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Mdm2 regulates HuR stability in human liver and colon cancer through Neddylation

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Abstract

Hu antigen R (HuR) is a central RNA-binding protein regulating cell dedifferentiation, proliferation and survival, well-established hallmarks of cancer. HuR is frequently overexpressed in tumors correlating with tumor malignancy, in line with a role for HuR in tumorigenesis. However, the precise mechanism leading to changes in HuR expression remains unclear. In the liver, HuR plays a crucial role in hepatocyte proliferation, differentiation, and transformation. Here, we unravel a novel mean of regulation of HuR expression in HCC and colon cancer. HuR levels correlate with the abundance of the oncogene Mdm2 in human HCC and colon cancer metastases. HuR is stabilized by Mdm2-mediated NEDDylation in at least three lysine residues, ensuring its nuclear localization and protection from degradation.

Conclusion—This novel Mdm2/NEDD8/HuR regulatory framework is essential for malignant transformation of tumor cells, which in turn unveils a novel signaling paradigm, pharmacologically amenable for cancer therapy.

Keywords

Liver; Hepatocarcinoma; NEDD8; NEDP1; protein stabilization

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Competing interest statement

*None of the authors have conflict of interest to declare.

HuR, a member of the Elav/Hu family, is a gene expression regulator with a central role in mRNA stabilization (1), HuR has been shown to increase cellular division by enhancing the stability of mRNAs encoding important proteins for cell cycle control and proliferation, as well as other factors that influence tumor cell growth (2, 3). HuR expression is elevated in colon, breast, prostate, pancreatic, oral, skin, brain, gastric, lung and ovarian cancers, and correlates with tumor malignancy, supporting a role for HuR in tumorigenesis (4–6). In the liver, HuR plays a crucial role in hepatocyte proliferation, differentiation, and HCC transformation (7). At the transcriptional level, NF- κ B can activate HuR transcription downstream of the PI3K-AKT signaling pathway (8). At the post-transcriptional level, the ubiquitin (Ub)-proteasome pathway has been implicated in HuR function, whereas the machinery involved in enhancing HuR protein stability remains obscure (9).

The ubiquitin-like molecule NEDD8 is a key regulator of cell growth, viability and development. NEDD8 is ubiquitously expressed and highly conserved among most eukaryotes. At the molecular level, only members of the cullin family of proteins, are well-characterized substrates for NEDDylation (10). However, through direct biological approaches or proteomic techniques, it is evident that NEDD8 proteome is widely diverse. Recent studies have shown that ribonucleoproteins are targets for NEDD8 conjugation which protect them from de-stabilization (11, 12). Conversely, cysteine protease (NEDP1) removes NEDD8 molecules from conjugated substrates (13). Mdm2 acts as an E3 NEDD8 ligase, promoting p53 NEDDylation and stabilization (14), and, of importance, Mdm2 is overexpressed in many human tumors, at least in part due to gene amplification (15). In this study, we show that HuR is overexpressed in proliferative HCC and colon cancer cells and biopsies through Mdm2-mediated NEDDylation. Our data suggests that HuR NEDDylation at K313 and K326 residues stabilize HuR. Mechanistically, Mdm2-mediated NEDDylation of HuR controls the nuclear localization of HuR and protects it from degradation. To our knowledge, this is the first report to implicate NEDDylation in the regulation of HuR stability and localization. Our findings show that NEDDylation is a novel mechanism for HuR regulation that might represent a useful tool for new therapeutic strategies for HCC and colon cancer.

Materials and Methods

Human Samples, Cell Lines and Primary Hepatocytes

Surgically resected specimens of 61 patients with metastatic colon cancer to the liver included in two Tissue Microarrays and 22 HCC (10 Hepatitis C, 10 ASH and 2 NASH) patients were examined. Normal human liver, normal colon mucosa and colon carcinoma primary tumors were used as controls for immunostaining. Patients gave informed consent to all clinical investigations, performed in accordance with the principles embodied in the Declaration of Helsinki. The data and type of biospecimen used in this project was provided by the Basque Biobank for Research. Mouse liver progenitor 29 cell line MLP29 was provided by Dr Ponzeto (16), human hepatoma cell line HepG2, and human colon cancer cell line RKO were obtained from ATCC. Mouse HCC cell line SAME-D was described previously (17). Primary mouse hepatocytes were isolated from male C57BL6 as described (18).

RNA interference

NEDD8, HuR and Mdm2 siRNAs were from Qiagen or SIGMA. Details are described in Supporting Information.

Sub-cloning of HuR-V5 and HuR mutants

The full-length cDNA of wild-type mouse HuR was purchased from GmbH. HuR-V5 was constructed by PCR using the 5' oligonucleotide containing the V5 tag sequence and subcloned into pCDNA 3.3 TOPO vector (Invitrogen). HuR mutants were constructed by site-directed mutagenesis using the QuickChange kit (Stratagene) and as template the pCDNA-V5-HuR plasmid.

Cell lines transfections are described in Supporting Information.

Protein-histidine affinity purification using Ni-NTA beads

Cells were transfected with 1 µg of HuR constructs and/or His₆-NEDD8 and His₆-Ubiquitin as described. For UVC experiments, 20 hours after transfection, cells were exposed to a 20 J/m² UVC using a CL-1000 Ultraviolet crosslinker (UVP). Six hours after irradiation, His₆-ubiquitinated or His₆-NEDDylated proteins were purified as described (19). Where Mdm2 was also transfected cells were lysed in buffers without imidazole.

RNA isolation and real-time PCR (qPCR)

RNA was isolated with Trizol (Invitrogen) and its concentration and integrity determined. PCRs were performed using BioRad iCycler thermocycler. For HuR-V5 and the mutants, a forward V5 primer and a HuR reverse primer were used. Ct values were extrapolated to a standard curve and data was then normalized (20) to the housekeeping expression (GAPDH) (21).

Cycloheximide treatment

MLP29 cells were transiently transfected as described before. Eighteen hours later, cycloheximide (50 µg/ml) was added and at the indicated times cells were lysed. Protein was analyzed by Western blotting using an anti-V5 antibody, quantified with Image J and presented as percentage of remaining protein. Data are representative from 3 independent experiments.

Ribonucleoprotein immunoprecipitation (RNP-IP)

For IP of endogenous mRNA–transfected HuR complexes, whole-cell lysate was incubated with a suspension of Protein A–Sepharose beads (Sigma-Aldrich), pre-coated with 5 µg of either IgG2α (BD Pharmingen) or anti-V5 (Invitrogen) antibodies and incubated with 500 µg of protein lysates. RNA was analyzed as described previously (22). Bound mRNA was measured by real-time PCR analysis, normalized to GAPDH mRNA bound in a non-specific manner to IgG2α.

Protein isolation and western blotting

Isolation of total, cytosol and nuclear proteins from cells was done as described (22) and antibodies are described in Supporting Information.

Immunocytochemistry

Cells were fixed with ethanol (for V5 antibody) or methanol (for HuR antibody), washed and blocked with PBS containing 0.1% BSA and 10% horse serum. Images were taken using a Leica Confocal Microscope.

Immunohistochemistry

Paraffin sections (5-µm thick) of FFPE liver and colon carcinomasamples were treated as described in the Supporting Information.

Apoptosis measurement

Caspase 3 activity was measured as described (23).

Cell cycle analysis

Cell cycle distribution was determined by measuring the cellular DNA content using flow cytometry. Details are described in the Supporting Information.

Image analysis

For HuR and Mdm2 antibodies, twelve images per colon carcinoma patient, and 5 images from primary HCC patients were taken with a 40x objective from an upright light microscope (Zeiss). Quantification of staining intensity in colon carcinoma metastasis was performed using ImageJ software and expressed as mean intensity and stained area percentage. For HCC samples, average sum of intensities and stained area percentage of each patient was calculated using FRIDA software.

Immunoprecipitation experiments

500 µg of total cellular protein extract were immunoprecipitated with 5 µg of IgG2α (BD Pharmingen) or anti-Mdm2 (Invitrogen) antibodies and protein A Sepharose beads (SIGMA).

Statistical analysis

All experiments were performed in triplicate. Statistical significance was estimated with Student's *t* test. For immunohistochemical analysis of human samples, Pearson correlation coefficient was calculated. *p* value < 0.05 was considered significant.

Results

HuR and Mdm2 are overexpressed in hepatoma and colon cancer cells and human HCC and colon carcinoma

Recent studies have shown that HuR and Mdm2 expression are significantly higher in malignant than in benign lung and gastric tumors (24). Whereas in normal liver tissues there was not a significant expression of Mdm2 (Supporting Fig. 1A) or HuR (7) we found, in a cohort of primary human HCC and in metastatic colon cancer to the liver, a significant correlation between Mdm2 and HuR levels (Fig. 1). Among all HCC samples analyzed a positive and significant correlation between the intensity of Mdm2 and HuR expression and patients with HCC from Hepatitis C was detected (Supporting Fig. 1B). MLP29 and SAME-D cells (17, 25, 26) also had significantly higher HuR and Mdm2 levels than primary mouse hepatocytes, correlating with expression of HuR targets like cyclin A and cyclin D1 (Fig. 2A). Over-expression of Mdm2 in MLP29 and RKO cells increased levels of endogenous HuR and transfected HuR V5 (Fig. 2B and C).

We studied HuR ablation in terms of cell response in MLP29, SAME-D, RKO and the human hepatoma cell line HepG2. HuR silencing induced a strong activation of caspase 3 and a lower percentage of cells entering into S phase in those cell lines (Fig. 2D). These data suggest that HuR ablation promotes apoptosis and cell cycle arrest.

Furthermore, Mdm2 silencing in MLP29, hepatoma and RKO cells led to a significant decrease in HuR levels and its target, cyclin A (Fig. 2E). In addition, in RKO cells the ablation of Mdm2 increased 9.7 times the activity of caspase 3 activity (Fig. 2F, left graphics), while no changes in this apoptotic response was detected in MLP29 and SAME-D cells 48 hours after transfection (data not shown). However, silencing of Mdm2 for a longer

period of time, 72 hours, rendered a significant increase of caspase 3 activity and cell cycle arrest in those cell lines (Fig. 2F). All these data suggest a crosstalk between HuR and Mdm2, where at least part of the oncogenic features linked to Mdm2 could be attributed to HuR functionality.

NEDDylation is linked to HuR stability

Mdm2 acts as an E3 ubiquitin ligase and can regulate its own stability through autoubiquitination, targeting itself for proteasomal degradation (27). Importantly, Mdm2 exhibits E3 NEDD8 ligase activity, promoting p53 stability (14). Since we find that Mdm2 can regulate HuR levels (Fig. 2E), we examined whether this occurred through NEDDylation. To this end, we overexpressed Mdm2 and His₆-NEDD8 in MLP29 cell line. IP of HuR revealed the appearance of high molecular bands with HuR immunoreactivity in the presence of either Mdm2 or NEDD8 (Fig. 3A). To gain insight about HuR NEDDylation, MLP29 cells were transfected with a plasmid expressing HuR-V5 along with His₆-tagged NEDD8 and the proteins conjugated to His₆-NEDD8 purified as described previously (28). We found that HuR NEDDylation in the presence of His₆-NEDD8 was increased by Mdm2 suggesting that Mdm2 regulates HuR NEDDylation (Fig. 3B).

To examine the influence of NEDDylation on HuR stabilization, we used siRNA to knockdown NEDD8 expression. Sequential transfections to silence NEDD8 completely destabilized HuR-V5, even in the presence of Mdm2 (Fig. 3C). To show the importance of Mdm2-mediated NEDD8 conjugation in HuR stabilization, we co-transfected HuR and Mdm2 with the cysteine protease (NEDP1), which removes NEDD8 molecules from conjugated substrates (13). Whilst Mdm2 alone induced accumulation of HuR-V5, co-expression with NEDP1 clearly decreased the amount of V5 expressed (Fig. 3D). In agreement with this, the expression of NEDP1 induced the destabilization of endogenous HuR in MLP29 cells and RKO cells (Fig. 3E).

Ubiquitin-mediated proteolysis of HuR by stress signals has been previously reported to regulate its stability (9). We thus examined if HuR ubiquitination following a stress signal such as UVC affected NEDDylated HuR-V5. NEDDylated and ubiquitinated HuR were isolated on Ni²⁺-NTA agarose and detected by Western blot. No conjugation was detected without the His₆ expression vector or in the presence of His₆-LacZ-V5 alone (data not shown). UVC treatment induced a switch from NEDDylated to ubiquitinated HuR in concordance with its decreased total content (Fig. 3F). In summary, the results indicate that Mdm2 regulates NEDDylation of HuR and therefore regulates its stability.

Lysines 283, 313 and 326 are important sites for HuR NEDDylation and stability

To map the possible residues in HuR that are subjected to NEDDylation, we performed lysine mutagenesis within the RRM3 and the C-terminus of HuR (Supporting Fig. 2A). Mutation of lysine residues 283, 313 and 326 to arginine affected HuR stability, with K326 exhibiting the most profound effect (Fig. 4A and Supporting Fig. 3). Notably, these three residues are conserved between species (Fig. 4A). Importantly, the triple mutant H(3KR)V5 (K283R/K313R/K326R) was highly unstable (Fig. 4B). The analysis of the half-life of the single mutant proteins revealed a decrease as shown in Fig. 4C in comparison to the HuR-V5, while the mRNA levels of the mutants were indistinguishable from those of WT HuR-V5 (Fig. 4B). This data suggest that post-translational modifications at lysines K283, K313, and K326 could regulate the stability of HuR. To further test this, we co-transfected HuR-V5 mutants in the presence of His₆-NEDD8 and/or Mdm2. We observed a decrease in high V5-immunoreactive bands in each mutant protein relative to HuR-V5 control after His₆-NEDD8 transfection and purification, with a stronger reduction of H(K326R)V5. Mdm2 overexpression increased each of these modifications (Fig. 4D). Finally, NEDD8

knockdown, consistent with its protective role, reduced the levels of both HuR-V5 and H(K326R)V5 (Supporting Fig. 2B).

We explored the susceptibility of HuR NEDDylation mutants to ubiquitination. We observed that the modification pattern between these proteins differed in the presence of Mdm2 after cotransfection of HuR-V5 (WT and mutants) with His₆-ubiquitin (Fig. 5A). Mdm2 produced an enrichment of the ubiquitinated forms in the HuR-V5 mutants compared to WT, excluding the possibility that these proteins are ubiquitination mutants and emphasizing the possibility that this accumulation is due to a lack of NEDDylation. Finally, we found that HuR-V5 was the most susceptible to UVC-triggered degradation (Fig. 5B).

NEDDylation is a well-established modification that affects protein functionality (14). Using RNP-IP assay, we found that HuR mutations did not affect binding to proliferative genes such as *PTMA* or *Cyclin D1* mRNAs, suggesting that lysine mutation of HuR did not interfere in its RNA-binding function (Fig. 5C). These data were reinforced with the lack of response in cell cycle progression and soft agar assays observed in the different cell lines transfected with HuR-V5 or H(3KR)V5 (Supporting 4A, B). In terms of apoptosis response even at basal levels H(3KR)V5 mutant sensitizes the different cells to this stimulus as shown by the increased of caspase 3 activity (Fig 5D). After UVC treatment in the more sensible cells lines RKO and HepG2 a robust caspase 3 activity increase was observed at 5 and 8 hours respectively in the presence of H(3KR)V5 mutant in comparison to HuR-V5. Similar results were obtained in MLP29 and SAME-D cells at 16 and 36 hours after UVC treatment (Fig. 5D). These data highlight the proapoptotic phenotype associated to H(3KR)V5 mutant and therefore to the lack of HuR neddylation.

Cytoplasmic NEDDylation of HuR is mediated by Mdm2

To further explore the mechanism by which HuR gets NEDDylated, we examined the interaction between Mdm2 and HuR by IP. Mdm2 interacts with both WT and H(K326R)V5 mutant (Fig. 6A). Also, HuR-V5 was co-transfected with Mdm2 WT and Mdm2 mutants (NLS and C464A). NLS, a Mdm2 mutant characterized by its exclusively cytoplasmic localization (29, 30), produced the same stabilization of HuR compared to Mdm2 WT (Fig. 6B), suggesting that Mdm2-mediated HuR NEDDylation takes place in the cytoplasm. The C464A Mdm2 mutant, residue required for Mdm2 function as an E3 ubiquitin ligase (27), had the same effect as WT Mdm2 (Fig. 6B). In summary, these data indicate that Mdm2 interacts with HuR in the cytoplasm and participates in its stabilization independently of Mdm2 ubiquitin ligase activity.

NEDDylation controls nuclear localization of HuR

HuR is a predominantly a nuclear protein (31). Using immunofluorescence we observed that HuR-V5 expression was mostly nuclear, similar to the endogenous protein, whereas HuR mutants had a more diffuse expression, with a predominantly cytoplasmic localization in the case of H(K326R)V5 (Fig. 6C upper panel). These results were confirmed by Western blot analysis (Fig. 6C lower panel). Interestingly the cysteine protease (NEDP1), which specifically removes NEDD8 molecules from conjugated substrates, reduced by approximately 50% the nuclear localization of HuR-V5 having no effect on cytoplasmic content. These data emphasize the role of NEDDylation in HuR nuclear localization (Fig. 6D).

Discussion

Given that HuR plays a central role in the post-transcriptional regulation of many critical proteins involved in fundamental processes in tumorigenesis (e.g., cell cycle, apoptosis and

survival, proliferation, pro-angiogenesis, etc.) we analyzed the mechanisms regulating HuR overexpression in HCC and colon cancer. It was previously reported that in gastric cancers HuR is transcriptionally upregulated via the NF κ B-PI3K axis (8). In liver cells, we reported that HuR expression levels increased proportionately to their transformation status (22). Here we observed a strong correlation in the levels of HuR and Mdm2 during the transformation from primary hepatocytes to hepatoma, in colon cancer cells and more importantly in a cohort of human metastatic colon cancer and HCC samples. We report that HuR is a NEDDylation substrate and that Mdm2 mediates this NEDDylation by acting as an E3 NEDD8 ligase in the cytoplasm. Consequently, knockdown of Mdm2 results in a decrease of endogenous HuR and cyclin A levels. Based on these results, we propose that HuR and Mdm2 expression cooperatively influences tumor cell growth, by controlling the expression of cell cycle regulators. Interestingly it has been previously reported that Mdm2 mRNA level is stabilized by HuR, establishing a regulatory network among these targets either at protein and mRNA levels and highlighting the tight balance that controls both (32).

The Ub-proteasome pathway has been implicated in HuR levels and function (9, 33). Interestingly, several NEDDylated proteins seem to be either substrates or components of ubiquitin E3s, revealing an intriguing relationship between ubiquitination and NEDDylation. NEDDylated-HuR-V5 shifts to ubiquitinated conjugation after UV treatment, which is accompanied by a decrease in HuR-V5 levels. In addition, endogenous HuR was also a target for NEDDylation, and Mdm2 was demonstrated to increase its stabilization due to a robust enrichment in this posttranslational modification.

Analysis of single amino acid substitutions in the RRM3 and C-terminal region of HuR demonstrated that three evolutionarily conserved lysines, K283, K313 and particularly K326, affected the stability of HuR. The three K $\rightarrow$ R mutants were less stable than the control HuR-V5, but their intrinsic ability to associate with target mRNAs (PTMA or cyclin D1) were not affected although the triple mutant H(3KR)V5 showed a clear proapoptotic phenotype. NEDD8 silencing reduces drastically the levels of HuR, and NEDDylation experiments showed that H(K326R)V5 is deficient for NEDD8 conjugation and this corresponds to increased destabilization of the mutant. Also, H(K326R)V5 showed increased ubiquitination in the presence of Mdm2 and ubiquitin, suggesting that suppression of NEDDylation renders HuR more susceptible to ubiquitination.

HuR is localized mainly in the nucleus and translocates to the cytoplasm in response to specific stimuli. It was previously reported that NEDD8 plays a role in the correct nuclear localization of L11, protecting it from degradation (29). We observed that NEDP1 transfection lowered the nuclear content of HuR-V5 and that the mutant H(K326R)V5 was mostly cytoplasmic. HuR export to the cytoplasm is associated with a reduction of this protein due to proteasomal degradation after UVC exposure (Supporting Fig. 5B). K313R and K326R mutants were degraded by the proteasome in the cytoplasm, since proteasome inhibition by MG132 treatment induced an accumulation of K313 and K326 HuR mutants (Supporting Fig. 5A). Also, Mdm2 NLS mutant, which is localized exclusively in the cytoplasm, and Mdm2 bearing a mutation in C464, a residue involved in Mdm2 ubiquitination, were able to promote HuR stabilization. All together these results support the hypothesis that cytoplasmic Mdm2-mediated NEDDylation of HuR is independent of its ubiquitinase activity, and that NEDDylation of HuR plays a role in HuR nuclear localization protecting it from cytoplasmic ubiquitination and degradation (Fig. 7).

Finally, our results show the importance of the Mdm2-NEDD8 network in HuR overexpression during malignant transformation, supporting the role of HuR in tumorigenesis. The potential of antitumor activity for the NEDD8-activating enzyme (NAE) inhibitor MLN4924 has been shown in human colon and lung tumor xenograft models in

immunocompromised mice (34). Taken together, HuR is a new target for NEDDylation and NEDD-dependent regulation plays a crucial role as principal conductor of a new regulatory mechanism. Our findings might represent a useful tool to uncover new therapeutic strategies for HCC and colon cancer. In closing, our results show that NEDDylation is a novel mechanism for HuR regulation, which broadly reveals its influence on the cellular post-transcriptional regulatory machinery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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List of Abbreviations

HuR	Hu antigen R
HCC	hepatocellular carcinoma
ASH	Alcoholic steatohepatitis
NASH	Non-alcoholic steatohepatitis
RNP-IP	ribonucleoprotein immunoprecipitation
qPCR	quantitative polymerase chain reaction
siRNA	small interfering RNA
Ni-NTA	nickel-nitrilotriacetic acid
UVC	ultraviolet light C
FFPE	formalin-fixed paraffin-embedded
Ub	ubiquitin
NLS	nuclear localization signal
RRM	RNA binding domain
HNS	HuR nucleocytoplasmic shuttling sequence
IP	immunoprecipitation
WT	wild type
NAE	NEDD8-activating enzyme
NPTII	Neomycin Phosphotransferase 2

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Figure 1.

Mdm2 and HuR protein levels in human colon cancer and HCCs. Upper panel, tissue sections from HCC (patient 1 ASH etiology and patient 2 Hepatitis C etiology) and colon cancer patients stained using HuR and Mdm2 antibodies. Original magnification, x40 and scale bar 50 μ m. Magnified images are shown as insets. Lower panel, representative plots for intensity and area staining of Mdm2 against HuR in the tumors. Pearson tests and scatterplots for the obtained data were developed.

Figure 2.

Increased expression of Mdm2-dependent HuR contributes to oncogenicity. A) Western blot of Mdm2, HuR and its targets in primary mouse hepatocytes, MLP29 and SAMe-D cell lines. B) Western blot and densitometric analysis of endogenous HuR in control and Mdm2-overexpressing MLP29 and RKO cells. (* $p < 0.05$ Mdm2 transfected vs Control). C) MLP29 cells immunofluorescence and Western blot of MLP29 and RKO cells transfected with HuR-V5 (alone or with Mdm2). D) Left and right panels: caspase 3 activity assay and representative flow cytometry plots and histograms for cell cycle of cell lines transiently transfected with control or HuR-directed siRNAs. *Fold changes are statistically significant $p < 0.05$. E) Western blot for MLP29, SAMe-D and RKO cell lines transfected with control or Mdm2-directed siRNAs. (F) Left and right panels: caspase 3 activity assay and representative flow cytometry plots and histograms for cell cycle of cell lines transiently transfected with control or Mdm2-directed siRNAs. *Fold changes are statistically significant $p < 0.05$.

Figure 3.

NEDDylation of endogenous HuR is dependent on Mdm2. A) Western blot analysis of endogenous HuR in IP reactions after overexpression of Mdm2 or NEDD8 in MLP29 cells. B) V5-immunoreactive proteins in MLP29 cells expressing HuR-V5 and His₆-NEDD8 and/or Mdm2. C) V5 and Mdm2 in MLP29 cells silenced with siControl or siNEDD8 twice, transfected with HuR-V5 and with or without Mdm2. D) Western blot analysis from MLP29 cells transfected with HuR-V5 and/or Mdm2 and/or NEDP1. E) NEDP1 overexpression in MLP29 and RKO cells. Numbers represent the HuR protein quantification normalized by β-actin. Graphs show quantification of endogenous HuR protein levels ($p < 0.05$ NEDP1 transfected vs Control). F) SAME-D cells were transfected HuR-V5, His₆-Ub and/or His₆-NEDD8 plasmids; twenty-four hours later, cells were irradiated with 20 J/m² UVC and V5-containing proteins purified using Ni²⁺-NTA and detected by Western blot. In B and F, lower blots show the input of the transfected HuR-V5. In A, B, and F, * indicates the localization of posttranslational modifications.

Figure 4.

Identification of HuR residues involved in NEDDylation. A) Upper panel, protein expression for HuR mutants after 24 hours of transfection in MLP29 cells using the expression of the Neomycin Phosphotransferase 2 as transfection efficiency control. Lower panel, schematic representation of the HuR-V5 unstable mutants. The scheme displays the HuR-V5 protein, RRM1, 2 and 3 (RNA binding domains) and HNS (HuR nucleocytoplasmic shuttling sequence). B) mRNAs levels of HuR-V5, and mutants 24 hours after transient transfection of plasmids in MLP29 cells. C) Stability of HuR-V5, H(K283R)V5, H(K313R)V5 and H(K326R)V5 proteins as percentage of protein level remaining after treated with cycloheximide (CHX). Densitometric analysis was obtained from Western blot data. Left graphic: HuR-V5 and mutants protein levels at different time points between 0 and 8 h after CHX treatment. Right graphic: HuR-V5, H(K283R)V5 and H(K313R)V5 protein levels at different time points between 10 and 24 hours after CHX treatment. Vertical bars are indicative of the calculated half-life for every protein. D) Ni^{2+} -NTA purification in MLP29 cells transfected with HuR-V5 and mutants and/or His₆-NEDD8 and/or Mdm2. Western blot was performed using an anti-V5 antibody. * indicates the posttranslational modifications.

Figure 5.

Characterization of HuR-V5 NEDDylation mutants. A) HuR-V5, and mutants, were transfected in MLP29 with or without His₆-Ubi and/or Mdm2. His₆-ubiquitinated proteins were purified as described before. * indicates the localization of posttranslational modifications. B) Upper panel: After transfection of MLP29 cell line with HuR-V5, H(K283R)V5, H(K313R)V5 and H(K326R)V5, cells were UVC irradiated and after 6 hours lysed. Bottom panel: Densitometric analysis shows the % of protein degradation 6h after UVC **p*<0.05. C) The activity of HuR-V5 and mutants was analyzed by RNP-IP. D) Caspase 3 activity assay for cell lines transfected with HuR-V5 or H(3KR)V5 and UVC (20 J/m²) irradiated. ** show differences between UVC (+) vs non-UVC (-) treatment for the same construct (*p*<0.05). * show differences between different constructs with the same UVC (+) or non-UVC (-) treatment (*p*<0.05).

Figure 6.

The complex Mdm2-HuR regulates HuR nuclear localization through its neddylation. A) IP of Mdm2 in whole-cell lysates from MLP29 cells overexpressing HuR-V5 or H(K326R)V5. B) Western blot of HuR protein from MLP29 cells after cotransfection with HuR-V5 together with Mdm2-WT, Mdm2-NLS or Mdm2(C464A). C) Localization of HuR WT and mutants. Upper panel: Representative images of the localization of HuR-V5, H(K283R)V5, H(K313R)V5 and H(K326R)V5 by immunofluorescence in MLP29 cells. DNA quantities were adjusted to obtain similar expression levels: HuR-V5 (0.1 μ g), H(K283R)V5, H(K313R)V5 and H(K326R)V5 (5 μ g). Lower panel: cytoplasmic localization of HuR after HuR-V5 and mutants transfection by Western blot and densitometry *Fold changes are statistically significant. D) MLP29 cells were transfected with HuR-V5 and/or NEDP1 plasmids. Western blot and densitometry using anti-V5 and anti-NEDP1 antibodies from cytosolic and nuclear proteins were developed. Two different exposures are presented.

Figure 7. Model for HuR NEDDylation. Mdm2 acts as an E3 NEDD8 ligase promoting HuR NEDDylation and stabilization. HuR NEDDylation occurs in the cytoplasm and is required for the correct localization of HuR in the nucleus. This post-translational modification maintains HuR out of the ubiquitination pathway.