5. Results: Cellular localization of MDM2 splice variants

5.1. Introduction

Of the >40 alternative and aberrant splice variants of MDM2 that have been described to date, the majority has lost both the nuclear localization signal and the nuclear export signal sequence. Therefore the goal of this study was to evaluate where splice variants might localize within the cell in order that potential functions might be predicted. The secondary goal of this study was to determine whether cellular localization of the splice variants is dependent upon expression of p53, p14ARF and full-length MDM2.

Three splice variants were chosen for this project (Figure 20), MDM2-B which is the most common found variant in human cancer, MDM2-A the most common found splice variant in pediatric rhabdomyosarcoma and MDM2-FB26 one of only two splice variants described to date that contains the complete p53 binding domain. MDM2-A and B both lack

![Figure 20](image-url)

Structure of MDM2 and the splice variants evaluated in this study, MDM2-A, B and FB26. NLS- nuclear localization signal, NES- nuclear export signal. Numbers represent exons, nucleotides or amino acids of MDM2 as indicated.
most of the p53-binding domain, the nuclear localization signal (NLS) and the nuclear export signal (NES). However, the MDM2-A mRNA sequence contains an additional 234bp compared to MDM2-B that includes the acidic domain. The acidic domain is the p300/CREB and ARF binding region. In contrast, splice variant MDM2-FB26 contains the complete p53-binding site and also the NLS. This variant lacks the C-terminus due to an out-of-frame sequence and a premature stop codon.

5.2. Generation of the Mdm2 splice variant constructs

The DNA of splice variant MDM2-FB26 cDNA was amplified from total mRNA of rhabdomyosarcoma samples as previously described and subcloned into helper plasmid pCR®2.1-TOPO® (Invitrogen). The cDNA of splice variant B was obtained from Dr. John Lunec (University Newcastle, UK). The cDNAs of MDM2-A and full-length MDM2 were provided by Dr. Frank Bartel (Martin-Luther-University, Halle-Wittenberg, Germany). Sequencing analysis of the splice variants and full-length MDM2 revealed mutations within various regions of the cDNA sequence (Table 6). Therefore, site-directed mutagenesis (Section 3.1.13) was performed to correct the base pair changes so that the MDM2 sequences would match the entries in the GenBank (Table 6). The oligonucleotides used in the mutagenesis reactions are listed in Section 2.8.

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). Large-scale plasmid DNA was isolated by Mrs. Misty D. Cheney, Technical Assistant, St. Jude as described in Method section 3.1.2.

<table>
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<tr>
<td>MDM2-FB26</td>
<td>T 394, T 491</td>
<td>G 394, C 491</td>
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Table 6
Detected mutations in the cDNAs of MDM2 splice variants and full-length MDM2. Oligonucleotides used for site-directed mutagenesis are listed under Section 2.8. bp-base pair.
The plasmids were stably transfected into knock out mouse embryonic fibroblasts that were either null for p53/MDM2 (double knock out, DKO); or null for p19ARF/p53/MDM2 (triple knock out, TKO). The plasmids contained either the Zeocin™ or the Blastcidin resistance genes for selection of the stable cell lines transfected with the splice variant or the full-length MDM2 vector, respectively. Cell lines were established that expressed each of the splice variants and full-length MDM2 independently. However, after several passages in culture expression of the MDM2 proteins was gradually reduced until expression could no longer be detected. For that reason, transiently transfected DKO and/or TKO were used in the following experiments. The cellular localization of the splice variants was visualized using fluorescence digital imaging microscopy.

5.3. Expression of MDM2 splice variants and full-length MDM2 in knockout mouse embryonic fibroblasts

In TKOs, the splice variants MDM2-A and B localized predominantly to the nucleus with faint expression in the cytoplasm (Figure 21A). FB26 localized to the nucleoplasm and was excluded from the cytoplasm and the nucleoli. As predicted, full-length MDM2 appeared to be exclusively expressed into the nucleoplasm and excluded from the nucleoli and the cytoplasm (Figure 21A). Similar results were observed when each of the four MDM2 proteins was separately expressed in DKO (Figure 21B). However, previously published studies are contradictory with regards to where in the cell MDM2 splice variants lacking the NLS are expressed 14, 97, 118, 119. One potential explanation for the results above is that the fusion tag used in these experiments might affect the cellular transport of MDM2 splice variants. However, it is also possible that there may be alternate nuclear localization signals within the different MDM2 splice variants sufficient for the nuclear import.
Figure 21
Cellular localization of MDM2 splice variants and full-length MDM2 in (A) MDM2/p53/ARF null murine fibroblasts (TKOs) and (B) MDM2/p53 null murine fibroblasts (DKOs).
**5.4. Fusion epitope tags do not influence the cellular localization**

To determine whether the fusion epitope tag can affect cellular localization of MDM2 splice variants, MDM2-FB26 protein was expressed as fusion protein with a V5 epitope tag, a FLAG epitope tag and without any fusion tag. In addition, MDM2 full-length was expressed with the MYC epitope tag and without fusion tag. The different proteins were either transiently transfected or transduced into the cells using retroviral vectors (see Method sections 3.2.3 and 3.2.4, respectively).

The fusion proteins FB26-V5, Flag-FB26 and FB26 were expressed predominantly in the nucleolus and excluded from the nucleoli and cytoplasm (Figure 22). These results suggest that the previously characterized nuclear localization signal (NLS) was responsible for the nuclear entry of FB26 and that the V5 epitope tag did not mediate nuclear localization.

As shown in Figure 22, the fusion protein full-length MDM2-MYC and untagged full-length MDM2 were both expressed exclusively in the nucleus and excluded from the nucleoli and cytoplasm. These data demonstrate that the MYC epitope did not facilitate nuclear entry of the full-length protein.

![Figure 22](image)

**Figure 22**
Cellular localization of MDM2-FB26 and full-length MDM2 with and without fusion epitope tags. Both MDM2 proteins were expressed in p53/MDM2/ARF null murine fibroblasts (TKOs).
5.5. Evaluation of alternate NLS sequences that could facilitate nuclear entry of MDM2 splice variants.

The nuclear localization of MDM2 splice variants MDM2-A and MDM2-B, which lack the previously characterized NLS, suggested that alternate sequences might facilitate their nuclear import. A nuclear localization signal has been confirmed within the N-terminal region of human full-length MDM2 (from now on called NLS1). However, a potential second nuclear localization signal appears to be contained within the RING finger domain (NLS2). A minimal feature of the majority of nuclear localization signals is the motif KXXXK/R (lysine-X-X-lysine/arginine). NLS1 consists of amino acid numbers 181-185 with the motif RKRHK. The second potential transport signal (NLS2) consists of amino acid 466-473 resulting in three overlapping motifs within the same sequence KKLK/RHK. To determine whether both nuclear location signals are functional in MDM2, we introduced two mis-sense mutations into both transport signals, which changed the protein sequences as follows K182N and R183G for NLS1; and K469N and K470E for NLS2 (Figure 23A).

The splice variant MDM2-A, which lacks NLS1 was mutated to introduce the described mutations into NLS2 to generate MDM2-A\textsubscript{NLS2}. The MDM2-A\textsubscript{NLS2} protein was expressed predominately within the nucleus with faint expression in the cytoplasm of TKOs (Figure 24A) in an identical manner to the non-mutated protein. In addition, splice variant

![Diagram](image)

**Figure 23**
Introduction of mutations into the nuclear localization signals of MDM2 proteins. (A) For MDM2 full-length two nuclear localization signals have been predicted, NLS1 and NLS2. (B) The out-of-frame region of MDM2-FB26 contains a motif (NLS3) similar to the nuclear localization signals NLS1 and NLS2. Numbers represent amino acid positions. The resulting amino acid changes are shown in bold. R-arginine, K-lysine, H-histidine, N-asparagine, G-glycine, Q-glutamine, L-leucine, A-alanine.
MDM2-B, which also had mutations incorporated into NLS2 and lacks the NLS1, similarly localized predominantly in the nucleoplasm with visible expression in the cytoplasm of TKO cells (Figure 24A), as seen with the non-mutant proteins. Splice variant FB26, which contained NLS1 but lacked NLS2, had mutations introduced within the NLS1 sequence (FB26\textsubscript{NLS1}). FB26\textsubscript{NLS1} localized predominantly within the nucleus, but nucleolar and cytoplasmic expression of FB26\textsubscript{NLS1} could also be detected (Figure 24A), which was not visible upon expression of wild-type FB26 (Figure 21A and B). MDM2 full-length\textsubscript{NLS1} and full-length\textsubscript{NLS2} each containing one intact localization signal expressed predominantly within the nucleus. Exclusion from the nucleoli was observed for each of these mutated full-length MDM2 proteins (Figure 24B). MDM2 full-length\textsubscript{NLS1+2} had mutations introduced within both NLS1 and NLS2. However, this protein was still expressed predominantly in the nucleoplasm but was excluded from the nucleoli and the cytoplasm (Figure 24B). These results demonstrate that introduction of these mutations into the NLS motifs could not inhibit protein transport to the nucleus. However, the mutations created within the NLS1 of MDM2-FB26 slightly altered its cellular localization but it was still predominant nuclear, suggesting that other domains of this protein might substitute for the loss of NLS1.

These data suggested either that additional sequences are important for nuclear entry, or that the NLS signals had not been disrupted by the mutations that had been introduced. Introduction of mutations into the nuclear localization signal of large-T antigen of SV40 virus showed that of three contiguous lysine residues (amino acids 127-129) only lysine 128 was essential for the nuclear localization suggesting that different amino acids are of different importance for nuclear transport\textsuperscript{122}. Therefore, additional site-directed mutagenesis was undertaken to further mutate NLS2 in the MDM2 splice variants to determine whether additional changes could interrupt the cellular transport of the splice variants to the nucleus. In addition to the amino acid changes at position 469 and 470 in NLS2 (Figure 23A, mutant sequence No.1), lysine residues 466 and 467 were also mutated (Figure 23A, mutant sequence No.2). The MDM2-A\textsubscript{NLS2} protein sequence was changed as follows K466N, K467Q to generate MDM2-A\textsubscript{NLS2+}. These additional mutations changed the cellular localization of MDM2-A. In TKOs, MDM2-A\textsubscript{NLS2+} was now expressed predominantly in the cytoplasm with faint nuclear staining, which was not observed upon wild-type MDM2-A or mutant MDM2-A\textsubscript{NLS2} expression. These data demonstrate that additional mutations within NLS2 were necessary to inhibit nuclear entry of MDM2-A\textsubscript{NLS2+}. 
Figure 24
Cellular localization of MDM2 splice variants and full-length MDM2 mutated in their nuclear localization signals. (A) MDM2-A and B both lack the first nuclear localization signal (NLS1) and mutations have been introduced within a second nuclear localization signal (NLS2). MDM2-FB26 lacks NLS2 and is mutated within the first nuclear localization signal (NLS1). (B) Full-length MDM2 was expressed containing either a mutation within one of the two potential localization signals or within both. (C) Additional mutations were introduced into NLS2 of MDM2-A (NLS2+).
The splice variant FB26 is a rare MDM2 splice variant, which to date has only been detected in pediatric rhabdomyosarcomas. Evaluation of the C-terminal out-of-frame region of aberrant splice variant FB26 revealed a novel protein sequence consisting of seven amino acids KEAKEKE (MDM2-FB26 accession number AF385323). This novel sequence revealed a motif for a potential nuclear localization (NLS3?) consisting of amino acids KEAKEK (Figure 5.4B), which could have been responsible for nuclear localization of FB26. However, this novel sequence does not occur in other aberrantly spliced MDM2 variants, and therefore, the contribution of this novel potential NLS (NLS3?) to the nuclear localization of MDM2-FB26 was not pursued any further.

5.6. Co-expression of MDM2 splice variants with full-length MDM2

Another goal of this report was to determine if binding partners such as full-length MDM2, p53 and p14ARF influence the cellular localization of MDM2 splice variants. To co-express full-length MDM2 with its splice variants, the plasmids encoding the different proteins as described in Section 5.2, were transiently transfected into mutant mouse embryonic fibroblasts (p53−/−, MDM2−/−, p19ARF−/−, TKOs). To distinguish between the different proteins when co-expressed, 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 (Section 3.3.6).

Dual staining to detect the three different MDM2 splice variants when co-expressed with full-length MDM2 revealed that as expected all three co-localized with full-length MDM2 in the nucleoplasm (Figure 25). Negative controls for antibody staining and staining of untransfected cells are shown in Figure 25 demonstrating that the observed staining was specific. In summary, full-length MDM2 co-localized with the MDM2 splice variant in the nucleoplasm.
Figure 25
Co-expression of MDM2 splice variants with full-length MDM2. The MDM2 splice variants co-localized with full-length MDM2 within the nucleoplasm.
5.7. Co-expression of MDM2 splice variants with p53

To determine the effect of p53 expression on the cellular localization of MDM2 splice variants, p53 was expressed in the mutant mouse embryonic fibroblasts (p53−/−, MDM2−/−, ARF−/− TKOs) together with the MDM2 splice variants. Expression of p53 was achieved by transduction with an adenoviral vector containing the p53 cDNA (refer to Method section 3.2.5). To visualize both proteins within the cell, a secondary antibody conjugated with FITC (green fluorescence) was used to detect the primary antibody against the V5-tagged splice variants, whereas a Texas Red (red fluorescence) conjugated secondary antibody was used to detected the primary antibody against p53 (Section 3.3.6). p53 expression was primarily detected in the nucleoplasm (Figure 26). As previously described splice variants A and B were predominately expressed within the nucleus, although these proteins were also detected in the nucleoli and the cytoplasm. MDM2-FB26 could only be detected in the nucleoplasm. Negative controls for antibody staining and negative staining of untreated cells are also shown in Figures 26 demonstrating that the observed staining was specific. These data indicate that p53 co-localizes with the different MDM2 isoforms within the nucleus of TKO cells.
Figure 26
Co-expression of MDM2 splice variants and p53 in knock out fibroblasts (TKOs). MDM2 splice variants co-localize with p53 within the nucleoplasm. Negative control antibodies were used to ensure that the immunostaining is specific.
5.8. Co-expression of MDM2 splice variants with p14ARF

To determine whether p14ARF expression influenced the localization of MDM2 splice variants, a retroviral vector containing the p14ARF cDNA was used to express ARF in knock out (p53-/-, MDM2-/-, ARF-/-) MEFs. To distinguish between ARF and the MDM2 proteins, a secondary antibody conjugated with FITC (fluoresces green at 520nm) was used to detect the primary antibody against the splice variant V5 tag, whereas a Texas Red (fluoresces red at 620nm) conjugated secondary antibody was used to detect the primary p14ARF antibody.

p14ARF expressed predominantly within the nucleoli of p53/MDM2/ARF null murine fibroblasts (TKO) (Figure 27). However, upon ARF expression, MDM2-A, B and FB26 maintained their localization predominantly in the nucleoplasm (Figure 27). As previously observed, MDM2-A and B could faintly be detected in the cytoplasm. In summary, the co-expression of ARF with the splice variants did not result in co-localization within the cell demonstrating that ARF expression did not affect the cellular localization of MDM2-A, B and FB26.

5.9. Immunoprecipitation of MDM2 splice variants upon ARF expression

MDM2-A is the only splice variant that still contains the acidic domain that p14ARF has previously been shown to bind. Therefore, immunoprecipitation analysis (IP) was undertaken to determine if MDM2-A and other MDM2 splice variants could form complexes with p14ARF. ARF protein was immunoprecipitated following retroviral transduction of TKOs as described in the Methods (Section 3.3.5). MDM2 proteins were detected using an MDM2 specific antibody.

Full-length MDM2 formed a complex with ARF, as shown in Figure 28A. However, ARF binding to the MDM2 splice variants including MDM2-A could not be detected. If ARF bound to MDM2-A and B, then these proteins would have been visible following the IP. Unfortunately, due to the cross-reaction of the Horseradish peroxidase-conjugated secondary antibody with the ARF antibody used for the IP, any ARF binding to FB26 would have been obscured. Expression levels of full-length MDM2 (90kDa), MDM2-A (~53KDa), MDM2-B (~45KDa) and FB26 (~35KDa) in the TKO cell extracts before the IP are shown in Figure 28B.
Co-expression of MDM2 splice variants and full-length MDM2 with p14^ARF in TKOs. ARF expressed predominantly within the nucleoli of the cell and was excluded from the nucleus and cytoplasm. MDM2-A, B and FB26 did not co-localize with ARF in the nucleoli. The splice variants maintained their predominantly nuclear localization.
Figure 28
(A) Immunoprecipitation of MDM2 following retroviral transduction of ARF into TKO cells. An ARF specific antibody was used to immunoprecipitate p14ARF protein. MDM2 antibody was used to visualize MDM2 proteins. Full-length MDM2 complexes with ARF. ARF binding to the MDM2 splice variants could not be detected. (B) MDM2 protein expression in the whole cell lysate before immunoprecipitation. FL- full-length MDM2; A- MDM2-A; B- MDM2-B; FB26- MDM2-FB26; UT- untreated and numbers represent the molecular weight markers (kDA-kilo Dalton).
5.10. Discussion

In this study, the cellular localization of human MDM2 splice variants was investigated in the presence and absence of potential binding partners; p53, p14ARF and full-length MDM2. The splice variants and full-length MDM2 where expressed in knockout mouse embryonic fibroblasts (DKO, TKO) and all of the MDM2 proteins, including those that lacked the previously characterized NLS signal, localized predominately to the nucleus. At first, we proposed that the epitope tags V5 or MYC might have influenced the nuclear localization of the proteins. However, this possibility was discounted after showing that FB26 and full-length MDM2 could localize to the nucleus independent from the fusion tags expressed. Then, the mechanism by which the proteins localized to the nucleus was evaluated. Splice variants MDM2-A and B both lack the well-characterized NLS and NES (Figure 20) and yet they were detected predominantly in the nucleoplasm. These results suggested that MDM2-A and B may contain an alternative signal that mediated the nuclear localization of these proteins.

The small motif KXXK/R (lysine XX 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. This motif has been suggested to be the minimal feature required for a nuclear transport signal. This protein sequence was found three times within the RING finger region as $^{466}$KKLKKRNK$^{473}$ of MDM2. In order to test the hypothesis that the potential nuclear localization signal in the RING finger domain influenced nuclear localization of MDM2 and its splice variants, multiple missense mutations were incorporated to disrupt all three possible transport motifs (NLS2) (Figure 23). The amino acid substitutions chosen were similar to mutations that had previously been introduced into the nuclear localization signal sequences of plant NPR1 and viral SV40 large T and were demonstrated to disrupt the ability of these proteins to localize to the nucleus. Two mutations were integrated into the original sequence of NLS2 ($^{466}$KKLKKRNK$^{473}$) of MDM2-A and B changing lysine residues 469 and 470 into asparagine and glutamic acid, respectively (mutant sequence $^{466}$KKLN$^{473}$, Figure 23). However, this first set of mutations within the NLS2 did not interrupt the nucleoplasmic import of MDM2-A and B (Figure 24A). The mutants MDM2-A$_{NLS2}$ and B$_{NLS2}$ remained predominantly localized to the nucleoplasm with visible cytoplasmic staining in the same way as observed for the wild-type MDM2-A and B proteins (compare Figures 24A and 21, respectively). These data indicated that the amino acid changes in the three motifs $^{466}$KKLN$^{469}$, $^{467}$KLNE$^{470}$, or $^{470}$ERNK$^{473}$ were not sufficient to disrupt the nuclear transport. Because the mutations within NLS2 of MDM2 were chosen so...
that each of the three motifs would be mutated, it was not expected that the MDM2 proteins enter the nucleus. Therefore, it was proposed either that alternate regions of MDM2 protein were responsible for the nuclear localization or that the different lysine residues within the nuclear localization sequence of MDM2 are of different importance.

In support of the second hypothesis, the introduction of two additional mutations into MDM2-A\textsubscript{NLS2} that changed lysine residues 466 and 467 to asparagine and glutamine, respectively (\textsuperscript{466}NQLNERNK\textsuperscript{473}) resulted in MDM2-A expression predominantly within the cytoplasm (Figure 24C). These data demonstrate that lysine 466 and lysine 467 are critical for nuclear localization of MDM2 proteins. This C-terminal NLS signal (NLS2) has previously been described, as a cryptic nucleolar localization signal that only becomes unmasked upon ARF binding to MDM2 facilitating nuclear entry \cite{119}. This sequence has not previously been demonstrated to be a nuclear localization signal for full-length MDM2. However, we have demonstrated that NLS2 can also act as a NLS for full-length protein because when NLS1 is mutated, the full-length protein still enters the nucleus (Figure 24B).

Following mutation of NLS1 the localization of FB26 changed from being predominately nucleoplasmic to nucleolar, nucleoplasmic and faintly cytoplasmic (Figure 24A). FB26 contains seven novel amino acids at its C-terminus resulting from aberrant splicing and use of a different open reading frame (\textsuperscript{189}KEAKEKE\textsuperscript{195}). As described above, a minimal feature for a nuclear localization signal of various proteins is the amino acid sequence K-X-X-K/R \cite{122}. This sequence can be found once in the out-of-frame region of FB26 (\textsuperscript{189}KEAK\textsuperscript{192}) and could be the reason for nuclear localization of mutant FB26\textsubscript{NLS1}. The same motif might also be responsible for the observed nucleolar localization of FB26\textsubscript{NLS1}. Comparison of nucleolar localization signals of proteins such as ARF \cite{60,124}, HIV tat \cite{125}, and ribosomal proteins L5 \cite{126} and S6 \cite{127} suggested that the minimal feature for a nucleolar signal contains the following motif K/R-K/R-X-K/R \cite{121}. However, this exact motif cannot be detected in the out-of-frame region of FB26 and therefore it is not clear whether it is responsible for the nucleolar localization of FB26\textsubscript{NLS1}.

Another possible explanation for why FB26\textsubscript{NLS1} localizes to the nucleoli of TKO cells might be that this out-of-frame region exerts a novel function. For example, this region could be a binding domain for an unknown protein that sequesters FB26 in the nucleoli. Because splice variant MDM2-FB26 is very rare and to date has only been detected in a single rhabdomyosarcoma tumor \cite{75}, the mechanism for nuclear and nucleolar localization of this protein was not pursued any further.
Our data were different compared to two previously published studies that evaluated cellular localization of MDM2 splice variants and truncated MDM2 proteins. Data from one of those studies showed predominately cytoplasmic expression of HDM2-ALT1 (human MDM2-B) in p53/MDM2 null mouse fibroblasts (DKO). In addition, truncated MDM2 isoforms, which lacked amino acids Δ150-230 including the nuclear localization signal, were expressed only in the cytoplasm in U2OS cells (p14\(^{ARF/-}\))\(^1\). However, our data are in agreement with two other studies that evaluated MDM2 splice variants and MDM2 deletion mutants. In one study, an EGFP-MDM2-B fusion protein localized to the nucleus in human HEK293 cells. In addition, the deletion mutant MDM2-Δ491-154 that does not contain NLS1 expressed predominantly in the nucleus of NIH3T3 cells. A potential reason for the differences could be that expression of MDM2 proteins is influenced by the different cellular backgrounds in which these investigations have been carried out. However, a second reason for the observed differences could be that the different MDM2 proteins evaluated vary in their deleted domains and therefore may have lost the ability to bind to other proteins that could influence cellular localization.

However, the different results observed for MDM2-B in the present study and MDM2-ALT1 (MDM2-B) in the study by Evans et al. (2001) are not easy to explain. In both cases, the splice variant was expressed in p53/MDM2 null mouse embryonic fibroblasts (DKO). However, in the present study MDM2-B predominantly expressed in the nucleoplasm whereas in contrast, in the study by Evans et al. (2001), MDM2-ALT1 primarily localized within the cytoplasm of DKO cells. One potential difference between the two studies is that the cells used differed in their passage number or in their clonal origin. These factors may influence the cellular localization of MDM2-B because they might change the characteristics of each cell line in culture. Also, the plasmid constructs used to express MDM2-B in both studies were not identical. In our study, MDM2-B was expressed with an V5 epitope tag and in the study by Evans et al. (2001), it appears that a CMV-His-MDM2-ALT1 plasmid construct was used. Therefore, different fusion tags used to express the same protein might be the cause for the observed differences in the cellular expression of MDM2-B. However, these suggestions are speculative and additional work would be required to determine the exact reasons for the differences.

To evaluate whether the cellular localization of MDM2 splice variants was influenced by p53, p14\(^{ARF}\) and full-length MDM2, each of these proteins was co-expressed with individual MDM2 splice variants in mutant MEFs (p53-/-, MDM2-/-, ARF-/- TKO). In a
previously published report\textsuperscript{97}, MDM2 splice variants with an intact C-terminal RING finger domain such as MDM2-B, bound full-length MDM2 and sequestered it in the cytoplasm\textsuperscript{97}. However, we have not observed a predominantly cytoplasmic localization for any of the splice variants evaluated in our study, and co-expression of the three variants analyzed was observed together with full-length MDM2 in the nucleus (Figure 25). We have confirmed that MDM2-A can interact with full-length MDM2 through its RING finger domain (refer to Section 4.7) and therefore it appears that full-length MDM2 and the splice variants can interact through their RING finger domains 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 occurs independent of the cellular compartment\textsuperscript{98}. Therefore, it was concluded that nuclear export is not necessary for the interaction of full-length MDM2 with MDM2-A and B, or for the activation of p53.

The p53 tumor suppressor protein was visualized in the nucleus as expected\textsuperscript{128, 129}, and co-localized with the MDM2 splice variants in the nucleoplasm (Figure 26). Full-length MDM2 has been shown to export p53 from the nucleus to the cytoplasm resulting in p53 proteasomal degradation\textsuperscript{61}, however, the majority of MDM2 splice variants remains nucleoplasmic. Because the splice variant FB26 contains the p53-binding site, co-localization of this protein with p53 could be indicative of protein binding. However, MDM2 A and B both lack the p53 binding domain, and therefore co-expression of MDM2 A and B with p53 in the nucleus is independent of their binding to one another.

To determine whether p14\textsuperscript{ARF} influences the cellular localization of MDM2 splice variants, p14\textsuperscript{ARF} was expressed in the MEFs using a retroviral vector. p14\textsuperscript{ARF} as well as its murine equivalent p19\textsuperscript{ARF} have been shown to localize to the nucleolus independent of either p53 or MDM2 expression\textsuperscript{60, 61}. However, upon elevated levels of ARF expression, for example by oncogenic activation, ARF mediates the translocation of full-length MDM2 to the nucleolus, thereby releasing control of p53\textsuperscript{60}. In this manner cells are protected from oncogene-induced transformation by induction of p53-mediated apoptosis. Therefore, the question remained whether p14\textsuperscript{ARF} could bind and transfer MDM2 splice variants into the nucleolus?

MDM2-A, B and FB26 were expressed within the nucleoplasm and this did not change upon ARF expression. Of the three splice variants analyzed; MDM2-A was the only one that contains the central highly acidic domain to which ARF has been shown to bind (amino acids 210-304) and so MDM2-A would be predicted to bind and co-localize with ARF in the nucleoli. However, immunoprecipitation analysis revealed that even though MDM2-A
contained the ARF binding site it could not bind to the ARF protein, thus explaining the maintenance of its predominantly nucleoplasmic cellular localization upon ARF expression. MDM2-B and 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 MDM2 splice variants evaluated predominantly localized to the nucleus in mouse embryonic fibroblasts. This mechanism appears to involve two nuclear localization signals within the protein. Even though the localization of the splice variants was not altered by the expression of full-length MDM2 or p53, their co-localization within the cell is probably important for their function. MDM2-A and B co-localization with full-length MDM2 in the nucleoplasm would allow their binding and subsequent activation of p53. FB26 does not contain the C-terminal RING finger domain but does contain the p53-binding site, which would facilitate its binding to p53 protein in the nucleoplasm. Like full-length MDM2, FB26 would be predicted to inactivate p53 protein, although this hypothesis was not tested. It was interesting that MDM2-A that contained the ARF-binding site did not bind ARF protein and localize to the nucleoli. However, maintenance of MDM2-A in the nucleoplasm would allow its binding to full-length MDM2 and subsequent activation of p53 as confirmed in Section 4.7.