The Mdm2 RING domain C-terminus is required for supramolecular assembly and ubiquitin ligase activity

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Mdm2, a key negative regulator of the p53 tumor suppressor, is a RING-type E3 ubiquitin ligase. The Mdm2 RING domain can be biochemically fractionated into two discrete species, one of which exists as higher order oligomers that are visible by electron microscopy, whereas the other is a monomer. Both fractions are ATP binding and E3 ligase activity competent, although the oligomeric fraction exhibits lower dependence on the E2 component of ubiquitin polymerization reactions. The extreme C-terminal five amino acids of Mdm2 are essential for E3 ligase activity *in vivo* and *in vitro*, as well as for oligomeric assembly of the protein. A single residue (phenylalanine 490) in that sequence is critical for both properties. Interestingly, the C-terminus of the Mdm2 homologue, MdmX (itself inert as an E3 ligase), can fully substitute for the equivalent segment of Mdm2 and restore its E3 activity. We further show that the Mdm2 C-terminus is involved in intramolecular interactions and can set up a platform for direct protein–protein interactions with the E2.

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Introduction

Human Mdm2 is a highly conserved homologue of the product of the murine *double minute* (*mdm2*) gene. A multi-domain nuclear phosphoprotein whose principal function in normal tissue is inhibition of the p53 tumor suppressor protein (Prives, 1998; Harris and Levine, 2005; Poyurovsky and Prives, 2006), Mdm2 controls p53 through two distinct mechanisms, by directly binding and masking the N-terminal

transactivation domain of p53 (Momand *et al*, 1992) and by promoting proteasomal degradation of p53 (Haupt *et al*, 1997; Kubbutat *et al*, 1997). Mdm2 is a RING-type E3 ubiquitin ligase that primarily regulates its own levels and the levels of p53 in cells through proteasome-mediated degradation (Fang *et al*, 2000; Honda and Yasuda, 2000). In response to various stress signals, the Mdm2–p53 interaction is rapidly uncoupled through multiple mechanisms, including phosphorylation, acetylation, redistribution of protein complexes and changes in subcellular localization (Prives and Hall, 1999; Moll and Petrenko, 2003).

RING-type E3s constitute the largest number of the RING-containing proteins. Approximately 50 of the 200 known RING proteins can be found in discrete subcellular structures that are visible by confocal microscopy (Saurin *et al*, 1996). The integrity of these structures depends on proper RING domain organization. For example, the RING domain of PML is required for the formation of PML nuclear bodies (Melnick and Licht, 1999). These observations, along with the fact that several RING domain proteins oligomerize in solution, have led to the hypothesis that RING domains serve as scaffolds for forming multi-protein complexes (Bailin *et al*, 1999; Borden, 2000; Brzovic *et al*, 2003). This view is supported by the fact that several unrelated RING domains self-assemble *in vitro* into supramolecular structures capable of scaffolding multiple interacting proteins on their surface and amplifying the specific activities of biochemical reactions (Kentsis *et al*, 2002a, b).

The Mdm2 RING domain is atypical when compared to canonical RING motifs both with respect to the spacing of Zn²⁺ coordinating cysteines and its multiple functions in cells (Boddy *et al*, 1994). First, the Mdm2 RING is essential for proper E3 ubiquitin ligase activity and mutation of any of the Zn²⁺ coordinating residues renders the protein inactive (Lai *et al*, 1998; Fang *et al*, 2000). Second, the Mdm2 RING domain was shown to bind to 5S RNA (Elenbaas *et al*, 1996). Third, this region of Mdm2 contains a cryptic nucleolar localization signal revealed upon protein interactions with p14^{ARF} (Lohrum *et al*, 2000). Fourth, we found that the Mdm2 RING domain binds nucleotides with a strong preference for ATP and although such binding does not contribute to its E3 ubiquitin ligase, it is important for sub-nuclear translocation of Mdm2 from the nucleoplasm to the nucleolus (Poyurovsky *et al*, 2003). Finally, the lysine residues within the RING domain of Mdm2 have been shown to be substrates for CBP/p300-mediated acetylation leading to inhibition of the ubiquitin ligase activity (Wang *et al*, 2004). Collectively, these data suggest multiple important functions and modes of regulation of the RING domain of Mdm2, although it is unclear how these activities are carried out by a single domain.

Here we demonstrate that the purified RING domain of Mdm2 can exist as two species in solution: as a monomer and

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as a spontaneously assembling structured supramolecular complex. Both species are capable of ATP binding and E3 ubiquitin ligase activity. Remarkably, the C-terminus of Mdm2 is required both for assembly and E3 activity, while being dispensable for nucleotide binding. We propose that intramolecular interactions made by the extreme C-terminus stabilize the RING structure, thereby providing a binding platform for E2. These data elucidate a previously undescribed aspect of the Mdm2 RING domain.

Results

Purification and behavior of Mdm2 RING domain constructs

We set out to purify and characterize the Mdm2 RING domain (residues 400–491). The purification scheme for the fusion protein as well as the untagged Mdm2 RING domain is outlined in Figure 1A and B. While yields of the fusion protein were quite large (20 mg/l in *Escherichia coli*, 100 mg/l from insect cells) and displayed minimal protease sensitivity, indicative of being well folded, analytical gel filtration revealed that this construct displayed polydispersity both as a fusion protein and upon cleavage from the GST fusion partner. A proportion of the GST-RING protein showed clear monodispersity in solution, however, and could be purified away from the polydisperse fraction through two consecutive gel filtration steps (Figure 1C). Furthermore, the behavior of the fusion protein during purification was virtually indistinguishable from the cleaved Mdm2 construct. Protein fractions from the second gel filtration step were 99% pure, as determined by SDS–PAGE (Figure 1D).

The peak fractions of the monodisperse form of the GST-Mdm2 RING domain were analyzed by static light-scattering immediately after purification and following storage at -80°C

and a thaw cycle (Figure 1E). The mass-proportional refractive index trace showed that more than 95% of the protein migrated as a single peak, indicating the presence of only one species in solution. The predicted molecular weight of the fusion protein is 36 kDa and light-scattering measurements estimated the molecular weight of the protein as ~ 38 kDa, suggesting that the predominant protein species in this, peak, Mdm2 was a monomer. It is difficult to estimate the exact size and stoichiometry of the large molecular weight RING fractions owing to the heterogeneity of this material and the limitations of the gel-filtration column resolution.

Mdm2 forms higher order structured oligomers in solution

Several RING domain proteins, including PML, BRCA1, BARD1, KAP-1 and arenaviral protein Z were shown to self-assemble into approximately 50 nm spherical structures *in vitro*, termed ‘bodies’, dependent on Zn^{2+} coordination (Kentsis *et al*, 2002a,b). The ability of Mdm2 to form bodies *in vitro* was assessed by negative staining electron microscopy (EM). Both the Mdm2 RING domain (Figure 2A, left panel) and the GST-Mdm2 RING (Figure 2A, middle panel) formed spherical structures similar in size to those previously described for arenaviral protein Z (Z) and BRCA1 RINGS. The mean diameter of the observed structures was about 50 nm; the slight physical heterogeneity is likely due to the differential grid adsorption or the potential influence of the fusion partner on the appearance of the body. Observed heterogeneity could also be attributed to the low (femtomolar) protein concentrations required for single-particle EM measurements that are well below the K_d calculated for association of Z bodies (Kentsis *et al*, 2002b). As expected, and consistent with previous results, the bodies were only observed only in the higher molecular weight fractions of

Figure 1 Purification of the RING domain of Mdm2. (A, B) Purification of Mdm2 protein. Flow-chart representations of the purification schemes for the RING domain of Mdm2 with (B) and without (A) a GST tag. (C) Mdm2 can be purified to homogeneity by size-exclusion chromatography. Mdm2 RING domain sample was prepared as outlined in (A). Upper panel: Superdex-200 gel-filtration absorbance profiles of Mdm2^{400–491} RING (#I); lower panel: peak fractions of around 16 ml were collected and subjected to a second round of gel filtration (#II). (D) Purified Mdm2 RING domain is a single species. Fractions eluted from the second Superdex-200 gel-filtration column (#II) were subjected to 12% SDS–PAGE gel and stained with Coomassie blue. (E) Analytical gel-filtration and static light-scattering analysis of the Mdm2 RING monomer fraction. The GST-Mdm2 peak fractions from the second gel filtration (#II) were injected onto an analytical gel-filtration column at 200 μM concentration and the effluent was monitored by refractive index (bottom trace, arrow) and 90° static light-scattering (top trace) detectors. Calculations from the Debye plot estimate a molecular mass of 36.1 kDa for the protein peak of the elution profile.

Figure 2 Supramolecular assembly of the Mdm2 RING domain. (A) The RING domain of Mdm2 self-assembles into spherical bodies. Peak fractions of the void and monomeric forms of Mdm2 RING and GST-Mdm2 RING protein, respectively, were subjected to single-particle EM at a nominal magnification of $\times 100\,000$ or $\times 80\,000$, using uranyl acetate counterstain. (B) Mdm2 gel-filtration profile juxtaposed with elution of sizing standards. Right panel: Superdex-200 gel-filtration elution profiles of gel filtration standards; left panel: Mdm2 RING domain sample from Figure 1C.

Mdm2, whereas none were associated with the monomer (Figure 2A, right panel). Comparison of the elution profile of the oligomerized material with that of gel filtration standards allowed us to estimate a molecular weight range associated with this peak. Mdm2 RING oligomers eluted as a broad peak from 100 to 600 kDa. Taking into account the migration profile of the monomer, we estimate that the elution range of the body fractions could be produced by complexes of 2–16 subunits (Figure 2B). Previous studies on whether RING supramolecular assemblies are biochemically functional entities have revealed that the BRCA1/BARD1 hetero-oligomeric assembly is not only able to support ubiquitin conjugation and polymerization, but is also substantially more active than the unassembled heterodimer (Kentsis *et al*, 2002a). Identification of supramolecular assembly mediated by the RING domain of Mdm2 posed interesting questions, such as which regions of the RING domain are involved in the interaction and is there functional significance of these higher order Mdm2 oligomers.

The C-terminal five amino acids of Mdm2 are required for body assembly

To identify the regions of the RING necessary for formation of the observed higher order oligomers, we focused on the hydrophobic C-terminus of Mdm2, as such regions are frequently implicated in protein–protein interactions. We deleted the last five or seven residues of the RING domain to generate two new recombinant proteins GST-Mdm2 (400–486) and GST-Mdm2 (400–484). The fusion proteins were then subjected to analytical gel-filtration chromatography

to determine their oligomeric state (Figure 3A and B). Unexpectedly, deletion of as few as five amino acids from the C-terminus of Mdm2 led to a striking change in the gel-filtration profile of the protein. Specifically, both proteins exhibited only one elution peak consistent in size with the Mdm2 RING monomer, and the oligomeric peak was virtually absent from the preparations. The solution behavior of the Mdm2- Δ C7 RING domain remained unchanged following removal of the fusion protein, and the resulting elution profile was indistinguishable from that of the monomer fraction of the Mdm2 RING (Figure 3C and D). These data indicate that the extreme C-terminus of Mdm2 is directly involved in Mdm2 body assembly.

Metal and ATP binding analysis of C-terminally deleted Mdm2 RING domains

To further study the anomalous behavior of the intact RING domain construct, we used inductively coupled plasma-mass spectrometry (ICP-MS) to determine the metal content of the void and monomer fractions of Mdm2 and Mdm2 RING with the last seven residues deleted (Mdm2- Δ C7) (Figure 3E). Metal analysis confirmed the presence of stoichiometric amounts of Zn^{2+} in all protein preparations, demonstrating that these proteins are capable of zinc coordination and that the anomalous gel-filtration profiles of our constructs are due to other determinants. The metal content was also roughly equivalent between the GST fusion proteins and those purified away from GST, demonstrating that zinc was specifically associated with the RING domain.

We also compared the nucleotide binding activity of the monomeric and oligomerized fractions of Mdm2, as well as the C-terminally deleted RING domain. All three Mdm2 preparations bound ATP to a similar extent (Figure 3F), and nucleotide binding by these preparations was lost following heat and guanidinium denaturation (data not shown). Thus, proper folding is required for nucleotide binding and these three forms of Mdm2 are likely to possess functional tertiary structure.

Ubiquitin ligase activity of different Mdm2 species

The ability of the Mdm2 body fraction to function as an E3 ubiquitin ligase was examined, as well as whether forced monomerization via the deletion of the C-terminus would have any effect on Mdm2 function. To this end, we purified a version of His-tagged ubiquitin with a PKA kinase site at the N-terminus, which allowed us to label it with ^{32}P , along with His-UbcH5c, the E2 shown to function with Mdm2 *in vivo* (Tan *et al*, 1999). The relative activities of body and monomer versions of the Mdm2 RING domain were then analyzed by performing ubiquitination assays *in vitro*, followed by resolution of the reaction products by SDS-PAGE and their visualization by autoradiography (Figure 4A).

Both monomeric and body fractions of Mdm2 RING domain showed activity in the polymerization assay over a broad range of E2 concentrations. We observed that the monomer was relatively more active over a range of E2 protein between 50 and 500 ng. However, monomers showed no detectable activity at 10 ng, whereas the Mdm2 body still exhibited some activity in the presence of only 10 ng of UbcH5c (Figure 4A, lanes 3 and 9; see Supplementary Figure 1 for a longer exposure). Based on this observation, we performed the ubiquitin polymerization experiment in the

Figure 3 Deletion of C-terminal residues alters the behavior of the Mdm2 RING domain in solution. (A) Deletion of amino acids 485–491 and 487–491 prevents body formation. SD-200 gel-filtration profile of GST-Mdm2 (400–484) (left panel) and GST-Mdm2 (400–486) (right panel). (B) GST-ΔC7-Mdm2-RING and GST-ΔC5-Mdm2-RING elute as single species on gel filtration. Identical peak fractions from GST-ΔC7-Mdm2-RING elution (top panel) and GST-ΔC5-Mdm2-RING (lower panel) were resolved on 12 % SDS-PAGE and visualized by Coomassie blue staining. (C) Removal of GST fusion protein did not alter the solution behavior of ΔC7-Mdm2-RING domain. Gel-filtration profile of the ΔC7-Mdm2-RING domain prepared according to the procedure in Figure 1A. (D) Mdm2 RING and ΔC7-Mdm2-RING elute in the same volume from an SD-200 column. Peak fractions from ΔC7-Mdm2-RING elution (top panel) and Mdm2 RING (lower panel) were resolved on 12 % SDS-PAGE and visualized by Coomassie blue staining. (E) Wild-type and C-terminally deleted forms of Mdm2 RING bind similar amounts of Zn²⁺. Inductively coupled plasma-mass spectrometry (ICP-MS) metal analysis of the void, monomer and Mdm2-ΔC7 RING fractions as well as purification buffer was performed under standard analytical conditions. The concentration of the protein preparations were ~5 μM in a volume of 2 ml each in buffer containing 50 mM bis-tris-propane (BTP), pH 6.8, 350 mM NaCl and 1 mM DTT. (F) C-terminally deleted Mdm2 and Mdm2-ΔC7 protein at indicated amounts were incubated with ATP-γ³²P for 10 min at 30°C, filtered through nitrocellulose and washed extensively with 25 mM Hepes buffer. ATP-bound proteins were quantified using a scintillation counter.

presence of limiting amounts of Ubch5c. Here, with sub-saturating amounts of Ubch5c, at every point, Mdm2 oligomers showed higher activity (Figure 4B). The ability of the Mdm2 bodies to participate in E2-dependent ubiquitin ligation along with the fact that they coordinate Zn²⁺ and bind ATP further supports our assumption that we have isolated properly folded yet structurally distinct Mdm2 proteins. Note that at higher saturating E2 concentrations, monomers exhibited somewhat higher activity than the bodies, possibly owing to the heterogeneity of the body fraction (Figures 4B

and 6A). All subsequent *in vitro* ubiquitination reactions were performed in the presence of saturating amounts (500 ng) of Ubch5c.

Far less predictable were our results with the C-terminally deleted Mdm2. Remarkably, loss of the last seven residues completely abrogated Mdm2 E3 activity under our conditions (Figure 4C). Thus, deletion of the hydrophobic tail led to the loss of the oligomeric fraction as well as Mdm2 E3 activity. We surmise that when E2 amounts are limiting (a situation likely to arise in the cell when there are many more E3s than

Figure 4 Mdm2 monomers, oligomers and C-terminally deleted Mdm2 display differential ubiquitin ligase activities. (A) Mdm2 RING bodies and monomers exhibit similar E3 activity at saturating amounts of E2 protein. Ubiquitin ligation assays were performed as indicated in Materials and methods. Reaction mixtures contained Mdm2 monomer and body (1.5 μg) per reaction, ~300 pmol ³²P-labeled ubiquitin and a 50-fold concentration range of the E2, as indicated. Following incubation at 37°C, reactions were terminated and resolved by 8% SDS-PAGE, followed by autoradiography. (B) Mdm2 bodies exhibit lower dependence on E2 in ubiquitination reactions *in vitro*. Reactions were performed as in (A) in the presence of 7, 15, 20 and 40 ng purified UbCH5c protein. Reactions were resolved by 8% SDS-PAGE and visualized by autoradiography. (C) Deletion of the RING C-terminus abrogates Mdm2 E3 activity. The ubiquitin ligation assay was performed as in (A). Mixtures containing UbCH5c protein (500 ng) ~300 pmol of ³²P-labeled ubiquitin and purified Mdm2 monomer, body and Mdm2-ΔC7 proteins (1, 3, 5 and 7 μg) were incubated as in (A) and then resolved by 8% SDS-PAGE and visualized by autoradiography. (D) Mdm2 RING species used in the ubiquitin polymerization reactions. A Coomassie blue-stained 12% SDS-PAGE gel was used to visualize 1, 3, 5 and 7 μg of Mdm2 monomer, oligomer and Mdm2-ΔC7 RING proteins.

E2s), Mdm2 bodies are more active E3 ligases than Mdm2 monomers. Further, the C-terminus plays an important role in the E3 ligase activity of the monomer and the body.

The last seven amino acids of MdmX are able to substitute for the Mdm2 C-terminus

Having established the requirement for an intact C-terminus in Mdm2 E3 activity, we set out to determine the potential function of this region in ubiquitin polymerization. To this end, we took advantage of the fact that MdmX, a closely related protein, possesses a homologous C-terminal RING domain but is devoid of E3 activity (Marine and Jochemsen, 2005). Initially, we confirmed this observation in our *in vitro* system with the purified MdmX RING domain and, as expected, did not observe E3 activity from the MdmX RING (Figure 5A).

Primary sequence comparison between the Mdm2 and MdmX C-termini revealed that both contain several hydrophobic residues, although there is only limited similarity between their last five amino acids. We performed a swap experiment where the last seven amino acids of Mdm2 and MdmX RING domains were exchanged (generating Mdm2-

XC7 and MdmX-2C7) and the resulting proteins were purified and assayed for the E3 activity (Figure 5B and Supplementary Figure 2). Substitution of the last seven residues of MdmX for those of Mdm2 did not impart ubiquitin ligase function on the MdmX RING domain. Thus, other parts of the Mdm2 RING must also be required for its E3 ligase function and, reciprocally, they must be absent from the MdmX RING domain. Importantly, however, the C-terminal seven amino acids of MdmX were able to substitute for the last seven residues of Mdm2, resulting in a fully functional chimeric protein (Mdm2-XC7) (Figure 5B). By contrast, mixing the full-length MdmX-2C7 and Mdm2-Δ7 RING did not rescue Mdm2-Δ7 RING ligase activity. Further, the MdmX-2C7 RING had no effect on the activity of Mdm2-XC7. Nevertheless, the fact that Mdm2-XC7 is fully functional as an E3 ligase suggests that residues within the C-termini of Mdm2 and MdmX that are essential for the E3 ligase activity of Mdm2 must be conserved between them. As gel-filtration profiles of the Mdm2-ΔC5 and Mdm2-ΔC7 constructs were similarly monomeric, it is likely that one (or more) key determinant(s) of Mdm2 activity must be contained in the last five residues (Figure 3A and B). Phenylalanine is the only common residue

Figure 5 The C-terminus of MdmX can substitute for the Mdm2 C-terminus in ubiquitin polymerization with a conserved phenylalanine residue being essential for this activity. (A) MdmX is not able to catalyze ubiquitin conjugation. Ubiquitin ligation assays were performed as in Figure 4 and described in Materials and methods. Equivalent aliquots of reaction mixtures with UbCh5c protein (500 ng), ~300 pmol of 32 P-labeled ubiquitin and purified Mdm2 monomer, body and MdmX RING domains (at the indicated amounts) were resolved by 8% SDS-PAGE and visualized by autoradiography. (B) The C-terminal residues of the Mdm2 RING domain are necessary but not essential for E3 activity. Mdm2 and MdmX C-terminal swap proteins; Mdm2XC7 (Mdm2-Δ7 fused to the last seven residues of MdmX) and MdmX2C7 (MdmX-ΔC7 fused to the last seven residues of Mdm2) were generated and purified as described in Materials and methods. Ubiquitin ligation assays were as in (A) with the above proteins at the indicated amounts. In lanes 11–14, 3 and 5 μ g curves of each protein were used. (C) A phenylalanine residue is the one common residue within the last five amino acids of Mdm2 and MdmX. Graphic representation of the C-terminal swap constructs used in (B) and (C), in which F490 of Mdm2 and F488 are circled as the single conserved residues. (D) Substitution of F490 for Q leads to altered behavior in solution of the Mdm2 RING domain. Comparison of the Superdex-200 elution profiles of wild-type Mdm2 RING domain and F490Q Mdm2 RING domain. (E) MdmX enhances E3 activity of the C-terminal mutant F490Q Mdm2. Ubiquitin ligation assays were performed as indicated in (A) with purified GST-Mdm2 RING (3 and 5 μ g), GST-MdmX RING (1, 3 and 5 μ g) and either no additional proteins, or Mdm2-F490Q (F490Q; 5 μ g) or Mdm2-ΔC7 (ΔC7; 5 μ g) proteins. Equivalent aliquots of each mixture were resolved by 8% SDS-PAGE and visualized by autoradiography (top panel). Reactions were quantitated by phosphorimaging and graphed as fold increase in activity over the no E3 control (lower panel).

within the last five amino acids of the two C-termini (F490 of Mdm2; F488 of MdmX) and so we focused on this residue in subsequent mutational analysis (Figure 5C).

Mutation of F490 diminishes oligomerization and E3 activity of Mdm2

We generated an F490Q Mdm2 RING mutant and, following bacterial expression and purification, subjected the resulting protein to gel-filtration chromatography. Consistent with our original observation that Mdm2 body formation is mediated by the C-terminus, the predominant F490Q species detected was the monomer, although some heterogeneous large molecular weight material was also present, in contrast to the completely monomeric C-terminally deleted Mdm2 RINGs (Figure 5D). Purified F490Q monomers were as inert as inactive Mdm2- Δ C7 (Supplementary Figure 3). By contrast, F490Q protein before gel filtration displayed a modest amount of E3 activity (Figure 5E, compare lanes 1 and 7).

Our observation about the necessity of the intact C-terminus for the ubiquitination activity of Mdm2 could be tested further with the F490Q mutant, which, as mentioned above, was impaired as an E3 ligase (Figure 5E, compare lanes 3 and 7, which contain equivalent amounts of wild-type and F490Q Mdm2 RING proteins). The activity of F490Q mutant was significantly increased by the addition of MdmX RING into the ubiquitin polymerization reaction (Figure 5E). Note that larger quantities of MdmX actually stimulated F490Q less well, probably owing to changed stoichiometry of complexes in favor of inactive MdmX. Thus, the C-terminus of MdmX is able to substitute for that of Mdm2 as long as the Mdm2 C-terminus is not deleted (Figure 5E). As noted above, it cannot be concluded that body formation is essential for E3 activity owing to the fact that the full-length monomers are also active in the reaction. Our data support a hypothesis that proper contacts made by the C-terminus (and specifically the interaction(s) made by F490) are essential for E3 activity, and that such interactions can support E3 activity whether they are formed in *cis* or in *trans*.

The extreme C-terminus of Mdm2 is required for its function in vivo

To extend these observations to Mdm2's activity in cells, we prepared Myc-tagged mammalian expression constructs encoding full-length Mdm2, Mdm2-F490Q and Mdm2- Δ C7. Following transfection of a range of DNA concentrations into H1299 cells, we observed that C-terminally deleted Mdm2 and F490Q mutant Mdm2 were far more stable than wild-type Mdm2, consistent with their defect in E3 ubiquitin ligase activity *in vitro* (Figure 6A). Furthermore, whereas wild-type Mdm2 reduced the levels of coexpressed p53, Mdm2- Δ C7 was incapable of targeting p53 for degradation. In fact, coexpression of p53 and Mdm2- Δ C7 led to marked stabilization of p53, possibly owing to protection of the latter from endogenous Mdm2 (Figure 6A). Mdm2-F490Q was partly impaired in p53 degradation, but failed to stabilize p53, a phenotype different from that of Mdm2- Δ C7, which is consistent with its retention of some activity in the *in vitro* assay (Figure 6A).

Further, we compared the half-lives of wild-type Mdm2 and Mdm2- Δ C7 in cyclohexamide chase experiments. In stark contrast to wild-type Mdm2, the overall protein levels

Figure 6 Deletion of the last seven amino acids of Mdm2 or mutation of F490 inhibits autodegradation and p53 degradation *in vivo*. (A) Mdm2- Δ C7 and F490Q are deficient in self- and p53 degradation *in vivo*. Increasing amounts (1, 2 and 4 μ g) of plasmids encoding Myc-tagged full-length Mdm2, Mdm2-F490Q or Mdm2- Δ C7 were transiently expressed in H1299 cells along with HA-tagged p53 (350 ng) in the presence of a GFP plasmid (150 ng) to normalize transfection efficiency. Cells were harvested 36 h after transfection and soluble proteins were resolved by 10% SDS-PAGE, followed by Western blotting with anti-HA antibody for p53, mixture of Smp14 and 2A10 antibodies for Mdm2 and anti-actin and anti-GFP antibodies for their respective targets. (B) Mdm2- Δ C7 is stable *in vivo*. Myc-tagged full-length Mdm2 and Mdm2- Δ C7 (4 μ g) were transiently coexpressed in H1299 cells in the presence of GFP. At 36 h after transfection, cells were treated with 25 μ g/ml cyclohexamide and harvested at the indicated time points. Mdm2, actin and GFP were detected as in (A). (C) Proteasome inhibition has little effect on the levels of Mdm2- Δ C7. Myc-tagged full length Mdm2 and Mdm2- Δ C7 (4 μ g) were transiently co-expressed in H1299 cells in the presence of GFP. At 24 h after transfection, cells were treated with 50 μ M LLnL and harvested at the indicated time points. Mdm2, actin and GFP were detected as in (A). Note that the upper band (arrow) is the ectopically expressed Myc-Mdm2. Longer exposure of the Mdm2 Western blot allows for the visualization of higher molecular weight forms of the proteins (predicted ubiquitin conjugates) only in lanes with wild-type Mdm2.

of Mdm2- Δ C7 were unchanged during the course of the experiment. Further, the proteasome inhibitor LLnL markedly increased the levels of wild-type Mdm2, but had

a much lesser impact on Mdm2- Δ C7, and LLnL treatment led to detectable ubiquitinated forms of only wild-type Mdm2 (Figure 6 B and C). These data support our *in vitro* observations and show that the last five or seven amino acids of Mdm2 are essential for its functions in the context of full-length protein *in vivo*.

The Mdm2 C-terminus directly interacts with the E2 ubiquitin-conjugating enzyme

The extreme C-terminus of Mdm2 is required for oligomerization and E3 activity of Mdm2 (Figures 3 and 5), although RING oligomerization is dispensable for the latter function (Figure 4). To determine whether the C-terminus may function as a molecular platform for E2, we investigated the ability of the Mdm2 RING domain to interact with the components of the ubiquitination machinery.

As contacts between ubiquitin, E2 and E3 components are likely to be transient, glutaraldehyde crosslinking was employed to detect possible interactions between ubiquitin and the Mdm2 RING with or without the E2 protein UbcH5c. We first compared the ability of ubiquitin to interact with the RING fusion constructs or GST alone by incubating each of these components with HA-ubiquitin in the presence of glutaraldehyde and then, after resolution by SDS-PAGE, performed Western blotting with anti-HA antibody (Figure 7A). Although one ubiquitin molecule could be cross-linked to GST (the $\sim$ 37 kDa species detected in

Figure 7A, lane 2, and $\sim$ 48 kDa species in lanes 3 and 4), there were no unique bands with the GST-RING constructs. This indicates that the Mdm2 RING domain does not form crosslinkable non-covalent contacts with ubiquitin. When UbcH5c was added to these components, however, novel polypeptide species were detected in a RING domain-dependent manner (Figure 7B). Specifically with full-length Mdm2 RING protein, a prominent $\sim$ 115 kDa species was detected as well as higher molecular weight species extending to the top of the gel (Figure 7B). We believe that the $\sim$ 82 kDa species seen with Mdm2- Δ C7 as well as with GST is the same nonspecific band observed in the absence of UbcH5 (see Figure 7A).

As ubiquitin did not interact with the Mdm2 RING directly, we postulate that UbcH5c mediates this interaction. Our ability to detect binding between ubiquitin and E2 is consistent with a number of current reports that describe non-covalent interactions between these proteins (Brzovic *et al*, 2006; Lewis *et al*, 2006). Of greater significance was the fact that under these conditions, ubiquitin crosslinking into high-molecular-weight species was greatly increased by the presence of the intact C-terminus (Figure 7B). These data support our hypothesis that the extreme C-terminus of Mdm2 is necessary for interaction of the RING domain with the components of the ubiquitination machinery and specifically identify this region as essential for proper E2 binding (Figure 8).

Figure 7 The extreme C-terminus of Mdm2 facilitates interaction of its RING domain with the components of the ubiquitination machinery. (A) Ubiquitin does not directly associate with the Mdm2 RING domain. HA-ubiquitin (10 μ g) and either GST-Mdm2 RING or GST-Mdm2- Δ C7 RING or GST alone (10 μ g) were incubated together in the ubiquitination buffer (total volume 100 μ l) containing 0.007% glutaraldehyde. Reactions were terminated by the addition of SDS-PAGE loading buffer at the indicated time points and resolved by 10% SDS-PAGE, followed by Western blotting with HA antibody to detect ubiquitin. (B) Ubiquitin crosslinking is stimulated in the presence of UbcH5c and Mdm2-RING. Crosslinking experiment was performed as in (A) in the presence of UbcH5c (10 μ g). Reaction mixtures were incubated for 3 and 7 min and terminated by addition of SDS sample buffer. Proteins were resolved by 10% SDS-PAGE, transferred to nitrocellulose membrane, followed by Western blotting with anti-HA antibody to visualize crosslinked ubiquitin. On the right is an expanded image of a darker exposure of the indicated region of the blot on the left. (C) Proteins used in the crosslinking reactions. The nitrocellulose membrane for the experiment shown in (B) was stained with Coomassie blue dye to show migration of non-crosslinked proteins, as well as to ensure that equivalent protein amounts were used in each reaction.

Figure 8 Schematic representation of the proposed mechanism of action for C-terminus of Mdm2. The extreme C-terminus of Mdm2 is able to form either intramolecular interactions forming a stable monomer that can interact with Ubch5c or similar interactions with another RING domain, which can lead to supramolecular assembly. Absence of C-terminal contacts with the RING domain does not disturb protein folding or ATP binding while being necessary for stabilization of a proper docking of E2 either with the monomer of the RING or the multimeric RING assembly.

Discussion

Multiple RING domain-containing proteins have been described as parts of these supramolecular complexes (Saurin *et al*, 1996). To acquire a more detailed biochemical examination of RING-containing bodies Kentsis *et al* (2002b) characterized bodies formed by a model RING protein Z through EM, analytical ultracentrifugation, light-scattering and circular dichroism spectroscopy. Their work revealed a mechanism for non-fibrillar self-assembly. Other non-related RING domains have been reported to form similar structures *in vitro* and in cells, and we show here that the Mdm2 RING is also able to undergo self-assembly (Figure 2).

Indeed, endogenous Mdm2 has been localized to discrete nuclear foci upon treatment with leptomycin B, a nuclear export inhibitor (Lain *et al*, 1999). Although it is tempting to speculate that body formation by the RING domain may have significant physiological function, as no such structures have been reported for full-length Mdm2 to date, we are hesitant to directly translate our findings into cellular context. However, it is possible that Mdm2 is forming bodies in cells that are below confocal resolution, and may even be difficult to detect by immuno-EM.

Because F490 (a large hydrophobic residue) is required for oligomerization and E3 activity, it may form key contacts with other residues of the RING domain. Such contacts could be established either *in cis* leading to the formation of a monomeric RING, or *in trans*, where RING domains are linked through their C-termini forming the higher order

oligomers that we observed. In fact, it is likely that both are occurring. This would explain both the observed behavior in solution and the fact that there is little or no inter-conversion between the bodies and the monomers of Mdm2 (data not shown).

There are numerous reports in the literature, that describe both homo-dimerization of Mdm2 and hetero-dimerization of the Mdm2 and MdmX RING domains (Sharp *et al*, 1999; Tanimura *et al*, 1999; Stad *et al*, 2001). Although these reports clearly demonstrate complex formation, none have reported the necessary experiments to derive the exact stoichiometry of the complex. Here, we present evidence that further defines the nature of the Mdm2 RING–RING complexes. A recent paper describing the NMR structure of a homodimeric complex between two Mdm2 RING domains supports our biochemical observations. Significantly, this structure clearly demonstrates the importance of the C-terminal amino acids of Mdm2 for proper complex formation and stabilization of the overall RING structure (Kostic *et al*, 2006). Our data are supportive of a mechanism where more than two RING domains could be linked to large structural complexes through the interaction of their C-termini. It is of note that RING–RING interactions mediated by sequences peripheral to the RING domain itself have been reported for cIAP/XIAP and BRCA1/BARD1 heterodimers (Brzovic *et al*, 2001; Silke *et al*, 2005).

There are several theories about the role of the RING domain in ubiquitin polymerization. The suggestion that RING E3s are able to allosterically activate E2 enzymes is unlikely, owing to the fact that the structures of the Ubch7 E2

are virtually identical in complex with the Cbl RING or with E6AP (a HECT domain E3) (Huang *et al*, 1999; Zheng *et al*, 2000). The prevailing view of RING E3 activity stipulates that RING-type E3s bring the target protein to the E2 bound to activated ubiquitin and potentially help to orient the substrate for optimal ubiquitin conjugation (Pickart, 2004). The exact placement of the reactants has to be achieved in order for proximity to have substantial effects on the rate of catalysis (Fersht, 1985). The fact that ubiquitination often takes place on multiple lysine residues of the substrate (Rodriguez *et al*, 2000), as well as the tens of angstroms that separate the bound E2 and the substrate in the existing crystal structures, makes it unclear whether the RING E3s can provide the necessarily stringent substrate orientation (Zheng *et al*, 2002; Orlicky *et al*, 2003). The proximity hypothesis is also challenged by the fact that mutations in the Ubr1 RING domain abolish ubiquitination, but have little effect on the ability of this protein to interact with Rad6p, its cognate E2 (Xie and Varshavsky, 1999).

Our results support the idea that RING E3s may induce ubiquitin polymerization by providing a scaffold for the reaction. Although the Mdm2 RING monomer is also capable of supporting ubiquitination, it is possible that in cells the reaction would take place in the absence of saturating amounts of E2, thus favoring the activity of the body to that of the monomer. Further, the question of polyubiquitin chain generation, which is currently poorly understood, is worth considering in the context of RING assembly. Processive enzymes perform multiple rounds of action before dissociation from the substrate; this is often achieved by the existence of platforms with multiple binding sites for the substrate (Breyer and Matthews, 2001). Thus, it is possible that Mdm2 bodies aid processivity not only by increasing the local concentration of reactants, but also by providing a binding surface for multiple E2s and allowing for chain elongation. A similar mechanism has recently been proposed based on structural data for the activity of UbcH5c in the context of BRCA1-directed ubiquitination (Brzovic *et al*, 2006).

Remarkably, a hypothesis very similar to ours is proposed in the accompanying study by Uldrijan *et al* (2006) based on a different experimental approach to the study of E3 function and interactions of full-length Mdm2, MdmX and C-terminally altered versions of Mdm2 in cells. These authors also present data, where deletion of the extreme C-terminus of Mdm2 leads to loss of E3 function and the ability to oligomerize. Importantly as well, Uldrijan *et al* (2006) show that proper Zn²⁺ coordination along with the intact C-terminus is required for Mdm2–MdmX interactions in cells. Their results not only support our findings of the direct involvement of the Mdm2 C-terminus in ubiquitination, but also provide evidence for a potential *in vivo* function of the supra-molecular assemblies we describe in our study.

Mdm2 is currently under much investigation as a potential target for therapeutic intervention in cancer therapy. Our observation that mutating a single hydrophobic residue (F490) in the C-terminus of the RING domain has a profound effect on Mdm2 E3 function is reminiscent of the interaction between p53 and Mdm2, which requires two hydrophobic residues (Kussie *et al*, 1996). The Mdm2–p53 interaction has been successfully disrupted by small compounds (Kussie *et al*, 1996; Vassilev *et al*, 2004). A similar approach

could conceivably be taken to disable Mdm2 RING function by identifying small molecules that inhibit formation of contacts made by the C-terminal residues of Mdm2.

Materials and methods

Plasmids and antibodies

Human wild-type Mdm2 and Mdm2-ΔC7 cDNAs were unidirectionally cloned with the addition of two N-terminal Myc tags between the *EcoRI* and *BamHI* sites of pCDNA3. All GST-Mdm2/MdmX RING deletion constructs were unidirectionally cloned between the *BamHI* and *EcoRI* sites of the pGEX-4T1 vector and used as a backbone for site-directed mutagenesis and generation of F490Q mutant (Quick Change, Stratagene). The Pc53-HA vector expresses full-length HA-p53 cDNA from the *CMV* promoter in pCDNA3. Constructs expressing PK-Ub and His-UbcH5c in pET-15b vectors have been previously described (Tan *et al*, 1999). Monoclonal antibodies against HA and Myc epitopes were purchased from HA11, BabCo and 9E10, SantaCruz.

Protein purification

GST fusion proteins were expressed in BL21 (DE3) cells. After induction for 12 h at 16°C with 0.4 M IPTG, soluble proteins were extracted by sonication in lysis buffer (50 mM Tris at pH 7.0, 350 mM NaCl, 0.1% aprotinin, 1 mM DTT, 0.5 mM PMSF). The soluble protein fraction was incubated with glutathione–Sepharose beads (Pharmacia) at 4°C for 1 h, and the bound protein was eluted with reduced glutathione. Purified GST-Mdm2 was subjected to thrombin cleavage at 4°C for 12 h, at a 1/100 thrombin/target ratio. Cleaved protein was bound to a MonoS column and eluted in buffer containing 50 mM Mes (pH 6.0), 500 mM NaCl and 1 mM DTT. Purified Mdm2 RING or GST-Mdm2 RING was further separated on a Superdex-200 gel-filtration column in 50 mM Tris–HCl, pH 7.0, 150 mM NaCl, 2 mM DTT in 10% glycerol. Purified proteins were stored at –80°C.

ATP binding experiments

Mdm2 proteins were incubated with 10 nM ATP and 0.5 μCi of [γ -³²P]ATP for 20 min at 23°C in ATP binding solution (40 mM creatine phosphate, 1 mM MgCl₂, 15 mM NaCl, 0.5 mM DTT, 10 μg BSA). Reaction mixtures were filtered through 25-mm nitrocellulose filters (Protran, Schleicher & Schuell, Keene, NH) under vacuum. Filters were washed three times with 5 ml of 25 mM Hepes buffer (pH 7.9), dried and counted by liquid scintillation.

Cell lines, transfections and immunoblotting

U2OS (osteosarcoma) cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS). Transfections were performed using Lipofectamine 2000 Reagent (Invitrogen) in accordance with the manufacturer's protocols. Immunoblotting was performed as previously described (Poyurovsky *et al*, 2003).

In vitro ubiquitination experiments

Reaction mixtures contained 50 mM Tris, pH 7.6, 5 mM MgCl₂, 5 mM ATP, 300 pM recombinant PK-ubiquitin and rabbit E1 (50 nM; BostonBiochem), His-UbcH5c (1.5 μM) and indicated amounts of Mdm2. Following a 1 h incubation at 37°C, reactions were terminated by the addition of 15 μl of protein sample buffer, and the reactions were boiled for 5 min at 100°C in SDS sample buffer. Products were resolved by SDS–PAGE and visualized by autoradiography.

PK-Ub and His-UbcH5c were prepared using Ni²⁺-NTA-based affinity purification procedures (Qiagen). Phosphorylation of PK-Ub, 10 μg each, was carried out in a reaction mixture (50 μl) containing 20 mM Tris–HCl (pH 7.4), 12 mM MgCl₂, 2 mM NaF, 50 mM NaCl, 25 μM ATP, 5 μCi of [γ -³²P]ATP, 0.1 mg/ml BSA and 1 U cAMP kinase (Sigma). The reaction mixture was incubated at 37°C for 30 min. To inactivate the kinase, the mixture was heated at 70°C for 3 min.

Electron microscopy

Formvar-coated nickel grids (Electron Microscopy Sciences, Fort Washington, PA) were glow-discharged, floated on sample drops for 60 s, blotted and air-dried. They were then negatively stained for

90 s with either 1% (w/v) aqueous uranyl acetate, 2% aqueous methylamine tungstenate (Nano-W, Nanoprobes, Yaphank, NY), or aqueous solution of 1% Nano-W and 1% methylamine (NanoVan, Nanoprobes). Grids were photographed by using a JEOL JEM 100-CX electron microscope under low-dose conditions at 80 kV.

Size-exclusion chromatography

Analytical measurements were carried out by using FPLC chromatograph and Superdex-200 (25 ml bed volume) column (Amersham Pharmacia). Void and total column volumes were measured by using Dextran blue 2000 and 1 M NaCl, respectively. Samples were injected directly onto the column and separated at 4°C at a rate of 0.5–1.0 ml/min while recording UV absorbance at 280 nm.

Analytical gel-filtration chromatography and static light-scattering measurements

A sample comprising Mdm2 at 10 mg/ml was injected onto a Shodex 802.5 silica-based gel-filtration column (Showa Denko, Tokyo, Japan) equilibrated at 4°C in 100 mM NaCl, 0.025% (w/v) NaN₃ and 100 mM Tris-HCl (pH 7.5). The column was fed by an Äkta Purifier FPLC system (Amersham Biosciences, Piscataway, NJ) contained in a cold box at 4°C, and the effluent passed through a UV detector (also at 4°C), a 15-channel Dawn static light-scattering detector at room temperature and finally an Optilab refractive index

detector with its sample chamber heated to 40°C. The latter two detectors were from Wyatt Technologies (Santa Barbara, CA). Integration of the refractive index trace indicated essentially 100% recovery of the protein injected onto the column.

Metal content analysis

Metal analysis on the purified Mdm2 protein was performed at the Chemical Analysis Laboratory at the University of Georgia by ICP-MS using the PlasmaQuad 3 instrument with a standard analytical procedure consisting of adding an internal standard. The concentration of the protein is ~5–10 µM in a volume of 2 ml each in buffer containing 50 mM bis-tris-propane (BTP), pH 6.8, 350 mM NaCl and 1 mM DTT.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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References

- Bailin T, Mo X, Sadofsky MJ (1999) A RAG1 and RAG2 tetramer complex is active in cleavage in V(D)J recombination. *Mol Cell Biol* **19**: 4664–4671
- Boddy MN, Freemont PS, Borden KL (1994) The p53-associated protein MDM2 contains a newly characterized zinc-binding domain called the RING finger. *Trends Biochem Sci* **19**: 198–199
- Borden KL (2000) RING domains: master builders of molecular scaffolds? *J Mol Biol* **295**: 1103–1112
- Breyer WA, Matthews BW (2001) A structural basis for processivity. *Protein Sci* **10**: 1699–1711
- Brzovic PS, Keefe JR, Nishikawa H, Miyamoto K, Fox III D, Fukuda M, Ohta T, Kleivit R (2003) Binding and recognition in the assembly of an active BRCA1/BARD1 ubiquitin-ligase complex. *Proc Natl Acad Sci USA* **100**: 5646–5651
- Brzovic PS, Lissounov A, Christensen DE, Hoyt DW, Kleivit RE (2006) A UbcH5/ubiquitin noncovalent complex is required for processive BRCA1-directed ubiquitination. *Mol Cell* **21**: 873–880
- Brzovic PS, Rajagopal P, Hoyt DW, King MC, Kleivit RE (2001) Structure of a BRCA1-BARD1 heterodimeric RING-RING complex. *Nat Struct Biol* **8**: 833–837
- Elenbaas B, Dobbstein M, Roth J, Shenk T, Levine AJ (1996) The MDM2 oncoprotein binds specifically to RNA through its RING finger domain. *Mol Med* **2**: 439–451
- Fang S, Jensen JP, Ludwig RL, Vousden KH, Weissman AM (2000) Mdm2 is a RING finger-dependent ubiquitin protein ligase for itself and p53. *J Biol Chem* **275**: 8945–8951
- Fersht A (1985) *Enzyme Structure and Mechanism*. New York: WH Freeman & Company
- Harris SL, Levine AJ (2005) The p53 pathway: positive and negative feedback loops. *Oncogene* **24**: 2899–2908
- Haupt Y, Maya R, Kazaz A, Oren M (1997) Mdm2 promotes the rapid degradation of p53. *Nature* **387**: 296–299
- Honda R, Yasuda H (2000) Activity of MDM2, a ubiquitin ligase, toward p53 or itself is dependent on the RING finger domain of the ligase. *Oncogene* **19**: 1473–1476
- Huang L, Kinnucan E, Wang G, Beaudenon S, Howley PM, Huijbregtse JM, Pavletich NP (1999) Structure of an E6AP-UbcH7 complex: insights into ubiquitination by the E2-E3 enzyme cascade. *Science* **286**: 1321–1326
- Kentsis A, Gordon RE, Borden KL (2002a) Control of biochemical reactions through supramolecular RING domain self-assembly. *Proc Natl Acad Sci USA* **99**: 15404–15409
- Kentsis A, Gordon RE, Borden KL (2002b) Self-assembly properties of a model RING domain. *Proc Natl Acad Sci USA* **99**: 667–672
- Kostic M, Matt T, Martinez-Yamout MA, Dyson HJ, Wright PE (2006) Solution structure of the Hdm2 C2H2C4 RING, a domain critical for ubiquitination of p53. *J Mol Biol* **363**: 433–450
- Kubbutat MH, Jones SN, Vousden KH (1997) Regulation of p53 stability by Mdm2. *Nature* **387**: 299–303
- Kussie PH, Gorina S, Marechal V, Elenbaas B, Moreau J, Levine AJ, Pavletich NP (1996) Structure of the MDM2 oncoprotein bound to the p53 tumor suppressor transactivation domain. *Science* **274**: 948–953
- Lai Z, Freedman DA, Levine AJ, McLendon GL (1998) Metal and RNA binding properties of the hdm2 RING finger domain. *Biochemistry* **37**: 17005–17015
- Lain S, Midgley C, Sparks A, Lane EB, Lane DP (1999) An inhibitor of nuclear export activates the p53 response and induces the localization of HDM2 and p53 to U1A-positive nuclear bodies associated with the PODs. *Exp Cell Res* **248**: 457–472
- Lewis MJ, Saltibus LF, Hau DD, Xiao W, Spyropoulos L (2006) Structural basis for non-covalent interaction between ubiquitin and the ubiquitin conjugating enzyme variant human MMS2. *J Biomol NMR* **34**: 89–100
- Lohrum MA, Ashcroft M, Kubbutat MH, Vousden KH (2000) Identification of a cryptic nucleolar-localization signal in MDM2. *Nat Cell Biol* **2**: 179–181
- Marine JC, Jochemsen AG (2005) Mdmx as an essential regulator of p53 activity. *Biochem Biophys Res Commun* **331**: 750–760
- Melnick A, Licht JD (1999) Deconstructing a disease: RARalpha, its fusion partners, and their roles in the pathogenesis of acute promyelocytic leukemia. *Blood* **93**: 3167–3215
- Moll UM, Petrenko O (2003) The MDM2–p53 interaction. *Mol Cancer Res* **1**: 1001–1008
- Momand J, Zambetti GP, Olson DC, George D, Levine AJ (1992) The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. *Cell* **69**: 1237–1245
- Orlicky S, Tang X, Willems A, Tyers M, Sicheri F (2003) Structural basis for phosphodependent substrate selection and orientation by the SCFCdc4 ubiquitin ligase. *Cell* **112**: 243–256
- Pickart CM (2004) Back to the future with ubiquitin. *Cell* **116**: 181–190
- Poyurovsky MV, Jacq X, Ma C, Karni-Schmidt O, Parker PJ, Chalfie M, Manley JL, Prives C (2003) Nucleotide binding by the Mdm2 RING domain facilitates Arf-independent Mdm2 nucleolar localization. *Mol Cell* **12**: 875–887
- Poyurovsky MV, Prives C (2006) Unleashing the power of p53: lessons from mice and men. *Genes Dev* **20**: 125–131
- Prives C (1998) Signaling to p53: breaking the MDM2–p53 circuit. *Cell* **95**: 5–8
- Prives C, Hall PA (1999) The p53 pathway. *J Pathol* **187**: 112–126
- Rodriguez MS, Desterro JM, Lain S, Lane DP, Hay RT (2000) Multiple C-terminal lysine residues target p53 for ubiquitin-proteasome-mediated degradation. *Mol Cell Biol* **20**: 8458–8467

- Saurin AJ, Borden KL, Boddy MN, Freemont PS (1996) Does this have a familiar RING? *Trends Biochem Sci* **21**: 208–214
- Sharp DA, Kratowicz SA, Sank MJ, George DL (1999) Stabilization of the MDM2 oncoprotein by interaction with the structurally related MDMX protein. *J Biol Chem* **274**: 38189–38196
- Silke J, Kratina T, Chu D, Ekert PG, Day CL, Pakusch M, Huang DC, Vaux DL (2005) Determination of cell survival by RING-mediated regulation of inhibitor of apoptosis (IAP) protein abundance. *Proc Natl Acad Sci USA* **102**: 16182–16187
- Stad R, Little NA, Xirodimas DP, Frenk R, van der Eb AJ, Lane DP, Saville MK, Jochemsen AG (2001) Mdmx stabilizes p53 and Mdm2 via two distinct mechanisms. *EMBO Rep* **2**: 1029–1034
- Tan P, Fuchs SY, Chen A, Wu K, Gomez C, Ronai Z, Pan ZQ (1999) Recruitment of a ROC1-CUL1 ubiquitin ligase by Skp1 and HOS to catalyze the ubiquitination of I kappa B alpha. *Mol Cell* **3**: 527–533
- Tanimura S, Ohtsuka S, Mitsui K, Shirouzu K, Yoshimura A, Ohtsubo M (1999) MDM2 interacts with MDMX through their RING finger domains. *FEBS Lett* **447**: 5–9
- Uldrijan S, Pannekoek W, Vousden KH (2006) An essential function of the extreme C-terminus of Mdm2 can be provided by MdmX. *EMBO J* [E-pub ahead of print: 14 December 2006; doi:10.1038/sj.emboj.7601469]
- Vassilev LT, Vu BT, Graves B, Carvajal D, Podlaski F, Filipovic Z, Kong N, Kammlott U, Lukacs C, Klein C, Fotouhi N, Liu EA (2004) *In vivo* activation of the p53 pathway by small-molecule antagonists of MDM2. *Science* **303**: 844–848
- Wang X, Taplick J, Geva N, Oren M (2004) Inhibition of p53 degradation by Mdm2 acetylation. *FEBS Lett* **561**: 195–201
- Xie Y, Varshavsky A (1999) The E2–E3 interaction in the N-end rule pathway: the RING-H2 finger of E3 is required for the synthesis of multiubiquitin chain. *EMBO J* **18**: 6832–6844
- Zheng N, Schulman BA, Song L, Miller JJ, Jeffrey PD, Wang P, Chu C, Koepp DM, Elledge SJ, Pagano M, Conaway RC, Conaway JW, Harper JW, Pavletich NP (2002) Structure of the Cul1–Rbx1–Skp1–F boxSkp2 SCF ubiquitin ligase complex. *Nature* **416**: 703–709
- Zheng N, Wang P, Jeffrey PD, Pavletich NP (2000) Structure of a c-Cbl–UbcH7 complex: RING domain function in ubiquitin-protein ligases. *Cell* **102**: 533–539