. *Hepatology* 47(2), 511-20 (2008)

31. Veerakumarasivam, A., H.E. Scott, S.F. Chin, A. Warren, M.J. Wallard, D. Grimmer, K. Ichimura, C. Caldas, V.P. Collins, D.E. Neal, and J.D. Kelly, High-resolution array-based comparative genomic hybridization of bladder cancers identifies mouse double minute 4 (MDM4) as an amplification target exclusive of MDM2 and TP53. *Clin Cancer Res* 14(9), 2527-34 (2008)

32. Gilkes, D.M., L. Chen, and J. Chen, MDMX regulation of p53 response to ribosomal stress. *Embo J* 25(23), 5614-25 (2006)

33. Markey, M. and S.J. Berberich, Full-length hdmX transcripts decrease following genotoxic stress. *Oncogene* 27(52), 6657-66 (2008)

34. Rallapalli, R., G. Strachan, B. Cho, W.E. Mercer, and D.J. Hall, A novel MDMX transcript expressed in a variety of transformed cell lines encodes a truncated protein with potent p53 repressive activity. *J Biol Chem* 274(12), 8299-308 (1999)

35. Rallapalli, R., G. Strachan, R.S. Tuan, and D.J. Hall, Identification of a domain within MDMX-S that is responsible for its high affinity interaction with p53 and high-level expression in mammalian cells. *J Cell Biochem* 89(3), 563-75 (2003)

36. de Graaf, P., N.A. Little, Y.F. Ramos, E. Meulmeester, S.J. Letteboer, and A.G. Jochemsen, Hdmx protein stability is regulated by the ubiquitin ligase activity of Mdm2. *J Biol Chem* 278(40), 38315-24 (2003)

37. Li, C., L. Chen, and J. Chen, DNA damage induces MDMX nuclear translocation by p53-dependent and -independent mechanisms. *Mol Cell Biol* 22(21), 7562-71 (2002)

38. Bartel, F., J. Schulz, K. Blumke, M. Kappler, M. Bache, H. Schmidt, and H. Taubert, [HDMX amplification and high levels of HDMX-S splice variant are correlated with a poor prognosis in soft tissue sarcomas]. *Verh Dtsch Ges Pathol* 88 199-206 (2004)

39. Prodosmo, A., S. Giglio, S. Moretti, F. Mancini, F. Barbi, N. Avenia, G. Di Conza, H.J. Schunemann, L. Pistola, V. Ludovini, A. Sacchi, A. Pontecorvi, E. Puxeddu, and F. Moretti, Analysis of human MDM4 variants in papillary thyroid carcinomas reveals new potential markers of cancer properties. *J Mol Med* 86(5), 585-96 (2008)

40. Giglio, S., F. Mancini, F. Gentiletti, G. Sparaco, L. Felicioni, F. Barassi, C. Martella, A. Prodosmo, S. Iacovelli, F. Buttitta, A. Farsetti, S. Soddu, A. Marchetti, A. Sacchi, A. Pontecorvi, and F. Moretti, Identification of an aberrantly spliced form of HDMX in human tumors: a new mechanism for HDM2 stabilization. *Cancer Res* 65(21), 9687-94 (2005)

41. Stad, R., N.A. Little, D.P. Xirodimas, R. Frenk, A.J. van der Eb, D.P. Lane, M.K. Saville, and A.G. Jochemsen, Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms. *EMBO Rep* 2(11), 1029-34 (2001)

42. Stad, R., Y.F. Ramos, N. Little, S. Grivell, J. Attema, A.J. van Der Eb, and A.G. Jochemsen, Hdmx stabilizes Mdm2 and p53. *J Biol Chem* 275(36), 28039-44 (2000)

43. Chandler, D.S., R.K. Singh, L.C. Caldwell, J.L. Bitler, and G. Lozano, Genotoxic stress induces coordinately regulated alternative splicing of the p53 modulators MDM2 and MDM4. *Cancer Res* 66(19), 9502-8 (2006)

44. Bommer, G.T., I. Gerin, Y. Feng, A.J. Kaczorowski, R. Kuick, R.E. Love, Y. Zhai, T.J. Giordano, Z.S. Qin, B.B. Moore, O.A. MacDougald, K.R. Cho, and E.R. Fearon, p53-mediated activation of miRNA34 candidate tumor-suppressor genes. *Curr Biol* 17(15), 1298-307 (2007)

45. Chang, T.C., E.A. Wentzel, O.A. Kent, K. Ramachandran, M. Mullendore, K.H. Lee, G. Feldmann, M. Yamakuchi, M. Ferlito, C.J. Lowenstein, D.E. Arking, M.A. Beer, A. Maitra, and J.T. Mendell, Transactivation of miR-34a by p53 broadly influences gene expression and promotes apoptosis. *Mol Cell* 26(5), 745-52 (2007)

46. He, L., X. He, L.P. Lim, E. de Stanchina, Z. Xuan, Y. Liang, W. Xue, L. Zender, J. Magnus, D. Ridzon, A.L. Jackson, P.S. Linsley, C. Chen, S.W. Lowe, M.A. Cleary, and G.J. Hannon, A microRNA component of the p53 tumour suppressor network. *Nature* 447(7148), 1130-4 (2007)

47. He, X., L. He, and G.J. Hannon, The guardian's little helper: microRNAs in the p53 tumor suppressor network. *Cancer Res* 67(23), 11099-101 (2007)

48. Raver-Shapira, N., E. Marciano, E. Meiri, Y. Spector, N. Rosenfeld, N. Moskovits, Z. Bentwich, and M. Oren, Transcriptional activation of miR-34a contributes to p53-mediated apoptosis. *Mol Cell* 26(5), 731-43 (2007)

49. Tarasov, V., P. Jung, B. Verdoodt, D. Lodygin, A. Epanchintsev, A. Menssen, G. Meister, and H. Hermeking, Differential regulation of microRNAs by p53 revealed by massively parallel sequencing: miR-34a is a p53 target that induces apoptosis and G1-arrest. *Cell Cycle* 6(13), 1586-93 (2007)

50. Zhu, N., L. Gu, H.W. Findley, and M. Zhou, Transcriptional repression of the eukaryotic initiation factor 4E gene by wild type p53. *Biochem Biophys Res Commun* 335(4), 1272-9 (2005)

51. Constantinou, C. and M.J. Clemens, Regulation of the phosphorylation and integrity of protein synthesis initiation factor eIF4GI and the translational repressor 4E-BP1 by p53. *Oncogene* 24(30), 4839-50 (2005)

52. Horton, L.E., M. Bushell, D. Barth-Baus, V.J. Tilleray, M.J. Clemens, and J.O. Hensold, p53 activation results in rapid dephosphorylation of the eIF4E-binding protein 4E-BP1, inhibition of ribosomal protein S6 kinase and inhibition of translation initiation. *Oncogene* 21(34), 5325-34 (2002)

53. Tilleray, V., C. Constantinou, and M.J. Clemens, Regulation of protein synthesis by inducible wild-type p53 in human lung carcinoma cells. *FEBS Lett* 580(7), 1766-70 (2006)

54. Marine, J.C. and A.G. Jochemsen, Mdmx as an essential regulator of p53 activity. *Biochem Biophys Res Commun* 331(3), 750-60 (2005)

55. Bottger, V., A. Bottger, S.F. Howard, S.M. Picksley, P. Chene, C. Garcia-Echeverria, H.K. Hochkeppel, and D.P. Lane, Identification of novel mdm2 binding peptides by phage display. *Oncogene* 13(10), 2141-7 (1996)

56. Jackson, M.W. and S.J. Berberich, MdmX protects p53 from Mdm2-mediated degradation. *Mol Cell Biol* 20(3), 1001-7 (2000)

57. Kawai, H., V. Lopez-Pajares, M.M. Kim, D. Wiederschain, and Z.M. Yuan, RING domain-mediated interaction is a requirement for MDM2's E3 ligase activity. *Cancer Res* 67(13), 6026-30 (2007)

58. Bergamaschi, D., Y. Samuels, S. Zhong, and X. Lu, Mdm2 and mdmX prevent ASPP1 and ASPP2 from stimulating p53 without targeting p53 for degradation. *Oncogene* 24(23), 3836-41 (2005)

59. Kadakia, M., T.L. Brown, M.M. McGorry, and S.J. Berberich, MdmX inhibits Smad transactivation. *Oncogene* 21(57), 8776-85 (2002)

60. Sabbatini, P. and F. McCormick, MDMX inhibits the p300/CBP-mediated acetylation of p53. *DNA Cell Biol* 21(7), 519-25 (2002)

61. Jin, Y., H. Lee, S.X. Zeng, M.S. Dai, and H. Lu, MDM2 promotes p21waf1/cip1 proteasomal turnover independently of ubiquitylation. *Embo J* 22(23), 6365-77 (2003)

62. Jin, Y., S.X. Zeng, X.X. Sun, H. Lee, C. Blattner, Z. Xiao, and H. Lu, MDMX promotes proteasomal turnover of p21 at G1 and early S phases independently of, but in cooperation with, MDM2. *Mol Cell Biol* 28(4), 1218-29 (2008)

63. Zhang, Z., H. Wang, M. Li, S. Agrawal, X. Chen, and R. Zhang, MDM2 is a negative regulator of p21WAF1/CIP1, independent of p53. *J Biol Chem* 279(16), 16000-6 (2004)

64. Strachan, G.D., K.L. Jordan-Sciutto, R. Rallapalli, R.S. Tuan, and D.J. Hall, The E2F-1 transcription factor is negatively regulated by its interaction with the MDMX protein. *J Cell Biochem* 88(3), 557-68 (2003)

65. Wunderlich, M., M. Ghosh, K. Weghorst, and S.J. Berberich, MdmX represses E2F1 transactivation. *Cell Cycle* 3(4), 472-8 (2004)

66. Strachan, G.D., R. Rallapalli, B. Pucci, T.P. Lafond, and D.J. Hall, A transcriptionally inactive E2F-1 targets the MDM family of proteins for proteolytic degradation. *J Biol Chem* 276(49), 45677-85 (2001)

67. Okamoto, K., Y. Taya, and H. Nakagama, Mdmx enhances p53 ubiquitination by altering the substrate preference of the Mdm2 ubiquitin ligase. *FEBS Lett* 583(17), 2710-4 (2009)

68. Uchida, C., S. Miwa, T. Isobe, K. Kitagawa, T. Hattori, T. Oda, H. Yasuda, and M. Kitagawa, Effects of MdmX on Mdm2-mediated downregulation of pRB. *FEBS Lett* 580(7), 1753-8 (2006)

69. Yam, C.H., W.Y. Siu, T. Arooz, C.H. Chiu, A. Lau, X.Q. Wang, and R.Y. Poon, MDM2 and MDMX inhibit the transcriptional activity of ectopically expressed SMAD proteins. *Cancer Res* 59(20), 5075-8 (1999)

70. Kawai, H., D. Wiederschain, H. Kitao, J. Stuart, K.K. Tsai, and Z.M. Yuan, DNA damage-induced MDMX degradation is mediated by MDM2. *J Biol Chem* 278(46), 45946-53 (2003)

71. Pan, Y. and J. Chen, MDM2 promotes ubiquitination and degradation of MDMX. *Mol Cell Biol* 23(15), 5113-21 (2003)

72. Lopez-Pajares, V., M.M. Kim, and Z.M. Yuan, Phosphorylation of MDMX mediated by Akt leads to stabilization and induces 14-3-3 binding. *J Biol Chem* 283(20), 13707-13 (2008)

73. Okamoto, K., K. Kashima, Y. Pereg, M. Ishida, S. Yamazaki, A. Nota, A. Teunisse, D. Migliorini, I. Kitabayashi, J.C. Marine, C. Prives, Y. Shiloh, A.G. Jochemsen, and Y. Taya, DNA damage-induced phosphorylation of MdmX at serine 367 activates p53 by targeting MdmX for Mdm2-dependent degradation. Mol Cell Biol 25(21), 9608-20 (2005)

74. Pereg, Y., S. Lam, A. Teunisse, S. Biton, E. Meulmeester, L. Mittelman, G. Buscemi, K. Okamoto, Y. Taya, Y. Shiloh, and A.G. Jochemsen, Differential roles of ATM- and Chk2-mediated phosphorylations of Hdmx in response to DNA damage. Mol Cell Biol 26(18), 6819-31 (2006)

75. Jin, Y., M.S. Dai, S.Z. Lu, Y. Xu, Z. Luo, Y. Zhao, and H. Lu, 14-3-3gamma binds to MDMX that is phosphorylated by UV-activated Chk1, resulting in p53 activation. Embo J 25(6), 1207-18 (2006)

76. LeBron, C., L. Chen, D.M. Gilkes, and J. Chen, Regulation of MDMX nuclear import and degradation by Chk2 and 14-3-3. Embo J 25(6), 1196-206 (2006)

77. Wang, L., G. He, P. Zhang, X. Wang, M. Jiang, and L. Yu, Interplay between MDM2, MDMX, Pirh2 and COP1: the negative regulators of p53. Mol Biol Rep, Mar 24 (2010)

78. Jackson, M.W., M.S. Lindstrom, and S.J. Berberich, MdmX binding to ARF affects Mdm2 protein stability and p53 transactivation. *J Biol Chem* 276(27), 25336-41 (2001)

79. Ghosh, M., K. Weghorst, and S.J. Berberich, MdmX inhibits ARF mediated Mdm2 sumoylation. *Cell Cycle* 4(4), 604-8 (2005)

80. Pan, Y. and J. Chen, Modification of MDMX by sumoylation. *Biochem Biophys Res Commun* 332(3), 702-9 (2005)

81. Cummins, J.M., C. Rago, M. Kohli, K.W. Kinzler, C. Lengauer, and B. Vogelstein, Tumour suppression: disruption of HAUSP gene stabilizes p53. *Nature* 428, 6982 (2004)

82. Allende-Vega, N., A. Sparks, D.P. Lane, and M.K. Saville, MdmX is a substrate for the deubiquitinating enzyme USP2a. *Oncogene* 29(3), 432-41 (2010)

83. Gentiletti, F., F. Mancini, M. D'Angelo, A. Sacchi, A. Pontecorvi, A.G. Jochemsen, and F. Moretti, MDMX stability is regulated by p53-induced caspase cleavage in NIH3T3 mouse fibroblasts. *Oncogene* 21(6), 867-77 (2002)

84. Pereg, Y., D. Shkedy, P. de Graaf, E. Meulmeester, M. Edelson-Averbukh, M. Salek, S. Biton, A.F. Teunisse, W.D. Lehmann, A.G. Jochemsen, and Y. Shiloh, Phosphorylation of Hdmx mediates its Hdm2- and ATM-dependent degradation in response to DNA damage. *Proc Natl Acad Sci U S A* 102(14), 5056-61 (2005)

85. Chen, L., D.M. Gilkes, Y. Pan, W.S. Lane, and J. Chen, ATM and Chk2-dependent phosphorylation of MDMX contribute to p53 activation after DNA damage. *Embo J* 24(19), 3411-22 (2005)

86. Meulmeester, E., M.M. Maurice, C. Boutell, A.F. Teunisse, H. Ovaa, T.E. Abraham, R.W. Dirks, and A.G. Jochemsen, Loss of HAUSP-mediated deubiquitination contributes to DNA damage-induced destabilization of Hdmx and Hdm2. *Mol Cell* 18(5), 565-76 (2005)

87. Meulmeester, E., Y. Pereg, Y. Shiloh, and A.G. Jochemsen, ATM-mediated phosphorylations inhibit Mdmx/Mdm2 stabilization by HAUSP in favor of p53 activation. *Cell Cycle* 4(9), 1166-70 (2005)

88. Chen, L., C. Li, Y. Pan, and J. Chen, Regulation of p53-MDMX interaction by casein kinase 1 alpha. *Mol Cell Biol* 25(15), 6509-20 (2005)

89. Zuckerman, V., K. Lenos, G.M. Popowicz, I. Silberman, T. Grossman, J.C. Marine, T.A. Holak, A.G. Jochemsen, and Y. Haupt, c-Abl phosphorylates Hdmx and regulates its interaction with p53. *J Biol Chem* 284(6), 4031-9 (2009)

90. Elias, B., A. Laine, and Z. Ronai, Phosphorylation of MdmX by CDK2/Cdc2(p34) is required for nuclear export of Mdm2. *Oncogene* 24(15), 2574-9 (2005)

91. Migliorini, D., D. Danovi, E. Colombo, R. Carbone, P.G. Pelicci, and J.C. Marine, Hdmx recruitment into the nucleus by Hdm2 is essential for its ability to regulate p53 stability and transactivation. *J Biol Chem* 277(9), 7318-23 (2002)

92. Ohtsubo, C., D. Shiokawa, M. Kodama, C. Gaiddon, H. Nakagama, A.G. Jochemsen, Y. Taya, and K. Okamoto, Cytoplasmic tethering is involved in synergistic inhibition of p53 by Mdmx and Mdm2. *Cancer Sci* 100(7), 1291-9 (2009)

93. Mancini, F., G. Di Conza, M. Pellegrino, C. Rinaldo, A. Prodosmo, S. Giglio, I. D'Agnano, F. Florenzano, L. Felicioni, F. Buttitta, A. Marchetti, A. Sacchi, A. Pontecorvi, S. Soddu, and F. Moretti, MDM4 (MDMX) localizes at the mitochondria and facilitates the p53-mediated intrinsic-apoptotic pathway. *Embo J* 28(13), 1926-39 (2009)

94. May, P. and E. May, Twenty years of p53 research: structural and functional aspects of the p53 protein. *Oncogene* 18(53), 7621-36 (1999)

95. Vassilev, L.T., B.T. Vu, B. Graves, D. Carvajal, F. Podlaski, Z. Filipovic, N. Kong, U. Kammlott, C. Lukacs, C. Klein, N. Fotouhi, and E.A. Liu, In vivo activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* 303(5659), 844-8 (2004)

96. Xia, M., D. Knezevic, C. Tovar, B. Huang, D.C. Heimbrook, and L.T. Vassilev, Elevated MDM2 boosts the apoptotic activity of p53-MDM2 binding inhibitors by facilitating MDMX degradation. *Cell Cycle* 7(11), 1604-12 (2008)

97. Bo, M.D., P. Secchiero, M. Degan, D. Marconi, R. Bomben, G. Pozzato, G. Gaidano, G. Del Poeta, F. Forconi, G. Zauli, and V. Gattei, MDM4 (MDMX) is overexpressed in chronic lymphocytic leukaemia (CLL) and marks a subset of p53 CLL with a poor cytotoxic response to Nutlin-3. *Br J Haematol*. 150(2), 237-9 (2010)

98. Bottger, V., A. Bottger, C. Garcia-Echeverria, Y.F. Ramos, A.J. van der Eb, A.G. Jochemsen, and D.P. Lane, Comparative study of the p53-mdm2 and p53-MDMX interfaces. *Oncogene* 18(1), 189-99 (1999)

99. Hu, B., D.M. Gilkes, and J. Chen, Efficient p53 activation and apoptosis by simultaneous disruption of binding to MDM2 and MDMX. *Cancer Res* 67(18), 8810-7 (2007)

100. Phan, J., Z. Li, A. Kasprzak, B. Li, S. Sebti, W. Guida, E. Schonbrunn, and J. Chen, Structure-based design of high affinity peptides inhibiting the interaction of p53 with MDM2 and MDMX. *J Biol Chem* 285(3), 2174-83 (2010)

101. Popowicz, G.M., A. Czarna, and T.A. Holak, Structure of the human Mdmx protein bound to the p53 tumor suppressor transactivation domain. *Cell Cycle* 7(15), 2441-3 (2008)

102. Popowicz, G.M., A. Czarna, U. Rothweiler, A. Szwagierczak, M. Krajewski, L. Weber, and T.A. Holak,

Molecular basis for the inhibition of p53 by Mdmx. *Cell Cycle* 6(19), 2386-92 (2007)

103. Kallen, J., A. Goepfert, A. Blechschmidt, A. Izaac, M. Geiser, G. Tavares, P. Ramage, P. Furet, K. Masuya, and J. Lisztwan, Crystal Structures of Human MdmX (HdmX) in Complex with p53 Peptide Analogues Reveal Surprising Conformational Changes. *J Biol Chem* 284(13), 8812-21 (2009)

104. Harker, E.A., D.S. Daniels, D.A. Guarracino, and A. Schepartz, Beta-peptides with improved affinity for hDM2 and hDMX. *Bioorg Med Chem* 17(5), 2038-46 (2009)

105. Pazgier, M., M. Liu, G. Zou, W. Yuan, C. Li, C. Li, J. Li, J. Monbo, D. Zella, S.G. Tarasov, and W. Lu, Structural basis for high-affinity peptide inhibition of p53 interactions with MDM2 and MDMX. *Proc Natl Acad Sci U S A* 106(12), 4665-70 (2009)

106. Hayashi, R., D. Wang, T. Hara, J.A. Iera, S.R. Durell, and D.H. Appella, N-acylpolyamine inhibitors of HDM2 and HDMX binding to p53. *Bioorg Med Chem* 17(23), 7884-93 (2009)

107. Reed, D., Y. Shen, A.A. Shelat, L.A. Arnold, A.M. Ferreira, F. Zhu, N. Mills, D.C. Smithson, C.A. Regni, D. Bashford, S.A. Cicero, B.A. Schulman, A.G. Jochemsen, R.K. Guy, and M.A. Dyer, Identification and characterization of the first small molecule inhibitor of MDMX. *J Biol Chem* 285(14), 10786-96 (2010)

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