Nuclear Localization Signal

Nucleoplasm Mediated by a COOH-Terminal Nuclear Localization Signal

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Abstract

Of the >40 alternative and aberrant splice variants of MDM2 that have been described to date, the majority has lost both the well-characterized nuclear localization signal (NLS1) and the nuclear export signal (NES) sequence. Because cellular localization of proteins provides insight regarding their potential function, we determined the localization of three different MDM2 splice variants. The splice variants chosen were the common variants MDM2-A and MDM2-B. In addition, MDM2-FB26 was chosen because it is one of the few variants described that contains the complete p53-binding site. All three splice variants predominantly localized to the nucleus. Nuclear localization of MDM2-A and MDM2-B was controlled by a previously uncharacterized nuclear localization signal (NLS2), whereas nucleoplasmic localization of MDM2-FB26 was mediated by NLS1, p53 and full-length MDM2 colocalized with the splice variants in the nucleus. MDM2-A and MDM2-B both contain a COOH-terminal RING finger domain, and interaction with full-length MDM2 through this domain was confirmed. MDM2-FB26 was the only splice variant evaluated that contained a p53-binding domain; however, interaction between MDM2-FB26 and p53 could not be shown. p14ARF did not colocalize with the splice variants and was predominantly expressed within the nucleoli. In summary, nuclear localization signals responsible for the nucleoplasmic distribution of MDM2 splice variants have been characterized. Colocalization and interaction of MDM2-A and MDM2-B with full-length MDM2 in the nucleus have important physiologic consequences, for example, deregulation of p53 activity.


Introduction

To date, more than 40 splice variants of MDM2 have been detected in several different tumor types, including tumors of the ovary and bladder (1), brain (2), soft tissues (3-5), and the mammary gland (6). Expression of alternative MDM2 splice variants often correlates with a more advanced tumor stage (1, 2), and in a study of invasive breast cancers, the presence of aberrant splice forms was associated with decreased patient survival (6).

Splicing of the pre-mRNA of MDM2 occurs either through the use of alternative existing intron-exon borders or by an aberrant mechanism that results in splicing within introns and exons (6, 7). As a result, splice variants of the MDM2 gene encode proteins that lack important domains of the protein and that probably display altered biological activity.

The functions of MDM2 splice variants are unclear. Several studies have evaluated the cellular localization of various human (8-11) and murine (12) MDM2 proteins to predict potential activities. In a study by Evans et al. (9), the tumor splice variant MDM2-B was excluded from the nucleus and was visualized predominantly within the cytoplasm of MEFs. In another study, MDM2-Δ150-230 localized to the cytoplasm of U2OS cells (11). Both of these MDM2 proteins lacked part of the NH2-terminal region that contains a nuclear localization signal (NLS), suggesting that loss of this signal prevented the nuclear entry of these two proteins. However, two other studies showed that the loss of this NLS did not prevent MDM2 entry into the nucleus of NIH3T3 and 293 cells, respectively (8, 10). Furthermore, an MDM2 splice variant (V3) lacking most of the p53-binding domain but containing the complete NLS was excluded from the nucleus and predominantly localized to the cytoplasm of MEF (12). These data show that cellular localization of MDM2 proteins is complex and seems to depend on factors other than the well-characterized NH2-terminal NLS (NLS1).

Because of the contradicting published information, our goal was to evaluate the cellular localization of three tumor MDM2 splice variants in vitro to (a) determine whether the lack of the p53 binding domain or the NH2-terminal NLS influenced the localization of MDM2; and (b) evaluate whether the MDM2 isoforms colocalized with known binding proteins; full-length MDM2, p53, or p14ARF (ARF).

Results

Three splice variants were chosen for this project (Fig. 1): MDM2-A, which was the most common splice variant found in pediatric rhabdomyosarcoma; MDM2-B, which is the most
common variant found in human cancer; and MDM2-FB26 (5), one of only two splice variants described to date that contains the complete p53 binding domain. MDM2-A and MDM2-B both lack most of the p53-binding domain, the well-characterized NLS1 and the NES. However, the MDM2-A protein contains an additional 78 amino acids compared with MDM2-B that includes the central acidic domain (Fig. 1). In contrast, splice variant MDM2-FB26 contains the complete p53-binding site and also NLS1. This variant lacks the COOH terminus due to an out-of-frame sequence and a premature stop codon.

MDM2 Splice Variants and Full-Length MDM2 Localize to the Nucleus

To determine where the individual MDM2 proteins localize within a cell, MDM2-A, MDM2-B, MDM2-FB26, and full-length MDM2 proteins were expressed with fusion epitope tags in triple knock-out MEFs (TKO; null for p53, MDM2, and ARF). The splice variants MDM2-A and MDM2-B localized predominantly to the nucleus with faint expression in the cytoplasm (Fig. 2). MDM2-FB26 localized to the nucleoplasm and was excluded from the cytoplasm and the nucleoli. As predicted, full-length MDM2 seemed to be exclusively expressed into the nucleoplasm and excluded from the nucleoli and the cytoplasm (Fig. 2). Similar results were observed when each of the four MDM2 proteins expressed independently in double knock-out MEFs (DKO; null for p53 and MDM2; data not shown).

Alternate NLS Sequences Facilitate Nuclear Entry of MDM2 Splice Variants

The nuclear localization of MDM2 splice variants A and B, which lack the previously characterized NLS1 (8, 9, 11), suggested that alternate sequences might facilitate their nuclear import. By analysis of the MDM2 amino acid sequence, a potential second NLS (NLS2) was identified within the RING finger domain, which has previously been described as cryptic nucleolar localization signal (11). A minimal feature of the majority of NLSs is the motif K-X-X-K/R (lysine-X-X-lysine/arginine; ref. 13). NLS1 consists of amino acid numbers 181 to 185 with the motif RKRHK. The second potential transport signal (NLS2) consists of amino acid 466 to 473, resulting in three overlapping motifs within the same sequence KKLKKRNK.

The splice variant MDM2-A, which lacks NLS1, was mutated to introduce mutant sequence 1 (Fig. 3A) into NLS2.
to generate MDM2-A_{NLS2}. The MDM2-A_{NLS2} protein was expressed predominantly within the nucleus with faint expression in the cytoplasm. Additional mutations were introduced into NLS2 of MDM2-A (NLS2+), MDM2-A–containing NLS2 mutant sequence 2 (MDM2–A_{NLS2+}) was excluded from the nucleus and localized predominantly in the cytoplasm. Full-length MDM2 with mutations in NLS1 resulted in cytoplasmic protein, whereas following the mutagenesis of NLS2 (NLS2+), the protein remained nuclear.

**FIGURE 3.** Mutagenesis of MDM2 NLSs (NLS1 and 2). A. Full-length MDM2 contains NLS1 and a putative NLS2. Mutations were introduced to eliminate each signal independently, as shown. MDM2-A lacks the first NLS (NLS1). Mutations were introduced into the second NLS (NLS2). B. MDM2-A containing NLS2 mutant sequence 1 (MDM2–A_{NLS2}) was detected in the nucleus with visible expression in the cytoplasm. Additional mutations were introduced into NLS2 of MDM2-A (NLS2+). MDM2-A–containing NLS2 mutant sequence 2 (MDM2–A_{NLS2+}) was excluded from the nucleus and localized predominantly within the cytoplasm. C. Full-length MDM2 with mutations in NLS1 resulted in cytoplasmic protein, whereas following the mutagenesis of NLS2 (NLS2+), the protein remained nuclear.

were necessary to reduce nuclear entry of MDM2-A_{NLS2+} and provide evidence for a second NLS in the COOH-terminal portion of the MDM2 protein. However, these data are in contrast to those obtained for full-length MDM2 protein. In full-length MDM2, only NLS1 functions as a NLS. When NLS1 was mutated, as shown in Fig. 3A, full-length MDM2 localized to the cytoplasm (Fig. 3C). In contrast, when NLS2 was mutagenized (NLS2+), full-length MDM2 remained nucleoplasmic (Fig. 3C). These data suggest that NLS2 only functions as a NLS in MDM2 splice variants. It seems that the change in conformation of the COOH-terminal RING finger in MDM2 splice variants lacking a central portion of the protein allows NLS2 to become unmasked.

**MDM2 Splice Variants Colocalize with Full-Length MDM2 in the Nucleus**

To coexpress full-length MDM2 with its splice variants, the plasmids encoding the different proteins were transiently transfected into mutant TKO mouse embryonic fibroblasts. To distinguish between the different proteins when coexpressed, a secondary antibody conjugated to FITC (green fluorescence) was used to detect the primary antibody against the V5 tag of the splice variants, whereas a Texas Red (red fluorescence)–conjugated secondary antibody was used to detect the primary antibody against the MYC-tagged full-length MDM2 protein.

Dual staining to detect the three different MDM2 splice variants when coexpressed with full-length MDM2 revealed...
that as expected, all three colocalized with full-length MDM2 in the nucleoplasm (Fig. 4A). Negative controls for antibody staining and staining of untransfected cells showed that the observed staining was specific.

MDM2 Splice Variants Bind Full-Length MDM2 through Their RING Finger Domains

Because MDM2-A, MDM2-B, and MDM2-FB26 colocalized with full-length MDM2 in the nucleus, further experiments

FIGURE 4. A. Coexpression of MDM2 splice variants with full-length MDM2. TKO cells were stained with anti-V5 antibody to detect the splice variants followed by a FITC-conjugated secondary antibody. Full-length MDM2 was visualized using an anti-Myc antibody and a Texas Red–conjugated secondary antibody. The DNA in the nuclei was stained with Hoechst 33342. The MDM2 splice variants colocalized with full-length MDM2 within the nucleoplasm. B. Immunoprecipitation of MDM2 splice variants following the expression of full-length MDM2 in TKO cells. Truncated MDM2 proteins were precipitated using a V5-specific antibody. Full-length MDM2 and splice variants were detected with an MDM2-specific antibody. MDM2-A and MDM2-B formed complexes with full-length MDM2. Splice variant MDM2-FB26 did not bind full-length protein. FL, full-length MDM2; A, MDM2-A; B, MDM2-B; FB26, MDM2-FB26; UT, untreated; and WCE, whole cell extract.
were undertaken to determine whether the MDM2 splice variants could bind full-length MDM2. The MDM2 splice variants and full-length MDM2 were coexpressed in TKOs and immunoprecipitated using an anti-V5 antibody (Fig. 4B).

As others have previously shown (9), MDM2-B was immunoprecipitated in complex with full-length MDM2 (Fig. 4B). In a similar manner, MDM2-A binding to full-length MDM2 was detected. The full-length MDM2 protein could not be detected in complex with FB26 (Fig. 4B). FB26 was the only variant evaluated that lacked the complete COOH-terminal region, including the RING finger domain necessary for MDM2/MDM2 interactions (Fig. 1), and therefore, our results are consistent with the idea that the interaction of MDM2-A and MDM2-B with full-length MDM2 occurred through the COOH-terminal RING domain.

**Coexpression of MDM2 Splice Variants with p53 in the Nucleus**

To determine the effect of p53 expression on the cellular localization of MDM2 splice variants, p53 was expressed in the mutant TKO mouse embryonic fibroblasts, together with the MDM2 splice variants. Expression of p53 was achieved by transduction with an adenoviral vector containing the p53 cDNA (as described in Materials and Methods). To visualize both proteins within the cell, a secondary antibody conjugated with FITC was used to detect the primary antibody against the V5-tagged splice variants; whereas a Texas Red–conjugated secondary antibody was used to detect the primary antibody against p53. As expected, p53 expression was primarily detected in the nucleoplasm and colocalized with the MDM2 splice variants MDM2-A, MDM2-B, and MDM2-FB26. These data are not shown but were identical to those in Fig. 4A for full-length MDM2.

**MDM2 Splice Variants Do Not Interact with p53**

MDM2-FB26 was the only splice variant evaluated that contained the complete p53-binding domain. MDM2-FB26 and the other splice variants colocalized with p53 in the nucleus, suggesting that they had the potential to form a complex with the p53 protein. Therefore, we evaluated whether MDM2-A, MDM2-B, and MDM2-FB26 could bind p53 protein by transient transfecting the cDNAs of different MDM2 isoforms into TKOs. To express the p53 tumor suppressor, cells were transduced with adenoviral particles containing the p53 cDNA. Immunoprecipitation assays were carried out as described in Materials and Methods.

As previously shown, full-length MDM2 coprecipitated with p53 (Fig. 5). Splice variants MDM2-A and MDM2-B were unable to bind p53 (Fig. 5). These results were expected because both isoforms lack the p53-binding domain. However, surprisingly, FB26 was also unable to bind p53 protein, although it contains the complete p53-binding domain (Fig. 5). These data suggest that the lack of the COOH-terminal region in MDM2-FB26 might influence the structure of the p53-binding domain, preventing its interaction with p53.

**Coexpression of MDM2 Splice Variants with ARF**

To determine whether ARF expression influenced the localization of MDM2 splice variants, a retroviral vector containing the ARF cDNA was used to express ARF in the TKO fibroblasts. To distinguish between ARF and the MDM2 proteins, a secondary antibody conjugated with FITC was used to detect the primary antibody against the splice variant V5 tag, whereas a Texas Red–conjugated secondary antibody was used to detect the primary ARF antibody.

ARF localized predominantly within the nucleoli of the TKO (Fig. 6A), and it did not influence the nuclear localization of the MDM2 splice variants.

**Immunoprecipitation of MDM2 Splice Variants with ARF**

MDM2-A was the only splice variant evaluated that contained the acidic domain to which ARF has previously been shown to bind. Therefore, immunoprecipitation analysis was undertaken to determine whether MDM2-A and other MDM2 splice variants bound ARF protein. ARF was immunoprecipitated following retroviral transduction of TKOs as described in Materials and Methods. MDM2 proteins were detected using an MDM2-specific antibody (anti-MDM2 rabbit, R&D Systems, Minneapolis, MN).

Full-length MDM2 formed a complex with ARF, as shown in Fig. 6B. However, ARF binding to the MDM2 splice variants could not be detected. Unfortunately, due to the cross-reaction of the horseradish peroxidase–conjugated secondary antibody with the ARF antibody used for the immunoprecipitation, any ARF binding to FB26 would have been obscured. Expression levels of full-length MDM2 (90 kDa), MDM2-A (~53 kDa), MDM2-B (~53 kDa), and FB26 (~45 kDa) in the TKO cell extracts before the immunoprecipitation are shown in Fig. 6B.

**Discussion**

In this study, the cellular localization of human MDM2 splice variants was investigated in the presence and absence of potential binding partners: p53, ARF, and full-length MDM2. The splice variants and full-length MDM2 were expressed in knock-out mouse embryonic fibroblasts (DKO, TKO). All of the MDM2 proteins, including those that lacked the previously
FIGURE 6. A. Coexpression of MDM2 splice variants and full-length MDM2 with ARF in TKOs. Cells were stained with anti-V5 antibody to detect the splice variants followed by an FITC-conjugated secondary antibody. ARF was visualized using an anti-ARF antibody and a Texas Red–conjugated secondary antibody. The DNA in the nuclei was stained with Hoechst 33342. ARF expressed predominantly within the nucleoli of the cell and was excluded from the nucleus and cytoplasm. MDM2-A, MDM2-B, and MDM2-FB26 did not colocalize with ARF.

B. Immunoprecipitation of MDM2 following retroviral transduction of ARF into TKO cells. An ARF-specific antibody was used to immunoprecipitate ARF protein. An MDM2 antibody was used to visualize MDM2 proteins (R&D Systems). Full-length MDM2 forms a complex with ARF. ARF binding to the MDM2 splice variants could not be detected.
characterized nuclear localization signal (NLS1), localized predominantly to the nucleus. Splice variants MDM2-A and MDM2-B both lack the well-characterized NLS1 and NES (Fig. 1), and yet they were detected predominantly in the nucleoplasm. After eliminating the possibility that the epitope tag may have played a role in the nuclear localization (data not shown), we suggested that MDM2-A and MDM2-B might contain an alternative signal that mediated the nuclear localization of these proteins.

The motif K-X-X-K/R (lysine-X-lysine/arginine) containing highly charged amino acids has been shown to mediate nuclear localization of several proteins, including Lamin A, human C-MYC, and SV40 large T (13). This motif is the minimal consensus sequence of the nuclear transport signals found in nuclear proteins. This motif was found thrice within the RING finger region as 466KKLKKRNK473 of MDM2 (1, 8, 11). This sequence has been previously described as a cryptic nuclear localization signal that facilitates nuclear localization of MDM2 upon ARF binding (11). However, the nuclear localization motif is very similar to that of the NLS R/K-R/K-X-R/K (arginine/lysine, arginine/lysine X arginine/lysine; ref. 14) and the MDM2 RING finger sequence contains both motifs. It is unclear what other factors may be required to direct the localization of proteins containing motifs that could potentially mediate either nuclear or nucleolar localization.

To test the hypothesis that the cryptic nuclear localization signal could also act as a NLS, multiple missense mutations were incorporated to disrupt all three possible transport motifs (NLS2; Fig. 3A). The amino acid substitutions chosen were similar to mutations that had previously been introduced into the NLS sequences of plant NPR1 (15) and viral SV40 large T (13) and which were confirmed to disrupt the ability of these proteins to localize to the nucleus. Initially, two mutations were generated in the NLS2 sequence (466KKLKKRNK473) of MDM2-A and MDM2-B changing lysine residues 469 and 470 into asparagine and glutamic acid, respectively (mutant sequence 1, 466KKLN473, Fig. 3A). However, this first set of mutations within NLS2 did not interrupt the nuclear localization of MDM2-A (Fig. 3B) and MDM2-B (data not shown). The mutants MDM2-A-NLS2 and B-NLS2 remained predominantly localized to the nucleoplasm identical to that observed for the wild-type MDM2-A and MDM2-B proteins (compare Figs. 2 and 3B). These data suggested that the amino acid changes in the three motifs 466KKLN467, 467KLNE470, or 470ERNK473 were not sufficient to disrupt the nuclear transport.

Because it has been previously shown that specific lysine residues can be very important in directing nuclear localization (13), we introduced two additional mutations into MDM2-A-NLS2 that changed lysine residues 466 and 467 to asparagine and glutamine, respectively (466NQLNERNK473). These additional mutations changed the localization of MDM2-A to be predominantly cytoplasmic (Fig. 3B). These data show that Lys466 and Lys467 are critical for nuclear localization of MDM2-A. The COOH-terminal NLS signal (NLS2) has previously been described as a cryptic nuclear localization signal that only becomes unmasked upon ARF binding to MDM2 (11). This sequence does not seem to facilitate nuclear localization of full-length MDM2 (Fig. 3C), but acts as a NLS only for MDM2 splice variants. The change in protein conformation that occurs when the central portion of MDM2 is deleted seems to unmask the NLS contained within the RING finger.

Our data are in contrast to two previously published studies that evaluated cellular localization of MDM2 splice variants and truncated MDM2 proteins (9, 11). Data from one of those studies (9) showed predominantly cytoplasmic expression of MDM2-ALT1 (human MDM2-B) in p53/MDM2 null mouse fibroblasts (DKO). In addition, truncated MDM2 isoforms, which lacked amino acids Δ150-230 including NLS1, were expressed only in the cytoplasm in U2OS cells (ARFΔ74; ref. 11). However, our data are in agreement with two other studies that evaluated MDM2 splice variants and MDM2 deletion mutants (8, 10). In the first study, the deletion mutant MDM2-Δ491-154 that does not contain NLS1 expressed predominantly in the nucleus of NIH3T3 cells (8). The second study described an enhanced green fluorescent protein-MDM2-B fusion protein that localized to the nucleus in human HEK293 cells (10). A potential reason for the differences could be that expression of MDM2 proteins is influenced by the different cellular backgrounds in which these studies have been carried out. It is also possible that the different MDM2 proteins, which varied in their deleted domains, may display differences in their conformation that could be critical for unmasking the COOH-terminal NLS.

However, these reasons do not explain why we observed nuclear localization for MDM2-B compared with the cytoplasmic localization observed by Evans et al. (9). In both cases, the splice variant was expressed in p53/MDM2 null mouse embryonic fibroblasts (DKO), although it is likely that the cells used were of different passage number, which might have changed the characteristics of the cells in culture. However, this suggestion is speculative, and additional work would be required to determine the exact reason for the observed differences.

Previous studies have shown that both full-length MDM2 and p53 predominantly localize in the nucleoplasm (16, 17). Furthermore, p53, nuclear ARF (18), and truncated MDM2 proteins have been shown to interact with full-length MDM2, resulting in an altered cellular localization. We therefore asked the question whether the splice variants evaluated in this study colocalized with full-length MDM2, p53, or ARF, and whether the MDM2 splice variant cellular localization was altered upon expression of these proteins.

Full-length MDM2, p53, and ARF were each coexpressed with individual MDM2 splice variants in mutant TKO fibroblasts. Full-length MDM2 and p53 were predominantly nuclear, whereas ARF was predominantly localized to the nucleoli (Figs. 4 and 6). Localization of the MDM2 splice variants did not change when coexpressed with these potential binding partners. In a previously published report, MDM2 splice variants with an intact COOH-terminal RING finger domain such as MDM2-B, bound full-length MDM2 and sequestered it in the cytoplasm (9). Our results are consistent with MDM2-A and MDM2-B interacting with full-length MDM2 through their RING finger domains (Fig. 4B) and suggest that full-length MDM2 and the splice variants can interact in the nucleus without being exported to the cytoplasm. This hypothesis is supported by a previous study that showed that the interaction of full-length MDM2 with its splice variants...
occurred independent of the cellular compartment (12). Therefore, data indicate that nuclear export is not necessary for the interaction of full-length MDM2 with MDM2-A and MDM2-B or for the activation of p53.

The p53 tumor suppressor protein was visualized in the nucleus as expected (19, 20) and colocalized with the MDM2 splice variants in the nucleoplasm (data not shown). Because the splice variant FB26 contains the p53-binding site, binding with p53 was predicted. However, no binding of the two proteins was observed (Fig. 5), suggesting either that the p53 binding domain of FB26 was not in the correct conformation, or that other domains within MDM2 might be needed to stabilize a p53/MDM2-FB26 complex. This result was surprising because a similar splice variant (MDM2-E) containing the p53-binding domain has previously been shown to bind p53 protein (1). With the exception of the NLS1 in FB26, the amino acid sequences that differ between MDM2-FB26 and MDM2-E contain no obvious motifs. Therefore, it is difficult to predict what other sequences may influence p53-MDM2 binding. MDM2-A and MDM2-B both lack the p53-binding domain, and as expected, these proteins did not bind p53 protein. Although expression of MDM2-A was very low in the Western blot analysis shown in Fig. 5, both MDM2-A and MDM2-B have previously been shown not to bind p53 (1). The interaction of MDM2 splice variants A and B with full-length MDM2 in the nucleus would likely inhibit the activity of full-length protein to appropriately regulate p53, thereby resulting in p53 activation.

To determine whether ARF influenced the cellular localization of MDM2 splice variants, ARF was expressed in the MEFs using a retroviral vector. ARF has been shown to localize to the nucleolus independent of either p53 or MDM2 expression (21, 22). However, once induced, for example, by oncogenic activation, ARF mediates the translocation of full-length MDM2 to the nucleolus, thereby releasing control of p53 (22). In this manner, cells are protected from oncogene-induced transformation by induction of p53-mediated apoptosis. Therefore, we asked the question whether elevated ARF expression could mediate the transfer MDM2 splice variants to the nucleolus. Upon ARF expression, MDM2-A, MDM2-B, and MDM2-FB26 remained within the nucleoplasm (Fig. 6A). Of the three splice variants analyzed, MDM2-A was the only one that contained the central highly acidic domain to which ARF has been shown to bind (amino acids 210-304). Therefore, MDM2-A would be predicted to bind and colocalize with ARF in the nucleoli. However, immunoprecipitation analysis revealed that although MDM2-A contained the ARF binding site, it could not bind to the ARF protein (Fig. 6B), thus explaining the maintenance of its nuclear localization upon ARF expression. MDM2-B and MDM2-FB26 both lack the complete central acidic domain (amino acids 222-300 and 222-437, respectively), and these proteins also remained nucleoplasmic upon ARF expression, as expected.

In summary, all three MDM2 splice variants evaluated predominantly localized to the nucleus in mouse embryonic fibroblasts. This mechanism seems to involve two NLSs within the protein. Although the localization of the splice variant was not altered by the expression of full-length MDM2 or p53, their colocalization within the cell is probably important for their function. MDM2-A and MDM2-B colocalization with full-length MDM2 in the nucleoplasm would allow their binding and subsequent activation of p53. MDM2-FB26 does not contain the COOH-terminal RING finger domain but does contain the p53-binding site, although binding to p53 protein was not observed. Therefore, MDM2-FB26 would not be predicted to inactivate p53 protein in a similar manner to full-length MDM2 protein. MDM2-A that contained the ARF-binding site was not shown to bind ARF protein or localize to the nucleoli. Therefore, upon ARF activation, only full-length MDM2 would be predicted to move to the nucleoli with ARF. The expression of MDM2 splice variants within such a cell would also enhance p53 activation independent of ARF activity.

Materials and Methods

Cell Culture

Mutant mouse embryonic fibroblasts were kindly provided by Dr. G. Lozano (MD Anderson, Houston, TX; p53/MDM2 null, DKO) and Dr. G. Zambetti (St. Jude Children’s Research Hospital, Memphis, TN; p53/MDM2/ARF null, TKO). Cells were grown in DMEM supplemented with 10% fetal bovine serum and 2% l-glutamine in 10% CO2.

Generation of the MDM2 Splice Variant Constructs

The cDNAs of splice variants MDM2-A and MDM2-FB26 were amplified from total mRNA of primary rhabdomyosarcoma tumor samples as previously described (5) and subcloned into helper plasmid pCR 2.1-TOPO (Invitrogen, Carlsbad, CA). The cDNA of splice variant MDM2-B was obtained from Dr. John Lunec (University of Newcastle, United Kingdom). The full-length MDM2 cDNA was provided by Dr. Frank Bartel (Martin-Luther-University, Halle-Wittenberg, Germany). The variants were cloned into a mammalian expression vector containing the sequence for the V5 epitope tag (pcDNA4/V5-his, Invitrogen). Full-length MDM2 was cloned into a mammalian vector and expressed as a fusion protein with the MYC epitope tag (pcDNA6/ MYC-his, Invitrogen). The plasmids were transiently transfected into mouse embryonic fibroblasts in which endogenous p53, MDM2, and ARF had been deleted (TKO). Some of the experiments as described in the text were carried out using MEFs in which only MDM2 and p53 were deleted (DKO).

Site-Directed Mutagenesis

The QuickChange Multi Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) was used to generate mutations or correct PCR-introduced mutations within a DNA sequence. Oligonucleotides were generated with a melting temperature (Tm) at or above 75°C. The formula Tm = 81.5 + 0.41(%)GC − 675/N − % mismatch (N = number of nucleotides in primer) was used to calculate the correct Tm. The Hartwell Center (St. Jude Children’s Research Hospital, Memphis, TN) synthesized the primers, which were phosphorylated at the 5’ end. The primers used for mutagenesis of the NLSs are for NLS1 MutAS182/183 5’-CGGGGTGAACGACAAAGAAA-3’ for NLS1 MutAS469/470 5’-CTGGTGAACGACAAAGAAA-3’. The primer ends are for NLS1 MutAS182/183 5’-CGGGGTGAACGACAAAGAAA-3’ for NLS1 MutAS469/470 5’-CTGGTGAACGACAAAGAAA-3’.
S'-GCAAAGAAGCTAAAAAGAAAGAATAAGCCCTGCCC-3', and MutASG466/467 S'-GGGCTGCTTTAACATGTCGACATCAGCTTAATGAAAAGG-3'. Mutagenesis reactions were carried out as described in the manufacturer's manual.

**Retroviral Transduction**

To create ecotropic retroviral particles, the MSCV-ires-GFP (23) or the pSRαMSVtkCD8 viral vector (Dr. Martine Roussel, St. Jude Children's Research Hospital, Memphis, TN) containing the cDNA of the gene of interest (MDM2 splice variants or ARF, respectively) was cotransfected with the helper plasmid pEQUECO (Peter Houghton, St. Jude Children's Research Hospital, Memphis, TN) into 293T packaging cells (Mary-Ann Bjornsti, St. Jude Children's Research Hospital, Memphis, TN). Replication-incompetent retroviral particles were collected 48 h post-transfection. Fresh media were added to the 293T cells, and new retroviral particles were collected after another 24 h. The media were either used immediately for transduction or stored at −80°C.

Fibroblasts were plated at a subconfluent density and allowed to attach before removal of the growth media. The supernatant containing retroviral particles was filtered with a Steriflip filter (0.45 μm) to remove all cell debris. The supernatant was diluted 1:2 with complete media and Polybrene (1 mg/mL; Sigma, St. Louis, MO) was added to a final concentration of 1 μg/mL to enhance virus-host interaction. After 24 h, cells transduced with MSCV-ires-GFP were analyzed for green fluorescent protein expression to confirm viral transduction and then incubated for another 24 h before Western blot analysis or immunofluorescence assay.

**Adenoviral Transduction**

Cells were plated at 70% confluency, and growth media were removed. Ad-p53 (Av1p53; Genetic Therapy Inc., Novartis Co., Gaithersburg, MD; ref. 24) containing the cDNA of p53 was diluted into 2 mL of adenoviral transduction media (2% fetal bovine serum) to a concentration of 100 plaque-forming units per cell before adding to the mouse embryonic fibroblasts. After a 2-h incubation at 10% CO2 and 37°C, complete growth media were added. The culture media were changed after 24 h, and the cells were incubated for a further 24 h before Western blot analysis or immunofluorescence assay.

**Immunofluorescence Assay**

MEF cells (3 × 10^4 cells per well) were plated into chamber slides (Nalge Nunc Intern. Rochester, NY) and incubated at 37°C and 10% CO2 for 24 h before analysis. Cells in chamber slides were fixed with 1% paraformaldehyde (Sigma) in FA-buffer (Difco, Fisher, Fair Lawn, NJ) and permeabilized with 0.25% Triton (Surfact-Amps X-100, Pierce, Rockford, IL) in FA at room temperature. The slides were blocked with 10% swine serum (DAKO, Carpinteria, CA) in FA and stained with antibodies. MDM2 proteins were visualized using the following primary antibodies: anti-V5 mouse (Invitrogen), anti-MYC mouse (Invitrogen), anti-Flag-M2 rabbit (R&D Systems), and anti-Flag-M2 mouse (Sigma) followed by the corresponding secondary antibodies: anti-mouse FITC-conjugated, anti-mouse Texas Red–conjugated and anti-rabbit Texas Red–conjugated (all from Jackson Inc., West Grove, PA). Isotype-matched negative control antibodies anti-immunoglobulin G2a (IgG2a), mouse, anti-IgG1 mouse, anti-IgG rabbit (all from Jackson Inc.), and anti-IgG mouse (Santa Cruz Biotechnology, Santa Cruz, CA) were used to determine if the staining was specific. The nuclei were visualized using a 30 μm/L Hoechst 33342 staining solution (Promega, Madison, WI). Cells were visualized with the fluorescence microscope Zeiss Axioplan 2 imaging at a magnification of 40 × or 63 × (Carl Zeiss Vision GmbH, Hallbermoos, Germany). Quantitations were done using Scanalytics Software (BD Biosciences, Rockville, MD).

To distinguish between ARF, p53, full-length MDM2, and the MDM2 splice variant proteins in the dual staining assays, a secondary antibody conjugated with FITC was used to detect the primary antibody against the MDM2 splice variant V5 tag, whereas a Texas Red–conjugated secondary antibody was used to detect the anti-p14ARF rabbit antibody (Novus Biologicals, Littleton, CO), the anti-p53 rabbit antibody (Santa Cruz Biotechnology), or the primary antibody against the full-length MDM2 Myc tag (anti-c–Myc-epitope rabbit, Affinity Bioreagents, Inc., Golden, CO).

**Immunoprecipitation Assay**

For immunoprecipitation, pelleted cells were resuspended in cooled immunoprecipitation-lysis buffer containing 20 mmol/L HEPES (Sigma; pH 7.5), 150 mmol/L sodium chloride, 0.1% Tween 20, and 10% glycerol. Samples were incubated on dry ice for 5 min and thawed for 5 min in a 37°C water bath (five times). A p14ARF-specific antibody, anti-p14 ARF (C-18) antibody (Santa Cruz Biotechnology), was used to precipitate ARF and its binding proteins from 200 μg of total protein for 1 to 2 h at room temperature. Immunoprecipitation complexes were washed thrice in ice-cold immunoprecipitation-lysis buffer. Proteins were separated by electrophoresis and transferred to polyvinylidene difluoride membranes.

**Western Blotting**

Samples containing 50 to 200 μg of total protein were electrophoresed using a Novex 4% to 20% gradient Tris-glycine gel (Invitrogen). Proteins were electroblotted to a polyvinylidene difluoride membrane. For immunodetection of proteins, membranes were washed thrice in 1× TBS-T [0.2 mol/L Tris (pH 7.6), 1.37 mol/L NaCl, and 10 mL of Tween] for 5 min each. Membranes were blocked with Biotto for 1 h at room temperature. Membranes were incubated with the primary antibodies diluted in Biotto (5% fat-free dry milk in 1× TBS-T [0.2 mol/L Tris (pH 7.6), 1.37 mol/L NaCl, and 10 mL of Tween]) between 1 h and overnight, and at room temperature or 4°C, respectively. The secondary antibodies conjugated with horseradish peroxidase were added, and the membranes were incubated at room temperature for 1 h. ECL Western blotting reagent kit (Amersham, Piscataway, NJ) was used for protein detection.

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References
MDM2 Splice Variants Predominantly Localize to the Nucleoplasm Mediated by a COOH-Terminal Nuclear Localization Signal

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