P14ARF promotes accumulation of SUMO-1 conjugated (H)Mdm2

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Abstract p14ARF tumour suppressor stabilises and activates p53 by directly interacting with (H)Mdm2 [(human) murine double minute 2 homologue] and inhibiting its E3 ubiquitin ligase activity. Here we demonstrate that p14ARF promotes accumulation of (H)Mdm2 conjugated to the small ubiquitin-like protein SUMO-1. Mutational analysis demonstrated that the N-terminus of Mdm2 is a target for p14ARF-mediated SUMO conjugation. SUMO modification requires residues 2–14 in p14ARF that interact with (H)Mdm2 and residues 82–101 in exon 2 involved in nucleolar localisation of p14ARF. These data suggest a novel role for p14ARF as a regulator of activity of (H)Mdm2, which could be related to its tumour suppressing activities.

Key words: (H)Mdm2; p14ARF; SUMO conjugation

1. Introduction

The product of the murine double minute 2 homologue (Mdm2) oncogene plays a vital role in regulation of the stability and activity of the p53 tumour suppressor protein. Initial genetic studies demonstrated that a critical role of Mdm2 is to negatively regulate the growth suppressing effects of p53 [1,2]. Biochemical and biological studies showed that Mdm2 can act as a ubiquitin E3 ligase [3], promoting ubiquitylation and proteasomal degradation of p53 [4–6]. Mdm2 can also regulate its own stability by promoting auto-ubiquitylation [7–9].

In mammals the INK4α-P14ARF locus encodes two different cell cycle inhibitors (p16INK4α and p14ARF) by alternative splicing [10,11]. The observation that p14ARF null mice develop tumours at an early stage and the requirement of wild-type p53 for p14ARF to induce G1 cell cycle arrest strongly suggest that p14ARF is acting upstream of p53 as a tumour suppressor [12].

At the molecular level, p14ARF has been shown to interfere with the ability of Mdm2 to suppress the stability and activity of p53 [13–16]. P14ARF directly interacts with (H)Mdm2 (Hdm2, human homologue of Mdm2), inhibiting ubiquitylation and resulting in p53 accumulation and induction of the p53 response [9,17,18].

Recently, the Mdm2 protein was demonstrated to become conjugated to small ubiquitin-like protein SUMO-1. This modification was proposed to affect the stability of Mdm2 and its ability to promote ubiquitylation of p53 [19]. SUMO is conjugated to protein substrates in a manner that is mechanistically similar to that of ubiquitin. In the case of SUMO, however, the SUMO activating enzyme (SAE) is a heterodimer of SAE1 and SAE2 and the E2 is Ubc9 (reviewed in [20,21]).

Here we demonstrate a role for p14ARF in SUMO-1 conjugation of (H)Mdm2. In the absence of p14ARF, (H)Mdm2 is modified by SUMO-1 at a very low level. However, in the presence of p14ARF, the levels of (H)Mdm2 conjugated to SUMO-1 increase dramatically. Mutational analysis demonstrated that the N-terminus of Mdm2 can be used for p14ARF-mediated SUMO-1 conjugation and that binding of p14ARF to (H)Mdm2 as well as sequences in exon 2 of p14ARF are required for this effect. These data propose a novel role for the p14ARF tumour suppressor as a regulator of SUMO conjugation, which may be related to its growth suppressing activities.

2. Materials and methods

2.1. Materials

Human and mouse Mdm2 were detected using the 4B2 mouse monoclonal antibody [22]. MG132 was purchased from Calbiochem. Plasmids used in this study are described elsewhere [18,23].

2.2. Transfections

H1299 cells were transfected with calcium-phosphate method as described before [18]. Where indicated 7 μg of H(M)dm2, 7 μg of p14ARF and 2 μg of His_6-SUMO-1 expressing plasmids were used.

2.3. Purification of His_6-SUMO-1 conjugates

Thirty-six hours post-transfection cells were harvested and His_6-SUMO-1 conjugates were purified as described before [18,23]. Twenty percent of cells were used for NP-40 lysis and Western blotting was performed as described in [18].

2.4. In vitro SUMO-1 conjugation assay

Wild-type Mdm2 or Mdm2 deletion mutants were expressed in vitro using the TnT T7 Coupled Wheat Germ Extract System from Promega according to manufacturer's instructions. One μl of ^{35}S-methionine-labelled protein was incubated in a 10 μl reaction with purified components required for SUMO modification and analysed by phosphorimaging as described [24].

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Abbreviations: Mdm2, murine double minute 2 homologue; Hdm2, human homologue of Mdm2

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3. Results

3.1. Expression of p14ARF promotes accumulation of SUMO-1 conjugated Hdm2 in vivo

Recently, it was reported that the Hdm2 protein could be modified with the ubiquitin-like protein SUMO-1 with consequences for the stability and E3 ubiquitin ligase activity of Hdm2 towards p53 [19]. We have recently demonstrated that expression of the p14ARF tumour suppressor stabilises Hdm2 without affecting its auto-ubiquitylation activity [18]. Therefore, we investigated the effect of p14ARF on SUMO-1 conjugation of Hdm2. H1299 cells (p53 null) were transfected with expression constructs for Hdm2, p14ARF and a His_6 version of SUMO-1. Cells were harvested and a fraction was used to monitor expression of Hdm2 by Western blotting. The remaining cells were lysed in guanidinium–HCl and His_6-SUMO-1 conjugated proteins were purified using Ni^{2+}-agarose beads. Proteins eluted from the Ni^{2+} beads were analysed by Western blotting with 4B2 anti-Hdm2 monoclonal antibody to detect SUMO-1-Hdm2 conjugation.

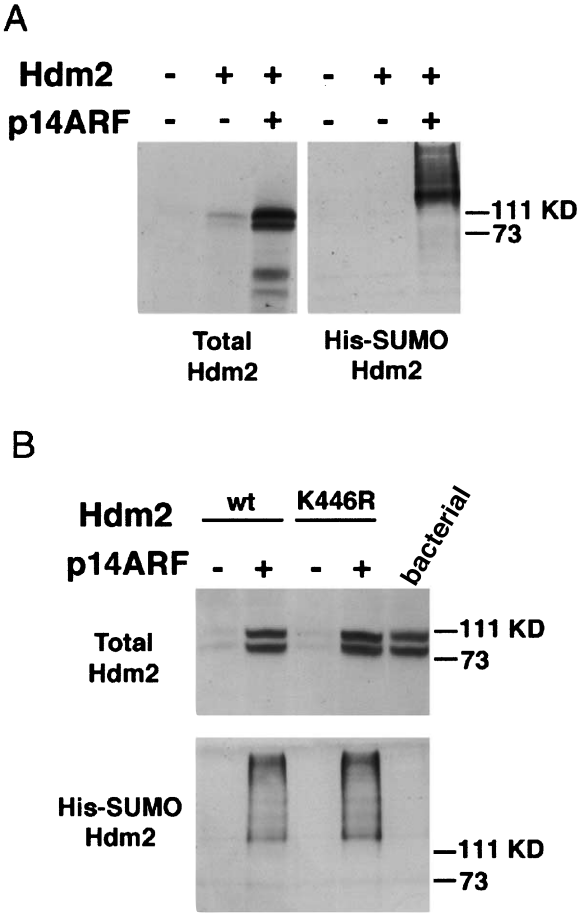

Fig. 1. A: Co-expression of p14ARF results in accumulation of SUMO-1 conjugated Hdm2. H1299 cells were transfected with Hdm2 alone (7 μg) or with p14ARF (7 μg) expression constructs. In all transfections 2 μg of His_6-SUMO-1 expression construct was included. Thirty-six hours post-transfection cells were harvested and analysed for total Hdm2 levels (left panel) or SUMO-1-Hdm2 (right panel) by Western blotting using the 4B2 anti-Hdm2 monoclonal antibody (1 μg/ml) as described in Section 2. B: P14ARF promotes SUMO-1 conjugation of the K446R Hdm2 mutant. Wild-type Hdm2 or the K446R mutant were expressed in the absence or presence of p14ARF and cell extracts were analysed as described above. Fifty ng of bacterial expressed Hdm2 [9] were also analysed by Western blotting.

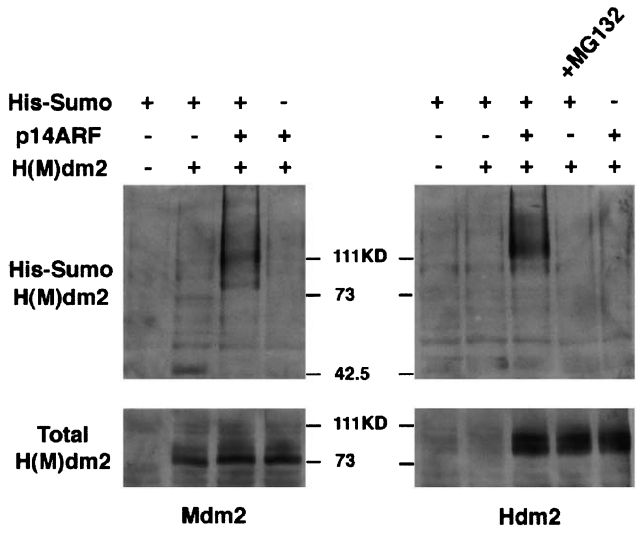

Fig. 2. P14ARF-mediated accumulation of SUMO-1-Hdm2 is not due to the stabilising effect of p14ARF on Hdm2. H1299 cells were transfected with the indicated constructs as described before. MG132 proteasome inhibitors were used for 3 h at 30 μM. Cells were harvested and analysed by Western blotting as described in Fig. 1.

Hdm2 expression generated species of 75 and 90 kDa, both of which were stabilised by expression of p14ARF (Fig. 1A, left panel). SUMO-1 modified Hdm2 was detected when p14ARF was co-expressed, but not when Hdm2 was expressed alone (Fig. 1A, right panel). The major form of SUMO-1-Hdm2 migrates as a 120 kDa species, which is consistent with the attachment of one or two SUMO-1 moieties. Furthermore, more slowly migrating forms of SUMO-1-Hdm2 were also detected, which could represent SUMO-1-Hdm2 with additional post-translational modifications. Alternatively, the slower migrating forms could result from the addition of multiple SUMO-1 molecules. It is worth noting that the His_6-SUMO-1 conjugated eluates do not contain any Hdm2 species of Mr 90 kDa, which was the reported size of Hdm2 after conjugation to a single SUMO-1 moiety [19].

It was previously reported that a single point mutation in the ring finger domain of Hdm2 (K446R) prevented SUMO-1 conjugation in vitro and in vivo [19]. However, we found that expression of p14ARF could also promote SUMO-1 conjugation of the K446R Hdm2 mutant as effectively as wild-type Hdm2 (Fig. 1B, lower panel). To our surprise expression of the K446R mutant generated the 75 and 90 kDa species observed with wild-type Hdm2 and expression of p14ARF stabilised both species (Fig. 1B, upper panel). This is in direct contrast to previous observations which reported that K446R Hdm2, deficient for SUMO-1 conjugation, migrates as a single 75 kDa species which was thought to represent the unmodified Hdm2. Critically, bacterially expressed Hdm2, which is not SUMO conjugated, also migrates as two species of 75 and 90 kDa (Fig. 1B, upper panel).

Detection of SUMO-1-Hdm2 after expression of p14ARF could be due to either the stabilising effect of p14ARF on the levels of Hdm2, or that p14ARF plays an active role in the SUMO pathway. To distinguish between these two possibilities the mouse homologue of Hdm2 (Mdm2) whose stability

is not significantly affected by p14ARF was employed. Expression of p14ARF did not affect the stability of Mdm2 but increased the amount of SUMO-1-Mdm2 detected (Fig. 2, left panel). In another experiment (Fig. 2, right panel) cells expressing Hdm2 were treated with MG132 proteasome inhibitor in the absence of p14ARF. Under these conditions Hdm2 protein is elevated to the same level as that observed in the presence of p14ARF. While p14ARF expression resulted in detection of SUMO-1-Hdm2, proteasome inhibitors did not stimulate formation of SUMO-1 modified Hdm2. Therefore, it appears that expression of p14ARF promotes accumulation of SUMO-1-(H)Mdm2 conjugates and it is not due to the stabilising effect of p14ARF on Hdm2.

3.2. *The N-terminus of Mdm2 is a target for p14ARF-mediated SUMO-1 conjugation*

To identify regions in (H)Mdm2 required for SUMO-1 modification in vivo, Mdm2 deletion mutants were expressed in H1299 cells either in the absence or presence of p14ARF and lysates were analysed for total Mdm2 protein levels or His_6-SUMO-1-Mdm2 isolated on Ni^{2+} beads as before. The p14ARF binding domain on Mdm2 is located between residues 212–244 [9]. The Δ26 mutant (residues 1–152) lacks the p14ARF binding site and no SUMO-1 conjugation was observed. Mdm2 deletion mutants Δ18 (residues 1–272) and Δ11 (residues 1–342) contain the p14ARF binding site and both are conjugated to SUMO-1 in the presence of p14ARF (Fig. 3A). A similar mutational analysis was performed using an in vitro Mdm2 SUMO-1 conjugation assay. ^{35}S-labelled Mdm2 was incubated with purified SAE, Ubc9 and SUMO-1 as described [24]. Mdm2 is modified with SUMO-1 only when all the necessary components are included (Fig. 3B). Using this assay, Mdm2 deletion mutants were analysed for their capacity to be modified by SUMO-1. This analysis also demonstrated that the N-terminal 212 residues of Mdm2 are a target for SUMO-1 conjugation (Fig. 3C). The 1–134 Mdm2 deletion mutant was not conjugated to SUMO-1, suggesting that lysine residues between amino acids 134 and 212 in Mdm2 are sites for SUMO-1 conjugation, although this does not exclude the possibility that the mutant is deficient in its interaction with modification enzymes such as Ubc9. Analysis of point mutations in this area did not provide clear evidence for involvement of a single lysine residue in SUMO-1 conjugation. P14ARF was also added to the in vitro SUMO-1 conjugation assay either by co-expression in vitro with Mdm2 or by adding bacterially expressed p14ARF. Neither of these additions altered SUMO-1 conjugation of Mdm2. While the in vitro SUMO-1 assay may be lacking factors present in vivo the presented data from the in vivo and in vitro analysis demonstrate that the N-terminus of Mdm2 can be used as a substrate for SUMO-1 conjugation.

3.3. *Sequences in exon 2 of p14ARF are required for SUMO-1 conjugation of H(M)dm2*

To identify the region of p14ARF required for enhancement of SUMO modification of (H)Mdm2 in vivo, previously described p14ARF deletion mutants were employed [18,25]. The Δ2–14 p14ARF mutant was shown to be deficient for (H)Mdm2 binding, whereas the Δ82–101 p14ARF mutant was defective for nucleolar localisation of p14ARF [26,27]. While wild-type p14ARF stabilised Hdm2 and promoted SUMO-1 conjugation, the Δ2–14 p14ARF mutant had no

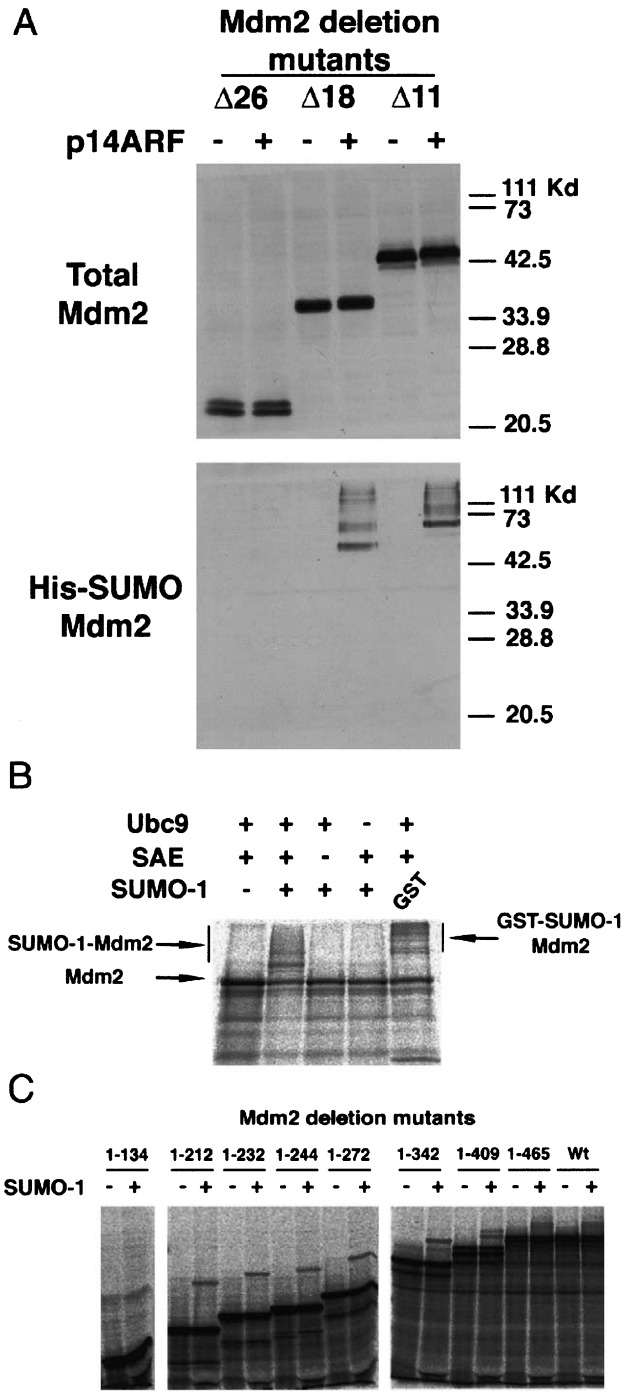

Fig. 3. A: The N-terminus of Mdm2 can be used for p14ARF-mediated SUMO-1 conjugation in vivo. Mdm2 deletion mutants were expressed in H1299 cells in the absence or presence of p14ARF and analysed as before. Δ26: 1–152, Δ18: 1–272, Δ11: 1–342. B: Mdm2 is SUMO-1 modified in vitro. Wild-type Mdm2 was expressed in vitro using wheat germ lysates and used in the in vitro SUMO-1 conjugation assay as described in Section 2. In one case GST-SUMO-1 was used in the in vitro reaction. C: The N-terminus of Mdm2 is SUMO-1 modified in vitro. Wild-type Mdm2 and Mdm2 deletion mutants were expressed in vitro and used in the in vitro SUMO-1 conjugation assay as above.

effect either on Hdm2 levels or the extent of SUMO-1 conjugation (Fig. 4). Although the Δ82–101 p14ARF could stabilise Hdm2, it was unable to induce SUMO-1 conjugation of Hdm2. To demonstrate that lack of SUMO-1 conjugation was

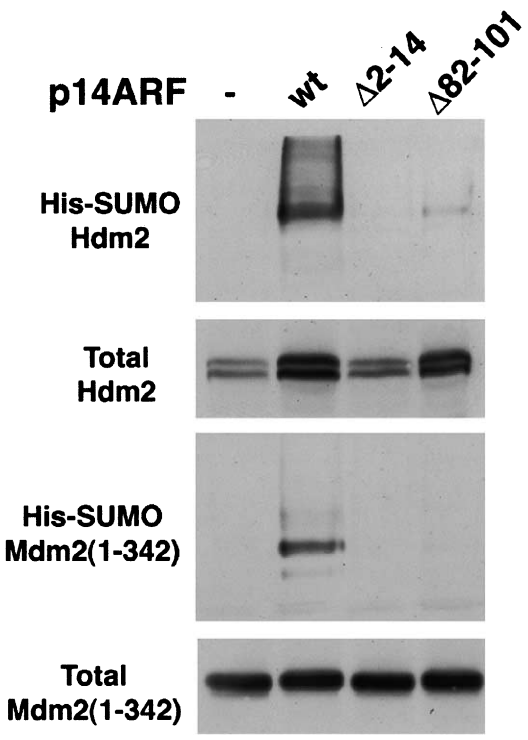

Fig. 4. Binding of p14ARF to (H)Mdm2 and sequences in exon 2 of p14ARF are required for p14ARF-mediated SUMO-1 conjugation of (H)Mdm2. Wild-type Hdm2 (upper two panels) or the Mdm2 deletion mutant Δ11 (amino acids 1–342, lower two panels) were expressed in H1299 cells in the absence or presence of wild-type p14ARF or deletion mutants Δ2–14 and Δ82–101. Cell extracts were analysed for total and SUMO-1 conjugated (H)Mdm2 as before.

not a consequence of Hdm2 stabilisation, Δ11 Mdm2 (residues 1–342), whose protein levels do not alter upon co-expression of p14ARF, was employed. Only co-expression of wild-type p14ARF could promote SUMO-1 conjugation of the Mdm2 mutant whereas neither the Δ2–14 nor the Δ82–101 p14ARF mutants had any effect on this modification (Fig. 4). These results demonstrate that both binding of p14ARF to H(M)dm2 and sequences in exon 2 of p14ARF are necessary for the ability of p14ARF to promote SUMO-1 conjugation of H(M)dm2.

4. Discussion

Here we demonstrate that p14ARF can promote accumulation of SUMO-1 conjugated (H)Mdm2. Three pieces of evidence support this statement: (1) While expression of p14ARF stabilised Hdm2 and led to the accumulation of SUMO-1-Hdm2, proteasome inhibitors failed to do so, despite the fact that Hdm2 was stabilised to very similar protein levels as with co-expression of p14ARF. (2) Co-expression of p14ARF with wild-type Mdm2 or Mdm2 deletion mutants lacking the C-terminus did not alter their protein levels but generated SUMO-1 conjugated species. (3) A p14ARF deletion mutant (Δ82–101) which could stabilise Hdm2, was deficient in generating SUMO-1-Hdm2 species. All these data strongly support an active role for p14ARF in accumulation of (H)Mdm2 conjugated to SUMO-1.

Previous studies reported that Hdm2 protein migrates as a 75 and 90 kDa species and that the 90 kDa species is Hdm2 modified with a single SUMO-1 moiety [19]. However, His_6-SUMO-1 conjugates purified through Ni^{2+} beads did not contain any 90 kDa Hdm2 species, despite the fact that this form of Hdm2 is constitutively expressed in mammalian cells. The major form of His_6-SUMO-1-Hdm2 purified with this approach migrated as a 120 kDa band suggesting the attachment of one or two SUMO-1 moieties on Hdm2. Furthermore, the K446R Hdm2 mutant, which was reported to be deficient for SUMO conjugation generated the same 75 and 90 kDa species, as wild-type Hdm2. More importantly, bacterially expressed Hdm2, which is not SUMO modified, generated the same 75 and 90 kDa species, observed in mammalian cells. These data demonstrate that the 90 kDa Hdm2 species is not a SUMO modified form and that the major SUMO-Hdm2 form migrates as a 120 kDa species.

Mutational analysis on p14ARF demonstrated that both binding to (H)Mdm2 and sequences in exon 2 of p14ARF are necessary for SUMO-1 conjugation of (H)Mdm2. Mutational analysis of Mdm2 did not provide evidence for the requirement of a single lysine residue in SUMO-1 conjugation although the N-terminus of Mdm2 can be used as a substrate for p14ARF-mediated SUMO-1 modification. Accumulation of SUMO-1 conjugated (H)Mdm2 mediated by p14ARF could result from either activation/recruitment of an E3-SUMO ligase or inhibition of a SUMO-specific protease.

The E3 ubiquitin ligase activity of (H)Mdm2 lies in the ring finger domain at the C-terminus of the protein [7–9]. The observation that p14ARF could promote SUMO conjugation of Mdm2 deletion mutants lacking the ring finger domain suggests that the E3 ubiquitin ligase activity of (H)Mdm2 is dispensable for p14ARF-mediated SUMO conjugation.

Overexpression of p14ARF changes the subcellular localisation of (H)Mdm2 by promoting its localisation to the nucleolus, which could be related with the ability of p14ARF to promote SUMO conjugation on (H)Mdm2 [28].

Expression of p14ARF protects p53 from (H)Mdm2-mediated ubiquitylation but has no effect on the auto-ubiquitylation activity of (H)Mdm2 [18]. As Δ82–101 p14ARF mutant could stabilise H(M)dm2 but was deficient in promoting SUMO-1 conjugation, this suggests that SUMO-1 modification of (H)Mdm2 does not interfere with the activity of (H)Mdm2 to regulate its own ubiquitylation and proteasomal degradation.

The correlation, between the differential role of p14ARF on the E3 ubiquitin ligase activity of (H)Mdm2 towards p53 and towards itself, and p14ARF-mediated SUMO conjugation of (H)Mdm2 could now be addressed along with the effect of this modification on the ability of (H)Mdm2 to down regulate the growth suppressing effects of p53.

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