Chapter

Identification of RNA Species That Bind to the hnRNP A1 in Normal and Senescent Human Fibroblasts

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Abstract

hnRNP A1 is a member of the hnRNPs (heterogeneous nuclear ribonucleoproteins) family of proteins that play a central role in regulating genes responsible for cell proliferation, DNA repair, apoptosis, and telomere biogenesis. Previous studies have shown that hnRNPA1 had reduced protein levels and increased cytoplasmic accumulation in senescent human diploid fibroblasts. The consequence of reduced protein expression and altered cellular localization may account for the alterations in gene expression observed during senescence. There is limited information for gene targets of hnRNP A1 as well as its *in vivo* function. In these studies, we performed RNA co-immunoprecipitation experiments using hnRNP A1 as the target protein to identify potential mRNA species in ribonucleoprotein (RNP) complexes. Using this approach, we identified the human double minute 2 (*HDM2)* mRNA as a binding target for hnRNP A1 in young and senescent human diploid fibroblasts cells. It was also observed that alterations of hnRNP A1 expression modulate *HDM2* mRNA levels in young IMR-90 cells. We also demonstrated that the levels of *HDM2* mRNA increased with the downregulation of hnRNP A1 and decrease with the overexpression of hnRNP A1. Although we did not observe a significant decrease in HDM2 protein level, a concomitant increase in p53 protein level was detected with the overexpression of hnRNP A1. Our studies also show that hnRNP A1 directly interacts with HDM2 mRNA at a region corresponding to its 3′ UTR (untranslated region of a gene). The results from this study demonstrate that hnRNP A1 has a novel role in participating in the regulation of HDM2 gene expression.

Keywords: senescence, fibroblasts, hnRNP A1, *hdm2*, RNP complexes

1. Introduction

Cellular senescence is best described as an inevitable irreversible proliferation arrest the phase of primary human fibroblasts in culture [1, 2]. The senescent phenotype is characterized by distinctive changes in morphology to become enlarged, flattened, and granular [2]. Multiple factors that activate senescence include various types of stress-related stimuli such as aberrant oncogenic signaling, oxidative stress, and DNA damage [2]. Moreover, the onset of senescence can be regulated by events such as epigenetic regulation, chromosome dynamics, protein degradation,

mitochondrial mechanisms, and metabolic pathways. The molecular pathways of senescence differ considerably among cell types as well as different species [3].

Alternative splicing of pre-mRNAs is a process in which varied mRNA transcripts are generated to provide a major source of protein diversity in higher eukaryotes. Pre-mRNA splicing is a nuclear process that can be constitutive or alternative [4, 5]. Constitutive splicing involves the removal of introns and the joining of adjacent exons in the order of their arrangement. One of the core proteins involved in splicing is hnRNPA1 [5, 6]. Consequently, a single protein may be produced from a single pre-mRNA in constitutive splicing [7]. In contrast, in alternative splicing, the variable use of splice sites permits two or more mature mRNAs to be generated from the same pre-mRNA. Among the nuclear complexes primarily responsible for alternative splicing are heterogeneous nuclear ribonucleoproteins, small nuclear ribonucleoproteins snRNPs, and SR proteins [8, 9]. Splicing factors that play a crucial role through concentration changes or alterations of their expression patterns have significant impacts on mRNA alternative splicing [9, 10].

We have previously found that hnRNP A1 is significantly downregulated in cellular senescence [10] and can regulate the levels of the alternatively spliced *INK*^{4a} locus that generates the mRNA isoforms, p16^{INK4a} and p14^{ARF} both of which are growth suppressors that are important in senescence [10, 11]. Increased expression levels of hnRNP A1 *via* over-expression can shift the expression pattern toward the p14^{ARF} mRNA isoform [10].

We initiated this study to identify novel targets of hnRNP A1 and to further explore the role of hnRNP A1 in the modulation of gene expression during cellular senescence. The experimental approach used in this study was to identify the *in vivo* RNA targets bound in hnRNP A1 RNP complexes isolated from human fibroblasts. RNP complexes were isolated by a brief co-immunoprecipitation step with the 4B10 hnRNP A1-specific monoclonal antibody [12]. RNA species in these complexes were then subjected to reverse transcription followed by amplification. The products were then cloned and sequenced. Our findings suggest that hnRNP A1 is involved in the regulation of *HDM2* gene expression. However, the involvement of hnRNP A1 in the regulation of *HDM2* gene expression remains to be elucidated.

2. Results

2.1 hnRNP A1-messenger ribonucleoprotein (mRNP) complexes

We sought to identify putative mRNA substrates for hnRNP A1 by identifying mRNA sequences that directly bind to the hnRNP A1 protein. We employed a modification of a procedure that had been used for the characterization of RNP complexes by Mili et al. [12]. We isolated hnRNP A1 protein complexes bound to their RNA targets from total young and senescent fibroblast cell lysates. To demonstrate that the complexes represented the majority of hnRNP A1-mRNP complexes found in cellular pools, we measured the mRNA levels of hnRNP A1 and actin in the isolated complexes by RT-PCR. Actin has been previously reported to be in hnRNP A1 RNP complexes; thus, we used actin as a positive control. The results in **Figure 1A**, show that actin and hnRNP A1 protein were present in lysates isolated from young and senescent IMR-90 fibroblasts. We assessed the protein level of hnRNP A1 in 4B10 RNP complexes isolated from young and senescent protein lysates. 4B10 RNP complexes reflect the results in panel A (**Figure 1B**) as hnRNP A1 was lower in senescent IMR-90 protein lysates following immunoprecipitation when compared with young cell lysates. These observations indicate that hnRNP A1 was present in the isolated RNP complexes. One known mRNA target of hnRNP A1 is its own RNA; therefore, we measured the level

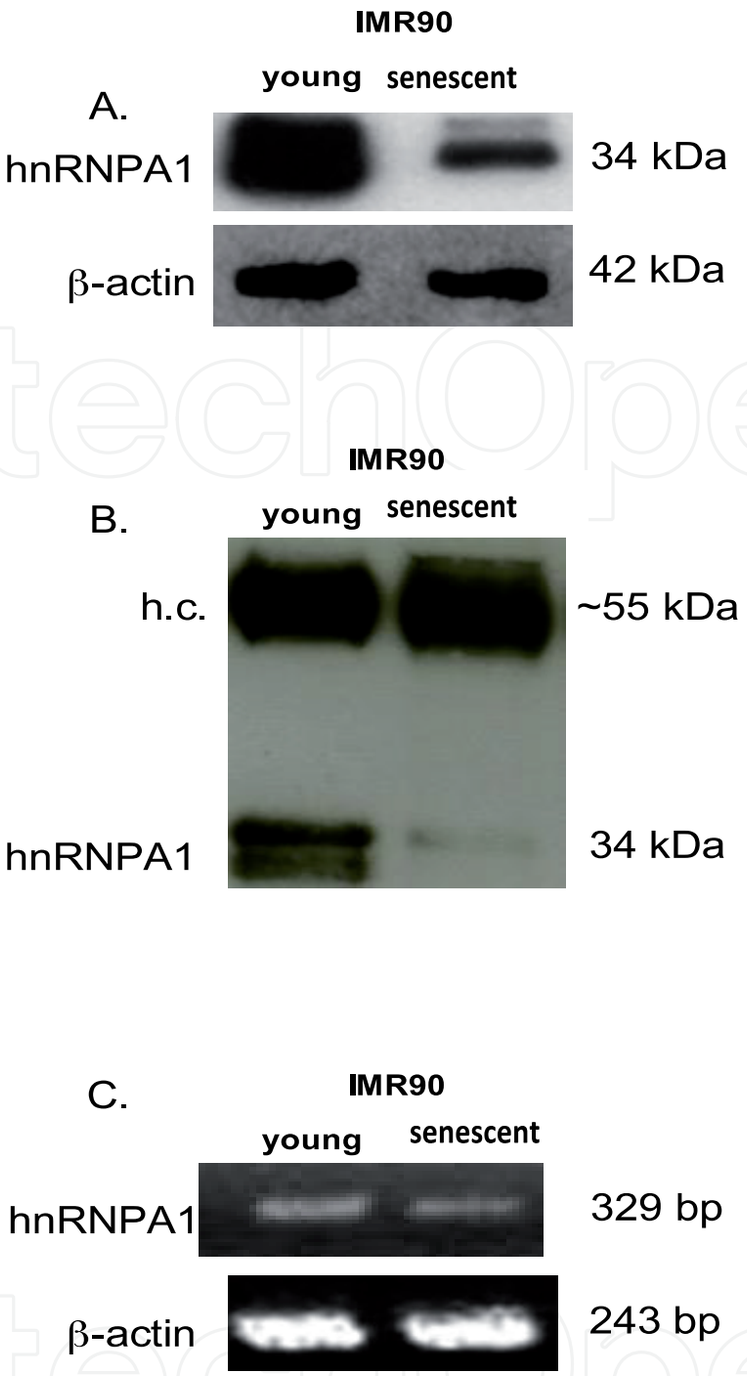

Figure 1.
A. Expression of hnRNPA1 in young and old IMR90 fibroblasts. The endogenous level of hnRNP A1 protein is significantly lower in old IMR90 fibroblasts (major band at ~34 kDa is hnRNPA1). Equivalent amounts of protein (20 μg) were loaded. B. Post-immunoprecipitation levels of hnRNPA1 were also low in old cells, see the band at ~34 kDa. C. RT-PCR on cDNA transcribed from RNA isolated complexes, using hnRNPA1 and β-actin primer sets. In both young and old lysates, there appear to be comparable amounts of hnRNPA1 mRNA species in the isolated hnRNPA1-containing complexes.

of hnRNP A1 mRNA by RT-PCR (**Figure 1C**). We found that the 4B10 monoclonal antibody immune-precipitated hnRNP A1 mRNA in RNP complexes from both young and senescent lysates. While the level of hnRNP A1 mRNA was lower in senescent RNP complexes, it was sufficient enough to determine putative mRNA targets.

2.2 Analysis of the hnRNPA1-mRNA complex

RNA that was isolated from the hnRNP A1-complexes was reverse transcribed and then amplified by PCR using random decamers to amplify all cDNA sequences.

We used two different concentrations of cDNA template for PCR as it was not always possible to visualize PCR products in the senescent samples because the cDNA abundance is typically lower in these cells. There was a correlative increase in PCR products as shown in **Figure 2** with an increase in cDNA template. PCR products were then immediately ligated into a pCR2.1cloning vector.

There may also be a differential availability of hnRNP A1 protein in old cells as hnRNPA1 is modified by post-translational events, such as phosphorylation and methylation [13, 14]. There is an additional possibility that rearrangements of individual components in hnRNPA1-mRNA ribonucleoparticles may change during senescence, which could alter specific and non-specific mRNA sequences bound in the complex.

2.3 Identification of RNA species in hnRNP A1-mRNP complexes

We then determined the identity of the cloned inserts by sequencing. We found that there were partial mRNA sequences for four human genes bound in young and senescent RNP complexes. The identity of genes was identical for both young and old cells. Scores were considered to be positive if the similarity score was more than 200 [15]. The genes identified were Homo sapiens *HDM2* gene (AF144029), intron 9 and exon 10, partial sequence; H. sapiens asthmatic clone 1 mRNA (AF095853), 3□ UTR; H. sapiens D15S1506 ca repeat region (AF018071), complete sequence, and H. sapiens partial *HR* gene for hairless protein (AJ277249). As these four human genes were the only gene candidates identified, we chose to score the sequences by the number of times a positive hit occurred in a (BLAST) similarity search for individually isolated clones. The sequence for *HDM2* that occurred most frequently was further analyzed to determine potential splicing enhancer/silencer elements by Zhang, et al. [16, 17] using the PESX utility (http://cubweb.biology.

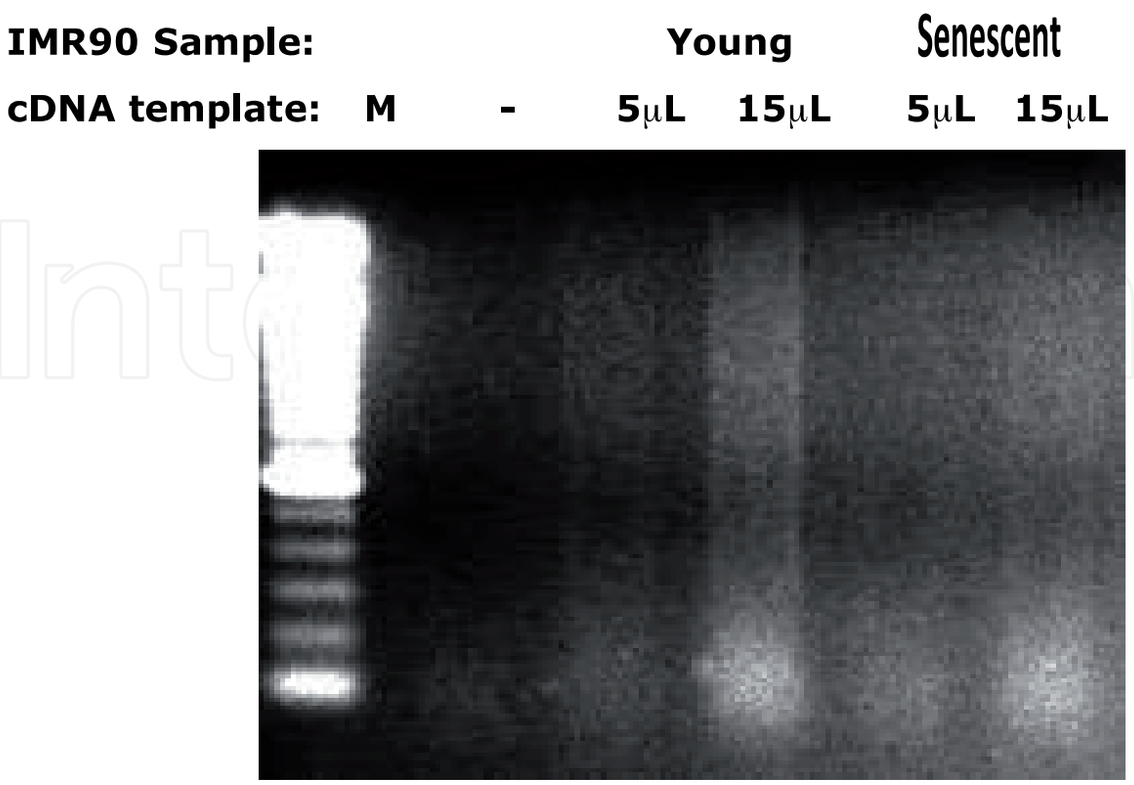

Figure 2.
Analysis of hnRNP A1 RNP complexes. A. RNA isolated from hnRNPA1 complexes was reverse transcribed to cDNA and amplified using random decamers as primers for the reaction. No amplification was observed in the absence of cDNA as indicated in the (−) lane.

columbia.edu/pesx/). **Figure 3** shows a schematic illustration of the *HDM2* mRNA sequence that binds to hnRNP A1. The binding site is between exon 9 and intron 10. The regions marked in red are putative silencer sequences. We found that there were several matches to these sequences within the isolated *HDM2* mRNA from hnRNPA1 RNP complexes.

The identification of the human double minute 2 gene (*HDM2*) was of particular interest to us. It is the human homolog of the *mouse double minute 2 (MDM2)* and is a known oncogene [18–20]. It is a protein of ~90 kDa size and is usually localized in the nucleus of cells. Overexpression of HDM2 causes cells to proliferate uncontrollably as it facilitates the proteasomal degradation of p53 by acting as a ubiquitin ligase [21, 22].

The human murine double minute 2 (*HDM2*) gene is a 33-KB nucleotide sequence located on chromosome 12 (q14.3-q15). The gene consists of 12 exons and 11 introns [18, 20]. Transcription of the *HDM2* gene is controlled by two different promoters, referred to as P1 and P2 that are P53-independent and P53-dependent, respectively [19]. The P1 promoter controls the basal expression of *HDM2* and is positioned upstream in the first exon of the *HDM2* gene [19, 20]. Transcription from the P2 promoter is highly regulated, responsible for the inducible expression of *HDM2*, and is found in the first intron [21]. The *HDM2* transcript is translated into a protein of 491 amino acids with multiple sizes ranging from about 50 to 90 kDa [22]. HDM2 can be regulated positively by AKT, a serine-threonine kinase, leading to the repression of p53 activity. AKT phosphorylates HDM2, which leads to its nuclear entry and subsequent attenuation of p53 activity and p53 degradation [23]. p14 (ARF), a protein product from the CDNK2A locus, is a negative regulator of *HDM2* [24]. The p14ARF protein binds to the central domain of HDM2, including the acidic region, leading to inhibition of the ability of HDM2 to act on p53 [25].

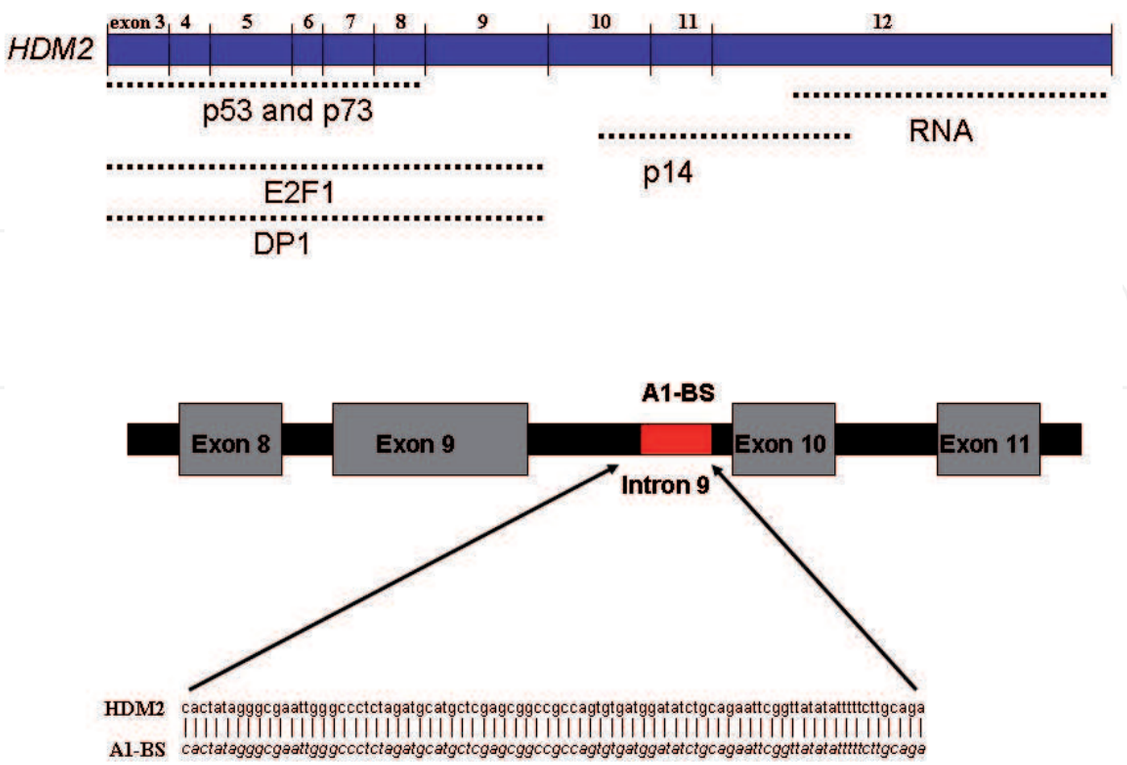

Figure 3.
PESX analysis. PESX (putative exon spliyancer/silencers) is a utility that can predict regions of an mRNA that may be involved in exon splicing to enhance the inclusion or exclusion of an exon. In addition to the identification of the putative splicing regulatory sequences, the sequences were also scanned for putative hnRNPA1-binding sites [UAG (G/a)] based on known binding sites.

To determine whether the full-length *HDM2* mRNA was bound to hnRNP A1 in RNP complexes, we isolated RNA from the complexes and used semi-quantitative to amplify full-length sequences. We found full-length *HDM2* in total RNA and complexes isolated from young cells (**Figure 4A**). To provide an estimation of the nature of the *HDM2* exons normally present in young fibroblasts, we amplified sequences from exon 1 to 2, exon 6 to 7, and exon 9 to 10. **Figure 4B** shows that of the regions amplified, the one that covered exon 9 to 10 was in the least abundance by a factor of 4 when compared with the most abundant region that spanned from exon 6 to 7. These results indicate that there may be preferential exon inclusion/exclusion in young fibroblasts in which hnRNP A1 is highly expressed.

2.4 Identification of p16INK4a mRNA in hnRNPA1 complexes

We have previously shown that changes in the expression of hnRNP A1 regulate the alternative splicing and mRNA levels of two mRNA isoforms of the INK4a locus known as $p14^{Arf}$ and p16(INK4a) [10]. Both protein isoforms are growth suppressors and knockout of the INK4a gene allows cells to escape cellular senescence [11]. Our previous studies have shown [9] that overexpression of hnRNA1 results in a preferential expression of the $p14^{Arf}$ mRNA isoform, and an increase in the mRNA levels of both isoforms, thus suggesting a role for hnRNP A1 in control of cell proliferation and senescence [10]. In this study, we assessed the ability of hnRNP A1 to directly bind to INK4a transcripts in hnRNPA1 complexes. For this, we used AR5 cells and PRNS-1 (SV40-transformed clones of HS74 primary human bone marrow fibroblasts) since these cells express high levels of INK4a transcripts as compared with normal IMR-90 fibroblasts [26].

Figure 5 shows that the p16 transcript was amplified from hnRNPA1RNP complexes indicating that hnRNP A1 directly binds to p16 mRNA. We also measured the ability of hnRNP A1 to bind to actin mRNA as a positive control for our co-immunoprecipitation studies. Actin mRNA has been previously identified in hnRNPA1-RNP complexes [12]. **Figure 5** shows that in addition to p16, we were also able to detect actin mRNA in the RNP complexes.

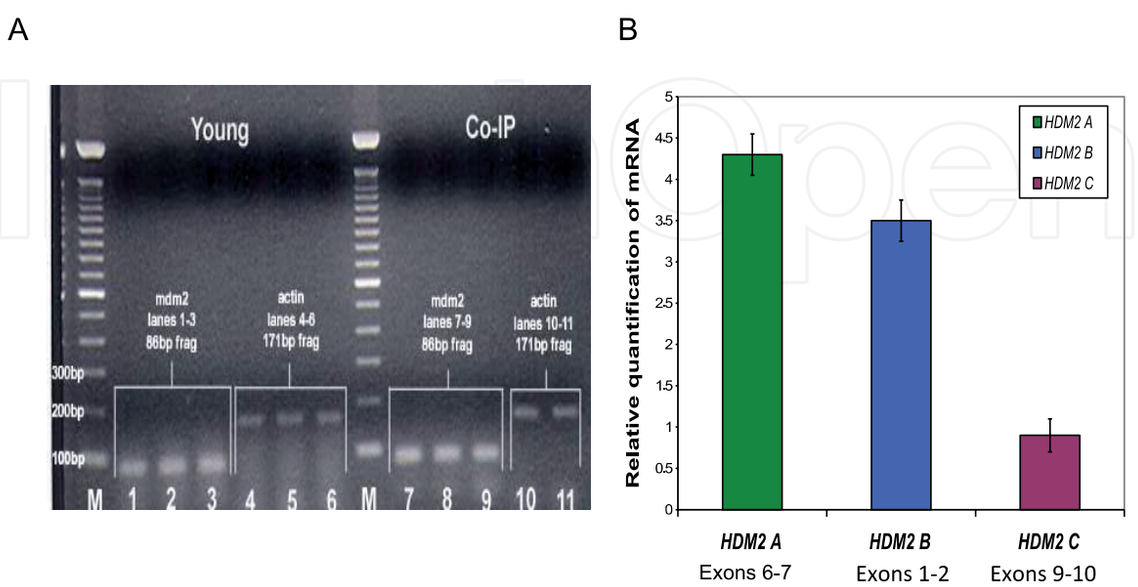

Figure 4.
HDM2 mRNA variant expression levels. RNA was isolated from co- immunoprecipitated hnRNPA1 protein complexes from young IMR90 fibroblasts and reverse transcribed. Three independent replicates of cDNA were used as templates for detecting HDM2 regions: Exons 6–7, HDM2A; exons 1–2, HDM2B and exons 9–10, HDM2C. Taqman primer-probe sets specific for these regions of the HDM2 gene were used in qRT-PCR assays and compared to actin mRNA levels.

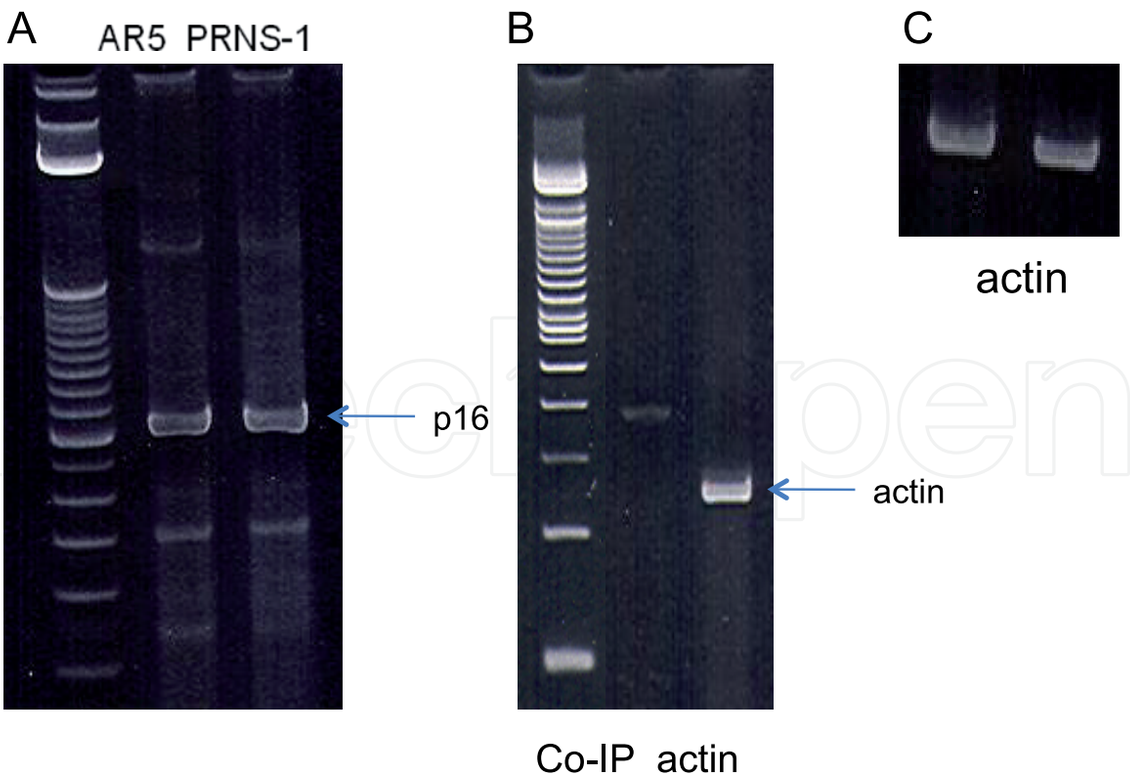

Figure 5.
Immunoprecipitation of specific mRNA hnRNP A1 RNP complexes. RNA was extracted from co-immunoprecipitated hnRNPA1 protein complexes isolated from AR5 and HS74-PRNS-1 cells and reverse transcribed. Primers set specific for p16 and actin were used to identify their respective mRNAs in hnRNP A1 RNP complexes. AR5 and HS74-PRNS-1 cells are SV40-transformed immortal cell lines.

2.5 Expression of *HDM2* is modulated by hnRNPA1 expression levels

We next sought to determine whether hnRNPA1 modulated the expression of *HDM2* mRNA levels. hnRNP A1 was overexpressed in young IMR-90 human fibroblast cells followed by real-time RT-PCR analysis using primer sets that amplified different regions of *HDM2* mRNA. We observed a significant decrease in the full-length *HDMd2* mRNA levels in cells overexpressing hnRNP A1 as compared with cells expressing the empty GFP Vector (**Figure 6B**). Downregulation of hnRNP A1 by siRNA transfection showed increased levels of *HDM2* mRNA (**Figure 6A**). These results indicate that hnRNP A1 protein levels modulate the mRNA levels of *HDM2*. Since we had previously shown that hnRNP A1 expression and its subcellular distribution were altered during cellular senescence [27], we compared the endogenous *HDM2* mRNA levels in young and senescent cells. We found that there was a significant increase in *HDM2* mRNA levels in senescent cells as compared with young cells (**Figure 6C**). These results show that endogenous levels of HDM2 were consistent with our overexpression and siRNA results discussed earlier.

We also investigated whether the protein level of HDM2 was modulated by the level of hnRNP A1 protein expression. To determine whether endogenous hnRNP A1 has an effect on HDM2 protein expression, scrambled siRNA or siA1 was transfected into IMR-90 fibroblast cells. We found that upon siRNA knockdown of hnRNP A1, the protein level of HDM2 was not altered as shown in **Figure 7A**. hnRNP A2, which has overlapping biochemical activity with hnRNP A1, when inhibited by siRNA interference, did not affect HDM2 expression. On the other hand, overexpression of hnRNP A1 in young cells transfected with GFP-A1 resulted in a slight decrease of HDM2 protein levels and an increase in p53 levels when compared with cells transfected with the GFP-Empty vector (**Figure 7B**). The increase in p53 protein levels may be a result of the decreased HDM2 expression. A direct

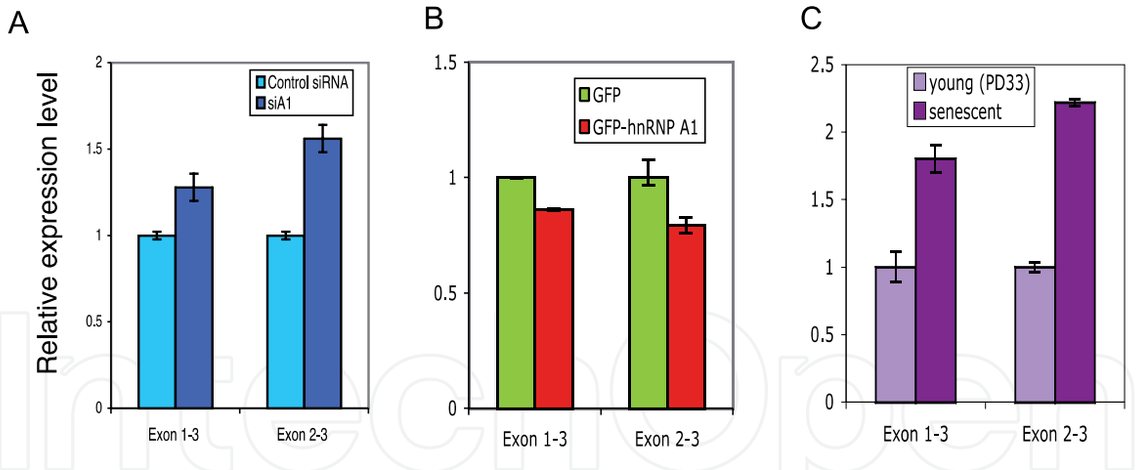

Figure 6.
Expression of HDM2 mRNA levels following alteration of hnRNP A1 expression. Panel a: Scrambled siRNA (control) or siA1 oligonucleotides were transfected into IMR-90 fibroblast cells. Real-time PCR was performed using primers for constitutive HDM2 mRNA (exon 1–3) and p53-inducible HDM2 mRNA (exon 2–3). RPLPO mRNA levels were used as an internal control. Panel B: Expression plasmids pEFGP empty vector or pEFGP-A1 were transfected into IMR-90 fibroblast cells. After 2 days of incubation, total RNA was extracted from cells and real-time PCR was performed with different sets of primers for detection of constitutive and p53-inducible HDM2 mRNA. RPLPO and GAPDH RNA levels were used as internal standards. Panel C: The steady-state endogenous levels of constitutive and p53-inducible HDM2 were measured in young and senescent mRNA.

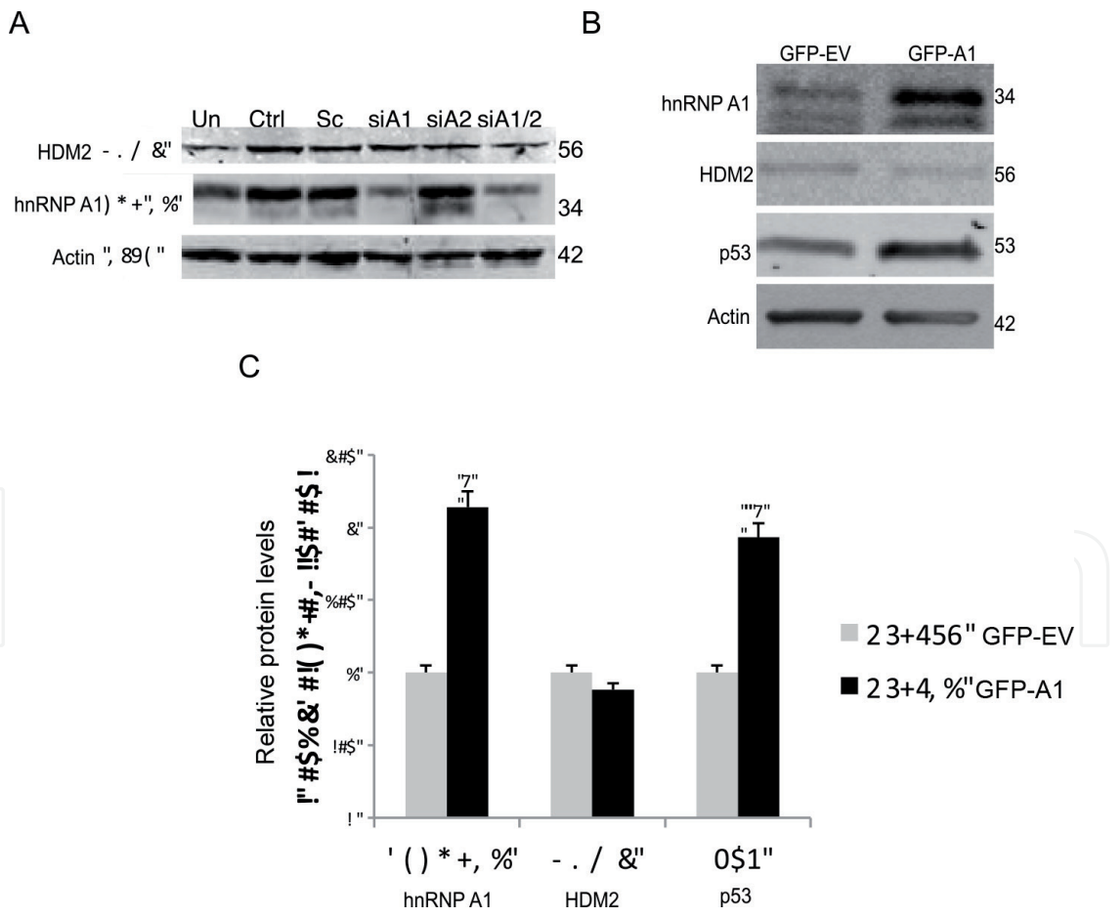

Figure 7.
*Effect of varying hnRNP A1 expression on HDM2 protein levels a. scrambled siRNA (control), siA1, siA2, or both oligonucleotides were transfected into IMR-90 fibroblast cells. About 30 μg of protein lysates were subjected to 12% SDS-PAGE and immunoblotted for hnRNPA1 and actin. The membranes were stripped and reprobed for total actin levels. B. IMR-90 cells were transiently transfected with GFP-hnRNP A1 and empty vector for 48 hours. Whole-cell lysates were prepared using RIPA lysis buffer. 30 μg of protein lysates were simultaneously subjected to 12% SDS-PAGE and immunoblotted for hnRNP A1, HDM2, p53 and actin. The membranes were stripped and reprobed for total actin levels. C. the relative protein levels was quantified by image density analysis software (image J), and the data were represented as mean value ± SEM (n = 3, and * $p < 0.05$ value).*

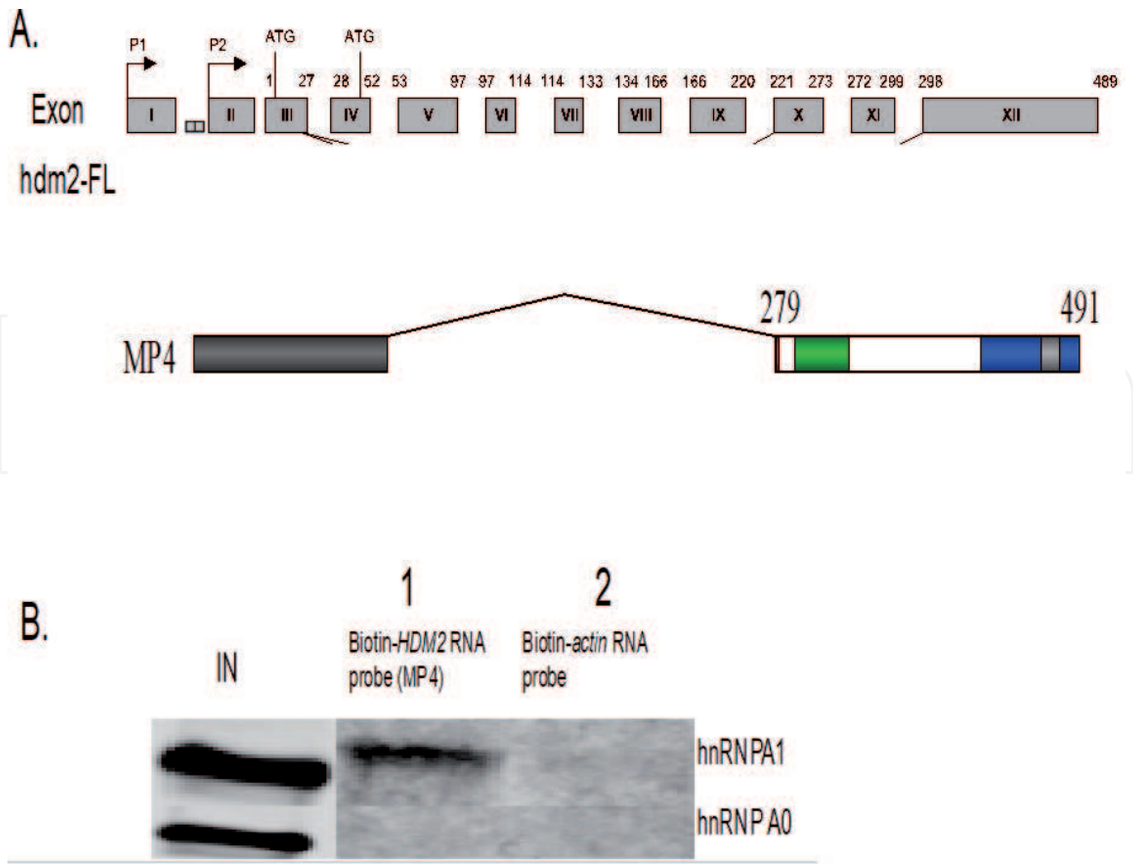

Figure 8.
HDM2 transcript (MP4) binds to hnRNP A1. A. Schematic representation of full-length HDM2 and mini-protein "MP" fragment of HDM2 MP4. B. the interaction between hnRNP A1 and biotinylated HDM2 mRNA (MP4) was examined by biotin pull-down assay. Biotinylated HDM2 and actin probes were incubated with IMR-90 cell lysates. Interactions were analyzed by Western blotting. The protein-RNA probe streptavidin complexes were subjected to electrophoresis on a 12% SDS-PAGE. The 4B10 monoclonal antibody and anti-actin antibody were used to detect hnRNP A1 and actin, respectively.

correlation between protein and mRNA levels for any given gene is complicated by varying processes. For instance, studies conducted by various groups such as Vogel et al. [28] pointed out that transcription, mRNA export, decay, translation, and protein degradation are key processes in determining steady-state protein concentration [28].

2.6 Identification of *HDM2* RNA sequences that bind to hnRNP A1

Our PESX analysis revealed that hnRNP A1 has a putative binding site within the intronic region between intron 9 and exon 10 of HDM2 (**Figure 4**). We obtained HDM2 constructs from Dr. Meek (University of Dundee). We performed biotin pull-down assay using MP4 construct that is similar to the MDM2-B isoform lacking p53-binding region followed by Western immunoblotting. We performed the biotin pull-down assay by first incubating the hnRNP A1 antibody (4B10) with Dynabeads Myone streptavidin. We also incubated the ^{Biotin}labeled *HDM2* mRNA (MP4 probe) or ^{Biotin}labeled *B*-actin RNA probe with IMR-90 cell lysate and the mixture was incubated overnight. We performed Western blot analysis as described in Material and Methods to investigate the interaction between ^{Biotin}labeled *HDM2* mRNA and hnRNP A1. As shown in **Figure 8**, hnRNP A1 is directly associated with ^{Biotin}labeled *HDM2* mRNA (MP4 probe). The direct association between hnRNP A1 and ^{Biotin}labeled *HDM2* mRNA appears to be specific since these interactions were not observed with similar RNA-binding protein hnRNP A0. Furthermore, associations

between hnRNP A1 and negative-control ^{Biotin}labeled *B*-actin were not observed. These results demonstrate that hnRNP A1 has specific binding to ^{Biotin}labeled *HDM2* mRNA (MP4 probe). These results also show that hnRNP A1 can bind to the 3′ end of HDM2 as MP4 is the 3′ of HDM2 which is also a region devoid of HDM2 sequences upstream of exon 11.

3. Discussion

The role of hnRNP A1 during cellular senescence is unclear. Significant alterations in its levels, localization, and activity in senescent cells suggest that hnRNP A1 may contribute to the senescent phenotype [27]. However, only a few gene targets are known for hnRNP A1 [12]. This prompted us to search for additional mRNA targets for hnRNP A1 in young and senescent IMR-90 cells. We used an RNA co-immunoprecipitation protocol [12] to identify mRNA new targets for the hnRNP A1 protein. We found that hnRNP A1 is bound to several mRNAs not previously identified. Of particular interest to us was the observation that hnRNP A1 bound to *HMD2* mRNA. Other RNA-binding proteins have been reported to bind to specific regions in *HDM2* mRNA. La antigen, an RNA-binding protein, was found to interact with HDM2 5′UTR in a BCR/ABL cell line resulting in increased HDM2 expression [29]. It was further demonstrated that translational regulation contributed to the increased HDM2 levels in BCR/ABL cells [29]. Nucleolin, a multifunctional nucleolar protein with defined roles in ribosomal RNA processing, has also been reported to bind to the NLS/NES and RING domain of HDM2 [30]. The expression of *HDM2* mRNA is transcriptionally regulated by p53 in response to stress such as DNA damage [31, 32]. We have found that the modulation of hnRNP A1 expression can regulate *HDM2* mRNA levels.

Posttranscriptional regulation of gene expression is important for the control of cellular processes such as cell proliferation, differentiation, development, and apoptosis [33]. RNA-binding proteins are the main regulators of post-transcriptional regulation [33]. hnRNP A1 is a multifunctional RNA-binding protein implicated in the regulation of major steps in posttranscriptional regulation of gene expression [14]. Upon observation that hnRNP A1 binds to *HDM2* mRNA, we sought to determine whether the modulation of the expression of hnRNP A1 had an effect on steady state *HDM2* mRNA levels. Given that our previous results demonstrated that overexpression of hnRNP A1 significantly decreased *HDM2* mRNA levels, we next asked whether the HDM2 protein levels were also lowered when hnRNP A1 was overexpressed. Our Western blot analysis data revealed that overexpression of GFP-A1 slightly decreased HDM2 protein levels (**Figure 7B**), whereas knockdown of hnRNP A1 did not have effect on HDM2 protein expression. We also observed that p53 levels increased upon GFP-A1 overexpression suggesting that the levels of p53 protein were increased by the decreased expression of HDM2 (**Figure 7B**). Previous studies have shown that HDM2 regulates p53 by targeting it for degradation [19]. Recent studies have demonstrated that overexpression of hnRNP A1 led to a reduction of *HDM2*-FL transcript levels in HaCat cells [34]. More importantly, it was also shown that UVB irradiation increased the binding of hnRNP A1 to *HDM2* pre-mRNA [34]. Our studies are consistent with these findings whereby we have found that overexpression of hnRNP A1 decreased *HDM2* transcript levels.

In this study, our findings suggest that hnRNP A1 binds to the ^{Biotin}labeled *HDM2* mRNA probe (MP4) that includes part of exons 11 and 12 of *HDM2* mRNA as shown in **Figure 8A**. Previous approaches as those used by S.J. Park et al. [35] demonstrated that hnRNP A1 associates with Drp1 mRNA at the 3′ UTR [35]. We applied this approach and found that hnRNP A1 binds to the *HDM2* MP4 mRNA

and that this binding was specific as hnRNP A0 did not bind to the *HDM2* MP4 probe (**Figure 8B**). Our sequencing data of the HDM2 MP4 reveal that it contains a putative G/AGAAG nucleotide sequence similar to the 5′AGAAG 3′ high-affinity binding site found in the purine-rich 3′ splice site of c-src mRNA exon N1 [36]. hnRNP A1 has been shown to bind to these sites in c-H-ras and HIV TAT [37, 38]. Overall, our RNA-protein interaction experiments data strongly suggest that hnRNP A1 interacts with a region of *HDM2* transcript corresponding to its 3′ UTR. It has been previously reported that RNA-binding protein RNPC1 binds to the 3′UTR region in *HDM2* transcripts and inhibits its expression [39]. Thus, the *HDM2* 3′ UTR is bound by different RNA-binding proteins that might either repress or induce its expression. For example, HuR, an RNA-binding protein, has been shown to bind and stabilize *HDM2 via* its 3′ UTR. From our studies, we found that hnRNP A1 modulated the mRNA expression of HDM2. Therefore, the MP4 sequence maybe partially contributing to this modulation. Our findings are significant when taken in the context of RNA-binding protein contributing to the aging phenotype. Both HuR and hnRNPA1 are involved in regulating the senescent phenotype [42, 43]. Inhibiting HuR expression induces the senescent phenotype [43]. hnRNP A1 has been recently shown to antagonize cellular senescence through the SIRT1 pathway [44]. The research findings of the research project are important because they can add to the knowledge of the regulation of HDM2 gene expression.

4. Materials and methods

4.1 Cell culture and generation of senescent fibroblasts

The human lung fibroblast cell strain IMR90 from Coriell, NJ, was subcultured from early passage to terminal passage as previously described by Hubbard and Ozer [40] in Dulbecco's modified Eagle's medium and Ham's F10 medium in a 1:1 mixture supplemented with 10% fetal bovine serum. IMR90 fibroblasts at population doubling <35 were used in all experiments and are considered comparable to young fibroblasts as determined by gene expression profiles previously performed [40]. Senescent IMR90 in all experiments was at a population doubling of 62.
For transfection experiments, once cells had reached 90% confluence, either the expression plasmid pEFGP (Control) or pEFGP-A1 was transfected into IMR-90 cells in DMEM/F10 media without FBS/penicillin using Lipofectamine 2000 (Invitrogen) and incubate at 37°C in a CO_2 incubator for 6 h.

4.2 RNA isolation and RNA-PCR to check for genomic contamination

After RNA was isolated as detailed above, to ensure that there was no genomic contamination, an RNA–PCR procedure was performed. 2 μL of template RNA was added to a PCR mixture using β-actin primers, which would detect genomic sequences if present. The total PCR reaction was composed of 2 μl of template RNA, 5 μl of 10× RT-PCR Buffer (100 mM Tris–HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 2.5 μl of dNTP mix (2.5 mM each dNTP), 0.25 μM each PCR primer, 0.5 unit of Thermostable DNA Polymerase (Novagen).

The primers for actin were: actin forward (5′CGCCGCCCTAGGCACCA3′) and actin reverse (5′TTGGCCTTAGGGTTCAGGGGGG 3′). For hnRNPA1, primer set were: hnRNP A1 forward (5′CTAAAGAGCCCGAACAGCTGAG 3′) and hnRNP A1 reverse (5′TCAGTGTCTTCTTTAATGCCACCA 3′). SYBR™ green stain. SYBR™ green can be visualized by blue fluorescence (Molecular Dynamics, Amersham) and quantified with ImageQuant software (Amersham).

5. Immunoblotting and protein analysis

Standard Western blotting protocols (Harlow et al. 1999) were to analyze specific proteins [41]. Protein extracts isolated from young and senescent fibroblasts were generated by washing cells were washed 3 times with 1X cold PBS and then, cultures were placed on ice. Cold RIPA (radioimmunoassay buffer containing NP-40 at 1%, sodium deoxycholate at 1%, sodium dodecyl sulfate at 0.1%, NaCl at 150 mM and Tris-HCl at 10 mM with protease inhibitors leupeptin at 0.1 μg/ml, pepstatin at 0.1 μg/ml, and phenylmethylsulfonyl fluoride at 1 mM) was added to culture dishes followed by scraping cells into cold microfuge tubes. The lysate was passed through a 21-gauge syringe needle to ensure complete lysis. Lysates were centrifuged at 10,000×g for 10 minutes at 4°C. The cleared lysate was collected and aliquots were prepared to estimate the amount of protein by the Bradford protein assay (Bio-Rad). Lysates were run on 8–12% acrylamide gels and then transferred in an electroblotting apparatus. Membranes (PVDF, Osmonics) were blocked with 5% non-fat milk in PBS. Monoclonal antibody 4B10 (1:10,000) was used to detect hnRNPA1/A1. An anti-actin monoclonal antibody (1:5000) was obtained from Chemicon. Antibodies specific for HDM2 were graciously provided by Dr. Jill Bargonetti. Secondary antibody, goat IgG, or mouse IgG conjugated with HRP were used for visualization of bands using the ECL kit (Amersham).

6. Overexpression of hnRNP A1 by transient transfection

Young IMR-90 cells were cultured in 10-cm plates. After approximately 24 h of incubation when the cells reached 90% confluence, the expression plasmid pEFGP (control) or pEFGP-A1 was transfected into IMR-90 cells in DMEM/F10 media without FBS/penicillin using Lipofectamine 2000 (Invitrogen) and incubated at 37°C in CO_2 incubator for 6 h. We changed the media to DMEM with FBS and without penicillin and incubated for 48 h at 37°C.

7. RNA co-immunoprecipitation protocol

The RNA co-immunoprecipitation protocol was a modified version published by Mili et al. [12] that included a short immunoprecipitation step that minimized degradation of protein-associated RNA.

8. Confirmation of gene expression using real-time PCR

Real-time PCR experiments on selected genes were performed using an Applied Biosystems 7500 real-time PCR system that utilizes TaqMan gene expression assays for the following genes: mdm2; Human GAPD (GAPDH) Endogenous Control FAM/MGB (4333764F). Reactions were performed according to standard methods using the universal 10X PCR TaqMan mix, at a final reaction volume of 25 μL (Applied Biosystems).

9. Cloning protocol and sequencing

PCR products were ligated into the pCR 2.1 (Invitrogen, TA cloning kit) cloning vector that utilizes the single dT overhangs that are a by-product of PCR reactions catalyzed by Taq polymerase. The ligation reaction was performed at 14°C overnight using T4 DNA ligase and 3 μL of fresh PCR product (Invitrogen protocols).

10. Sequence analysis using BLAST and PESX

Vector sequences were subtracted from the sequences obtained. The rest of the sequence was compared against known sequences using the BLAST tool (www.ncbi.nlm.nih.gov). Sequences were chosen based on being previously identified as human genes and either in the coding regions or in the flanking regions of the mRNA sequences of known genes.

11. Biotin pull-down assay

Biotinylated transcripts were obtained by reverse transcription with the Maxiscript Kit (Invitrogen) according to manufacturer instructions and as previously described above in Section 3.2. The biotin pull-down assay was performed by first incubating hnRNP A1 antibody (4B10) with Dynabeads Myone streptavidin (Invitrogen) for 1 hour at 4 C. Also, incubated ^{Biotin}labeled *HDM2* mRNA (MP4 probe) or B-actin RNA probe with 25 μg of IMR-90 cell lysate for 1 h at RT. Following this incubation, we added the biotinylated RNA probes and protein lysate mixtures to the Myone streptavidin beads coated with 4B10 (hnRNP A1 antibody) and performed a second overnight incubation, immediately subjected the protein-RNA complexes to Western blot analysis as described in Section 2.4 to detect specific proteins bound to biotinylated transcripts.

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Conflict of interest and financial disclosures

There are no conflicts of interest nor financial interest or benefits.

Abbreviations

HDM2	Human Double Minute 2
UTR	Untranslated region
hnRNPA1	Heterogeneous nuclear ribonucleoprotein A1
RNP	Ribonucleoprotein
MP4	Biotin-labeled HDM2 RNA probe
mRNP	Messenger ribonucleoprotein

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