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Identification and characterization of a novel *Mdm2* splice variant acutely induced by the chemotherapeutic agents adriamycin and actinomycin D

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Abstract

Mdm2, as the most important negative regulator of p53, plays an important homeostatic role in regulating cell division and the cellular response to DNA damage, oncogenic insult and other forms of cellular stress. We discovered that the DNA damaging agent adriamycin (doxorubicin) induces a novel aberrantly spliced *Mdm2* mRNA which incorporates 108 bp of intronic sequence not normally found in the *Mdm2* mature mRNA. Accordingly, we term this *Mdm2* splice variant *Mdm2*$^{+108}$. Importantly, this insertion introduces in-frame nonsense codons, thus encoding a profoundly truncated mdm2 protein lacking the C-terminal RING finger domain and the E3 ubiquitin ligase activity. A wide range of pharmacological testing revealed that *Mdm2*$^{+108}$ is induced, in mouse and rat cells, in specific response to Adriamycin and actinomycin D, but not other modes of DNA damage. Meanwhile, antibodies against the N-terminal region of mdm2 reveal a marked reduction in detectable mdm2 protein upon Adriamycin treatment, while p53 accumulates to strikingly high levels. We thus conclude that this alternative spicing of *Mdm2* may be an important mechanism to facilitate massive accumulation of p53 in response to genotoxic agents.

Keywords

mdm2; DNA damage; alternative mRNA splicing; adriamycin

Introduction

Mdm2 (called *HDM2* in humans) is among the most clinically relevant proto-oncogenes in the human genome and is found to be amplified in nearly 8% of human alignancies of a wide variety of tumor types, most often sarcomas.[1-4] Furthermore, overexpression without gene amplification is observed in >25% of human cancers and strongly correlates with poor prognosis.[4-6] Although the *HDM2* gene product has been postulated to function in tumorigenesis via several distinct mechanisms, the primary pathway for mdm2-induced cell

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transformation is through the negative regulation of the p53 tumor suppressor.[1,7-11] Given its critical function in responding to oncogenic insults, DNA damage and other forms of cellular stress, p53 has been called the “guardian of the human genome” and is directly mutated or deleted in over one-half of all human malignancies.[12-14]

In an unstressed cell, the primary mechanism of action of mdm2 as the negative regulator for p53 is through its E3 ubiquitin ligase activity, which targets p53 for proteolytic destruction by the 26S proteasome.[15-18] In turn, p53 transactivates both itself (*Tp53*) and the *Mdm2* gene.[19-24] This creates a negative feedback loop in which levels of p53 protein are kept very low despite relatively high levels of gene transcription.[8,9,16,25,26] In response to a wide variety of cellular stresses—including oxidative damage, nucleotide depletion, ionizing radiation, DNA damage and oncogene activation—the negative feedback loop is disrupted, usually by post-translational modification of p53, mdm2 or both.[2,8,16,27-29] Thus, the p53 protein is stabilized, rapidly accumulates, and initiates a coordinated cellular response to the stress conditions, leading to cell cycle arrest or apoptosis.[12,28]

As evidenced by the rarity of *HDM2* amplification co-existent with p53 inactivating mutations in cancer, the negative regulation of p53 is thought to be the principle oncogenic activity of mdm2.[4,30] However, many additional p53-indpendent roles of mdm2 in cell cycle progression, apoptosis and tumorigenesis have been described, and mutants of mdm2 unable to bind p53 retain the ability to transform cells.[31] Further, mdm2 has been shown to transform p53-null cells and accelerate tumorigenesis in $p53^{-/-}$ mice, and tumors with both p53 mutation and *HDM2* amplification, while rare, have been reported.[4,32,33] In summary, mdm2 is a potent oncoprotein in a variety of cellular contexts with multiple mechanisms of promoting oncogenic transformation.

Over 40 alternative or aberrant splice forms of mdm2 have been reported in both mice and humans.[34] Interestingly, nearly all of these alternate forms are observed exclusively in malignancies or transformed cells and the molecular events leading to the appearance of these alternative forms are entirely unknown.[34-38] It is also unknown how many of these alternative mRNAs result in stable mdm2 protein. However, the majority of these splice variants lack the p53 binding site, suggesting that uncoupling of the p53-mdm2 negative feedback loop is an important consequence of aberrant splicing.[34] In fact, transgenic mouse models have been developed which express tumor-derived alternative splice forms of mdm2.[39] Intriguingly, three of the four tested splice variant alleles promoted lymphoma development to the same degree as full-length mdm2.[39] The vast majority of observed mdm2 splice variants await detailed functional study.

Results

Discovery of an alternative splice form of *Mdm2*

In studies examining the effects of the DNA damage-inducing chemotherapeutic agent Adriamycin (Adr, also called Doxorubicin) on the expression of cell cycle genes, we observed an unexpected and dramatic alteration of the mdm2 mRNA transcript following Adr treatment. Specifically, one region of the *mdm2* transcript exhibited differences in electrophoretic mobility in Adr treated cells as compared to untreated controls (Fig. 1A). Importantly, analogous alterations were not found in any of the >20 other cell cycle-related genes examined in that study (data not shown). Since *Mdm2* is a known p53 target gene induced in response to DNA damage, we asked whether the induction of this transcript required functional p53. Our results show that this unexpected *Mdm2* splicing is observed in NIH3T3 cells that harbor a functional p53 protein as well as 3T3 cells derived from $p53^{-/-}$ and $p21^{-/-}$ mouse embryo fibroblasts (MEFs) (Fig. 1A). Intriguingly, we also observed the expected induction of *Mdm2* expression in NIH3T3 cells, but not $p53^{-/-}$ cells, presumably

through the stabilization and activation of p53 via the cellular response to DNA damage (compare post-Adr levels of mdm2 in NIH3T3 vs. $p53^{-/-}$ cells). Thus, this alteration of the *Mdm2* mRNA occurs irrespective of the DNA damage-induced, p53-mediated transactivation of *Mdm2*, and indeed, its appearance is p53-independent altogether.

Before proceeding to discover the nature of this aberrant *Mdm2* mRNA, we conducted a dose-response experiment in order to determine the optimum dose of Adriamycin for inducing the alternative splicing. We found that doses as little as 0.2 μg/mL produce detectable amounts of the alternative *Mdm2* RT-PCR amplicon, and we selected 0.75 μg/mL–1.0 μg/mL as the minimum optimal dose range for robust induction of the novel splicing phenomenon (Fig. 1B). Importantly, these doses are within the physiological range of Adriamycin experienced during cancer chemotherapy and are the doses commonly employed in DNA damage studies with this drug.

The mouse *Mdm2* gene has twelve exons, with ~40% of the protein encoded by exon 12 (Fig. 1C). There are two promoters within the *Mdm2* gene: the constitutive P1 promoter, responsible for steady-state expression of *Mdm2*; and the downstream p53-responsive P2 promoter, located within exon 2 of the full P1 transcript.[20,23] Because the start codon is located in exon 3, mdm2 protein translated from mRNAs generated from either of these two promoters is indistinguishable. Because the PCR primers that revealed the alteration in the *Mdm2* mRNA generate an RT-PCR amplicon that spans exons 9, 10, 11 and 12 of the mature *Mdm2* mRNA (Fig. 1B), we will focus our attention on this region of the *Mdm2* mRNA.

Following Adr treatment, the dominant *Mdm2* transcript contains additional sequence betweens exons 10 and 11

In order to discover why the *Mdm2* RT-PCR amplicon comprising exons 9–12 (x9-12) appears larger following DNA damage, we employed a panel of primer pairs corresponding to smaller segments within the x9-12 region of the *Mdm2* transcript. Using this approach, we observed that, in both NIH3T3 and $p53^{-/-}$ 3T3 cells, the *Mdm2* mRNA displays no alteration in size between exons 9 and 10 or between exons 11 and 12 upon treatment with Adr, although, as expected, there is a dramatic induction of *Mdm2* in NIH3T3s but not $p53^{-/-}$ 3T3s (Fig. 2A). However, amplicons spanning exons 9 through 12 and exons 10 through 12 reveal roughly the same reduced mobility following DNA damage by Adr (Fig. 2A). Thus, the region of the *Mdm2* transcript containing the additional sequence following Adr treatment resides within the region encompassed by the previously determined exon 10–exon 11. After a higher resolution analysis, we estimate that roughly 100 bp of additional mRNA sequence appears in the mature *Mdm2* transcript following treatment with Adriamycin (Fig. 2B).

Data in Figures 1A and 2A clearly demonstrate that the DNA damage-induced shift in amplicon size for *Mdm2* mRNA is independent of p53. However, the presence of intact p53 clearly leads to an induction of Mdm2, indicating that transcription through the *Mdm2* gene is predominantly via the p53-responsive P2 promoter located within exon 2. To confirm this, we performed RT-PCR reactions using a 5′ primer specific for intron 1, which would not be found in a P2-derived, p53-induced *Mdm2* transcript. As expected, following DNA damage by Adr in NIH3T3 cells, RT-PCR amplicons derived from exon 1 sequences are dramatically reduced (Fig. 2C), despite a strong overall induction of *Mdm2* (Figs. 1A, 2A and B). Thus, transcription of the *Mdm2* gene switches from the constitutive P1 promoter to the p53-responsive P2 promoter located within exon 2 following DNA damage by Adriamycin.

Adriamycin induces alternative splicing of *Mdm2* to include 108 bp of sequence from within intron 10

In order to discover the origin of the additional mRNA sequence contained in the *Mdm2* transcript following Adr treatment, we excised the gel bands shown in Figure 2B and purified the resulting DNA. The DNA sequence of the cloned *Mdm2* RT-PCR amplicon derived from untreated NIH3T3 cells corresponds perfectly to the expected cDNA sequence of *Mdm2* with exons 10 and 11 spliced normally (not shown). However, sequencing revealed that the *Mdm2* amplicon derived from Adr-treated cells contains 108 bp of cDNA sequence nestled between otherwise complete and unaltered exons 10 and 11 (Fig. 3A). Intriguingly, this 108 bp perfectly matches a sequence of *Mdm2* genomic DNA near the middle of intron 10 and is flanked on both sides by GG, a potential mRNA splicing signal (Fig. 3B). This indicates that splicing at the 3′ end of exon 10 (5′ end of intron 10) and the 5′ end of exon 11 (3′ end of intron 10) occurs normally but that additional splice events *within* intron 10 occur in response to the DNA damage agent Adriamycin. We have termed this alternatively spliced *Mdm2* mRNA $Mdm2^{+108}$.

Because 108 is a multiple of three, we initially raised the exciting possibility that this added sequence is actually a novel alternative exon for *Mdm2*, possibly conveying novel DNA damage-specific functions to the $Mdm2^{+108}$-encoded mdm2 protein. However, examination of the intronic sequence immediately reveals nonsense codons in all frames, including the presumed open reading frame (Fig. 3C). Thus, translation of $Mdm2^{+108}$ would generate an mdm2 protein that lacks exons 11 and 12, representing >40% of the protein, including the Zinc finger and RING finger domains, and the E3 ubiquitin ligase activity.

To directly confirm the presence of this intronic sequence in $Mdm2^{+108}$, and thus eliminate the possibility of a sequencing anomaly, we performed intron-specific PCR by designing RT-PCR primer pairs in which one member of the pair was complementary to a stretch of sequence within this 108 bp region of intron 10. As expected, we were not able to detect significant *Mdm2*signal by intron-specific PCR in untreated NIH3T3 cells (Fig. 3D). However, *Mdm2* intron 10-specific PCR reveals a very strong signal following Adr treatment (Fig. 3D). Much weaker intron-specific signals were detected by additional DNA damaging agents, including ionizing γ-radiation (IR), ultraviolet light (UV) and H_2O_2 (Fig. 3D), which will be discussed below.

$Mdm2^{+108}$ is induced in response to specific pharmacological agents

In an effort to elucidate the mechanism of $Mdm2^{+108}$ induction, we employed a large panel of pharmacological agents and treatments known to cause acute DNA damage. First, we examined the kinetics of $Mdm2^{+108}$ induction during a time-course of Adr treatment (Fig. 4A, lanes 1–7). We observed that maximal induction occurs within three to six hours, and levels remain steady through 24 hours, by which time the bulk of the population appears to undergo DNA damage-induced apoptosis (data not shown).

Further, we found that both the protein synthesis inhibitor cycloheximide and the transcription inhibitor actinomycin D reduced, but did not eliminate, the induction of $Mdm2^{+108}$ (Fig. 4A, compare lanes 6, 9, 10). Interestingly, 450 nM actinomycin alone also induces $Mdm2^{+108}$, despite an overall reduction in steady-state levels of *Mdm2*, presumably due to global inhibition of de novo RNA transcription (Fig. 4A, lane 11). Therefore, we conclude that, although the overall induction of *Mdm2* levels due to transactivation of the p53-responsive P2 promoter is sensitive to inhibitors of transcription and translation, the splicing process that gives rise to $Mdm2^{+108}$ does not require de novo RNA or protein synthesis.

To further characterize the appearance of *Mdm2*$^{+108}$, we exposed cells to diverse DNA damaging agents. First, at the doses tested, although etoposide, ionizing γ-radiation (IR), ultraviolet radiation (UV), hydrogen peroxide (H_2O_2), hydroxyurea (HU), aphidicolin and cisplatin all strongly induce *Mdm2* mRNA expression, none promotes alternative splicing to the *Mdm2*$^{+108}$ form (Fig. 4). Similar results were observed with camptothecin and methyl methanesulfonate (data not shown). In addition, while adriamycin effectively induces *Mdm2*$^{+108}$ at doses of 200 ng/mL and above (Fig. 1 and other data not shown), actinomycin D does so at 450 nM, but not 45 nM (Fig. 4A, D and E. Therefore, the induction of *Mdm2*$^{+108}$ is surprisingly specific and restricted to a narrow range of DNA damaging agents including Adriamycin (doxorubicin) and high doses of actinomycin D. Furthermore, we noticed that pre-treatment with caffeine, an inhibitor of multiple kinases in the DNA damage response pathway, almost completely blocks the induction of *Mdm2*$^{+108}$ by Adriamycin (Fig. 4B).

Induction of *Mdm2*$^{+108}$ correlates with enhanced accumulation of p53

If translated, *Mdm2*$^{+108}$ encodes a profoundly truncated mdm2 protein lacking the critical domains for the negative regulation of p53. Thus, we hypothesize that alternative splicing of *Mdm2* to generate *Mdm2*$^{+108}$ would result in a loss of mdm2 function. p53 is continually translated at a reasonably high rate but is markedly unstable, having a half-life of roughly 20 minutes in an unstressed cell, largely due to mdm2-directed, ubiquitin-mediated proteasomal degradation.[16-18,25,26,40-43] In the event of acute loss of the mdm2 E3 ubiquitin ligase function, p53 would be liberated from this negative restraint and we thus predict a rapid and robust accumulation of p53 protein upon induction of *Mdm2*$^{+108}$.

To test this hypothesis, we assessed the levels of detectable mdm2 and p53 protein following treatment of cells with DNA damaging agents that can or cannot induce *Mdm2*$^{+108}$. Because the C-terminus of mdm2 would be deleted when cells switch to the *Mdm2*$^{+108}$ transcript, we used antibodies directed against amino acids 154–167 of mdm2 (sc-965) and found that, surprisingly, mdm2 levels significantly drop in cells treated with Adr (Fig. 5). This is in sharp contrast to the other DNA damaging agents that do not stimulate *Mdm2*$^{+108}$, all of which cause a consistent *induction* of mdm2 protein levels, presumably due to increased transactivation of the P2 promoter by p53, which is stabilized by the DNA damage response (Fig. 5). This result is particularly intriguing in light of our observations regarding p53 protein levels. Briefly, although etoposide, IR, UV and actinomycin D all induce the stabilization and accumulation of p53 to varying degrees, Adr treatment induces an accumulation of p53 that far exceeds that for any other treatment tested (Fig. 5). [That the same massive accumulation of p53 is not seen in actinomycin D-treated cells stems from the fact that global de novo transcription is inhibited in these cells.] Thus, it appears that the end result of *Mdm2*$^{+108}$ induction is the loss of mdm2 function and a rapid and massive accumulation of p53 (see Discussion).

Induction of *Mdm2*$^{+108}$ occurs in multiple cell types

We have observed the induction of *Mdm2*$^{+108}$ by Adr in cells derived from MEFs of three different genetic backgrounds (Fig. 1A). However, we sought to further generalize this observation by testing a cell line from a different lineage: C2C12 cells, a mouse cell line derived from skeletal muscle satellite cells from young mice.[44] Here, we examined the induction of *Mdm2*$^{+108}$ in C2C12 mouse myoblasts and observed the same pattern of *Mdm2*$^{+108}$ induction seen in MEFs (Fig. 6). Thus, we have shown that this phenomenon is not restricted to the fibroblast lineage.

Because the induction of *Mdm2*$^{+108}$ displays some sensitivity to caffeine pre-treatment, which inhibits multiple kinases in the DNA damage pathway, we assessed whether

knockdown of ATR would abrogate the induction of *Mdm2*$^{+108}$. For this experiment, we employed shRNA-mediated knockdown of ATR in rat Rin1 cells, as this is a highly efficient and selective way to reduce ATR levels. ATR is a serine-threonine kinase with a crucial function in the cellular response to DNA damage and is potently inhibited by caffeine.[45] In this experiment, it is also made clear that induction of *Mdm2*$^{+108}$ is not limited to mouse cells, suggesting that this particular cellular response to DNA damage has been conserved through evolution. Despite observing greater than 95% reduction of ATR protein (assessed by western blotting, data not shown), no observable reduction in *Mdm2*$^{+108}$ expression was observed (Fig. 7). These results suggest that ATR is not involved in the induction of *Mdm2*$^{+108}$. Alternatively, there may be additional kinases that function redundantly with ATR to induce expression of this transcript.

Discussion

The discovery of the alternative *Mdm2* splice variant *Mdm2*$^{+108}$ was unexpected and has not been previously reported. Although as many as 40 different splice variants of *Mdm2* have been previously observed, it is important to point out that none harbors the intronic sequence found in *Mdm2*$^{+108}$ and none has been shown to be induced by DNA damage or any other acute treatment. In fact, most alternative splice forms of *Mdm2* have only been observed in malignant tissues and likely arise through sporadic mutation. Further, the function of the majority of these splice variants has yet to be explored in detail.

We report the induction of *Mdm2*$^{+108}$ as a rapid and specific response to adriamycin and actinomycin D leading to a general loss of mdm2 function. This loss of mdm2 breaks the mdm2-p53 negative feedback loop resulting in a massive accumulation of p53 and, presumably, irreversible entry into the apoptotic process. The benefit of such a response is clear: adriamycin, at the doses employed in this study (in the same range experienced during cancer chemotherapy), has a highly toxic effect on mitotic cells. Cells treated with this amount of adriamycin will experience extensive DNA damage. Thus, the specific induction of *Mdm2*$^{+108}$ could be a mechanism by which cells ensure immediate accumulation of p53, so that the damaged cell cannot proliferate further. As such, *Mdm2*$^{+108}$ could be an additional component of the DNA damage response, selective for certain types of damage, that functions even when other components of the DNA damage pathway, such as ATR, have been compromised.

Although we can reasonably hypothesize that the function of this alternative splicing event is to immediately abolish mdm2 function, the mechanism of *Mdm2*$^{+108}$ induction remains a mystery. Both adriamycin and higher doses of actinomycin D are capable of inducing *Mdm2*$^{+108}$, but many other DNA damaging agents are not, suggesting that the induction of *Mdm2*$^{+108}$ occurs through a mechanism of action that is common to these two agents but not shared by the others. Although the precise mechanism of adriamycin-mediated DNA damage is unclear, it has been shown to intercalate into DNA and halt topoisomerase I function after it has broken one strand of DNA.[46-48] This exact mechanism of DNA damage is not shared by any of the other agents tested in this study, including actinomycin D. However, intriguingly, actinomycin D has been shown to interfere with DNA strand transfer during reverse transcription by HIV reverse transcriptase.[49,50] This function of actinomycin D is thought to occur through its known intercalation of double stranded DNA and DNA:RNA hybrids.[51-53]

This raises the possibility that the common cellular response to adriamycin and actinomycin D in inducing *Mdm2*$^{+108}$ is not due to DNA damage per se but to the interaction of these agents with DNA itself. However, ethidium bromide, another molecule long known to intercalate DNA, does not induce *Mdm2*$^{+108}$ despite causing an overall induction of *Mdm2*

mRNA (data not shown). Nevertheless, it is worth noting that adriamycin and actinomycin D both bind efficiently to single stranded DNA, while ethidium bromide does not.[54,55] Short regions of single stranded DNA are routinely experienced as "bubbles" during transcription and DNA replication and it is conceivable that adriamycin and actinomycin D bind to these regions. Thus, *Mdm2*$^{+108}$ could be a cellular response to perturbations in transcription and/or DNA replication caused by these agents, and potentially, retroviral reverse transcription. Thus, the induction of *Mdm2*$^{+108}$ by alternative splicing of *Mdm2* could represent a novel pro-apoptotic response common to both certain genomic threats and retroviral infection.

Materials and Methods

Cell culture and drug treatment

NIH3T3 cells were obtained from ATCC and cultured exactly as recommended. Prior to all experiments, cells were split from a ~70% confluent dish at a density of 1:2 and allowed to grow for 12–18 hours prior to drug treatment.

RT-PCR analysis

RT-PCR analysis was performed precisely as previously described.[56] Briefly, cells (100 mm dish) were harvested in 1mL TRIzol™ reagent (Invitrogen) and total RNA was isolated according to manufacturer's protocol. 250 ng total RNA was used for first-strand cDNA synthesis, following manufacturer's protocol (Invitrogen). Serial dilutions of concentrated cDNA stocks were analyzed for normalization for actin RT-PCR signal before serving as a template for RT-PCR analysis. Normalized cDNA samples were then analyzed with primers indicated in figure legends. All primer sequences available on request.

Western blot analysis

Western Blot analysis was performed by harvesting in cells in SDS-PAGE loading buffer: 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.002% bromophenol blue, 0.1 M Tris-HCL (pH 6.8). Lysates were then resolved by SDS-PAGE (10% gel) and transferred to PVDF membrane by semi-dry transfer following the manufacturer protocol (Bio-Rad). Membranes were blocked with 10% milk overnight and probed with the following antibodies in 2% milk for one hour: α-mdm2 (SMP14 (sc-965), 1:000), α-p53 (DO-1 (sc-126), 1:1000). ECL Visualization and 2° antibodies were exactly as previously described.[57]

shRNA-mediated ablation of ATR

shRNA-mediated ablation of ATR was performed precisely as previously reported.[58] Briefly, rat Rin1 cells were infected with retroviruses expressing pSuper-ATR-shRNA or control retroviruses for 24 hours followed by 24 hour recovery. (Parallel infections were performed for confirmation of ATR knockdown by western blotting.) Then cells were incubated in the presence or absence of Adriamycin for six hours followed by RT-PCR analysis as normal.

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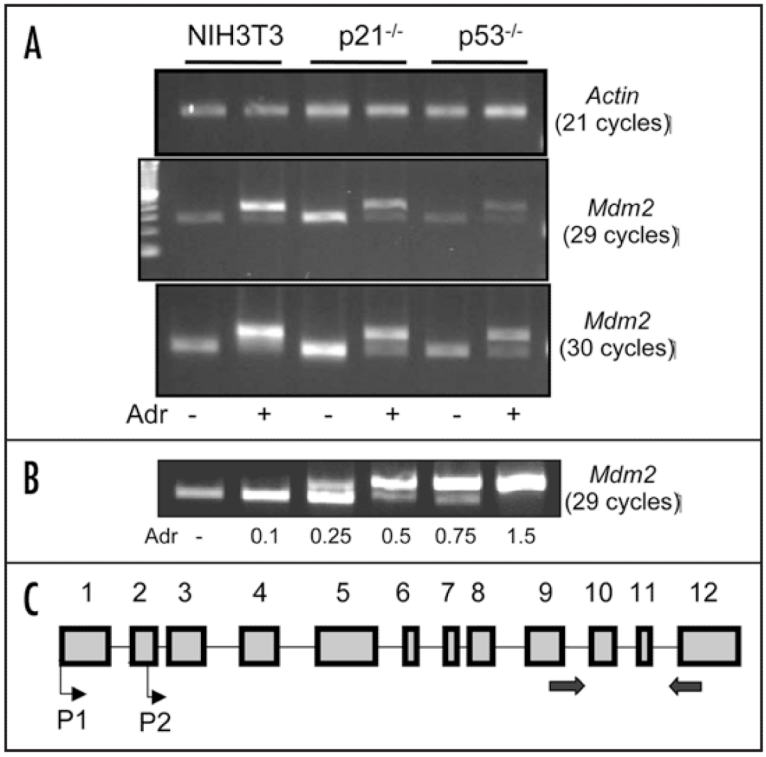

Figure 1.

Discovery of a DNA damage-induced alternative mRNA form of *Mdm2*. (A) Asynchronous NIH3T3, $p21^{-/-}$ 3T3 and $p53^{-/-}$ 3T3 cells were treated for eight hours with 1 μg/mL Adriamycin, followed by RT-PCR analysis (as described in materials and methods) with primers for *Actin* and *Mdm2* at indicated cycle numbers. (B) Asynchronous NIH3T3 cells were treated for eight hours with the indicated doses of Adriamycin (μg/mL) followed by RT-PCR analysis with primers for *Mdm2*. (C) Schematic of the *M. musculus Mdm2* gene indicating primers used for the *Mdm2* RT-PCR analysis in (A and B).

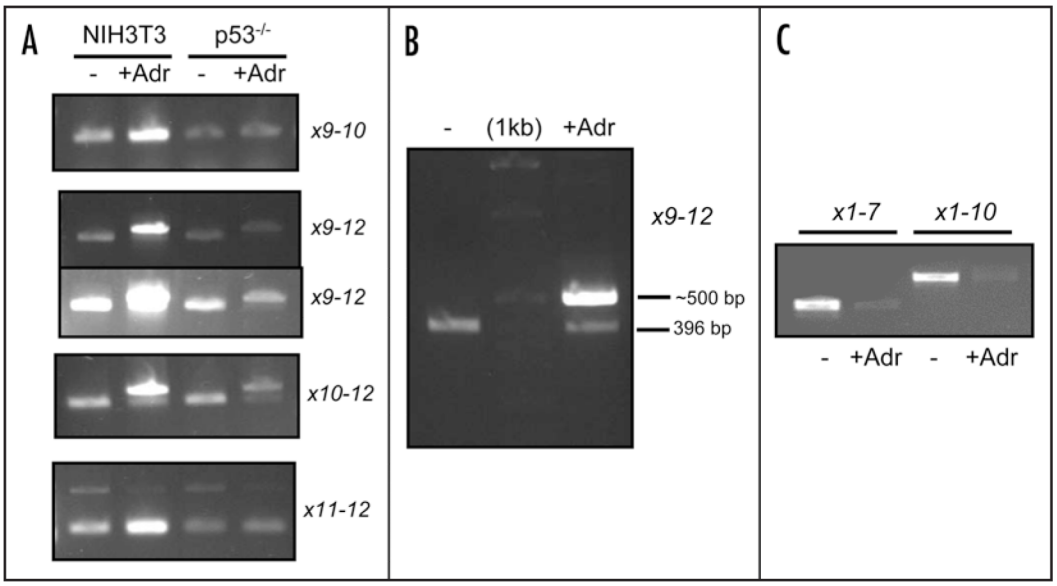

Figure 2.

~100 bp of additional sequence appears between exon 10 and exon 11 in *Mdm2* mRNA following Adr treament. (A–C) Asynchronous NIH3T3 (or $p53^{-/-}$ 3T3 cells in (A) only) were treated for eight hours with 1 μg/mL Adriamycin, followed by RT-PCR analysis with primers corresponding to various regions within the *Mdm2* mRNA. (x = exon; Two different exposures are shown for *x9-12*). (B) Higher-resolution picture. Labels indicate band sizes for the 1 kb DNA ladder.

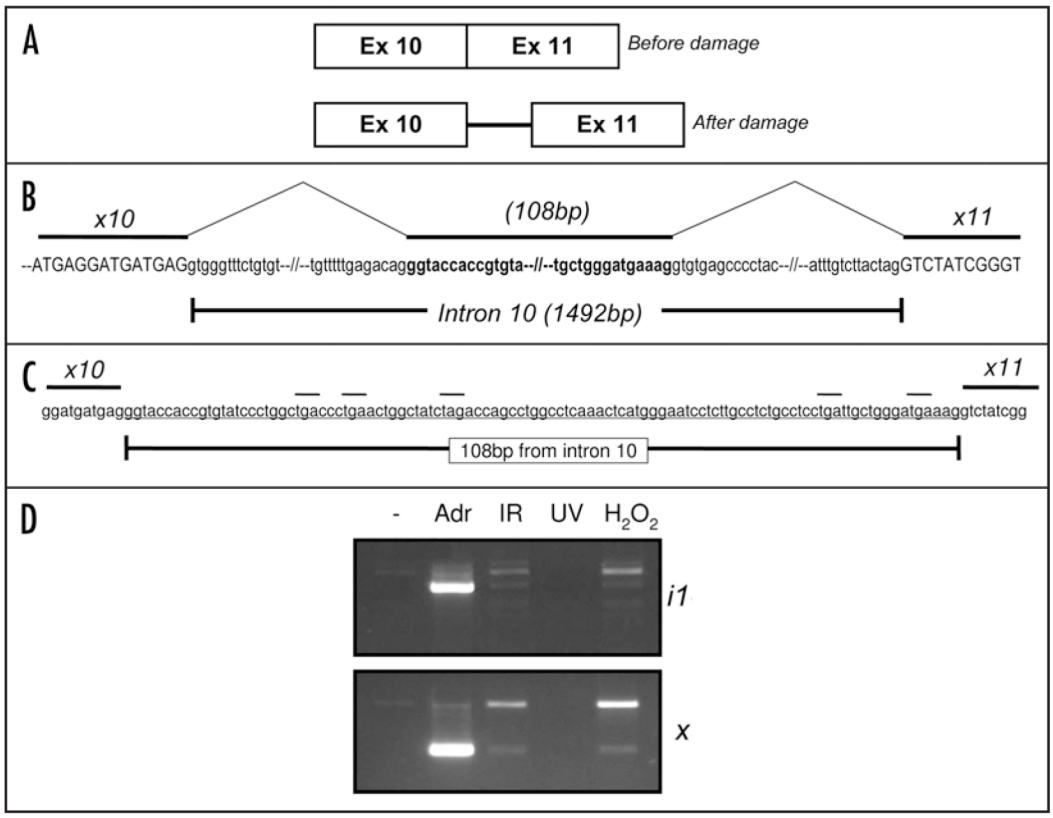

Figure 3.

Sequence of the intron 10-derived 108 bp insert in *Mdm2* mRNA following Adr treatment. (A) Diagram of the exon 10–exon 11 region of the *mdm2* mRNA before and after Adr treatment. (B) Partial genomic DNA sequence of the *mdm2* gene: intron 10 (lower-case letters); exons 10 and 11 (capital letters); the region of intron 10 found in mature *Mdm2* mRNA following Adr treatment (bold lower-case). Bars above the sequence indicate the apparent mRNA splicing events. (C) Complete sequence of the additional 108 bp found in *mdm2* cDNA following Adr treatment. Bars above indicate putative nonsense codons. D) NIH3T3 cells were treated exactly as in Figure 4C, followed by *Mdm2* RT-PCR using a 5′ primer (upper) or 3′ primer (lower) corresponding to sequences located within intron 10.

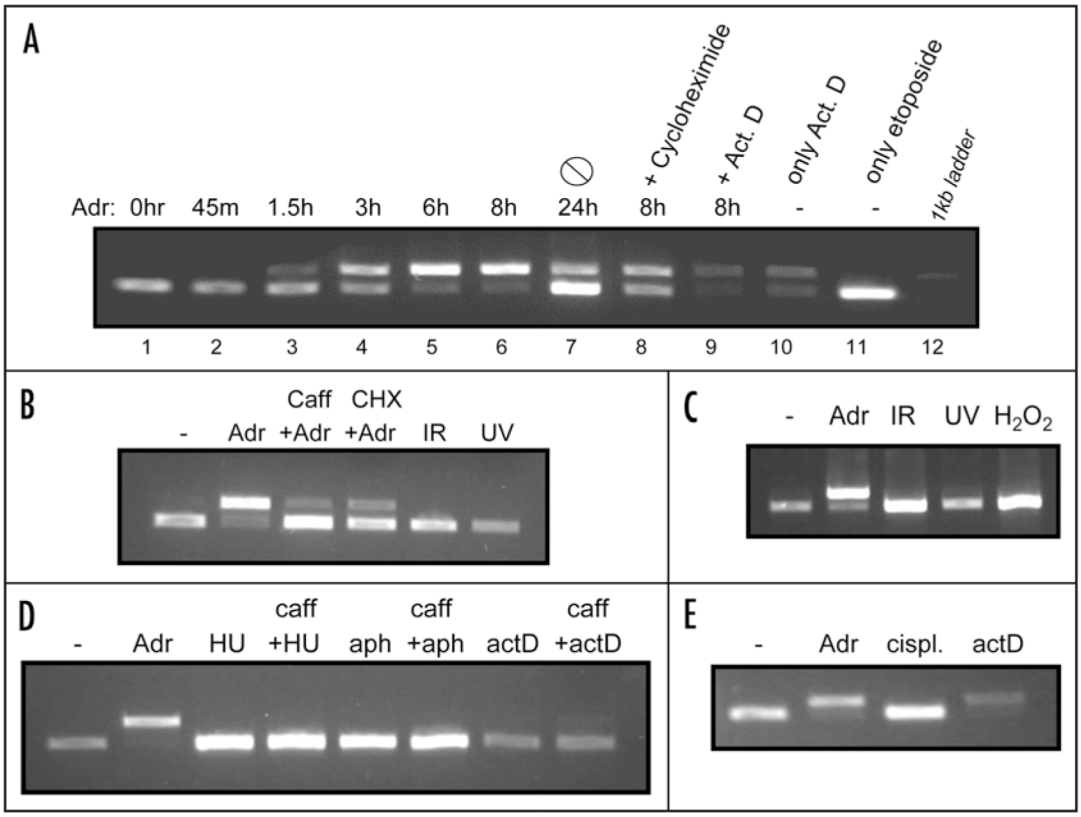

Figure 4.

Pharmacological characterization of *Mdm2*$^{+108}$. Following the indicated treatment of NIH3T3 cells, *mdm2* (x9-12) was analyzed by RT-PCR as previously. (A) Treatment with 1 μg/mL Adriamycin was for indicated lengths of time. [" ⊘" indicates that all cells appeared dead or dying at time of harvest.] Pre-treatment, where indicated, was for 30 minutes with 25 μg/mL Cycloheximide, or 500 nM Actinomycin D. Actinmycin D alone was 450 nM for 8 hours; etoposide alone was 25 μM for 8 hours. (B–E) All drug treatments were for six hours. (B) Adr, 750 ng/mL, alone or with 30 minute pre-treatment with 4 mM Caffeine or 100 μM Cycloheximide; ionizing radiation (IR), 10 Gy (6 h recovery); UV, 220 nm, 100 J/m^2 (6 h recovery). (C) Adr, 750 ng/mL; IR, 25 Gy (6 h recovery); UV, 220 nm, 250 J/m^2 (6 h recovery), H_2O_2, 1 mM. (D) Adr, 750 ng/mL; HU, 2 mM; aphidicolin, 5 μg/mL; actinomycin D, 45 nM; Caffeine pre-treatment, where indicated was 4 mM for 30 minutes. (E) Adr, 750 ng/mL; cisplatin, 20 μM; actinomycin D, 450 nM.

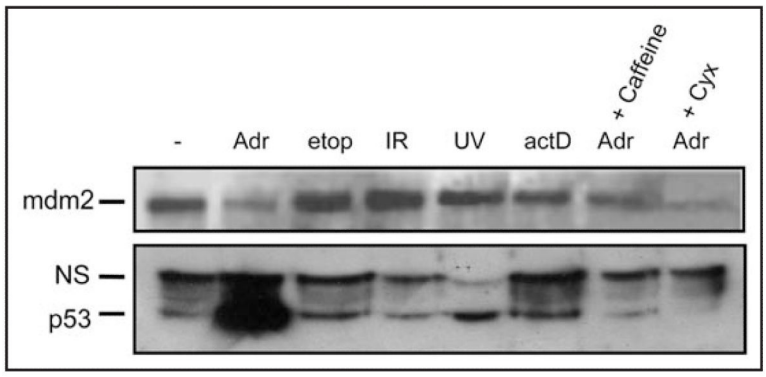

Figure 5.

Induction of *Mdm2*$^{+108}$ corresponds with ablation of mdm2 protein and accumulation of p53. NIH3T3 cells were treated for six hours with Adr (750 ng/mL), etoposide (25 μM), IR (10 Gy), UV (220 nM, 100 J/m^2), actinomycin D (450 nM), with or without 30 minute pre-treatment with Caffeine (4 mM) or Cycloximide (100 μM). Cells were then lysed and probed for antibodies to mdm2 or p53 by western blotting. NS = nonspecific band.

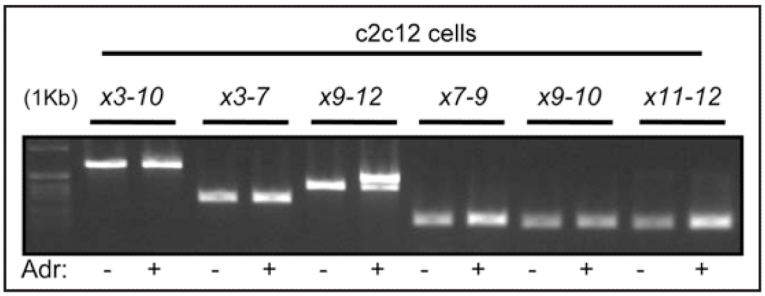

Figure 6.

Mdm2^{+108} is induced by Adr treatment in C2C12 myoblasts. Asynchronous C2C12 mouse myoblasts were treated with Adr for six hours prior to RT-PCR with a panel of primers to amplify the indicated regions of the *Mdm2* transcript.

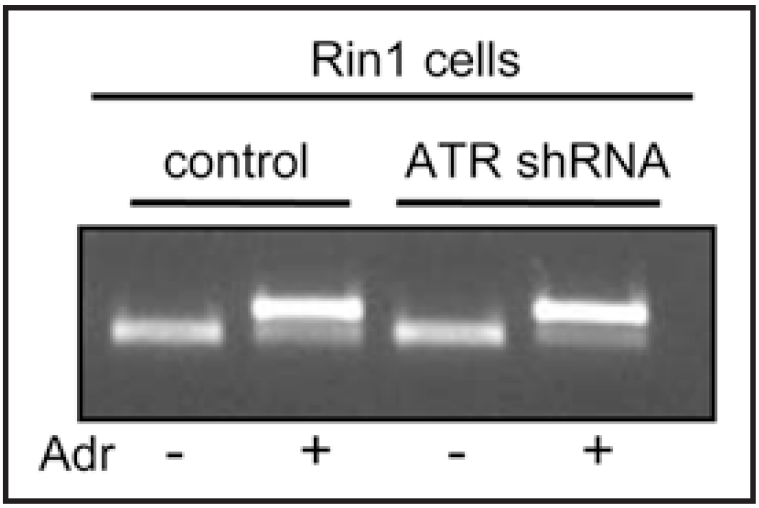

Figure 7.

Ablation of ATR has no effect on the induction of *Mdm2*$^{+108}$ by Adr. Rin1 cells were stably infected with retroviruses expressing pSuper-shRNAs directed against *Atr* or control retroviruses, followed by six hour Adr treatment and RT-PCR for *Mdm2* (x9-12).