The EMBO Journal (2007) 26, 102–112 | © 2007 European Molecular Biology Organization | All Rights Reserved 0261-4189/07
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THE
EMBO
JOURNAL

An essential function of the extreme C-terminus of MDM2 can be provided by MDMX

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MDM2 (HDM2) is a ubiquitin ligase that can target the p53 tumor suppressor protein for degradation. The RING domain is essential for the E3 activity of MDM2, and we show here that the extreme C-terminal tail of MDM2 is also critical for efficient E3 activity. Loss of E3 function in MDM2 mutants deleted of the C-terminal tail correlated with a failure of these mutants to oligomerize with MDM2, or with the related protein MDMX (HDMX). However, MDM2 containing point mutations within the C-terminus that inactivated E3 function retained the ability to oligomerize with the wild-type MDM2 RING domain and MDMX, and our results indicate that oligomers containing both wild-type MDM2 and a C-terminal mutant protein retain E3 function both in auto-degradation and degradation of p53. Interestingly, the E3 activity of C-terminal point mutants of MDM2 can also be supported by interaction with wild-type MDMX, suggesting that MDMX can directly contribute to E3 function.

The EMBO Journal (2007) **26,** 102–112. doi:10.1038/sj.emboj.7601469; Published online 14 December 2006
Subject Categories: proteins
Keywords: E3; MDM2; MDMX; p53; ubiquitin

Introduction

The p53 protein functions to inhibit the outgrowth of cells with malignant potential through the induction of cell cycle arrest and apoptosis in cells that have been stressed by genotoxic and other types of damage (Vogelstein *et al*, 2000). These activities are key to p53's ability to act as a tumor suppressor, and their loss is an important step in the development of most malignancies. Although these anti-proliferative activities of p53 are beneficial in tumor suppression, they are extremely deleterious to normal growth and development, and several mechanisms exist to keep p53 activity in check during normal proliferation (Ryan *et al*, 2001). One of these is the control of p53 protein stability, which allows for the maintenance of low levels of p53 protein in unstressed cells and the rapid increase in p53 levels owing to stabilization in response to stress. The degradation of p53 is principally through the proteasome, and a number of ubiquitin ligases have been described that can target the degradation of p53 through this pathway (Brooks and Gu, 2006). One of the most important ubiquitin ligases is MDM2 (also called HDM2), a RING domain E3 that targets a number of proteins, including p53, for degradation. The importance of MDM2 as a regulator of p53 has been demonstrated in a number of systems, where deletion of MDM2 leads to the stabilization and activation of p53 (Mendrysa *et al*, 2003; Grier *et al*, 2006).

Almost all tumor cells show defects in the p53 pathway, either through mutations within the p53 gene itself or by perturbations in the mechanisms that allow for the activation of p53 in response to stress (Vousden, 2002). Most p53-activating signals result in the stabilization of p53 through inhibition of MDM2, and in several tumor types failure to activate p53 has been associated with a failure to properly inactivate MDM2. This can result from amplification and overexpression of MDM2 (Momand *et al*, 1998), loss of kinases that phosphorylate p53 (Brooks and Gu, 2003) or MDM2 (Meek and Knippschild, 2003) to inhibit their interaction or function, or from defects in negative regulators of MDM2, such as $p14^{ARF}$ (Sharpless, 2005). These observations suggest that small molecule inhibitors of MDM2 might be beneficial in the treatment of cancers that retain wild-type p53 but cannot properly activate it (Buolamwini *et al*, 2005). The successful development of such drugs will depend on a clear understanding of how MDM2 functions to target p53 for degradation.

MDM2 belongs to the RING domain family of E3 ligases, and mutations in the core metal-coordinating residues within the RING domain result in the complete loss of MDM2's E3 function (Fang *et al*, 2000). In addition to p53, several other proteins are targeted for degradation by MDM2, including MDM2 itself and the MDM2-related protein MDMX (Mdm4 in mice) (de Graaf *et al*, 2003; Kawai *et al*, 2003a; Pan and Chen, 2003). While the isolated RING domain can be shown to possess some E3 activity, the ability to degrade p53 also depends on the integrity of other regions of MDM2, such as the central acidic domain (Argentini *et al*, 2001; Kawai *et al*, 2003b; Meulmeester *et al*, 2003). It has been shown that the ability of MDM2 to degrade itself can be separated from the ability to degrade p53, and some forms of genotoxic damage function to stabilize p53 by promoting the auto-degradation of MDM2, and so shifting the balance between the levels of the two proteins (Stommel and Wahl, 2004). Unlike MDM2, MDMX possesses no intrinsic E3 activity, despite a strong similarity between the two proteins within the RING domains (Jackson and Berberich, 2000; Stad *et al*, 2001). However, MDMX can form an interaction with MDM2 through the RING domain (Sharp *et al*, 1999; Tanimura *et al*, 1999), and whereas at high levels of expression this interaction can inhibit the ability of MDM2 to degrade p53 (Jackson and Berberich, 2000; Stad *et al*, 2000), at physiological levels

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Received: 23 May 2006; accepted: 26 October 2006; published online: 14 December 2006

MDMX has been shown to be important for the negative regulation of p53 (Parant *et al*, 2001; Migliorini *et al*, 2002b). Clearly, the ability of MDMX to bind directly to p53 and inhibit p53's transcriptional activity plays a major role in the negative regulation of p53 by MDMX (Shvarts *et al*, 1996), and this function of MDMX is independent of MDM2 (Francoz *et al*, 2006; Xiong *et al*, 2006). The current data most strongly support a model in which MDM2 regulates p53 by targeting it for degradation, whereas MDMX functions by directly inhibiting the transcriptional activity of p53 by binding to its N-terminal transactivation domain (Marine *et al*, 2006). However, in addition to these independent and synergistic activities of MDM2 and MDMX, there is evidence that interactions between these two proteins also play an important role in their mutual regulation, and thereby the regulation of the p53 response. A large part of this interconnection reflects the regulation of MDMX stability by MDM2. In response to DNA damage, phosphorylation of MDMX enhances the degradation of MDMX by MDM2 (Chen *et al*, 2005)—an effect that may be related to reduced binding to the deubiquitinating enzyme HAUSP (Meulmeester *et al*, 2005) or an increase in 14-3-3 binding (Okamoto *et al*, 2005; Lebron *et al*, 2006)—leading to the stabilization and activation of p53. MDM2 also promotes the nuclear accumulation of MDMX (Stad *et al*, 2001; Li *et al*, 2002; Migliorini *et al*, 2002a), although MDM2-independent nuclear localization of MDMX is also seen in response to DNA damage (Li *et al*, 2002; Lebron *et al*, 2006). In addition to the ability of MDM2 to regulate MDMX, there is some evidence that MDMX can function to enhance the ability of MDM2 to degrade p53, either by promoting the E3 activity of MDM2 against both itself and p53 (Linares *et al*, 2003) or by inhibiting the auto-degradation of MDM2, so allowing sufficient MDM2 accumulation to degrade p53 (Stad *et al*, 2001; Gu *et al*, 2002). Despite these observations, the overall contribution of MDMX to p53 or MDM2 stability remains unclear. Studies in mice have indicated that an effect of MDMX on p53 stability, through the modulation of MDM2, can be seen in some but not all tissues (Marine *et al*, 2006).

It is evident that there is a complex and dynamic relationship between MDM2 and MDMX, which is key to the regulation of p53 stability and function. Understanding the functions of these proteins, and how they might interact with each other, will be critical for exploiting them as potential therapeutic targets. Here, we identify a new region in the extreme C-terminus of MDM2 and MDMX that plays an important role in regulating MDM2 E3 activity.

Results

Contribution of the C-terminal tail of MDM2 to p53 degradation

The RING domain of MDM2 is located very close to its C-terminus, with the last cysteine of the zinc coordination motif 14 amino acids from the end of the protein. A comparison of the sequence of this C-terminal tail showed that this region is highly conserved through evolution (Figure 1A). In order to assess any role that this region might have in MDM2 activity, we tested two MDM2 mutants, deleting the C-terminal 9 or 12 amino acids (MDM2Δ9 and MDM2Δ12), for their ability to degrade and ubiquitylate p53 (Figure 1B). In cotransfection experiments, both mutants were defective

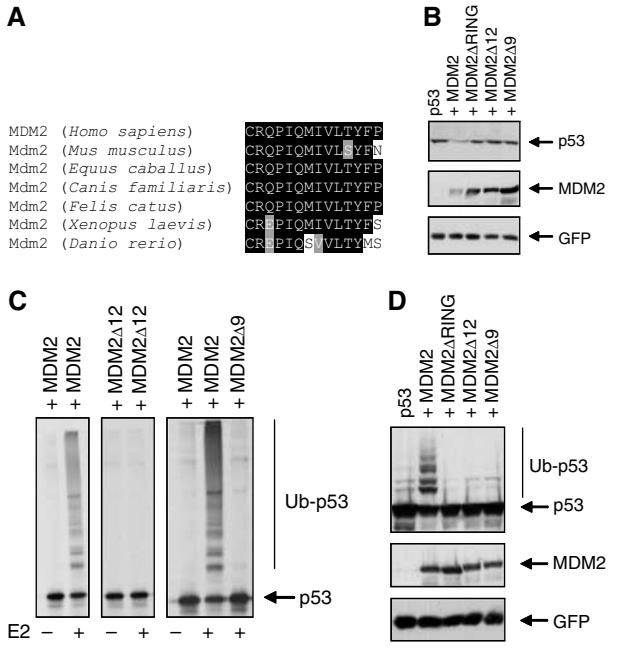

Figure 1 C-terminal tail of MDM2 is required for MDM2-mediated p53 degradation and ubiquitylation. (**A**) C-terminal tail sequences of MDM2 proteins were aligned using BOXSHADE 3.21 software at http://www.ch.embnet.org/software/BOX_form.html. (**B**) MDM2 C-terminal deletions are not able to target p53 for degradation. U2OS cells were transiently cotransfected with FLAG-p53, GFP and MDM2 C-terminal deletions and analyzed by Western blotting. (**C**) MDM2 C-terminal tail deletions prevent efficient p53 ubiquitylation *in vitro*. Bacterially expressed wild-type and mutant GST-MDM2, *in vitro*-translated p53, recombinant ubiquitin, E1 and E2 enzymes were incubated in the reaction buffer at 37°C for 2 h. A negative control reaction was prepared without the E2 enzyme. Reaction products were resolved by SDS–PAGE and analyzed by Western blotting with anti-p53 DO-1. (**D**) MDM2 C-terminal deletions prevent efficient p53 ubiquitylation *in vivo*. U2OS cells, transiently cotransfected with FLAG-p53 (0.2 μg) and MDM2 C-terminal deletion mutants (1.5 μg), were treated with 10 μM MG132 24 h after transfection and analyzed by Western blotting.

for the degradation of p53 compared with wild-type MDM2, and both mutants were expressed at higher levels, suggesting a failure to auto-degrade (Figure 1B). This defect in p53 degradation was also reflected in an inability of the MDM2 mutants to ubiquitylate p53 in an *in vitro* assay (Figure 1C). Loss of the C-terminal tail also prevented the enhanced ubiquitylation of p53 seen following expression of MDM2 in cells (Figure 1D), similar to the effect of a much larger C-terminal deletion that also removes the RING domain (MDM2ΔRING).

The C-terminal region of MDM2 contains threonine (serine in mouse Mdm2) and tyrosine residues at amino acids 488 and 489 that are potential targets for phosphorylation. Although these residues do not lie within predicted consensus sequences for kinase recognition sites, we made mutants of MDM2 carrying substitutions of these amino acids to non-phosphorylatable (T488A, Y489F) and phospho-mimetic (T488D, Y489D) alternatives (Figure 2A). Mutation of threonine 488 to alanine (T488A) or tyrosine 489 to phenylalanine (Y489F) did not affect the ability of MDM2 to degrade p53, compared with the wild-type protein (Figure 2B). However, mutation to a phospho-mimetic amino acid (T488D and Y489D) resulted in a loss of p53-degrading activity,

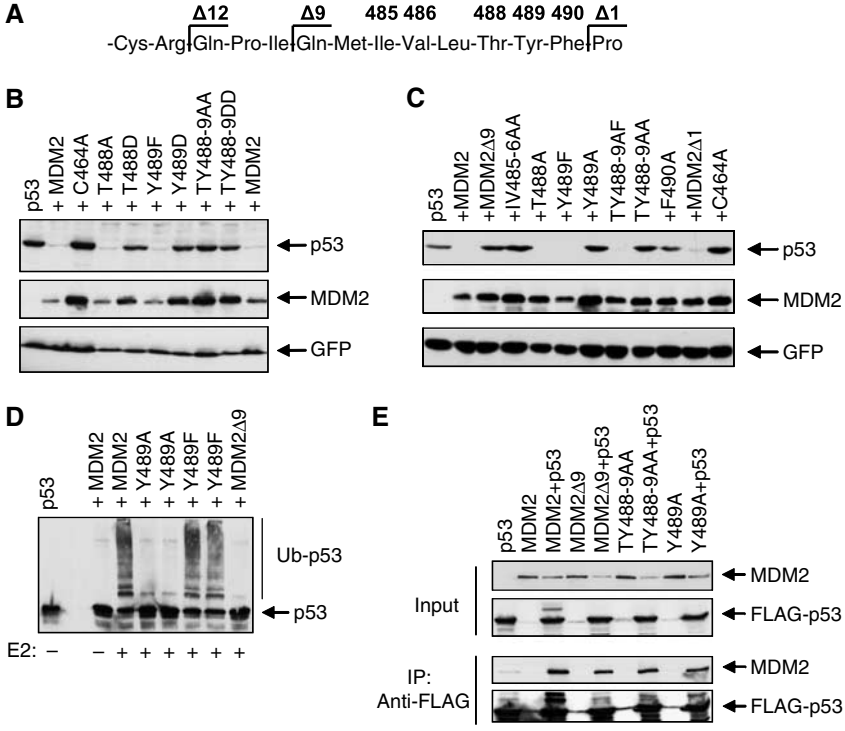

Figure 2 Point mutations within the C-terminal tail inhibit MDM2 function. (**A**) Location of mutants generated within MDM2 C-terminal tail. (**B**) MDM2 T488D and Y489D phospho-mimicking mutants do not degrade p53. U2OS cells were transiently cotransfected with FLAG-p53, wild-type or mutant MDM2 and pEGFP-N1 for 30 h and analyzed by Western blotting. (**C**) Aromatic residue at position 489 of MDM2 is required for p53 degradation. U2OS cells were cotransfected with FLAG-p53 and wild-type or C-terminal tail mutants of MDM2 for 30 h, then analyzed by Western blotting. (**D**) Aromatic residue at position 489 of MDM2 is required for p53 ubiquitylation *in vitro*. Bacterially expressed wild-type and mutant GST-MDM2, *in vitro*-translated p53, recombinant ubiquitin, E1 and E2 enzymes were incubated in the reaction buffer at 37°C for 2 h. Reactions containing Y489 mutants were performed in duplicate. (**E**) C-terminal tail is not required for MDM2 binding to p53. HEK293 cells, transiently cotransfected with FLAG-p53 and wild-type or C-terminal tail mutants of MDM2 for 36 h, were treated with proteasome inhibitor MG132 for 4 h and p53-MDM2 protein complexes were immunoprecipitated with anti-FLAG antibody M2. Immunoprecipitated proteins were analyzed by Western blotting.

comparable to that seen in an MDM2 mutant carrying a substitution of one of the key RING domain cysteines (C464A). Interestingly, each of the MDM2 mutants that failed to degrade p53 also showed evidence for increased stability, suggesting that these mutants also failed to target themselves for degradation. Although these results suggest that phosphorylation of either threonine 488 or tyrosine 489 may inhibit the ability of MDM2 to degrade p53, a double mutant substituting alanine at both positions (TY488-9AA) also lost the ability to degrade p53 (Figure 2B), suggesting that the retention of an aromatic residue at position 489 (either tyrosine or phenylalanine) is important for MDM2 activity. We made a number of further mutations affecting the C-terminal tail and examined their ability to degrade p53 (Figure 2C). As predicted, substitution of tyrosine for alanine (Y489A) destroyed the p53-degrading activity, whereas a double substitution conserving the aromatic nature of the residue at position 489 (TY488-9AF) retained this function. In support of the suggestion that the aromatic residues in this region of MDM2 are important for activity, substitution of the phenylalanine at position 490 to alanine (F490A) also prevented p53 degradation. Deletion of the last amino acid (MDM2Δ1) did not affect the ability to degrade p53 (Figure 2C). Substitutions of the two conserved hydrophobic amino acids within the C-terminus of MDM2 (IV485-6AA) also prevented the function of MDM2 in degrading p53 (Figure 2C), further supporting the importance of the integrity of the C-terminal tail for MDM2 function.

To confirm that the loss of function of some of these C-terminal MDM2 mutants directly reflected a loss of E3 activity, we tested the effect of mutations of tyrosine 489 on the ability of MDM2 to ubiquitinate p53 in an *in vitro* assay. In agreement with the degradation results, mutation of the tyrosine to phenylalanine (Y489F) did not affect E3 function, whereas substitution of alanine at this position (Y489A) destroyed this activity (Figure 2D).

Contribution of the C-terminal tail of MDM2 to p53 binding

Although the p53-binding region of MDM2 has been clearly mapped to the N-terminus of the protein, recent studies have shown that the central region of MDM2 also provides another interaction site for p53 (Yu *et al*, 2006), and it is possible that alterations in the C-terminus of the full-length MDM2 protein might affect the p53 interaction. However, we were unable to detect any defect in their ability to bind p53 (Figure 2E), indicating that failure of some of these mutants to degrade p53 is not the result of a defect in the ability to interact with p53.

Oligomerization of C-terminal MDM2 mutants

Previous studies have shown that MDM2 can oligomerize through RING/RING interactions (Tanimura *et al*, 1999; Dang

et al, 2002; Linares *et al*, 2003), and that this interaction may be important for MDM2's E3 activity. We therefore tested whether the defect in E3 activity exhibited by the C-terminal MDM2 mutants was a result of a failure to homo-oligomerize. Initial co-precipitation experiments showed no significant difference in the ability of the C-terminal MDM2 mutants to interact with a GFP-RING domain (data not shown). However, it has been shown that MDM2 can form interactions through both the RING and the acidic domains (Dang *et al*, 2002), and it is possible that binding between the RING and acidic domains was masking differences in the RING/RING interactions. Therefore, in order to look only at the interaction of MDM2 through the RING domains, we examined the ability of a GFP-tagged MDM2 RING only protein (GFP-RING) to bind an MDM2 protein deleted of the acidic domain (amino acids 245–295; MDM2ΔAD) and carrying the C-terminal mutations. Either co-precipitation through the GFP-RING protein or the reciprocal co-precipitation through the MDM2ΔAD protein (Figure 3A) showed a clear reduction in the interaction between the wild-type RING

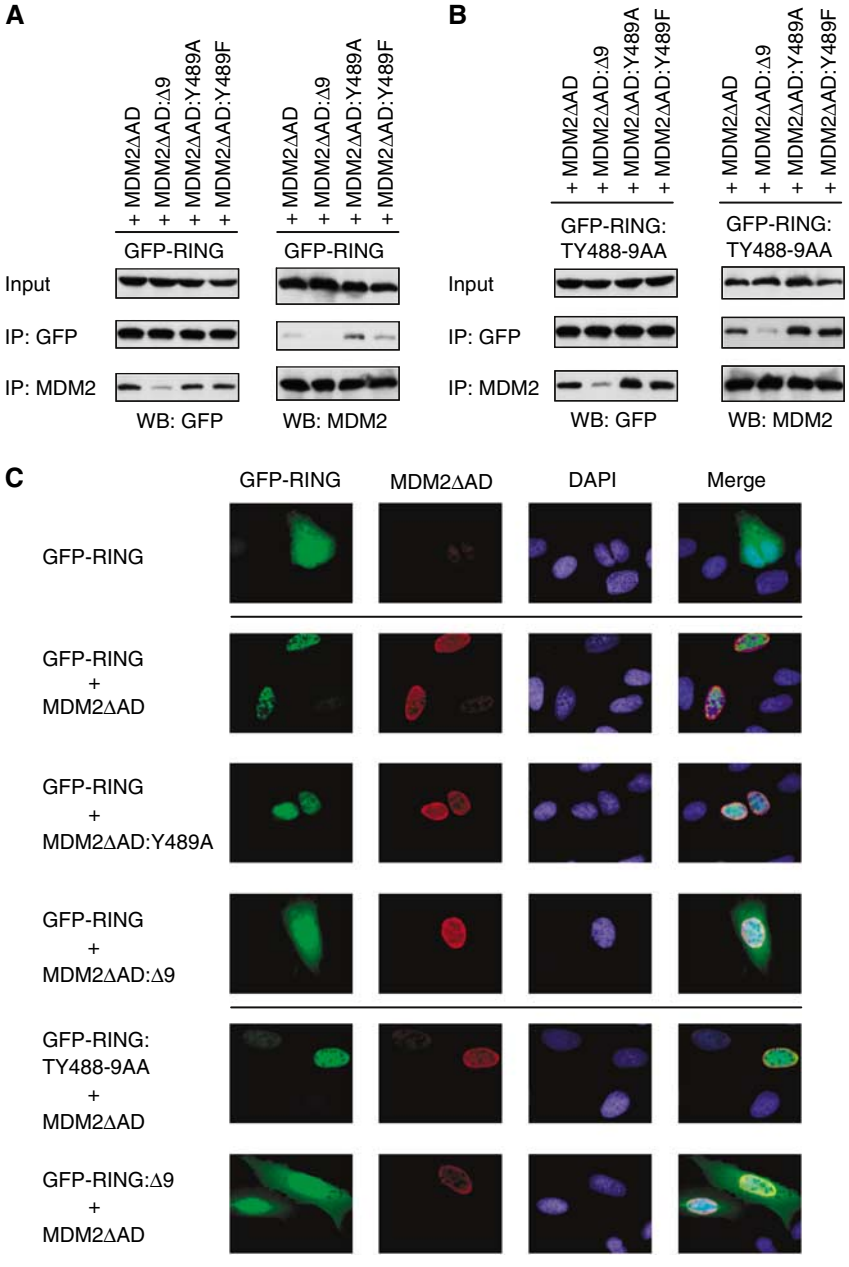

Figure 3 Point mutants of MDM2 within the C-terminal tail retain the ability to homo-oligomerize through RING–RING interactions. C-terminal tail mutants in the context of MDM2 lacking the acidic domain (MDM2ΔAD) were transiently transfected into HEK293 cells together with GFP-RING (**A**) or GFP-RING:TY488-9AA (**B**). Cells were treated 36 h post-transfection with proteasome inhibitor MG132 for 4 h, lysed and immunoprecipitated with anti-MDM2 (Ab-1) and anti-GFP (7.1/13.1) antibodies, then analyzed by Western blotting. (**C**) Wild-type RING domains of MDM2 can homo-oligomerize *in vivo* with the MDM2 C-terminal tail point mutants, but not with the C-terminal tail deletion mutants. U2OS cells were cotransfected with constructs coding for GFP-tagged MDM2 RING (lacks nuclear localization signal (NLS); diffuse pattern of subcellular localization) and MDM2ΔAD (contains NLS; nuclear protein) with wild-type or mutant C-terminal tail. MDM2ΔAD-induced translocation of GFP-RING into the nucleus was used as an indicator of the interaction between the two MDM2 proteins.

domain of the MDM2Δ9 deletion mutant. However, there was no detectable defect in the binding of the wild-type RING to the point mutants Y489A or Y489F. Interestingly, a very similar pattern of interaction was seen following coexpression of the C-terminal mutants (in the context of MDM2ΔAD) with a GFP-RING protein carrying the TY488-9AA double mutant (Figure 3B). These results therefore show that although the loss of p53 degradation activity shown by MDM2Δ9 may be a reflection of an inability to oligomerize through the RING domain, such a defect in oligomerization is not obviously the cause of the lack of p53 degradation activity of the Y489A mutant. To further confirm the oligomerization capacities of the MDM2 mutants, we examined their interaction in cells by immunofluorescence (Figure 3C). A GFP-RING protein, which lacks the MDM2 nuclear localization signals (NLS), localized diffusely in the cell nucleus and cytoplasm. However, following oligomerization with coexpressed MDM2ΔAD protein (which retains the NLS), the GFP-RING protein was also relocalized to the nucleus. Using this assay, we were able to confirm that point mutations in the C-terminal tail of MDM2 (Y489A or TY488-489AA) did not affect homo-oligomerization through the RING domain, whereas this ability was lost with the deletion of the C-terminal tail (MDM2Δ9).

As the Y489A mutant fails to target p53 for degradation, but retains the ability to oligomerize with the wild-type MDM2 RING domain, we were interested in determining whether this mutant might function as a dominant negative, and so inhibit the p53-degrading activity of wild-type MDM2. Interestingly, coexpression of the Y489A or Y489D mutants with wild-type MDM2 resulted in an efficient rate of p53 degradation (Figure 4A). A reduction in the degradation of p53 is not apparent until a high ratio of mutant to wild-type MDM2 is expressed, and only when mutant MDM2 is expressed alone is a complete failure to degrade p53 apparent. These results suggest that the Y489A and Y489D mutants do not function as dominant negatives, and that although a homo-oligomer of these mutant MDM2 proteins is inactive in the degradation of p53, a hetero-oligomer containing wild-type and mutant proteins is still functional. To compare the activities of different MDM2 mutants, we carried out a similar experiment using the MDM2Δ9 mutant (Figure 4B). Unlike either the Y489A or IV485-6AA mutants, which did not impede degradation of p53 by wild-type MDM2, coexpression of the MDM2Δ9 mutant was able to block p53 degradation in the presence of wild-type MDM2. This inhibition of wild-type MDM2 by the MDM2Δ9 mutant, which shows a defect in the RING/RING interaction, presumably results from the acidic domain interaction or by competing for p53 binding, and the extent of inhibition was dependent on the ratios of wild-type and MDM2Δ9 expressed. Taken together, these results suggest that the Y489A mutant can retain some function in p53 degradation when oligomerized with wild-type MDM2.

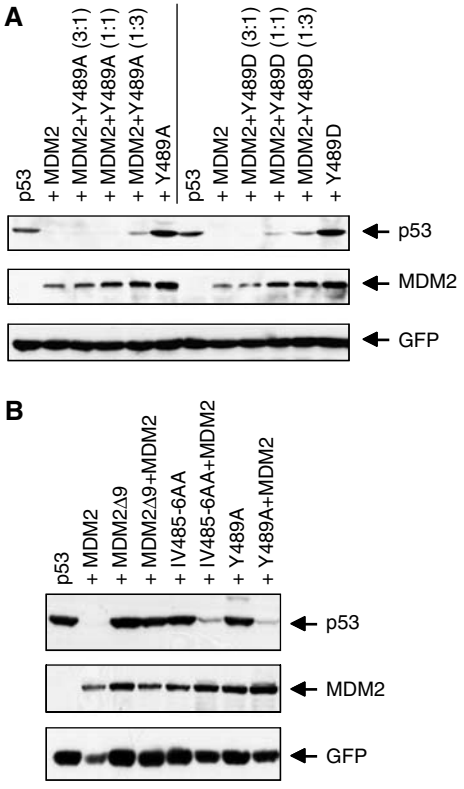

Figure 4 C-terminal tail point mutants can function in p53 degradation if oligomerized with wild-type MDM2. (**A**) U2OS cells were transiently transfected with FLAG-p53, GFP and different ratios of wild-type MDM2 to Y489A or Y489D mutants (to give a constant total amount of transfected MDM2 plasmid of 1.6 μg) and analyzed by Western blotting. (**B**) FLAG-p53 was transiently cotransfected into U2OS cells with wild-type MDM2 and C-terminal tail mutants in a 1:1 ratio.

Contribution of the C-terminal tail of MDM2 to MDMX degradation

Each of the C-terminal MDM2 mutants that was defective for p53 degradation also showed elevated expression, suggesting that they are also defective for auto-degradation. This effect is similar to that seen with RING domain mutants and might suggest that these mutations completely inactivate the E3 activity of the MDM2 protein. To examine this more closely, we tested the MDM2 mutants for their ability to drive the degradation of MDMX, another MDM2 target protein. Surprisingly, none of the MDM2 C-terminal point mutants showed any reduction in the ability to degrade MDMX (Figure 5A), although the MDM2Δ9 deletion mutant, like the RING domain mutant C464A, lost this activity. Therefore, despite their defects in auto-degradation and p53 degradation, the IV485-6AA, Y489A, TY488-9AA and F490A mutants retained the ability to target the degradation of MDMX.

Reactivation of MDM2 mutants by MDMX

Previous studies have suggested that MDMX can enhance the ability of MDM2 to degrade p53, possibly by preventing the auto-degradation of MDM2. As we had shown that some of the inactive C-terminal point mutants of MDM2 appear to retain function as part of a complex with wild-type MDM2, we examined whether these mutant MDM2 proteins showed any activity with MDMX (Figure 5B and C). Although high levels of ectopic MDMX expression can inhibit MDM2 activity, at lower relative expression levels, MDMX does not clearly affect p53 degradation by wild-type MDM2 or the active MDM2 mutants Y489F and T488A. As expected, the RING domain MDM2 mutant C464A failed to degrade p53 both in the absence and presence of MDMX (Figure 5B and C). Surprisingly, the MDM2 mutants T488D, Y489A, Y489D and a double mutant TY488-9DD, which have

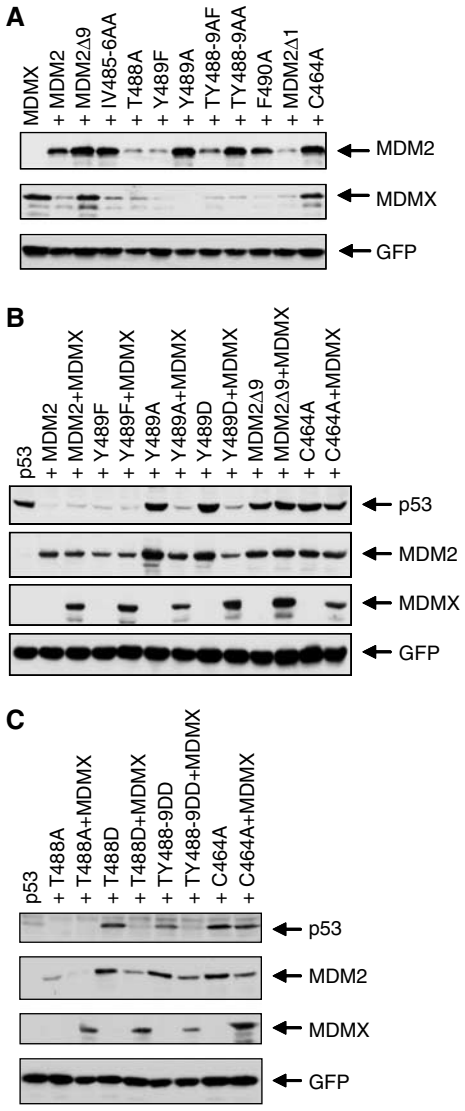

Figure 5 C-terminal tail of MDM2 contributes to MDMX degradation. (**A**) MDM2 C-terminal point mutants retain the ability to degrade MDMX. U2OS cells were transfected with Myc-MDMX and MDM2 C-terminal tail mutants and analyzed by Western blotting. (**B, C**) Reactivation of MDM2 C-terminal tail mutants by MDMX. U2OS cells were transfected with FLAG-p53 and MDM2 mutants and Myc-MDMX in a 1:1 ratio and analyzed by Western blotting.

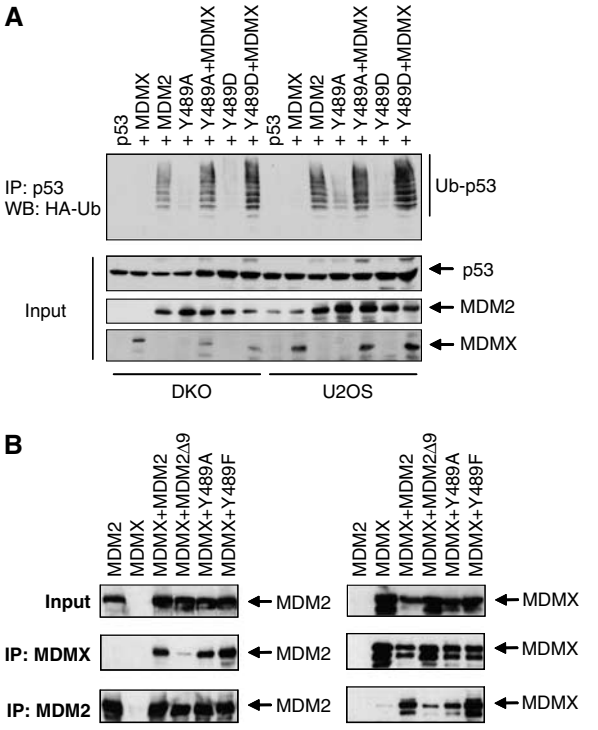

Figure 6 (**A**) MDMX restores p53 ubiquitylation by MDM2 C-terminal tail point mutants *in vivo*. DKO or U2OS cells, transiently cotransfected with FLAG-p53, HA-ubiquitin, MDM2 Y489 mutants and Myc-MDMX for 24 h, were treated with proteasome inhibitor MG132 for 3 h before lysis. Following immunoprecipitation of p53 with DO-1 antibody, ubiquitin-conjugated p53 was detected by Western blotting using anti-HA tag rabbit polyclonal antibody Y11 and p53 levels in the input for IP using DO-1 antibody. (**B**) Loss of the MDM2 C-terminal tail, but not point mutation of Y489, interferes with MDMX binding. HEK293 cells, transiently cotransfected with Myc-MDMX and MDM2 C-terminal tail mutants for 36 h, were treated with MG132 for 4 h, lysed and protein complexes containing wild-type or mutant MDM2 and Myc-MDMX were immunoprecipitated with anti-MDM2 antibody Ab-1 and anti-Myc antibody 9E10, resolved by SDS–PAGE and analyzed by Western blotting with anti-MDM2 (Ab-1) and anti-Myc (9E10) antibodies.

all lost the ability to degrade p53, regained p53 degradation function when coexpressed with MDMX. However, MDM2 carrying a complete deletion of the C-terminal tail (MDM2Δ9) was not reactivated for p53 degradation following coexpression of MDMX (Figure 5B). These results show that MDM2 C-terminal point mutants that retain the ability to degrade MDMX (Figure 5A) also regain the ability to degrade p53 in the presence of MDMX (Figure 5B). These unexpected results suggested that MDMX might be able to restore the E3 activity of the inactive MDM2 point mutants. To test this possibility, we performed an *in vivo* p53 ubiquitylation assay in two different cell lines DKO and U2OS (Figure 6A). As expected, MDMX was inactive in this assay and MDM2 mutants Y489A and Y489D alone were also unable to efficiently ubiquitylate p53. However, the strong increase in p53 ubiquitylation upon coexpression of both MDMX and the MDM2 mutants confirmed that MDMX is indeed capable of restoring the ubiquitin ligase activity of MDM2 C-terminal tail point mutants (Figure 6A).

We have shown that the C-terminal point mutants, but not the C-terminal deletion mutants, retain the ability to interact with the MDM2 RING (Figure 3). In light of the differential activity of these two classes of mutants following coexpression with MDMX, we examined their ability to interact with MDMX. In co-immunoprecipitation experiments, wild-type MDM2 associated with MDMX (Figure 6B), as previously described (Sharp *et al*, 1999; Tanimura *et al*, 1999). Deletion of the C-terminal tail of MDM2 (MDM2Δ9) significantly reduced the interaction with MDMX, whereas the point mutants (Y489A and Y489F) retained binding (Figure 6B). However, the Y489A mutant (which is inactive for p53 degradation) showed some variability in the strength of interaction with MDMX (e.g., Figure 6B, RHS). Therefore, to confirm the significance of this interaction, we tested the ability of the MDM2 mutants to translocate MDMX from the cytoplasm to the nucleus, a function that has been shown to be dependent on the RING domains of both MDM2 and MDMX (Gu *et al*, 2002; Migliorini *et al*, 2002a). These studies

confirmed that wild-type MDM2 can drive nuclear localization of MDMX, which is cytoplasmic in the absence of additional MDM2 (Figure 7). As expected, the RING domain MDM2 mutant (C464A) and the C-terminal deletion (MDM2Δ9), which fail to interact with MDMX, also failed to translocate MDMX. However, all the C-terminal point mutants (TY488-9AA, Y489A and Y489D) allowed translocation of MDMX to the nucleus (Figure 7), confirming the significance of the interaction of these mutants with MDM2.

The C-terminal tail of MDMX is important for the cooperation with MDM2

The ability of MDMX to cooperate with C-terminal MDM2 mutants in the degradation of p53 suggests that a hetero-oligomer of MDM2 and MDMX, which is capable of degrading MDMX, can also target the degradation of p53. Although MDMX does not have E3 activity by itself, it contains a RING domain and C-terminal region that is similar to MDM2 (Figure 8A). Using immunofluorescence to examine the ability of MDM2 to relocalize MDMX mutants to the nucleus, we found that as with MDM2, a point mutation within the MDMX C-terminal tail in MDMX(F488A) did not prevent the interaction with MDM2, and so, relocalization (Figure 8B). However, deletion of the last five amino acids of the C-terminal tail of MDMX in MDMX(Δ5) prevented the interaction and relocalization with MDM2.

To determine whether the C-terminal tail of MDMX is necessary for the ability to cooperate with MDM2 to degrade p53, we examined the ability of various MDMX mutants to

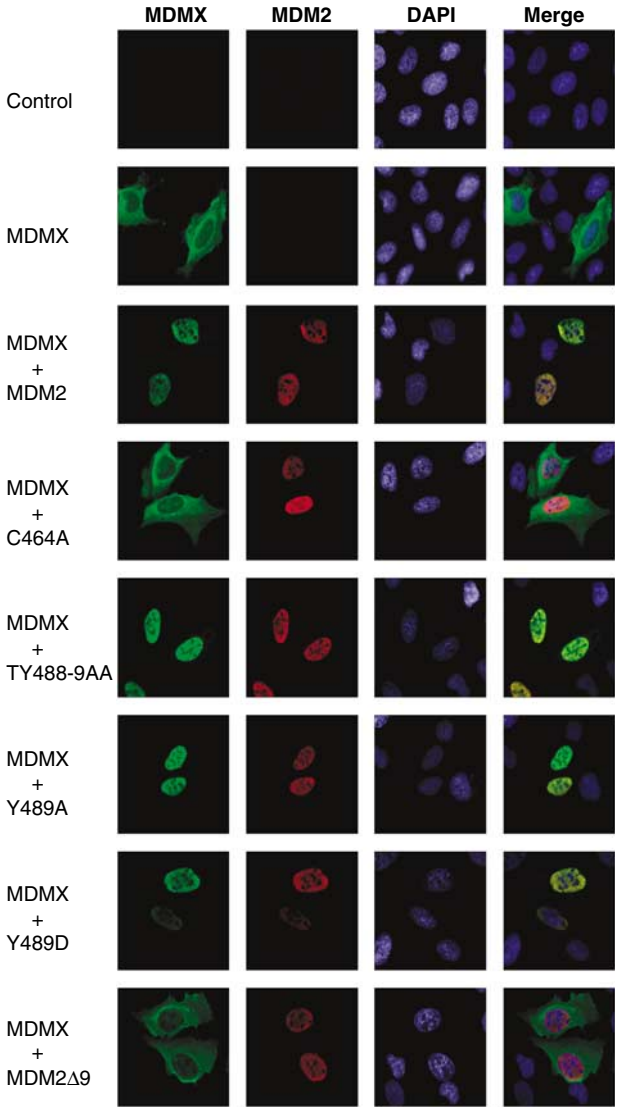

Figure 7 Subcellular translocation of MDMX by coexpression of MDM2 C-terminal tail mutants. U2OS cells were cotransfected with Myc-tagged MDMX and MDM2 C-terminal tail mutants, then proteins detected by immunofluorescence using a mixture of anti-Mdm2 mouse monoclonal IF2 (Ab-1, Calbiochem) and anti-Myc rabbit polyclonal (A14, Santa Cruz Biotechnology) antibodies.

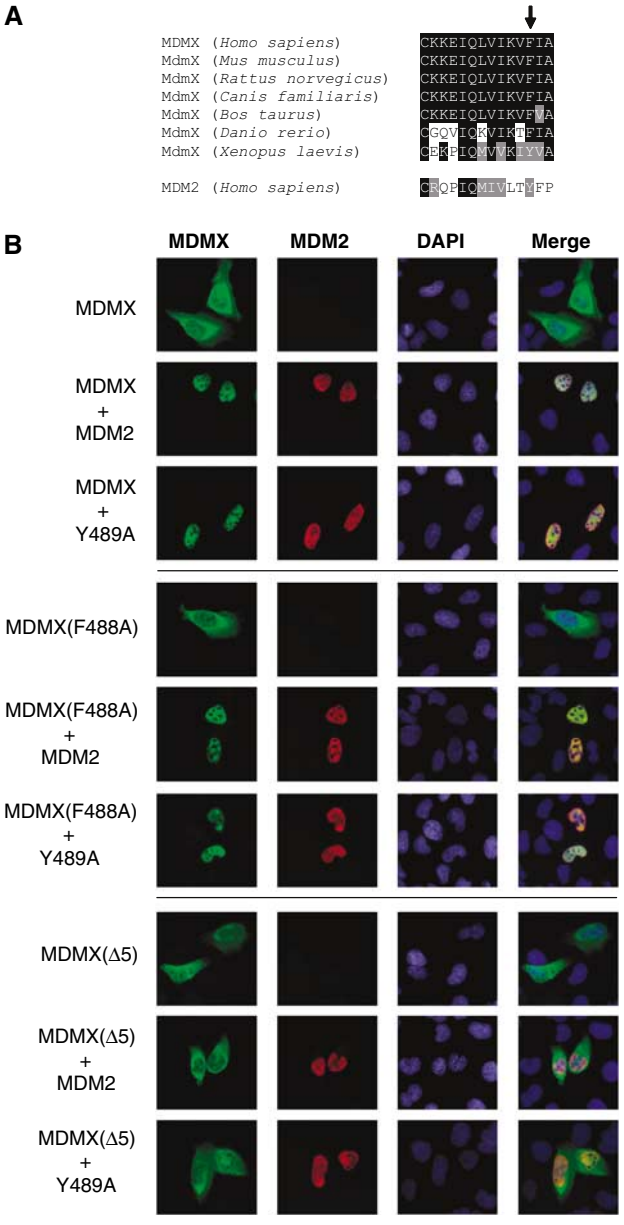

Figure 8 C-terminal tail of MDMX participates in the MDM2–MDMX interaction. (**A**) C-terminal tail sequences of MDM2 and MDMX were aligned using BOXSHADE 3.21 at http://www.ch.embnet.org/software/BOX_form.html. (**B**) U2OS cells were cotransfected with MDM2 and Myc-tagged MDMX C-terminal tail mutants. MDM2-mediated nuclear translocation of MDMX was detected as described in Figure 7.

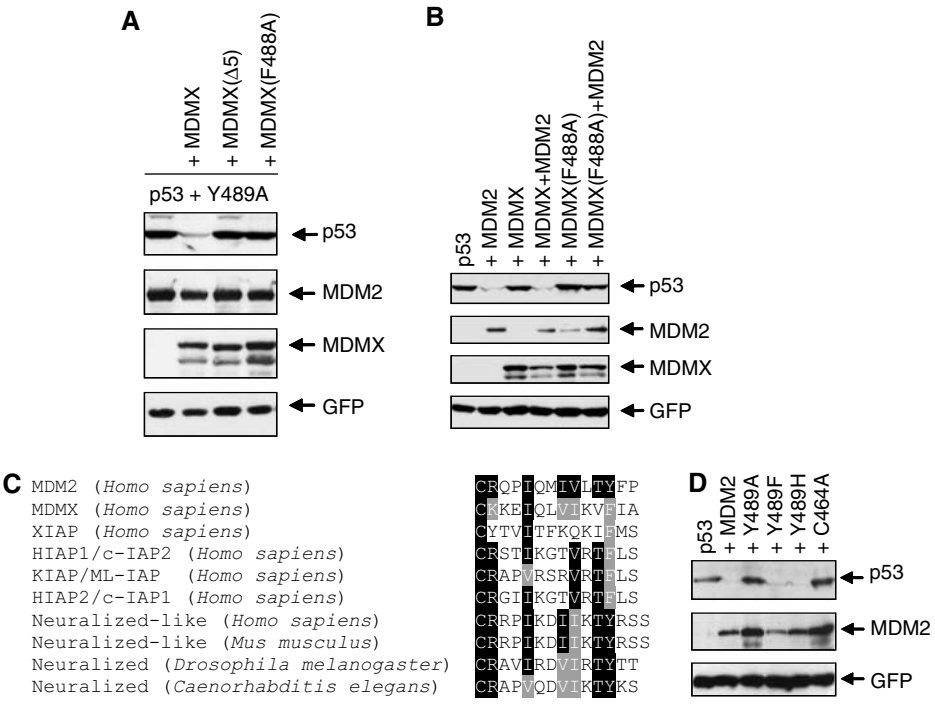

Figure 9 (**A**) Reactivation of MDM2 C-terminal tail mutants by MDMX requires intact MDMX C-terminal tail. U2OS cells were transfected with MDM2 Y489A mutants and wild-type Myc-MDMX or MDMX C-terminal tail mutants in a 1:1 ratio, together with FLAG-p53 and GFP and analyzed by Western blotting. (**B**) Interaction with MDMX C-terminal tail point mutant F488A inhibits MDM2 function. The experiment was performed using wild-type MDM2 in the way described in (A) for Y489A mutant. (**C**) C-terminal tail sequences of MDM2, MDMX, neuralized and IAP proteins were aligned using BOXSHADE 3.21 at http://www.ch.embnet.org/software/BOX_form.html. (**D**) Y489 can be substituted for other aromatic amino acids. U2OS cells were transiently cotransfected with FLAG-p53 and MDM2 Y489A, Y489F and Y489H point mutants and analyzed by Western blotting.

cooperate with the MDM2 Y489A mutant (Figure 9A). These analyses showed that mutations in the C-terminal region of MDMX equivalent to those shown to be important for MDM2's ability to degrade p53 (MDMXΔ5 and MDMX F488A) disrupted the ability of MDMX to participate in the degradation of p53 (Figure 9A). Interestingly, although wild-type MDMX can restore degradation by the MDM2 Y489A mutant, wild-type MDM2 is unable to form a functional complex with the MDMX(F488A) mutant (Figure 9B). This suggests that the RING and tail domains of MDM2/MDMX function *in trans*, and that a functional RING must be present on a molecule different from the functional tail. In the MDMX(F488A) plus MDM2 combination, only MDM2 has both functional RING and tail. However, as the MDMX(F488A) protein can still oligomerize with MDM2, it functions as a dominant negative. In support of this model, we also found that MDMX(Δ5)—which cannot oligomerize with MDM2 (Figure 8B)—cannot inhibit MDM2-mediated degradation of p53 (data not shown).

Our results indicate that MDMX may play an active role in the degradation of p53 beyond allowing the stabilization of MDM2. Interestingly, an aromatic residue (tyrosine or phenylalanine) is found at a similar position (11 residues from the last RING domain cysteine) in a number of E3 ligases with C-terminal RING domains (Figure 9C), suggesting that the presence of an aromatic amino acid at this position may be important. We have shown that either tyrosine or phenylalanine can function in this RING $+11$ position within MDM2 (Figure 2), and further analysis demonstrated that MDM2 activity in the degradation of p53 was also retained following substitution of this position with histidine, another aromatic amino acid (Figure 9D). Taken together, our observations suggest that the C-terminal tail of MDM2 can function in intra- or inter-molecular interactions, partly mediated by aromatic stacking.

Discussion

Although the importance of MDM2 in the regulation of p53 stability and function has been illustrated in many systems, how the E3 ligase activity of MDM2 is regulated and the role (if any) of MDMX in the regulation of MDM2 function are much less well understood. The RING domain of MDM2 is essential for E3 activity, and mutations in this region prevent all E3 activity of MDM2. However, there is clear evidence that the activity of MDM2 can be differentially modulated, and that the auto-degradation activity can be separated from the ability to drive the degradation of p53. For example, the acidic domain in MDM2 has been shown to be important for the ubiquitylation and degradation of p53, although this region is not required for the E3 activity of the RING domain (Kawai *et al*, 2003b; Meulmeester *et al*, 2003). The MDM2-related protein MDMX also plays an important role in the regulation of p53, both by binding directly to p53 and through interaction with MDM2 (Marine *et al*, 2006). It has been suggested that MDMX can stabilize MDM2 to enhance the degradation of p53, and that MDM2 can negatively regulate MDMX by promoting its degradation. In this study, we show the importance of the extreme C-terminal tail of MDM2 in targeting the degradation of p53, and provide evidence that

this region of MDMX can cooperate with MDM2 to promote E3 activity.

Although the isolated RING domain from MDM2 has been shown to retain E3 function, the portions of MDM2 examined in these studies included the short region C-terminal to the end of the RING domain. We show here that deletion of part or all of this C-terminal region inactivates MDM2, possibly because such deletions prevent the formation of RING/RING oligomers. Our observations are strongly supported by the accompanying study (Poyurovsky *et al*, submitted) that also identifies the C-terminal tail as a region critical for both MDM2 oligomerization and E3 activity. The C-terminal deletion mutants of MDM2 also fail to degrade MDMX, with evidence that this correlates with the loss of both MDM2/MDM2 homo-oligomerization and hetero-oligomerization between MDM2 and MDMX. Our observations are strongly supported by a recent study, which presented the solution structure of MDM2 RING domain and suggested that the C-terminal tail forms a part of the interface between two bound RING domains in both the MDM2/MDM2 homo-dimer and the MDM2/MDMX hetero-dimer (Kostic *et al*, 2006). A similar contribution of the C-terminal-flanking sequences in mediating the RING–RING interaction between cIAP and XIAP has also recently been demonstrated (Silke *et al*, 2005), suggesting that this is a common mechanism to mediate RING–RING binding. In contrast to the complete deletion of this C-terminal tail, we show that point mutations within this region also perturb the activity of MDM2, but do not detectably affect the RING–RING interaction. Interestingly, the defect in E3 activity exhibited by these mutants appears to be apparent only under conditions where both MDM2 proteins in the oligomer are mutant. Titration experiments suggest that oligomers containing mutant and wild-type MDM2 proteins retain the ability to degrade p53. Although we do not, as yet, understand fully the role of the C-terminus in MDM2 function, previous structural studies have suggested that the tyrosine at position 489 in MDM2 is close to the first metal-coordinating center of the RING domain (Lai *et al*, 1998), implying that this C-terminal tail folds back into the RING domain. We have shown that whereas the substitution of aromatic amino acids is compatible with the retention of function, alterations to other types of amino acids prevents activity, potentially by preventing the interaction between the tail and the RING domain. The fact that only one intact tail is required for function might suggest that the C-terminal tail from one MDM2 molecule in the oligomer interacts with the RING domain of another. Of particular interest is the possibility that modification of one of the critical C-terminal amino acids can inhibit MDM2 function. Phosphorylation of threonine 488 or tyrosine 489 is a clear candidate, although we have, as yet, been unable to detect phosphorylation at these sites of MDM2 (data not shown).

Our observations that oligomers containing wild-type and C-terminal tail mutants of MDM2 can retain E3 activity are further supported by the activity of these mutant MDM2s in the degradation of MDMX. Deletion of the C-terminus of MDM2, which prevents MDM2/MDM2 and MDM2/MDMX interactions, also prevents the formation of an active E3 and so results in an inability to degrade p53, MDM2 or MDMX. However, the C-terminal point mutants of MDM2, which are inactive by themselves but function in complex with wild-type MDM2, retain the ability to degrade MDMX. This, together with our observations that MDMX restores p53 ubiquitylation and degradation by inactive MDM2 C-terminal tail point mutants, suggests that hetero-oligomers between C-terminal tail mutants of MDM2 and wild-type MDMX retain E3 activity.

Taken together, our results are consistent with a model in which MDM2 proteins form an oligomeric complex where the extreme C-terminus of one MDM2 molecule adopts a conformation bringing it into close proximity with the RING domain of another MDM2. Such cross-interaction between the RING and tail domains of MDM2 is required to activate the E3 function and degradation of p53, potentially by allowing E2 binding. A similar model has been developed independently through biochemical analyses that showed a role for the C-terminal tail of MDM2 in supramolecular assembly and E3 activity (Poyurovsky *et al*, submitted). Most interestingly, we have shown that a hetero-oligomer between mutant MDM2 and MDMX also retains the ability to ubiquitylate p53 and target it for degradation, suggesting that the C-terminal tail of MDMX can substitute for that of MDM2, and transactivate the E3 function of an MDM2 protein mutated in this C-terminal domain. These results support the previous observation that MDMX can actively contribute to MDM2's E3 activity *in vitro* (Linares *et al*, 2003), rather than simply functioning to stabilize MDM2.

MDM2 is an attractive target for cancer therapeutics, as inhibition of the ability to degrade p53 could result in the activation of p53-dependent cell death in tumor cells that retain wild-type p53. Exciting progress has been made in the identification of small molecules that inhibit the interaction of p53 with MDM2 (Vassilev *et al*, 2004), and a class of compounds that directly block the E3 activity of MDM2 has also been described (Yang *et al*, 2005). Our studies suggest that other approaches to inhibition of MDM2 function might also include modulation of the function of the extreme C-terminus of the protein.

Materials and methods

Cell culture

Human U2OS and HEK293 cells, and p53/mdm2 double knock-out (DKO) MEFs (Jones *et al*, 1995) were cultured at 37°C/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum, 2 mM L-glutamine, 50 U/ml penicillin G and 50 μg/ml streptomycin sulfate.

Plasmids and mutagenesis

Plasmids coding for FLAG-tagged human wild-type p53 (pcDNA3-FLAG-p53), wild-type MDM2 (pCHDM1A), MDM2 mutants C464A and 1–440 (MDM2ΔRING), glutathione-*S*-transferase (GST)-tagged MDM2 (pGEX-MDM2) and Myc-tagged MDMX have been described (Chen *et al*, 1995; Sharp *et al*, 1999; Fang *et al*, 2000; Weber *et al*, 2005). Plasmid GFP-RING coding for GFP-tagged human MDM2 (amino acids 384–491) was generated by cloning *Hin*dIII/*Avr*I fragment from pCHDM1A into *Hin*dIII/*Xba*I sites of pEGFP-C1 (Clontech). All mutations within the C-terminal tail of MDM2 and MDMX were generated by site-directed mutagenesis using Pfu DNA polymerase (Stratagene) and verified by DNA sequencing.

MDM2-mediated p53 degradation

U2OS cells grown in 60-mm dishes were transfected with 0.2 μg of pcDNA3-p53 and 1.6 μg of pCHDM1A or derived mutants (1.5 μg in experiments presented in Figures 1B, 2B and C, and 4B) using Effectene transfection reagent (Qiagen). For experiments studying the role of MDMX in MDM2-mediated p53 degradation, the total amount of pCHDM1A (or MDM2 mutants) and Myc-MDMX transfected either separately or in combination was kept at

1.6 μg. Each transfection mixture also contained 0.2 μg of pEGP-N1 (Clontech) (0.1 μg in experiments presented in Figures 1B, 2B and C, and 4B) to control for transfection efficiency, and pcDNA3.1 (Invitrogen) was used to bring the total amount of transfected DNA to 2 μg (1.8 μg in experiments presented in Figures 1B, 2B and C, and 4B). Cells were cultured for an additional 24–30 h, washed with PBS and lysed with 0.3 ml of SDS sample buffer. Proteins were resolved by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and analyzed by Western blotting with anti-FLAG antibody M2 (Sigma), anti-MDM2 Ab-1 (Calbiochem), anti-Myc 9E10 and anti-GFP 7.1/13.1 (Roche).

Immunoprecipitation

HEK293 cells were transfected with 6 μg of DNA per 100-mm plate using Lipofectamine 2000 reagent (Invitrogen). Cells were treated with proteasome inhibitor (10 μM MG132 in cell culture medium) 24–36 h post-transfection, 4 h later washed with PBS and lysed in Triton X-100 lysis buffer (1% Triton X-100, 150 mM NaCl and 50 mM Tris pH 8.0) containing protease inhibitors (Complete, Roche). Lysates were pre-cleared with 50 μl of protein G-Sepharose (Sigma). Immunoprecipitations were performed with 1–2 μg of an antibody bound to 50 μl of protein G-Sepharose for 2 or 8 h at 4°C. MDM2 was immunoprecipitated with anti-MDM2 antibody IF2 (Ab-1, Calbiochem), Myc-MDMX with anti-Myc antibody 9E10, p53 with DO-1 or anti-FLAG M2 and GFP-RING with anti-GFP 7.1/13.1 (Roche). Immunoprecipitated proteins were washed with lysis buffer and resuspended in 2 × SDS sample buffer. Proteins from whole-cell extracts and immunoprecipitations were resolved by SDS–PAGE and analyzed by Western blotting with anti-p53 polyclonal antibody CM1, anti-MDM2 Ab-1, anti-Myc 9E10 or anti-GFP 7.1/13.1.

Immunofluorescence

U2OS cells grown on coverslips were transfected with GFP-RING (0.05 μg) and MDM2 C-terminal tail mutants (0.75 μg) or Myc-tagged MDMX (0.4 μg) and MDM2 C-terminal tail mutants (1.2 μg) using Effectene transfection reagent (Qiagen) and DMEM was changed 4 h later. Twenty-four hours after transfection, cells were treated with 15 μM MG132 in DMEM for 3 h, washed with PBS and fixed in 4% paraformaldehyde for 10 min at room temperature. After fixation, cells were washed with PBS and permeabilized with PBS containing 0.2% Triton X-100 for 5 min. Cells were blocked with PBS containing 0.5% bovine serum albumin at room temperature for 30 min and then incubated for 2 h at room temperature with anti-MDM2 monoclonal IF2 (Ab-1, Calbiochem) and anti-c-Myc polyclonal (A14, Santa Cruz Biotechnology) antibodies in blocking solution. Cells were washed with PBS and incubated for 1 h at room temperature with a mixture of fluorescein isothiocyanate (FITC)-conjugated donkey anti-rabbit and Cy3-conjugated donkey anti-mouse antibodies (Jackson Immuno-Research) in blocking solution containing 1 μg/ml DAPI (Sigma). Cells were washed with PBS and mounted with Vectorshield hard set (Vector Laboratories). Images were taken with a Zeiss Axioplan 2 microscope using Isis software (Metasystems).

MDM2-mediated p53 ubiquitylation in vitro

The expression of recombinant GST-tagged MDM2 (GST-MDM2) was induced in 10 ml culture of exponentially growing *Escherichia coli* BL21 cells (OD_{600} 0.6) by 0.3 mM IPTG for 3 h. GST-MDM2 was purified on 100 μl glutathione-Sepharose beads (Amersham), mixed with 20 μl *in vitro*-translated p53 (TNT Quick Coupled Transcription/Translation System, Promega) and incubated at 4°C for 1 h. The beads were washed with 50 mM Tris (pH 7.5) and incubated with 50 ng mammalian E1 (Affiniti), 200 ng human recombinant UbcH5B E2 (Affiniti) and 5 μg ubiquitin (Sigma) in reaction buffer (50 mM Tris pH 8, 2 mM DTT, 5 mM $MgCl_2$, 2 mM ATP). The reaction was incubated at 37°C for 2 h and then stopped by the addition of 2 × SDS sample buffer. Reaction products were resolved by SDS–PAGE and analyzed by Western blotting with anti-p53 DO-1.

MDM2-mediated p53 ubiquitylation in vivo

DKO or U2OS cells grown in 60-mm dishes were transiently transfected with FLAG-p53 (0.5 μg), hemagglutinin (HA)-ubiquitin (0.3 μg), MDM2 (or MDM2 mutants) and Myc-MDMX (0.8 μg each when transfected separately or 0.4 + 0.4 μg when combined) using Effectene transfection reagent (Qiagen) and cultivated for further 24 h. Cells were treated for 3 h with proteasome inhibitor MG132 (10 μM) and lysed in 300 μl 0.5% SDS. Lysates were boiled for 5 min to disrupt protein–protein interactions and to inactivate deubiquitinating enzymes, vortexed, cooled down to room temperature and diluted with 1 ml Triton X-100 lysis buffer. Immunoprecipitation of p53 was performed for 30 min using 1 μg of protein G-bound anti-p53 monoclonal antibody DO-1, followed by SDS–PAGE and Western blotting. Ubiquitylated p53 was detected using anti-HA-tagged rabbit polyclonal antibody Y11 (Santa Cruz Biotechnology); p53, MDM2 and MDMX levels in the input were detected using anti-p53 DO-1, anti-MDM2 Ab-1 (Calbiochem) and anti-Myc 9E20 antibody, respectively.

MDM2-mediated MDMX degradation

U2OS cells grown in 60-mm dishes were transfected with 1 μg of Myc-MDMX and 6 μg of pCHDM1A (or derived mutants) using Lipofectamine 2000 transfection reagent (Invitrogen) and DMEM was changed 4 h later. Each transfection mixture also contained 0.1 μg of pEGP-N1 (Clontech) as a transfection control. Empty plasmid pcDNA3.1 (Invitrogen) was used to bring the total amount of transfected DNA to 7.1 μg. Cells were cultured for additional 30 h, washed with PBS and lysed with 0.3 ml of SDS sample buffer. Proteins were resolved by SDS–PAGE and analyzed by Western blotting with anti-Myc 9E10, anti-MDM2 Ab-1 (Calbiochem) and anti-GFP 7.1/13.1 (Roche) antibodies.

Acknowledgements

We thank Arnie Levine, Donna George, Mary Hanlon and Allan Weissman for the MDM2, MDMX and ubiquitin expression plasmids. We also thank Robert Ludwig for his technical help. This work was funded by Cancer Research UK.

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