MDMX: from bench to bedside

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Summary

The tumor suppressor protein p53 is negatively regulated by Mdm2, a ubiquitin ligase protein that targets p53 for degradation. Mdmx (also known as Mdm4) is a relative of Mdm2 that was identified on the basis of its ability to physically interact with p53. An increasing body of evidence, including recent genetic studies, suggests that Mdmx also acts as a key negative regulator of p53. Aberrant expression of MDMX could thus contribute to tumor formation. Indeed, MDMX amplification and/or overexpression occurs in several diverse tumors. Strikingly, recent work identifies MDMX as a specific chemotherapeutic target for treatment of retinoblastoma. Specific MDMX antagonists should therefore be developed as a tool to ensure activation of 'dormant' p53 activity in tumors that retain wild-type p53.

Key words: p53, Mdmx, Cancer, Nutlin

The p53-Mdm2 network

In response to cellular stresses such as DNA damage or oncogene activation, the tumor suppressor protein p53 becomes stabilized and modulates transcription of target genes (Vousden and Lu, 2002). These drive a variety of cellular responses to stress, including DNA repair, cell-cycle arrest, senescence and apoptosis. Key targets are the proapoptotic genes *Bax*, *Puma* and *Noxa* (Michalak et al., 2005), the genes encoding the cell cycle regulators p21 (el-Deiry et al., 1993) and Ptprv (Doumont et al., 2005) and the senescence-inducing gene *Plasminogen activator inhibitor 1* (Kortlever et al., 2006).

In the absence of stress signals, the activity of the p53 protein is kept in check to allow normal cell proliferation and to maintain cell viability. Crucially important for this process is the product of the *murine double minute* gene *Mdm2*. *Mdm2* was originally identified by virtue of its amplification in a spontaneously transformed mouse BALB/c cell line (3T3-DM) (Cahilly-Snider et al., 1987). Momand et al. later reported that Mdm2 physically associates with p53 and inhibits p53-mediated transcriptional activation (Momand et al., 1992), providing a simple explanation for its transforming potential. Amplification of *MDM2* (also known as *HDM2*) has been observed in approximately one-third of human sarcomas that retain wild-type p53 (Oliner et al., 1992), which indicates that overexpression of MDM2 is a molecular mechanism by which cells can inactivate p53 during tumor formation.

Genetic experiments have demonstrated the importance of the Mdm2-p53 interaction. Mdm2-deficient mice die very early in development prior to implantation, whereas mice lacking both p53 and Mdm2 are viable and indistinguishable from mice lacking only p53 (Montes de Oca Luna et al., 1995; Jones et al., 1995). Similarly, Zdm2-deficient zebrafish embryos, generated by injection of antisense morpholinos, exhibit widespread apoptosis, which leads to developmental arrest. As in Mdm2-deficient mice, simultaneous inactivation of Zp53 in Zdm2-deficient zebrafish embryos rescues this developmental defect (Langheinrich et al., 2002). In addition, mice possessing a hypomorphic *Mdm2* mutation exhibit defects in thymus development, metabolism, bone marrow production and intestinal cell production (Mendrysa et al., 2003). Conditional inactivation of an $Mdm2^{Lox}$ allele in cardiomyocytes (Grier et al., 2006), neuronal progenitor cells (Xiong et al., 2006) and smooth muscle cells (SMCs) of the gastrointestinal (GI) tract (Boesten et al., 2006) leads to p53-dependent cell death. Finally, conditional expression of p53 in neuronal progenitor cells and post-mitotic neurons of Mdm2-null mice leads to dramatic p53 activation and cell death (Francoz et al., 2006). Mdm2 thus appears to be an essential p53 antagonist in the developing embryo and in mature differentiated cells.

Transfection studies suggest that Mdm2 inhibits p53 by multiple mechanisms. Mdm2 is a RING-finger-containing protein that acts as an E3 ligase, which is essential for ubiquitylation and subsequent degradation of p53 (Haupt et al., 1997; Kubbutat et al., 1997). Recent studies show that Mdm2 is required to maintain p53 at low levels both in proliferating and in post-mitotic cells (reviewed by Marine et al., 2006). Genetic evidence supports the notion that constitutive degradation of p53 in vivo strictly depends on Mdm2. This is particularly interesting since several other ubiquitin ligases that target p53, such as Pirh2, Cop-1 or ARF/BP1, have been discovered and shown to function in an Mdm2-independent manner (Leng et al., 2003; Dornan et al., 2004; Chen, D. et al., 2005). However, none of these proteins can sufficiently, if at all, compensate for loss of Mdm2 function in vivo.

Mdm2 binds to the transactivation domain of p53; this interaction might also interfere with the recruitment of the basal transcription machinery and/or essential co-activator(s) (Thut et al., 1997). Moreover, Mdm2 has been reported to promote conjugation of NEDD8 to p53, a modification that

inhibits its transcriptional activity (Xirodimas et al., 2004). Finally, Mdm2 induces mono-ubiquitylation of histone H2B surrounding p53-response elements, which results in transcriptional repression (Minsky and Oren, 2004). Recent genetic studies are not entirely consistent with a role for Mdm2 in the regulation of p53 transcriptional activity per se, however (Marine et al., 2006). Further genetic studies, such as knockin mutations, will be necessary to resolve whether Mdm2 antagonizes p53 only through protein degradation or through repression of transcriptional activation as well.

Mdmx, an Mdm2-related protein

Mdmx (also known as Mdm4) was originally isolated as a novel p53-interacting protein from a mouse cDNA expression library (Shvarts et al., 1996). The human ortholog, MDMX, was identified later (Shvarts et al., 1997). Both Mdmx and MDMX interact with p53 in cells, and Mdmx overexpression inhibits p53-activated transcription (Shvarts et al., 1996). cDNAs encoding MDMX were independently identified in a yeast two-hybrid screen for MDM2-associated proteins (Sharp et al., 1999; Tanimura et al., 1999). Indeed, the two related proteins interact in vivo. Hetero-oligomerization between Mdmx and Mdm2 appears to be much more stable than homo-oligomerization of each protein (Tanimura et al., 1999). Mdmx is thus implicated in the regulation of the p53-Mdm2 axis.

MDMX and MDM2 are structurally related proteins of 490 and 491 amino acids, respectively (Fig. 1). The greatest similarity between the two proteins is at the N-terminus, a region encompassing the p53-binding domain. The residues required for interaction with p53 are strictly conserved in MDM2 and MDMX (Shvarts et al., 1996), and the same residues in p53 are required for both MDMX-p53 and MDM2-p53 interactions (Bottger et al., 1999). Another well-conserved region common to MDMX and MDM2 is a RING-finger domain, located at the C-terminus of each protein. The integrity of the RING-finger domain is essential for MDMX-MDM2 heterodimerization (Sharp et al., 1999; Tanimura et al., 1999). Both MDM2 and MDMX also contain an additional zinc-finger domain. The function of this domain is largely unknown, but recent results suggest that an intact zinc finger, together with a central acidic domain, is essential for interaction between MDMX and casein kinase 1 alpha (CK1-α) (Chen, L. et al., 2005b). The central regions of MDM2 and MDMX show no significant similarity, but both regions are rich in acidic residues.

The post-translational modifications of Mdmx that have been characterized to date include phosphorylation, ubiquitylation and SUMOylation. Ubiquitylation and phosphorylation are both important factors in regulation of Mdmx stability and activity (see below). Pan and Chen showed in transient transfection studies that Mdmx is conjugated with SUMO-1 on K254 and K379 (Pan and Chen, 2005), but conversion of K254 and K379 to arginine has no effect on MdmX function in the assays used by these authors. We have extended these studies and could demonstrate that endogenous MDMX is modified by SUMO-2 on K254 and K379 (E. Meulmeester, M. Groenewoud and A.G.J., unpublished data). However, the biological relevance of these modifications remains unclear.

Mdmx, another key gatekeeper of the guardian

Because of its similarity to Mdm2 and its ability to inhibit p53-induced transcription following overexpression, Mdmx was hypothesized to act as a negative regulator of p53 (Shvarts et al., 1996; Migliorini et al., 2002a). This view has been

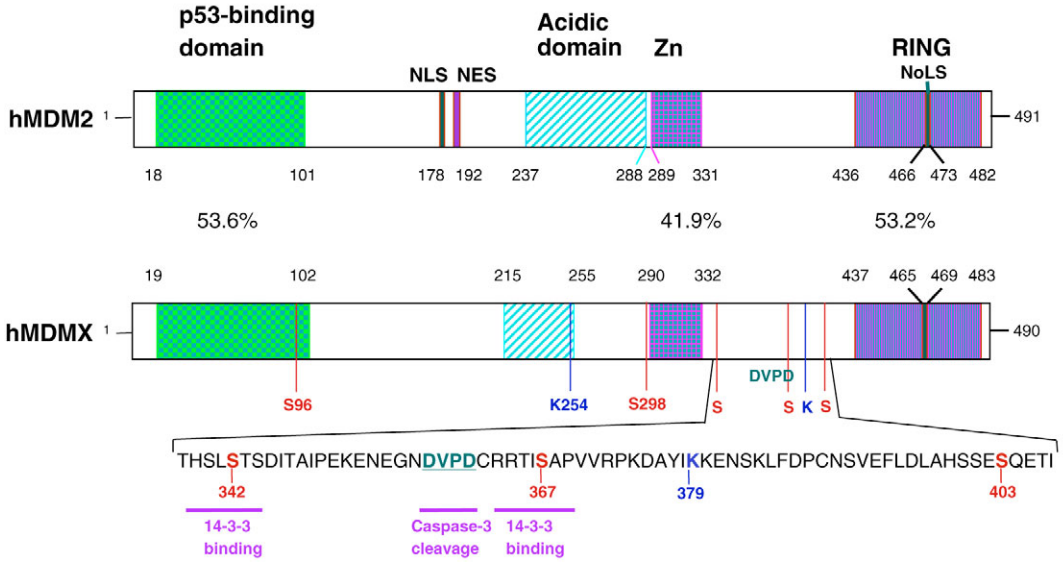

Fig. 1. Comparison of the MDM2 and MDMX primary structures. Several functional domains are highlighted. The p53-binding domain, zinc (Zn) finger and RING finger [containing the nucleolar location signal (NoLS)] are conserved. The percentage identity shared between these domains is indicated. Although both MDM2 and MDMX contain an acidic domain, no significant conservation of amino acid sequence is found, and the acidic domain of MDMX is smaller than that of MDM2. Part of the MDMX amino acid sequence (338-407) is shown to indicate the functional domains and modification sites. Serines (S) indicated in red are validated phosphorylation sites, whereas lysines (K) indicated in blue are targets for SUMO conjugation. DVPD, caspase-3 cleavage site; NES, nuclear export signal; NLS, nuclear localization signal.

confirmed by loss-of-function studies in two mutant mouse lines, which concluded that, similarly to Mdm2, Mdmx acts in vivo as an essential, non-redundant, negative regulator of p53 during embryonic development. Indeed, inactivation of p53 rescues the developmental defects that otherwise occur in Mdmx-deficient mice (Parant et al., 2001; Migliorini et al., 2002b; Finch et al., 2002) (reviewed by Marine and Jochemsen, 2004).

Unfortunately, because of the early embryonic lethality associated with Mdm2-null and Mdmx-null mutations, it has been difficult to assess the physiological contributions of Mdm2 and Mdmx to the regulation of p53 levels and activity. However, conditional alleles have recently been developed that yield further insight into how and in what cell types Mdm2 and Mdmx regulate p53 (Grier et al., 2002; Steinman and Jones, 2002; Mendrysa et al., 2003; Grier et al., 2006).

To test whether Mdm2 and Mdmx are required to restrain p53 activity in a single cell type, Xiong et al. conditionally inactivated both *Mdm2* and *Mdmx* in neuronal progenitors (Xiong et al., 2006). Meanwhile, Francoz et al. conditionally expressed p53 in neuronal progenitor cells or in post-mitotic cells of mice lacking *Mdm2* and/or *Mdmx* (Francoz et al., 2006). Loss of *Mdmx* or *Mdm2* leads to distinct phenotypes (see below) but, importantly, all phenotypes disappear in the absence of *p53*. Both Mdm2 and Mdmx are thus required to inhibit p53 activity in the same cell type, and these results confirm the notion that physiological levels of Mdm2 cannot compensate for Mdmx loss in vivo, at least in the abovementioned cell types.

Mdmx has also been conditionally inactivated in cardiomyocytes (Grier et al., 2006) and SMCs of the GI tract (Boesten et al., 2006). In contrast to loss of *Mdm2*, loss of *Mdmx* leads to only minor defects in histogenesis and tissue homeostasis. The data suggest that inhibition of p53 by Mdmx is required only in a restricted number of cell types and/or under certain physiological conditions. However, interpretation of these results is complicated. Even in cells in which Mdmx function is crucial, such as neuronal progenitor and post-mitotic cells, in contrast to *Mdm2*, loss of *Mdmx* consistently leads to only a moderate increase in p53 activity in vivo. This difference can be explained, at least in part, by the fact that p53 activates the transcription of *Mdm2* (Barak et al., 1993; Wu et al., 1993) but not *Mdmx*. Thus, in the absence of *Mdmx*, p53 transcriptional activity is enhanced, leading to the stimulation of the p53-Mdm2 negative feedback loop. Indeed, *Mdmx* loss leads to a moderate increase in Mdm2 protein levels in vitro and an increase in *Mdm2* transcription in vivo (Xiong et al., 2006; Francoz et al., 2006; Toledo et al., 2006). Note also that overexpression of an *Mdm2* transgene rescues the embryonic lethality associated with Mdmx-deficiency (Steinman et al., 2005), indicating that high levels of Mdm2 compensate for *Mdmx* loss. Thus, increased Mdm2 levels might better compensate for *Mdmx* loss in specific cell types, which would represent an alternative to a more simplistic view of a tissue-specific function of Mdmx. Nevertheless, at the molecular level, the difference in the severity of the Mdm2-null and Mdmx-null phenotypes is probably as a result of the fact that loss of Mdm2 leads to dramatic accumulation of the p53 protein, whereas loss of *Mdmx* does not significantly increase p53 levels in vivo (see below).

The molecular details of the role of Mdmx in the control of p53 and Mdm2 stability also remain unclear. Mdmx has been reported to act as a ubiquitin ligase in vitro (Badciong and Haas, 2002), but Mdmx overexpression in cells does not lead to p53 ubiquitylation and degradation (Jackson and Berberich, 2000; Stad et al., 2000; Migliorini et al., 2002a). However, Mdmx might regulate p53 stability indirectly by stabilizing Mdm2. Indeed, transfection studies suggest that Mdmx stabilizes Mdm2, perhaps by interfering with its auto-ubiquitylation (Gu et al., 2002; Stad et al., 2001). Another report, however, suggests that Mdmx stimulates not only Mdm2-mediated ubiquitylation of p53 but also Mdm2 self-ubiquitylation (Linares et al., 2003). p53 levels stay below the limit of detection when it is conditionally expressed in progenitor and post-mitotic neuronal cells of Mdmx-null mice (Francoz et al., 2006). Similarly, p53 is not detectable in E10.5 neural progenitor cells in which Mdmx is conditionally inactivated. By contrast, clear p53 staining is observed in Mdm2-deficient cells at the same stage of development (Xiong et al., 2006). Moreover, loss of both *Mdm2* and *Mdmx* does not lead to any further increase in p53 levels compared with loss of *Mdm2* alone. This suggests that Mdmx does not participate in the regulation of p53 stability independently of Mdm2 (Francoz et al., 2006). However, whether it does so in an Mdm2-dependent manner remains unclear. The analysis of mice encoding a mutant p53 that lacks the proline-rich domain ($p53^{\Delta P}$) also enabled evaluation of Mdmx function (Toledo et al., 2006). This hypomorphic *p53* mutant can fully rescue *Mdmx* deficiency. The consequences of *Mdmx* loss can therefore be observed in a compromised p53 context in the absence of Cre expression. In the absence of Mdmx, the transcription of *Mdm2* is stimulated to some extent, leading to slightly increased Mdm2 protein levels. Mdmx thus does not seem to affect Mdm2 protein stability significantly.

The contribution of Mdmx to the regulation of p53 transcriptional activity has become clearer. Genetic evidence indicates that Mdmx inhibits p53 transcriptional activity independently of Mdm2. Loss of *Mdmx* in cells lacking *Mdm2* causes an increase in p53 activity in cultured mouse embryonic fibroblasts (MEFs) without a concomitant increase in p53 levels (Francoz et al., 2006). Analyses of $p53^{\Delta P}$ regulation by Mdm2 and Mdmx produced similar results (Toledo et al., 2006). p53 interacts with the p300 histone acetyltransferase and this interaction appears to be essential for p53-dependent activation of the promoter of the p53-target gene *p21* (Liu et al., 2003). p53 itself is acetylated by p300 on several lysine residues, and this modification is thought to increase its transcriptional activity (Prives and Manley, 2001). Interestingly, Mdmx decreases p300-mediated acetylation of p53 (Danovi et al., 2004; Sabbatini and McCormick, 2002), and endogenous p53 acetylation is increased in Mdmx-null cells (Migliorini et al., 2002b). Regardless, the exact nature of the mechanism through which Mdmx attenuates p53 transcriptional activity awaits further investigation. Moreover, one additional caveat is that all these studies were performed in cultured cells (MEFs) in which Mdmx protein levels were not examined, mainly owing to lack of high-affinity antibodies. The stability of Mdmx could be affected by manipulation of Mdm2 levels because Mdm2 can ubiquitylate Mdmx, which leads to its degradation (discussed below). In cells lacking both p53 and Mdm2, the Mdmx protein levels are increased compared with cells lacking p53 only (Meulmeester et al.,

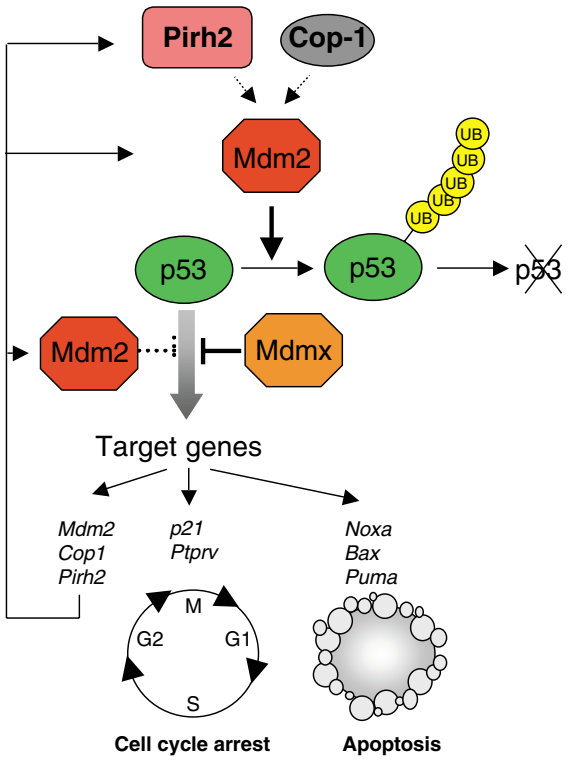

Fig. 2. A model for cooperative control of the p53 pathway by Mdm2 and Mdmx. In the absence of stress signals, the primary function of Mdm2 is to maintain p53 at low levels, whereas Mdmx contributes to the overall inhibition of p53 independently of Mdm2. Mdmx inhibits p53 transcriptional activity, whereas the contribution of Mdm2 to the regulation of p53 transcriptional activity per se is still unclear and a matter of debate. The role of Cop-1 and Pirh2 in the regulation of p53 levels and activity in vivo is unclear, but recent data suggest that, if they participate in the regulation of this pathway, they can only do so in an Mdm2-dependent manner. See text for details.

2005). It will, therefore, be essential in the future to assess endogenous Mdmx protein levels in such experiments.

Mdmx and Mdm2 thus appear to cooperate to antagonize p53 (Fig. 2). This has been confirmed in various in vivo settings. Mice lacking both *Mdm2* and *Mdmx* in the CNS exhibit a phenotype that is more severe and appears earlier than that of Mdm2-null mice (Xiong et al., 2006). Similarly, the extent of p53-mediated apoptosis is significantly greater in the neuroepithelium and in post-mitotic cells in mice lacking both *Mdm2* and *Mdmx* than in mice lacking *Mdm2* alone (Francoz et al., 2006). The cooperation of Mdm2 and Mdmx to limit p53 activity is therefore irrespective of the proliferation and/or differentiation status of the cells.

Both Mdm2 and Mdmx have been implicated in regulation of the stability and/or activity of several other proteins that control cell proliferation, such as the retinoblastoma protein pRb, the heterodimer E2F1-DP1, Numb and Smads (Ganguli and Wasylyk, 2003; Marine and Jochemsen, 2005). However, the relevance of these interactions has not been firmly established genetically. Moreover, several lines of evidence argue against p53-independent functions of Mdm2 and Mdmx under physiological conditions. They do not exclude the possibility, however, that supra-physiological expression levels of these two proteins affect the activity of other proteins and p53-independent pathways. This possibility is interesting, since both proteins are aberrantly expressed in several human primary tumors (see below).

Regulation of Mdmx expression and activity

As mentioned above, p53 is stabilized following cellular stress, such as DNA damage. Many mechanisms have been proposed (mostly involving phosphorylation) to regulate Mdm2-p53 interactions and/or Mdm2 activity (reviewed by Wahl, 2006). Only recently has insight into Mdmx regulation after DNA damage been obtained. In contrast to *Mdm2*, there is no evidence so far that transcription of *Mdmx* is affected by DNA damage, mitogens or any other cellular stress. Mdmx instead appears to be regulated mainly at the protein level. Several groups have shown that Mdm2 can bind to and ubiquitylate Mdmx to stimulate its proteasome-dependent degradation (Pan and Chen, 2003; de Graaf et al., 2003; Kawai et al., 2003).

Interestingly, in normal proliferating cells, MDM2 does not play a major role in regulation of MDMX stability. The MDMX protein is very stable, and knocking down MDM2 in cultured cells has little effect on the levels of MDMX (reviewed by Marine and Jochemsen, 2005). However, following DNA damage, MDMX levels rapidly decline in an MDM2-dependent manner. The Ataxia telangiectasia mutated (ATM) protein and checkpoint kinase 2 (Chk2) are key regulators of biological responses to DNA damage. Efficient degradation of MDMX following DNA damage requires ATM-dependent phosphorylation on S342 and S367 by Chk2 and S403 by ATM (Pereg et al., 2005; Chen, L. et al., 2005a; Okamoto et al., 2005; Pereg et al., 2006). Furthermore, UVC treatment results in Chk1-mediated phosphorylation of S367 (Jin et al., 2006). Phosphorylation of MDMX reduces its affinity for the deubiquitylating enzyme (DUB) HAUSP/USP7 (Meulmeester et al., 2005). Expression of HAUSP is essential for maintenance of both MDM2 and MDMX protein levels (Cummins et al., 2004; Li et al., 2004). MDM2 destabilization following DNA damage (Stommel and Wahl, 2004) is also the result of decreased HAUSP binding, whereas binding of p53 to HAUSP is not affected (Meulmeester et al., 2005). The destabilization of both MDMX and MDM2 is essential for proper p53 activation following DNA damage. The mechanism by which MDMX phosphorylations affect the MDMX-HAUSP interaction has not been elucidated. Clearly, loss of HAUSP binding might not be the only mechanism involved. For example, phosphorylation of both S342 and S367 creates binding sites for 14-3-3 protein. Interaction of 14-3-3 with MDMX is necessary for DNA-damage-induced nuclear accumulation and degradation of MDMX (LeBron et al., 2006; Pereg et al., 2006). It might also, however, affect binding to HAUSP.

Basal phosphorylation of Mdmx on Ser96 and Ser289 by CDK1 and CK1-α, respectively, has also been reported (Elias et al., 2005; Chen, L. et al., 2005b). Phosphorylation of Ser96 is proposed to regulate Mdm2 localisation, whereas the CK1-α-mediated phosphorylation stimulates the Mdmx-p53 interaction by an unknown mechanism. Mdmx thus appears to be regulated primarily by post-translational modifications that affect its stability, subcellular localization and protein-protein interactions.

MDMX contributes to tumorigenesis

Disruption of the p53 tumor suppressor pathway is as a result of mutation of the *p53* gene in approximately 50% of cases. Tumors retaining wild-type p53 are thought to have defects either in effector target genes or in the expression of p53 regulators. One of the best examples of the latter class is *MDM2*, levels of which can be increased by gene amplification, enhanced transcription or increased translation. Several studies now implicate MDMX in tumor formation. A study on a large series of gliomas revealed that *MDMX* is amplified/overexpressed in 5/208 tumor samples (Riemenschneider et al., 1999) and that *MDMX* is the common amplified gene in the large amplicons (Riemenschneider et al., 2003). Furthermore, in approximately 30% of the tumor cell lines tested *MDMX* is either overexpressed or alternatively transcribed, and in general this correlates with the presence of wild-type p53 (Ramos et al., 2001). A recent analysis of a large series of tumors also revealed overexpression of *MDMX* in a significant percentage of several tumor types – for example, 19% of breast carcinomas (Danovi et al., 2004). In all cases, amplification of *MDMX* correlated with wild-type p53 status and lack of *MDM2* amplification.

The importance of enhanced MDMX expression has been tested in the MCF-7 breast tumor cell line, which contains wild-type p53. Knocking down endogenous *MDMX* increases expression of p21, a p53-responsive gene product that negatively regulates progression through the cell cycle, without a significant increase in p53 levels. Colony assays showed that knocking down *MDMX* blocks proliferation of MCF-7 cells unless p53 levels are simultaneously decreased. Constitutive expression of Mdmx immortalizes MEFs in the absence of p53 mutation or loss of expression of ARF, a nucleolar protein that antagonizes Mdm2 functions (Sherr and Weber, 2000). Furthermore, Mdmx prevents oncogenic-Ras-induced premature senescence, and cells expressing Mdmx and activated Ras (Ras^{V12}) are oncogenic in nude mice (Danovi et al., 2004). MDMX thus functions as an oncogene when constitutively overexpressed, which can act as an alternative to p53 mutation in human tumors.

Many tumors contain aberrantly and/or alternatively spliced MDM2 variants. The effects of these variants are still unknown, but their expression is more common in high-grade than in low-grade tumors (Bartel et al., 2004). A systematic analysis of *MDMX* splice variants in large tumor sets is still lacking. However, two variants have been identified and partially characterized. The MDMX-S variant comprises only the p53-binding domain and a few alternative C-terminal amino acids. It is detected both in untransformed and transformed cells, and its expression is elevated when cells are stimulated to enter S-phase (Rallapalli et al., 1999). Owing to a higher affinity than full-length MDMX for p53 and to its increased nuclear localization, MDMX-S appears to be a very efficient inhibitor of p53 (Rallapalli et al., 1999; Rallapalli et al., 2003). MDMX-S is also more stable than MDMX, possibly because it can no longer interact with MDM2 and is, therefore, protected from MDM2-mediated degradation. Interestingly, an elevated MDMX-S/MDMX ratio has been reported in high-grade gliomas (Riemenschneider et al., 2003). Moreover, analysis of soft-tissue sarcomas indicated that high MDMX-S levels correlate with decreased survival and an increased risk of tumor-related death (Bartel et al., 2005).

Another splice variant, MDMX211, results from splicing between the exon 2 donor site and a cryptic splice acceptor site within exon 11 (Giglio et al., 2005). The resulting protein lacks the p53-binding domain but retains the RING-finger domain. Transfection and RNAi studies indicate that this protein has oncogenic activity possibly as a result of stabilization of MDM2. Although MDMX211 variant has been identified in 2/16 analyzed non-small-cell lung tumors, further studies are needed to establish its significance in human cancer.

MDMX as a drug target in retinoblastoma

Retinoblastoma is a rare childhood cancer of the retina that initiates in utero during fetal development because of inactivation of the *RB1* gene (Dyer and Harbour, 2006). Although the initiating genetic event (biallelic inactivation of *RB1*) is well established, the subsequent genetic events that contribute to retinoblastoma progression have not been well characterized. The status of the p53 pathway has been a topic of considerable debate in the field. Early studies on human tumors demonstrated that p53 is wild-type in retinoblastoma (Kato et al., 1996). However, exogenous p53 can induce cell death in retinoblastoma cell lines (Nork et al., 1997). In HPV-E7 transgenic mouse models of retinoblastoma, tumor development is greatly enhanced when p53 is inactivated (Howes et al., 1994). Recently, Zang et al. developed the first knockout mouse model of retinoblastoma (Zhang et al., 2004) by conditionally inactivating *Rb1* in the developing retina of $p107^{-/-}$ mice. As in the HPV-E7 transgenic mouse models, inactivation of p53 in retinal progenitor cells lacking *Rb1* and *p107* leads to an aggressive invasive form of retinoblastoma in mice that more faithfully recapitulates the human disease (Dyer et al., 2005). Inactivation of the p53 pathway is therefore likely to be an important step in retinoblastoma progression, but the *p53* gene itself remains intact.

BAC-CGH, fluorescence in situ hybridization (FISH) and immunohistochemistry (IHC) studies reveal an increased *MDMX* copy number in 65% of human retinoblastomas, and *MDM2* is amplified in an additional 10% of these tumors (Laurie et al., 2006). Genetic amplification of *MDMX* correlates with increased mRNA and protein levels and suppression of p53 target genes such as *p21*. MDMX regulates cell death and cell cycle exit in cultured retinoblastoma cells in a p53-dependent manner. Moreover, ectopic expression of *MDMX* in mouse *Rb*-null *p107*-null retinal progenitor cells leads to a reduction in p53-mediated apoptosis and clonal expansion of tumor cells. Similar studies of human fetal retinae demonstrate that ectopic expression of MDMX rescues p53-mediated cell death as a result of activation of the ARF oncogenic stress response pathway following *RB1* gene inactivation. These experiments clearly show that the p53 pathway is suppressed in retinoblastoma cells following biallelic inactivation of *RB1* and that a majority of tumors inactivate the p53 pathway through *MDMX* gene amplification (Fig. 3). In addition, they show that retinoblastoma does not arise from an intrinsically death-resistant cell as previously believed (Dyer and Bremner, 2005).

These findings not only challenge the long-standing belief that retinoblastoma is the exception to the general principle that the Rb and p53 pathways must be inactivated in cancer, but also provide a specific target for chemotherapy. Nutlin-3, a small-molecule inhibitor of the MDM2-p53 interaction

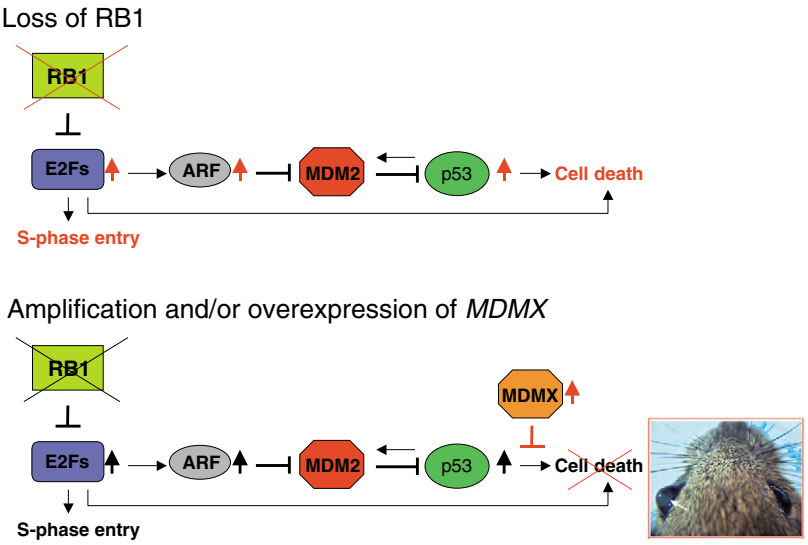

Fig. 3. Interactions between Rb, MDMX and p53 during retinoblastoma formation. (A) Biallelic inactivation of the *RB1* gene is the initiating oncogenic event in retinoblastoma. As a consequence, the activity of transcription factors of the E2F family is unleashed, leading to increased S-phase entry and induction of ARF expression. ARF is induced in response to oncogenic stress and a direct E2F3a transcription target. ARF functions as an Mdm2 antagonist and therefore activates the p53 pathway. High ARF expression was indeed recently detected in RB1-deficient retinoblasts and, as a consequence, a high proportion of these cells undergo p53-mediated apoptosis. (B) The p53 pathway is suppressed during the formation of retinoblastoma and the majority of tumors inactivate this pathway through selective amplification and/or overexpression of MDMX.

(Vassilev et al., 2004), was shown to act primarily through MDM2 even in cells expressing high levels of MDMX (Patton et al., 2006). However, we have recently shown that nutlin-3 also binds to MDMX, even if it does so with a 40-fold lower affinity than for MDM2 (Laurie et al., 2006). This is consistent with structural and functional studies suggesting that MDM2 antagonists may not be optimal as MDMX antagonists (Bottger et al., 1999; McCoy et al., 2003). Nevertheless, nutlin-3 can induce p53-mediated cell death in *Mdm2*-deficient MEFs in a manner that is dependent on Mdmx. Moreover, nutlin-3 reduces the MDMX-p53 interaction and efficiently kills retinoblastoma cells (Laurie et al., 2006). Furthermore, combining nutlin-3 with topotecan synergistically increases tumor cell killing through the p53 pathway. Given the efficacy of such a treatment in rodents, we propose that subconjunctival administration of these two drugs could achieve the same synergistic effect in patients without causing the side effects associated with prolonged systemic exposure. Not only is retinoblastoma a good model for suppression of p53-mediated cell death by *MDMX* amplification but it is an ideal system to study local delivery of chemotherapy targeted to the ARF-MDM2/MDMX-p53 pathway.

Concluding remarks and perspectives

Mdmx primarily inhibits p53 by interfering with its transcriptional activity. Even if it is now clear that direct interaction between Mdmx and p53 appears essential, the molecular mechanism by which Mdmx regulates p53 activity has not been fully elucidated and should be. Mdmx not only interacts with p53 but also heterodimerizes with Mdm2. The relevance of this interaction in vivo has to be further examined since the existing mouse models have failed, so far, to support most transfection studies, which indicates that this interaction regulates Mdm2 and Mdmx protein stability. In view of its high degree of sequence conservation and its similarity with Mdm2, it is intriguing that the RING-finger domain of Mdmx appears incapable of inducing ubiquitylation and subsequent degradation of p53. It will be interesting to assess whether or not this domain is required only for the formation of Mdmx-Mdm2 complexes or if it is required for ubiquitylation of other substrates. In this context, it would be worth checking whether this domain can promote binding of Mdmx to E2 ubiquityl conjugases. Another major unknown is the relative abundance of the Mdm2 and Mdmx proteins and the stoichiometry of the different complexes that can be formed, such as p53-Mdm2, p53-Mdmx, Mdm2-Mdmx and possibly p53-Mdm2-Mdmx, in normal cells and cells under stress. In order to resolve this important issue, new tools will have to be generated, such as high-affinity anti-Mdmx antibodies and more elaborate mouse models – for instance, *Mdm2* and *Mdmx* alleles expressing tagged proteins from the respective endogenous promoters. Knockin mutations should also be used to test the relevance of the ATM- and Chk2-phosphorylation sites described above.

There is now clear genetic evidence indicating that Mdmx contributes to the regulation of p53 independently of Mdm2 and that both proteins act synergistically to keep p53 in check (Francoz et al., 2006; Marine et al., 2006). Thus, activation of 'dormant' p53 tumor suppressor activity in tumors with wild-type p53 is expected to be more efficient if one uses specific antagonists that can target both MDM2 and MDMX. Since nutlin-3 has only a poor affinity for Mdmx, we propose that new, specific Mdmx antagonists should be developed. Alternatively, if nutlin-3 can be delivered locally at a high enough concentration to inhibit both MDM2 and MDMX, then this treatment may be sufficient. A particularly clear illustration of the latter approach is the efficacy with which retinoblastoma development is impaired in a rat xenograft model upon subconjunctival delivery of nutlin-3. Moreover, by combining MDM2/MDMX antagonists with drugs that induce a p53 response through DNA damage (e.g. topotecan) this anti-tumor effect may be further enhanced.

One of the most common ways that the p53 pathway is inactivated in retinoblastomas is by increased MDMX expression through gene amplification. It is intriguing that the frequency of *MDMX* amplification is high in retinoblastoma compared with other tumor types (Danovi et al., 2004). This observation may be explained by the difference in the ability of ARF to bind to MDM2 and MDMX. Biochemical studies have shown that ARF can bind to MDM2 but not MDMX (Wang et al., 2001). Considering that ARF is directly regulated by RB1 (Aslanian et al., 2004), retinal cells lacking RB1 may have a greater degree of induction of ARF than tumors initiated

by other disruptions in the Rb pathway – for instance, those involving p16, cyclin D1 or CDK4 (Sherr and McCormick, 2002). *MDM2* amplification should therefore not lead to efficient inhibition of the p53 pathway in *RB1*-deficient retinal cells. In contrast, despite high levels of ARF, *MDMX* amplification would be expected to efficiently block the p53 cell death pathway in retinoblastoma because ARF does not bind MDMX. Additional experiments, including the generation of conditional Mdm2 and Mdmx mouse models, will be necessary to test further this hypothesis.

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