

The phenotype of MDM2 auto-degradation after DNA damage is due to epitope masking by phosphorylation

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Key words: MDM2, auto-degradation, SMP14, 2A10, epitope masking, phosphorylation, DNA damage

It is widely accepted that DNA damage induces rapid degradation of MDM2 through phosphorylation, resulting in a transient reduction of MDM2 level. Elimination of MDM2 is a logical mechanism that stabilizes p53. This phenomenon has been reproduced by many independent studies and is frequently referenced. Here we present evidence that only phosphorylation-sensitive antibodies SMP14 and 2A10, but not other MDM2 antibodies, can detect robust downregulation of MDM2 after DNA damage. Therefore, we conclude that DNA damage does not accelerate MDM2 auto-degradation. SMP14 and 2A10 are frequently used to detect human and mouse MDM2, respectively. While it is not clear whether the discrepancy is entirely due to the use of these antibodies, our results suggest that epitope masking by phosphorylation should be an important consideration when interpreting results of MDM2 analysis by SMP14 and 2A10.

Background

The p53 pathway is critical for maintaining genomic stability and preventing development of cancer in higher organisms. A key feature of p53 is its stabilization and activation after exposure to a wide range of stress signals such as oncogene activation, abnormal ribosome biogenesis and DNA damage. These responses may be essential for its tumor suppressor function.¹ In normal cells, p53 is present at low levels due to degradation by the ubiquitin-dependent proteasome pathway. p53 turnover is regulated by MDM2, which binds p53 and functions as an E3 ligase to promote p53 ubiquitination.²⁻⁴ Additional ubiquitin E3 ligases such as Pirh2 and Cop1 have also been implicated as regulators of p53 turnover.^{5,6} However, current evidence suggests that MDM2 is a major and indispensable regulator of p53 level.^{7,8}

Elucidating how p53 is stabilized and activated after DNA damage is the subject of many studies. A number of mechanisms have been proposed. p53-MDM2 binding is essential for inhibition of p53 and is a point of regulation by DNA damage signaling. Several studies showed that DNA double strand breaks induce phosphorylation of p53 S15 by DNA-PK and ATM.⁹⁻¹² ATM also activates Chk2, which in turn phosphorylates p53 on S20 which is part of the MDM2 binding site.^{9,12-14} These findings suggest that p53 phosphorylation disrupts MDM2 binding and results in p53 stabilization. However, mouse model studies suggested that other mechanisms are needed to mediate robust p53 stabilization after DNA damage.¹⁵⁻¹⁷ Other studies have suggested that dephosphorylation of the MDM2 acidic domain,^{18,19} and phosphorylation of MDM2 C terminal region by c-Abl and

ATM regulate MDM2 E3 ligase activity during DNA damage response.²⁰⁻²³

Another potential mechanism of p53 stabilization is through elimination of MDM2. MDM2 undergoes self-ubiquitination and has a short half-life (< 30 min) in cultured cells. It has been reported that MDM2 undergoes accelerated degradation within 1–2 h after DNA damage by Neocarzinostatin (NCS), ultraviolet light and ionizing irradiation (IR),²⁴ suggesting that p53 stabilization is due to elimination of MDM2. Treatment with NCS reduces MDM2 half-life by seven-fold. DNA damage also causes a decrease in the steady state level of MDM2 at early time points. These effects are partially dependent on ATM and other PI-3 family kinases.²⁴ Subsequently, many studies from other groups also showed that DNA damage induces reduction in MDM2 level. Therefore, it is widely accepted that DNA damage promotes MDM2 degradation, which contributes to p53 stabilization. The mechanism of accelerated MDM2 degradation after DNA damage has been addressed in more recent studies.²⁵

However, MDM2 is a well-established transcriptional target for p53.^{26,27} Numerous studies reported induction of MDM2 level shortly (2–4 h) after DNA damage due to activation of p53. Other studies showed high levels of MDM2 and stabilized p53 coexisting in the cell after gamma irradiation (IR) and found no change in MDM2 half-life.²⁸ Although differences in experimental systems and the means of DNA damage induction may be responsible for the discrepancy, it is noteworthy that an earlier report showed that ATM-dependent phosphorylation of MDM2 causes a loss of reactivity to the antibody SMP14 due to epitope masking.²⁹ SMP14 is widely used for the detection of human

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Submitted: 02/01/11; Revised: 02/21/11; Accepted: 02/22/11
DOI: 10.4161/cc.10.7.15249

MDM2, therefore it may produce results that are interpreted as accelerated MDM2 degradation. Although it is difficult to determine whether all conclusions of MDM2 self-degradation were based on SMP14, it is apparent that most studies do not take into account its sensitivity to MDM2 phosphorylation. Therefore, widespread use of SMP14 can potentially lead to misinterpretation of certain experiments.

Results and Discussion

In an attempt to address the issue, we tested whether use of different MDM2 antibodies will produce the discrepancy on MDM2 downregulation after DNA damage. We tested several commercially available MDM2 monoclonal antibodies including SMP14,³⁰ 2A10, 2A9, 3G9, 4B2 and 4B11.³¹ Their epitope locations on MDM2 are summarized in **Figure 1A**. Human osteosarcoma cell line U2OS was treated with 10 Gy IR and analyzed over a wide range of time points for MDM2 level using the 3G9 antibody for western blot. The result showed that IR treatment induced a fraction of MDM2 to migrate faster on SDS-PAGE as previously observed (**Fig. 1B**), which was due to ATM-mediated phosphorylation.^{23,32} However, no significant decrease of MDM2 level was observed at any time point. MDM2 level increased significantly 4 h after IR with similar kinetics as p53 accumulation, as expected for a p53 target gene. The moderate decrease of MDM2 band intensity at the 0.3, 0.6 and 1.5 h time points were likely due to phosphorylation-induced spreading of the bands rather than a true decrease in total MDM2 level.

When SMP14 was used to detect MDM2, a significant reduction of MDM2 level was observed 0.5–1 h after IR as previously reported (**Fig. 1C**).²⁴ In contrast, antibodies 3G9 and 4B2 showed no significant change or only minor decrease using the same samples. These results suggest that the decrease in MDM2 level was a phenomenon specific to SMP14. To rule out a tumor cell-specific effect, we repeated the experiment using a primary human foreskin fibroblast cell line (HFF). SMP14 also showed a dramatic decrease in MDM2 level shortly after IR, whereas three different antibodies (3G9, 4B2 and 4B11) showed no significant decrease in MDM2 (**Fig. 1D**). Similar results were obtained using NCS to induce DNA damage instead of IR (**Fig. 1E**). Therefore, MDM2 downregulation by DNA damage was only observed using SMP14. We should note that some reduction of band intensity is visible in certain experiments using 4B2 (e.g., **Fig. 1B and D**, 4B2 parts). This may be partially due to phosphorylation-induced band diffusion and, in our experience, is not a robust and tractable phenotype.

Since the phosphorylation-sensitive MDM2 antibody 2A10 is frequently used for analyzing mouse MDM2,³² we also used 2A10 to detect MDM2 in MEFs after IR. The results showed a reduction of MDM2 in 1–2 h after IR using 2A10, whereas 4B2 showed no significant loss of MDM2 (**Fig. 2A**). The 2A10 antibody also detected a loss of human MDM2 in U2OS after IR (**Fig. 2B**). Therefore, the C terminal 2A10 epitope (a target of ATM mediated phosphorylation) may provide the majority of signal in 2A10 western blot. If SMP14 and 2A10 signal loss after DNA damage was due to degradation of MDM2, inhibition

of the proteasome should rescue MDM2 signal. However, we found that the proteasome inhibitor MG132 did not prevent the reduction of MDM2 signal by 2A10 or SMP14 after IR (**Fig. 2A and B**), suggesting that the signal loss was not due to degradation.

To test whether phosphorylation of MDM2 after DNA damage prevents detection by SMP14, the irradiated cell extract was treated with phosphatase (CIP). SMP14 signal was effectively restored by the phosphatase treatment (**Fig. 2C**). Reprobing of the same membrane using 4B2 clearly showed the inability of SMP14 to detect MDM2 in the irradiated sample prior to phosphatase treatment (**Fig. 2C**). Furthermore, inhibition of ATM kinase using a specific inhibitor KU55933 prevented the loss of SMP14 signal after IR, without changing MDM2 level as detected by 4B2. These results demonstrated that the reduction in SMP14 signal after DNA damage was caused by phosphorylation-dependent epitope masking.

Previous study showed that one of the two 2A10 epitopes on MDM2 (255-DSEDYLS-262 and 390-ESDDYSQ-396) was blocked by ATM phosphorylation of S395.^{20,32} The SMP14 epitope (154-SRPSTSSRRRAISE-167) contains multiple S and T residues, with S166 being a known AKT target site.^{33,34} Analysis of MDM2 using a commercial PS166 antibody showed no change in reactivity after IR (data not shown), ruling out S166 phosphorylation as the cause of SMP14 epitope masking. Our mass spectrometric analysis of MDM2 purified from irradiated cells was uninformative due to poor coverage of the SMP14 epitope. Therefore, it is unclear which residue within or near the SMP14 epitope is phosphorylated after DNA damage that interferes detection by SMP14. Although SMP14 was generated using a human MDM2 peptide,³⁰ it has been shown to coincidentally cross-recognize mouse MDM2 through a different epitope that includes T216. Interestingly, Cyclin A-CDK2 mediated phosphorylation of T216 also blocked mouse MDM2 detection by SMP14.³⁵ Therefore, SMP14 binding appears to be inherently sensitive to phosphorylation of its epitopes.

Together, our findings reinforce prior reports that MDM2 detection by SMP14 and 2A10 are blocked by DNA-damage induced epitope masking.^{29,36} Our results showed that DNA damage does not cause accelerated MDM2 degradation to a significant degree. Since there is no decrease in MDM2 level immediately after DNA damage, and there is robust induction of MDM2 at later time points that coexist with stabilized p53, other mechanisms such as regulation of MDM2 E3 ligase activity or p53 de-ubiquitination may be responsible for p53 stabilization.

How can we reconcile our finding with many reports that DNA damage induces MDM2 degradation or downregulation? We speculate that this discrepancy arises because SMP14 and 2A10 are widely used to detect human and mouse MDM2, often individually or in antibody cocktails. Unfortunately, commercial vendors do not provide adequate information on the phosphorylation-sensitive nature of these antibodies, and the original publications on these antibodies are dated and may be overlooked. As such, the results of MDM2 analysis using these antibodies are rarely discussed in terms of their sensitivity to epitope masking. Although neglecting this information is inconsequential in most

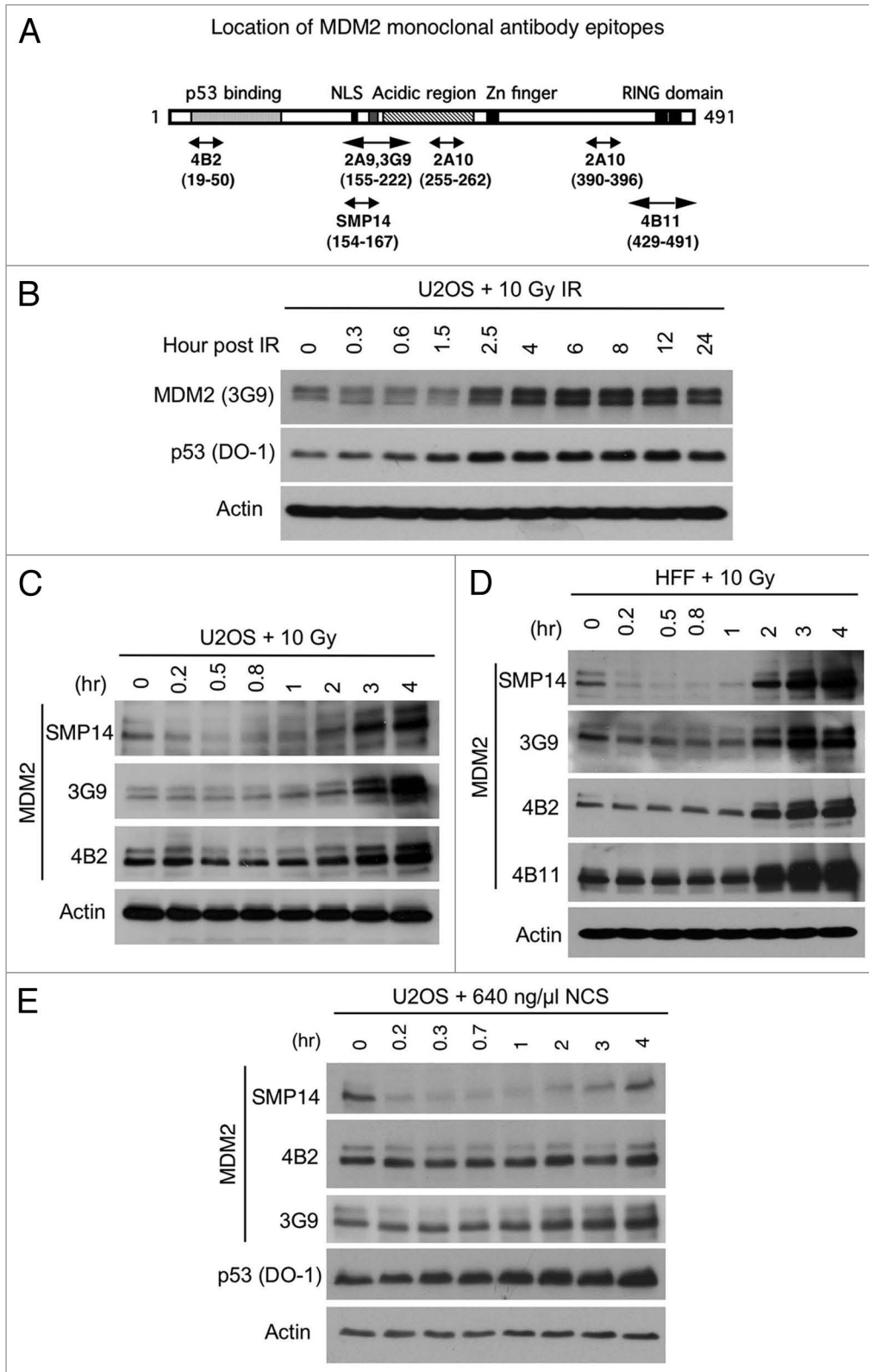


Figure 1. DNA damage reduces MDM2 reactivity to SMP14 antibody. (A) Diagram of MDM2 and epitope locations of the monoclonal antibodies used in this study. (B) U2OS osteosarcoma cells were treated with 10 Gy IR and analyzed for MDM2 level at indicated time points by western blot using 3G9. Identical amount of whole cell extract was used in each lane. (C and D) U2OS and normal human fibroblasts were treated with 10 Gy IR and analyzed for MDM2 level at indicated time points by western blot using different antibodies. (E) U2OS cells were incubated with Neocarzinostatin and analyzed for MDM2 level at indicated time points by western blot.

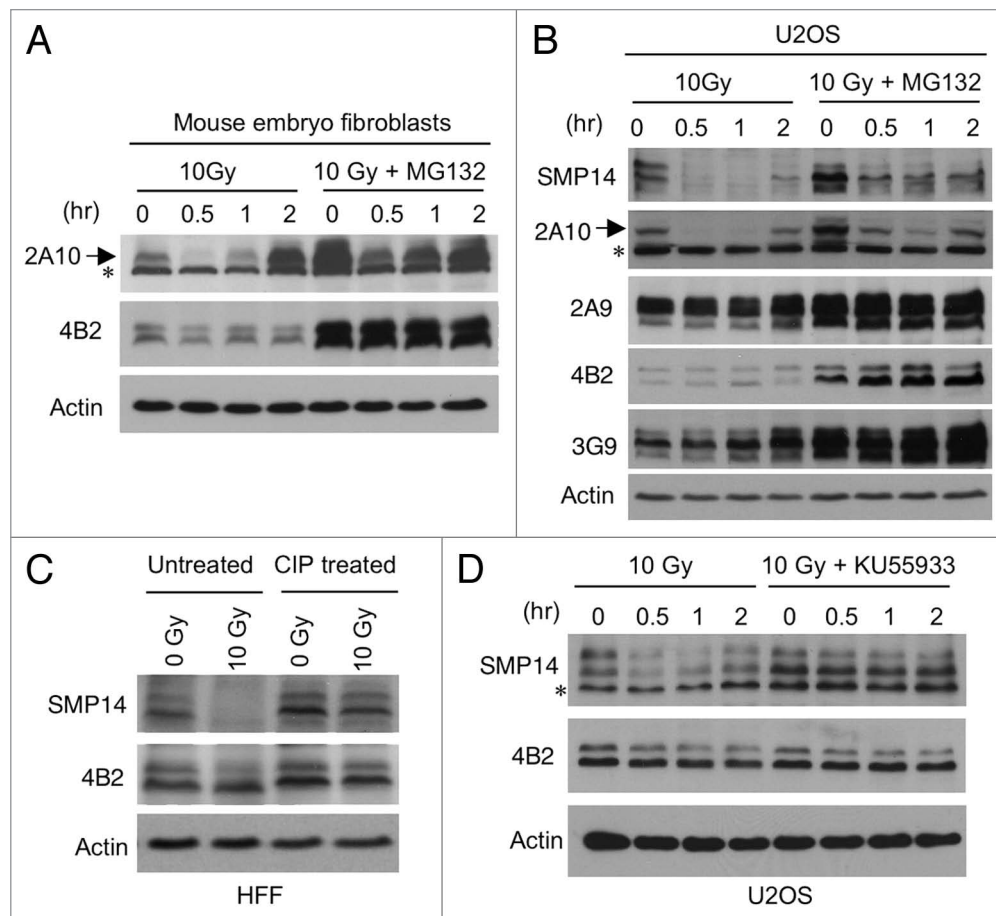


Figure 2. Loss of SMP14 signal after DNA damage due to epitope masking. (A and B) Mouse embryo fibroblasts and U2OS were treated with 10 Gy IR in the absence or presence of 30 μ M MG132. MDM2 level was analyzed by western blot at indicated time points using 2A10. Arrow indicates MDM2 band detected by 2A10. A background band is marked by *. (C) Normal human fibroblasts were irradiated with 10 Gy and after 1 h whole cell extract was treated with phosphatase (CIP) or incubated without CIP. Samples were analyzed by SMP14 western blot. The membrane was stripped and reprobed using 4B2. (D) U2OS cells were irradiated with 10 Gy in the absence or presence of 10 μ M ATM inhibitor KU55933. Samples were analyzed by SMP14 western blot. A background band is marked by *.

cases when analyzing MDM2 level at late time points, it may compromise the conclusion of certain experiments. Our results suggest that MDM2 detection using SMP14 and 2A10 should be interpreted with caution.

Materials and Methods

Cell lines and antibodies. Human foreskin fibroblast (HFF) was provided by Dr. Jack Pledger. Primary mouse embryo fibroblasts were produced in a previous study in reference 37. DNA damaging agent Neocarzinostatin was provided by the NCI. ATM inhibitor KU55933 was purchased from Calbiochem and used at 10 μ M. SMP14 was purchased from Santa Cruz Biotechnology. DO-1 was purchased from BD Bioscience. PS166 antibody was purchased from Cell Signaling. MDM2 antibodies 2A9, 2A10, 4B2, 4B11 and 3G9 were produced from hybridomas generated in a previous study in reference 31.

Protein analysis. To detect proteins by western blot, cells were lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, 150 mM NaCl, 0.5% NP40, 1 mM PMSF, 50 mM NaF] and centrifuged for 5 min at 10,000 g. Cell lysate (10–50 μ g protein) was fractionated by SDS-PAGE using gradient gel and transferred to Immobilon P filters (Millipore). The filter was blocked for 1 h with phosphate-buffered saline (PBS) containing 5% non-fat dry milk, 0.1% Tween-20. The filter was developed using ECL-plus reagent (Amersham). Dephosphorylation reaction was performed by incubating 30 μ g whole cell extract with 1 U calf intestinal phosphatase for 1 h at 37°C. Control was performed by incubation without phosphatase.

Acknowledgements

We would like to thank Dr. Wei Gu and Dr. Yanping Zhang for helpful discussions. This work was supported in part by grants from the National Institutes of Health to J.C. (CA141244).

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