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HDM2-Binding Partners: Interaction with Translation Elongation Factor EF1 α

Rebecca Frum^{†,‡}, Scott A. Busby[§], Mahesh Ramamoorthy[†], Sumitra Deb[†], Jeffrey Shabanowitz[§], Donald F. Hunt^{§,||}, and Swati P. Deb^{*,†}

Department of Biochemistry and the Massey Cancer Center, Virginia Commonwealth University, P.O. Box 980614, Richmond, Virginia 23298, Department of Chemistry, University of Virginia, Charlottesville, Virginia 22904, and Department of Pathology, University of Virginia, Charlottesville, Virginia 22904

Abstract

To understand the cellular functions of HDM2, we attempted to identify novel HDM2-interacting proteins by proteomic analysis. Along with previously identified interactions with the ribosomal proteins, our analysis reveals interactions of HDM2 with the ribosomal translation elongation factor EF1 α , 40S ribosomal protein S20, tubulins, glyceraldehyde 3-phosphate dehydrogenase, and a proteolysis-inducing factor dermicitin in the absence of tumor suppressor p53. Because a CTCL tumor antigen HD-CL-08 has high degree of homology with EF1 α , we confirmed interaction of HDM2 with EF1 α by immunoprecipitation and Western blot analysis in transformed as well as near normal diploid cells. Endogenous HDM2- EF1 α complex was detected in cancer cells overexpressing HDM2, suggesting a possible role of this interaction in HDM2-mediated oncogenesis. Consistent with their interaction, colocalization of HDM2 and EF1 α can be detected in the cytoplasm of normal or transformed cells. Amino acid residues 1–58 and 221–325 of HDM2 were found to be essential for its interaction with EF1 α , suggesting that the interaction is independent of its other ribosomal interacting proteins L5, L11, and L23. Overexpression of HDM2 did not affect translation. Because EF1 α has been implicated in DNA replication and severing of microtubules, interaction of HDM2 with EF1 α may signify a p53-independent cell growth regulatory role of HDM2.

Keywords

HDM2; EF1 α ; proteomics analysis

Introduction

Amplification of the mouse double minute-2 (mdm2) gene has been shown to enhance tumorigenic potential of murine cells.^{1,2} Consistent with this finding, the human homologue

*To whom correspondence should be addressed. Address: Goodwin Research Laboratory, P.O. Box 980035, 401 College Street, Virginia Commonwealth University, Richmond, VA 23298. Phone: 804-828-9541. Fax: 804-827-1427. spdeb@vcu.edu.

[†]Virginia Commonwealth University.

[‡]Present Address: Pathology and Laboratory Medicine, University of North Carolina, Chapel Hill, NC 27514.

[§]Department of Chemistry, University of Virginia.

^{||}Department of Pathology, University of Virginia.

of the *mdm2* gene is frequently overexpressed in various human breast tumors and carcinomas, soft tissue sarcomas, and other cancers, suggesting that human MDM2 (HDM2) overexpression may be one of the common causes of oncogenesis.^{3–6} HDM2 has also been shown to alter drug resistance of transformed cells.⁷ In contrast to its oncogenic function, HDM2 harbors G1-arrest function in nontransformed cells.⁸ Downstream mutations in cancer cells inactivate the G1-arrest function of HDM2⁹.

HDM2 harbors multiple biochemical properties.^{5,10–12} HDM2 interacts with several growth suppressors and other proteins, including the tumor suppressor p53, the retinoblastoma susceptibility gene product Rb, and the growth suppressor p14. HDM2 recognizes the transactivation domain of p53, inactivates p53-mediated transcriptional activation,^{13–17} and targets p53 to ubiquitination.^{18–21} However, mutants of HDM2 lacking the E3 ubiquitin ligase activity can efficiently bind with wild-type p53 and inhibit p53-mediated transcriptional activation,¹⁷ suggesting that HDM2-mediated inhibition of p53-dependent transcription is a function distinct from its p53 degrading property.

Apart from inhibiting p53-mediated transcriptional activation, HDM2 can directly repress basal transcription in the absence of p53. It inhibits telomerase RNA gene.²² On the other hand, HDM2 induces expression of NF κ B/p65 transcriptionally²³. Interaction of HDM2 with histone²⁴ and histone deacetylases²⁵ may account for its transcription regulatory functions. Furthermore HDM2 interacts with ribosomal proteins,^{26–30} which has been implicated in its p53 degrading functions. These reports suggest that HDM2 is a multifunctional protein capable of modulating several biochemical pathways.

To understand the mechanisms of various functions of HDM2, we attempted to identify its binding partners using mass spectrometry. In this report, we show that along with previously known interaction of HDM2 with ribosomal proteins,^{26–28} HDM2 interacts with several other factors such as 40S ribosomal protein S20, tubulins, glyceraldehyde 3-phosphate dehydrogenase, and a proteolysis-inducing factor dermicidin and translation elongation factor EF1 α . Our data show frequent interaction of HDM2 with the translation elongation factor EF1 α . Endogenous HDM2-EF1 α complexes were detected in cancer cells overexpressing HDM2. HDM2 and EF1 α were found to colocalize in the cytoplasm of normal or transformed cells. Because HDM2 shuttles from nuclei to cytoplasm, this shuttling is required for its p53 regulatory functions, and EF1 α localizes predominantly in the cytoplasm, our observation suggests that EF1 α interacts with cytoplasmic HDM2. Two domains of HDM2, an N-terminal and a central domain, are needed for its interaction with EF1 α . These domain requirements are different from the domains needed for interaction with L5, L11, and L23,^{26–29} suggesting that the interaction is independent of its interaction with other ribosomal proteins. Overexpression of HDM2 did not affect cellular translation.

Experimental Section

Chemicals

Chemicals were mostly purchased from Sigma. Nonidet P 40 (NP40) was purchased from Fluka Biochemica. Protease inhibitors were from Calbiochem. Radioactive (³⁵S) methionine

was purchased from ICN. Growth media were from Cellgro. Fetal bovine serum was purchased from Atlanta Biologicals. Trypsin was purchased from Promega.

Plasmids and HDM2 Deletion Mutants

The HDM2 cDNA was a generous gift from Bert Vogelstein.¹⁴ Construction of plasmids expressing the full-length HDM2 has been described earlier in detail.^{8,15,17}

Cells and Transfections

Normal diploid WI38 and H1299 lung cancer cells were purchased from American Type Culture Collection and were maintained in media as suggested by the suppliers. OsACL cell line was a kind gift from A.T. Look.³¹

WI38 or H1299 cells were seeded at $0.5\text{--}1 \times 10^6$ cells per 10 cm dish and either transfected by the calcium phosphate method^{8,15,17} or nucleofected using a nucleofector kit from Amaxa Biosystems using supplier's protocol. The cells were transfected with plasmids (5–10 μg) that express wild-type HDM2 or the deletion mutants as described earlier. Twenty-four hours after transfection, the cells were collected for immunoprecipitation experiments.

Cycloheximide Treatment

Actively growing H1299 cells were exposed to cycloheximide (10 $\mu\text{g}/\text{mL}$) or the vehicle dimethyl sulfoxide (DMSO) for 16 h prior to metabolic labeling.

Preparation of Cell Lysates

To prepare lysates, cells were harvested by trypsinization, washed in phosphate buffered saline (PBS), and suspended in a buffer A containing 10 mM Hepes (pH 7.9), 10 mM KCl, 1.5 mM MgCl_2 , 0.34 M sucrose, 10% glycerol, 1 mM DTT, 0.1 mM PMSF, and 1 $\times$ protease inhibitor cocktail (Calbiochem, set III). Cells were then lysed by the addition of Triton X-100 (final concentration 0.1%) for 5 min on ice. Nuclei were collected by low-speed centrifugation at 1300g for 5 min at 4 °C. Cell lysis and nuclei were visualized under light microscope. The supernatant was further clarified by high-speed centrifugation (15 min, 20 000g at 4 °C) to remove cell debris and insoluble aggregates. Nuclei were washed once in buffer A and then lysed in 3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, and protease inhibitors as described above. The nuclear extract was then diluted with a buffer to make the final concentration 50 mM HEPES, pH 7.9, 250 mM NaCl, 0.3% NP 40, 0.1% Triton X-100, 50 mM sodium phosphate (pH 7.0), 5 mM NaF, 1 mM DTT, 0.1 mM PMSF, and 1 $\times$ protease inhibitor cocktail (Calbiochem, set III).

Metabolic Labeling

Sixteen to twenty-four hours after transfection, cells were starved in methionine-free media for 1 h and were metabolically labeled with ³⁵S methionine for 2 h. After labeling, cells were washed three times with PBS and were lysed in a buffer containing 50 mM HEPES, pH 7.9, 250 mM NaCl, 0.3% NP 40, 0.1% Triton X-100, 50 mM sodium phosphate (pH 7.0), 5 mM NaF, 1 mM DTT, 0.1 mM PMSF, and 1 $\times$ protease inhibitor cocktail (Calbiochem, set III). An aliquot of the extracts was treated with alkali and subsequently

precipitated with trichloroacetic acid (TCA) to determine the incorporated acid insoluble radioactivity. The precipitates were collected on glass fiber filters and were washed subsequently with TCA and ethanol to remove adhered radioactivity. Filters were then dried and counted using a scintillation counter. Extracts containing equal amounts of acid precipitable radioactivity were used for immunoprecipitation experiments.

Immunoprecipitation

Cell (or cytoplasmic and nuclear) extracts were clarified with IgG-coupled agarose (Santa Cruz) and were subjected to immunoprecipitation using an anti-HDM2 antibody directly coupled with agarose (Santa Cruz, SMP14). Background interactions (negative control, Table 1) were determined by immunoprecipitation with a mouse isotype IgG coupled with agarose. Immunoprecipitates were washed in the respective binding buffers three times, one time with 0.8 M LiCl followed by one wash with 10 mM phosphate buffer, pH 7.0. The beads were either boiled in Laemmli loading buffer for gel electrophoresis or processed for mass spectrometric analysis.

Elution of HDM2-Bound Proteins

For mass spectrometric analyses, immunoprecipitated agarose beads were equilibrated with 10 mM phosphate buffer (pH 6.8), and the bound proteins were eluted using 100 mM glycine (pH 2.5). Eluants were immediately neutralized using 0.1 volume of 0.5 M phosphate buffer (pH 8.0). The mixtures were then desalted using a Microcon filter device (molecular weight cut off 3kDa, Millipore), and proteins were collected in 0.1 M ammonium bicarbonate.

Mass-Spectrometry

Proteins eluted from the nuclear and cytoplasmic HDM2 immunoprecipitation as well as the IgG only negative control were digested with trypsin (substrate: enzyme ratio of 20:1) for 16 h at 25 °C. Peptides generated from trypsin digestions were loaded onto capillary precolumns (360 μ m o.d. $\times$ 75 μ m i.d., Polymicro Technologies Phoenix, AZ) packed with irregular C18 resin (5–20 μ M, YMC Inc., Wilmington, NC) and washed with 0.1% acetic acid at a flow rate of 5 μ L/min for 10 min. The precolumns were connected by Teflon tubing to an analytical column (360 μ m $\times$ 50 μ m i.d., Polymicro Technologies, Phoenix, AZ) packed with C18 resin (5 μ m, YMC Inc, Wilmington, NC) constructed with an electrospray emitter as previously described.³² The samples were analyzed by nanoflow HPLC-microelectrospray ionization on a Finnigan LCQ Deca XP (Thermo Electron, San Jose, CA). The gradient used on a model 1100 series HPLC (Agilent, Palo Alto, CA) consisted of 0–60% B in 60 min (solvent A = 0.1% acetic acid, solvent B = 70% acetonitrile in 0.1% acetic acid). The LCQ was operated in data-dependent mode throughout the gradient, where the mass spectrometer acquired 1 full MS spectrum and 5 subsequent MS/MS spectra for the 5 most abundant parent ions. All MS/MS spectra were searched against the NCBI human protein database using the SEQUEST searching algorithm (Thermo Electron, San Jose, CA), and the data were collated and peptide and protein probabilities were generated using the Scaffold program (Proteome Software Inc., Portland, OR).^{33,34}

Western Analysis

Western blot analysis was carried out as previously described.^{8,15} After, polyacrylamide gel electrophoresis proteins were transferred to 0.45 μ m nitrocellulose paper. EF1 α was detected using an antibody from Upstate (05–235). HDM2 was detected by AB1 (Calbiochem). β -actin antibody was purchased from Sigma (A-5441). The blots were developed using ECL purchased from Amersham.

Immunofluorescent Staining and Confocal Imaging

Immunofluorescent staining and confocal imaging were performed following a method reported by Lohrum et al.²⁷ Briefly, normal diploid WI38 or lung cancer H1299 cells were seeded on sterile coverslips in a 6-well culture dish and were transfected with 2 μ g HDM2 expression plasmid per coverslip. The cells were fixed 48 h after transfection using 4% paraformaldehyde for 10 min at room temperature. The cells were then washed thrice in phosphate buffered saline (PBS), permeabilized in ice-cold PBS containing 0.2% Triton X-100 for 5 min, and blocked in PBS containing 0.5% bovine serum albumin at room temperature for 30 min. The fixed cells were consecutively immunostained overnight at 4 °C with a FITC-coupled anti-HDM2 antibody (N20, Santa Cruz) followed by staining with an anti-EF1 α primary antibody (Upstate, 05–235) and a rhodamine-coupled goat anti-mouse secondary antibody from Jackson ImmunoResearch (115–295-146) in blocking solution. The slides were then washed thrice in PBS and once in water, air-dried, and mounted with Prolong Gold Antifade with DAPI (4',6'-diamidino-2-phenylindole hydrochloride, molecular probes). The coverslips were then analyzed under a confocal microscope (Zeiss) under 63 $\times$ magnification.

Results and Discussion

Immunoprecipitation of HDM2-Interacting Proteins and Releasing the Proteins from the Immune Complex

The primary aim of this study was to identify novel binding partners of HDM2. To enhance the amount of HDM2-protein complexes, HDM2 was overexpressed in H1299 cells by transfecting an expression plasmid that expresses HDM2 from a CMV promoter.^{8,17} To enrich for cytoplasmic and nuclear complexes, the cytoplasmic and nuclear fractions were separated after cell lysis. After clarification, extracts were subjected to immunoprecipitation with an anti-HDM2 antibody directly coupled with agarose. After immunoprecipitation, the agarose beads were washed to remove adhering or nonspecific interactions. Proteins bound to HDM2 antibody were then eluted with glycine at an acidic pH, neutralized, and desalted. An aliquot of this eluted protein was tested by Western blot analysis (Figure 1A) for the predominant presence of HDM2 in comparison to coeluted IgG. For comparison, agarose beads boiled with Laemmli loading buffer were also analyzed (Figure 1A, lanes 1 and 2). The figure shows that coelution of IgG heavy and light chains were insignificant compared to the amount of HDM2 (Figure 1A, lanes 3 and 4). Interestingly, the nuclear fraction lacked two truncated forms of HDM2 (lane 4). These data suggested successful immunoprecipitation of HDM2 and its elution from the antibody coupled agarose. Lack of the truncated forms of HDM2 in the nuclear extract suggests enrichment of cytoplasmic and nuclear complexes in the respective extracts.

Identification of HDM2 Interacting Proteins

The eluted proteins from the HDM2 immunoprecipitations were digested with trypsin and subjected to analysis by nanospray HPLC mass spectrometry.³² The tryptic peptides were identified by searching them against the human NCBI protein database,^{33,34} and the identified proteins are shown in Table 1. A number of proteins were conclusively identified in the HDM2 immunoprecipitation fractions in addition to the bait protein HDM2 itself. The protein identified with the most number of unique peptide hits was the eukaryotic elongation factor EF1 α , and this result was confirmed by manual examination of spectra identified as EF1 α (Figure 1B).

Confirmation of Binding of HDM2 with EF1 α

As shown in Table 1, our mass spectrometric analysis detected several known HDM2 interacting protein such as L5, L11, and p14 ARF. Because H1299 cells are devoid of endogenous p53, we did not detect HDM2-p53 interaction. Thus, the identified interactions are p53-independent. Among the unknown interactions of HDM2, translation elongation factor EF1 α seemed to be most frequent. Several less frequent interacting proteins such as tubulins, 40S ribosomal protein S20, a proteolysis inducing factor dermicidin and glyceraldehyde 3-phosphate dehydrogenase were also detected (Table 1). Because interaction of HDM2 with EF1 α was most frequent, and a CTCL tumor antigen HD-CL-08 has a high degree of homology with EF1 α lacking only 77 amino acid residues of EF1 α at the N-terminus,³⁵ we wished to confirm interaction of HDM2 with EF1 α . H1299 cells were transfected with HDM2 expression plasmid or vector plasmid. Cells were harvested 42–48 h after transfection, and extracts were made. Cell extracts were subjected to immunoprecipitation with an anti-EF1 α antibody or mouse IgG and protein A agarose as described in the Materials and Methods. The immunoprecipitates were analyzed by SDS polyacrylamide gel electrophoresis and Western blot analysis. The results (Figure 2A) show that anti-EF1 α antibody coimmunoprecipitates HDM2 from HDM2 expression plasmid transfected cell extracts (lane 2). HDM2 or EF1 α was not immunoprecipitated by IgG (lane 1) as expected, suggesting that HDM2 coimmunoprecipitation is not a nonspecific binding with IgG. Also, vector transfected cells did not show significant amount of HDM2 coimmunoprecipitation (lane 3) as H1299 cells express negligible amount of HDM2 caused by the absence of p53.

Next, we wished to determine if HDM2 complexes with EF1 α endogenously. For this purpose, OsACL osteosarcoma cells that overexpress HDM2 were metabolically labeled with ³⁵S methionine. Cell extracts were subjected to immunoprecipitation with either an anti-HDM2 polyclonal antibody or preimmune serum. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis and autoradiography (Figure 2B). The data show that anti-HDM2 rabbit antiserum coimmunoprecipitates EF1 α (lane 2), whereas the preimmune serum cannot (lane 1). This data suggests that HDM2 forms a stable complex with EF1 α endogenously.

Because we detected interaction of HDM2 with EF1 α in transformed cells, we wished to determine if this interaction occurs in cells that are not transformed. For this purpose, WI38 cells were transfected with HDM2 expression plasmid or vector. Transfected cells were

metabolically labeled with ^{35}S methionine, and extracts were subjected to immunoprecipitation with anti-HDM2 antibody or anti-EF1 α antibody. Analysis of the immunoprecipitates by SDS polyacrylamide gel electrophoresis and autoradiography (Figure 3A–C) showed that anti-HDM2 antibody coimmunoprecipitates EF1 α in the presence of HDM2 (Figure 3A, lanes 2 and 5, B lane 3, C lane 2). Extracts prepared from vector-transfected cells did not show significant coimmunoprecipitation of EF1 α (lane 1 in Figure 3A–C), as amount of endogenous HDM2 in cells is low. As expected, the anti-EF1 α antibody coimmunoprecipitates HDM2 (Figure 3B, lane 5). An IgG isotype of the anti-EF1 α antibody was used for immunoprecipitation to detect background bands (Figure 3B, lane 6).

HDM2 is a multifunctional protein, and it interacts with many cellular factors, including ribosomal proteins. The domains of these functional interactions are known. To determine if interaction of HDM2 is related to one of its known function, or if the interaction is through its interaction with ribosomal proteins, we determined the domains of HDM2 required for its interaction with EF1 α . WI38 cells were transfected with plasmids expressing wild type or C- or N-terminal deletion mutants of HDM2 (or empty vector) and were metabolically labeled with ^{35}S methionine. The extracts were immunoprecipitated with appropriate anti-HDM2 antibodies that can recognize the deletion mutants. The immunoprecipitates were then analyzed with SDS polyacrylamide gel electrophoresis and autoradiography.

The results (Figure 3A) show that the C-terminal deletion mutants of HDM2, HDM2 Del 491–452 (lane 3), and HDM2 491–325 (lane 7) can interact with EF1 α , whereas HDM2 Del 491–221 cannot (Figure 3B, lane 4). These data suggest that the 324–221 amino acid residues of HDM2 are required for its interaction with EF1 α . A similar coimmunoprecipitation experiment was performed by transfecting plasmids expressing N-terminal deletion mutants of HDM2, HDM2 Del 1–58, and HDM2 Del 1–120. The result (Figure 3C) of our coimmunoprecipitation experiment showed that an anti-HDM2 antibody immunoprecipitated the deletion mutants Del 1–58 and Del 1–120 yet could not immunoprecipitate EF1 α (lanes 4 and 5), suggesting that either of these deletion mutants were incapable of interacting with HDM2. In a control experiment, the same antibody immunoprecipitated wild-type HDM2 and coimmunoprecipitated EF1 α (Figure 3C, lane 2).

These experimental data show that HDM2 requires two domains for its interaction with EF1 α , one situated within N-terminal 58 amino acid residues, whereas the other within a central domain, 220–325 residues (Figure 3D). Because the N-terminal 58 amino acid residues are not required for interaction of HDM2 with the ribosomal proteins, interaction of HDM2 with EF1 α should be independent of its interaction with ribosomal proteins.

Interaction of HDM2 with EF1 α is a Cytoplasmic Event

Because HDM2 localizes in the nucleus and shuttles from nucleus to cytoplasm³⁶ and EF1 α is predominantly localized in the cytoplasm,³⁷ we wished to determine whether HDM2-EF1 α complex is formed in the cytoplasm or nucleus. To achieve this goal, normal diploid WI38 cells were seeded on coverslips and transfected with HDM2 expression plasmid. Forty-eight hours after transfection, cells were fixed and subsequently stained with an FITC coupled anti HDM2 antibody, followed by anti-EF1 α primary antibody and a rhodamine

coupled secondary antibody. Nuclei were stained with DAPI and were then analyzed under a confocal microscope.

Results of this experiment (Figure 4) show that although the HDM2 (detected by green fluorescence) is predominantly localized in the nucleus and EF1 α (red fluorescence) in the cytoplasm, co-localization of HDM2 and EF1 α can be detected (yellow fluorescence) in the cytoplasm. This result suggests that HDM2 interacts with EF1 α in the cytoplasm.

HDM2 Does Not Alter Protein Synthesis

Because EF1 α is a translation elongation factor,³⁷ we tested if its interaction with HDM2 controls protein synthesis. WI38 or H1299 cells were transfected with HDM2 expression plasmid (or vector) harboring a neomycin resistant gene. After transfection, the cells were subjected to neomycin selection. Selected cells were then metabolically labeled with ³⁵S methionine for 2 h. Because neomycin selected cells were few, the actin content of the cell extracts was determined by Western blot analysis and densitometry to ensure equal amounts of protein used for TCA precipitation. As a control experiment, H1299 cells were treated with cycloheximide (10 μ g/mL) for 16 h and metabolically labeled with ³⁵S methionine as described above to determine whether our assay method is sensitive enough to detect cycloheximide-mediated inhibition of translation. Cell extracts containing equal amounts of protein were treated with alkali to degrade aminoacyl tRNA and were then precipitated with TCA. The precipitates were collected on glass fiber filters and were washed subsequently with TCA and ethanol to remove adhered radioactivity. Filters were then dried and counted using a scintillation counter. The result of this experiment shows that the ability of HDM2 overexpressing cells to synthesize protein (as measured by ³⁵S methionine incorporation) is very similar to that of vector transfected cells (Figure 5A). As expected, cycloheximide treatment inhibits cellular translation as evidenced by a decrease in ³⁵S methionine incorporation in TCA precipitates (Figure 5B). This result suggests that HDM2 overexpression through its interaction with EF1 α does not alter protein synthesis in cells.

Conclusion

Although HDM2 is a multi-functional protein and its interaction with cellular proteins relevant to its known functions have been reported, so far there are no reports to identify the major binding partners of HDM2 in normal or transformed cells to decipher unknown functions of HDM2. In this communication, we report several HDM2-binding partners formed in lung cancer H1299 cells in the absence of the tumor suppressor p53 identified by mass spectrometric analysis. We isolated HDM2-protein complexes by immunoprecipitation of HDM2 and determined the presence of coimmunoprecipitated proteins that were not immunoprecipitated by IgG coupled agarose.

Our analysis detected several previously reported and several novel HDM2-interacting proteins. Interactions of HDM2 with ribosomal proteins L5, L11, and p14ARF have been reported earlier.^{4,5,10} Ribosomal proteins L5 and L11 are thought to modulate p53-degrading functions of HDM2.²⁷⁻³⁰ Our analysis suggests that apart from its interaction with L5, L11, and L23, HDM2 can interact with 40S ribosomal protein S20. It is possible

that HDM2 interacts with ribosome. These interactions are consistent with a previous notion that HDM2 may have a role in ribosomal biogenesis.²⁶

We also detected interaction of HDM2 with tubulins $\alpha 6$, $\beta 1$, and 2 chains. Our mass spectrometric analysis detected interactions of HDM2 with the translation elongation factor EF1 α and glyceraldehyde 3-phosphate dehydrogenase (GAPDH), and both have been reported to be associated with actin depolymerization. A CTCL tumor antigen HD-CL-08 is highly homologous to EF1 α and lacks only 77 amino acids of EF1 α at the N-terminus.³⁵ We further analyzed the interaction of HDM2 with EF1 α . Our data suggests that HDM2 interacts with EF1 α in normal diploid or transformed cells. Two domains of HDM2, one at the N-terminus and the other at the central part, are required for this interaction (Figure 3D). Because the N-terminal EF1 α -binding domain overlaps with the p53-interaction domain of HDM2, this interaction may prevent HDM2 from interacting with p53 in the cytoplasm. HDM2-EF1 α -complex was detected in cancer cells such as OsACL or JEG3 overexpressing HDM2 (Figure 2B, data not shown). Co-occurrence of HDM2 and EF1 α in the cytoplasm suggests that the interaction is a cytoplasmic event (Figure 4). HDM2 did not show general inhibition or activation of translation, suggesting that its interaction with the translation elongation factor EF1 α does not alter EF1 α 's translation function (Figure 5).

As discussed in the Introduction, a wide range of activities has been shown to be associated with HDM2. Similarly, EF1 α has also been associated with several functions other than translation elongation, such as actin filament depolymerization, apoptosis, and ubiquitin-mediated protein degradation, etc.^{37,38} Upregulation of EF1 α has been associated with cell death.^{38,39} Our finding that HDM2 interacts with EF1 α suggests that they may modulate each others function. A recent publication³⁹ described a role of EF1 α in activation of a transcription factor heat shock factor 1 (HSF1). which induces expression of heat shock proteins and other cryoprotective proteins. This finding raises the possibility that MDM2 may regulate heat shock response of cells such as translational shutdown and cytoskeleton collapse of cells.

Interestingly, EF1 α , GAPDH, and tubulins have been shown to be associated with cellular cytoskeleton and are involved in microtubule formation or depolymerization.^{37,38}

Translation elongation factor EF1 α has been shown to induce fast depolymerization of the microtubule network. Because HDM2 is an oncogenic protein, it may regulate microtubule formation through its interaction with these factors. However, it is possible that HDM2 modulates the individual functions of its interacting proteins. Thus, identification of these interacting proteins will lead to a better understanding of the function of HDM2.

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Figure 1.

(A) Immunoprecipitation of HDM2-bound protein complexes. Cytoplasmic or nuclear extracts of H1299 cells transfected with an HDM2 expression plasmid were immunoprecipitated with an anti-HDM2 antibody directly coupled to agarose beads. After immunoprecipitation, the beads were either boiled in Laemmli loading buffer (shown as bound) or the immunoprecipitated proteins were eluted with glycine from the conjugated agarose (shown as eluted) and subjected to Western blot analysis with the same anti-HDM2 antibody to ensure immunoprecipitation of HDM2 and exclusion of IgG. The figure shows elution of HDM2 (shown by arrowheads), and the levels of immunoglobulin (shown by arrows) in the eluted fractions are below the level of detection by Western analysis. Migration of molecular weight markers is shown at the left. (B) Tandem mass spectrum of tryptic peptide from eukaryotic elongation factor 1 α (EF1 α). MS/MS spectrum recorded on the $[M + 2H]^{2+}$ ion (m/z 513.09) corresponding to a tryptic peptide identified in the digest of proteins associated with HDM2 immunoprecipitated from H1299 cells.

Figure 2.

(A) Confirmation of HDM2-EF1α interaction by immunoprecipitation and Western analysis. H1299 cells were transfected with HDM2 expression plasmid (or vector plasmid), and cell lysates containing equal amounts of protein were subjected to immunoprecipitation with either IgG or an anti-EF1 α antibody and protein A agarose. The immunoprecipitates were subjected to Western blot analysis to identify EF1α and coimmunoprecipitated HDM2 (shown by arrows). (B) Immunoprecipitation of endogenous HDM2-EF1α complex from OsACL cancer cells that overexpresses HDM2. OsACL cells were radiolabeled with S^{35} methionine. Cell lysates containing equal amounts of TCA precipitable radioactivity were subjected to immunoprecipitation with either a polyclonal anti-HDM2 antibody or preimmune serum and protein A agarose. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis and autoradiography. An arrowhead shows position of HDM2, and an arrow shows the position of EF1α. Migration of molecular weight markers is shown at the right.

Figure 3.

EF1α requires an N-terminal and a central region of HDM2 for its interaction. WI38 cells were transfected with plasmids expressing full-length or deletion mutants of HDM2 or vector plasmid. Forty-eight hours after transfection, cells were metabolically labeled with S^{35} methionine. Cell lysates were immunoprecipitated with an anti-HDM2 antibody coupled with agarose. The immunoprecipitates were analyzed by polyacrylamide gel electrophoresis and autoradiography (A–C). Expression plasmids used for transfection are shown at the top. Migration of EF1α is shown by arrow in each lane. Arrowheads indicate migration of HDM2 or its deletion mutants. Migration of molecular weight markers is shown at the left. The EF1α-binding domain predicted from the immunoprecipitation experiments are shown in Figure 3D.

Figure 4.

Confocal imaging analysis shows that EF1 α localizes in the cytoplasm and, thus, colocalizes with cytoplasmic HDM2. WI38 cells were nucleofected with 5 μ g HDM2 expression plasmid as indicated in Materials and Methods. Nuclei were stained with DAPI (blue). EF1 α was detected as red cytoplasmic staining of cells by an EF1 α antibody and a rhodamine-coupled secondary antibody. HDM2 expression was detected as green nuclear and cytoplasmic staining by an FITC coupled anti-HDM2 antibody. Colocalization can be seen as yellow fluorescence in the cytoplasm after overlaying FITC and rhodamine stained cells. Two representative fields are shown.

Figure 5.

HDM2 does not inhibit translation by interacting with the translation elongation factor EF1 α . H1299 cells were transfected with vector or HDM2 expression plasmids harboring neomycin resistant gene. Transfected cells were selected with neomycin and radiolabeled with ³⁵S methionine. Cell lysates containing equal amounts of protein were treated with alkali to degrade aminoacyl transfer RNA and subjected to TCA precipitation to determine incorporation of radioactive methionine in protein. Actin content was determined by Western blot analysis and densitometry. The bar graphs compare methionine incorporation in (A) HDM2 expression plasmid (or vector plasmid) transfected H1299 cells and (B) cycloheximide- (or DMSO-) treated H1299 cells. HDM2 expression was determined by Western blot analysis as shown in the right panel of (A).

Table 1

HDM2-Interacting Proteins Identified from Complexes Immunoprecipitated from H1299 Cells by Mass Spectral Analysis^a

		Probability Legend:													
		<div>over 95%</div>		<div>80% to 94%</div>		<div>50% to 79%</div>		<div>20% to 49%</div>		<div>0% to 19%</div>					
# Bio View:															
# Identified Proteins (25)				Accession		Weight						neg_control		nuclear	
1	mouse double minute 2 protein human homolog [Homo Sapiens]	gi	266516	55kDa											15
2	eukaryotic elongation factor 1 alpha 1 [Homo Sapiens]	gi	4503571	50kDa											15
3	chain L, Fab fragment of monoclonal antibody (Lmk-2)	gi	15826312	24kDa									5	3	
4	dermcidin preprotein [Homo Sapiens]	gi	16751921	11kDa											3
5	tubulin, beta,2 [Homo Sapiens]	gi	4507729	50kDa											4
6	tubulin, beta-1 chain [Homo Sapiens]	gi	88947	50kDa											4
7	keratin 14 [Homo Sapiens]	gi	17512236	52kDa									4		
8	40S ribosomal protein S20 [Homo Sapiens]	gi	4506697	13kDa											4
9	60S ribosomal protein L5 [Homo Sapiens]	gi	1362932	34kDa											4
10	glyceralehyde 3-phosphate dehydrogenase [Homo Sapiens]	gi	7869492	36kDa											3
11	60S ribosomal protein L1/L14-associated antigen KIW-12 [Homo Sapiens]	gi	51707295	20kDa											2
12	cyclin-dependent kinase inhibitor (tumor suppressor p14) [Homo Sapiens]	gi	30582879	19kDa											2
13	tubulin alpha 6 [Homo Sapiens]	gi	20455322	50kDa											2
14	gelsolin isoform b [Homo Sapiens]	gi	38044288	81kDa											2
15	cytokeratin 9 [Homo Sapiens]	gi	435476	62kDa									2	5	12
16	anti-human CD-19 immunoglobulin gamma 1 heavy chain	gi	9968332	51kDa									2	3	6
17	anti-human immunoglobulin light chain	gi	12278354	12kDa											2
18	similar to hornrin [Homo sapiens]	gi	88978872	282kDa											2
19	keratin 8 type 2 cytoskeleton [Homo Sapiens]	gi	4504919	54kDa											2
20	chain L, crystal structure of the Fab fragment of the mouse anti-human Fas antibody	gi	18655521	24kDa											2
21	actin beta [Homo Sapiens]	gi	4501885	41kDa										2	
22	vimentin [Homo Sapiens]	gi	37850	54kDa											2
23	cytokeratin 1 [Homo Sapiens]	gi	17318569	66kDa									5	10	22
24	KRT 10 protein [Homo Sapiens]	gi	21961605	59kDa									10	3	15
25	keratin 2a [Homo Sapiens]	gi	4557703	66kDa									13		10