

Tumor Suppressive MicroRNAs miR-34a/c Control Cancer Cell Expression of ULBP2, a Stress-Induced Ligand of the Natural Killer Cell Receptor NKG2D

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Abstract

Malignant cells express ligands for the natural killer cell immunoreceptor NKG2D, which sensitizes to early recognition and elimination by cytotoxic lymphocytes and provides an innate barrier against tumor development. However, the mechanisms that control NKG2D ligand (NKG2DL) expression in tumor cells remain unknown. We recently identified the NKG2DL ULBP2 as strong prognostic marker in human malignant melanoma. Here, we provide evidence that the tumor-suppressive microRNAs (miRNA) miR-34a and miR-34c control ULBP2 expression. Reporter gene analyses revealed that both miRNAs directly targeted the 3'-untranslated region of ULBP2 mRNA and that levels of miR-34a inversely correlated with expression of ULBP2 surface molecules. Accordingly, treatment of cancer cells with miRNA inhibitors led to upregulation of ULBP2, whereas miR-34 mimics led to downregulation of ULBP2, diminishing tumor cell recognition by NK cells. Treatment with the small molecule inhibitor Nutlin-3a also decreased ULBP2 levels in a p53-dependent manner, which was due to a p53-mediated increase in cellular miR-34 levels. Taken together, our study shows that tumor-suppressive miR-34a and miR-34c act as ULBP2 repressors. These findings also implicate p53 in ULBP2 regulation, emphasizing the role of the specific NKG2DL in tumor immune surveillance. *Cancer Res*; 72(2); 460–71. ©2011 AACR.

Introduction

NKG2D is a receptor of natural killer (NK) cells and cytotoxic T lymphocytes (CTL), involved in the detection of abnormal self, as it occurs upon infection or malignant transformation. Ligands of NKG2D are surface molecules, structurally related to classical MHC class I molecules that in humans belong to either the MIC (MICA, MICB) or the ULBP (ULBP1–6) molecule family (1). The importance of the NKG2D receptor-ligand system for the recognition and suppression of tumors has clearly been shown in different mouse models (2–7). Tumor cells modified to express NKG2D ligands (NKG2DL), when

grafted into mice, were rejected while nonmodified cells formed tumors (2–4). Evidence for an involvement of NKG2D in early immune surveillance of malignancies was provided by Raulet and colleagues who observed an increased incidence of spontaneous tumors in NKG2D-deficient mice (6). Accordingly, Unni and colleagues reported an early induction of NKG2DL surface expression during spontaneous murine tumorigenesis (5).

In humans, NKG2DL have been detected on tumor cells from different entities, sensitizing them to killing by NK cells and CTLs (8–10). Cancer cells often express a variety of NKG2DL, like melanoma cells that show a predominant expression of MICA and ULBP2 detectable *in vitro* and *in situ* (10–12). However, tumor cells can escape from NKG2D immune surveillance by an enhanced proteolytic shedding of NKG2DL (13–15). This shedding is most likely causative for the increased levels of soluble ligands in sera of cancer patients (16–18). Recently, we identified elevated levels of soluble ULBP2 in sera from melanoma patients as strong independent predictor of poor prognosis (12).

Despite the importance of NKG2DL in tumor immune surveillance, the mechanisms that control their expression in cancer cells are largely unknown. In general, NKG2DL surface expression is elicited under conditions associated with cellular stress like infection, heat shock and DNA damage (19–21). Notably, mRNA expression of NKG2DL has been detected in several normal tissues that, however, lack NKG2DL proteins, pointing toward a tight regulation of

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mRNA translation (1, 22). MicroRNAs (miRNA) recently emerged as central regulators of mRNA degradation and/or translation (23), involved in the control of different fundamental biological processes. miRNAs are noncoding small RNAs of 20 to 23 nucleotide length, binding to complementary sequences within the 3'-untranslated region (3'-UTR) of specific mRNAs (23).

Our observation on ULBP2 as a strong prognostic marker in malignant melanoma prompted us to study its regulation in tumor cells that so far remained elusive. Focusing on post-transcriptional mechanisms, we provide evidence herein that members of the tumor-suppressive miR-34 family directly control ULBP2 expression. Furthermore, our data also point to p53 as a regulator of ULBP2 indicating that different tumor suppressors are involved in the control of this specific NKG2DL.

Materials and Methods

Cells

Melanoma cell lines UKRV-Mel-02, UKRV-Mel-15a, Mel592 have been described (10, 24), Ma-Mel-48a, Ma-Mel-47, Ma-Mel-123, and Ma-Mel-100a were established from tumor metastases. Patient tumor samples were collected after written informed consent. Studies on human material were approved by the Institutional Review Board of the University Medicine Mannheim (Mannheim, Germany). HCT116 p53^{+/+} and HCT116 p53^{-/-} cells were kindly provided by Bert Vogelstein (John Hopkins University). All cell lines were cultured in RPMI 1640 or Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum (FCS).

Plasmid constructs and reagents

Different fragments of the ULBP2 3'-UTR were amplified by PCR from cDNA of Ma-Mel-47 cells and subsequently cloned into the dual luciferase vector psiCHECK2 (Promega), leading to the following constructs: pULBP2-3.1 (complete ULBP2 3'-UTR), pULBP2-3.2 (proximal half of the 3'-UTR with miR-34-binding site) and pULBP2-3.3 (distal half of the 3'-UTR, no miR-34-binding site). Site-specific mutation of the miR-34-binding site in pULBP2-3.1 was carried out with the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene), yielding the construct pULBP2-3.1 Mut. Primers used for amplification and site-specific mutagenesis are listed in Supplementary Table S1. The miScript miRNA mimics and Allstars control siRNA were purchased from Qiagen (Supplementary Table S2) and used at concentrations of 2.5 nmol/L (for HEK293, UKRV-Mel-02) and 20-40 nmol/L (for UKRV-Mel-15a). A specific, chemically modified single-strand nucleic acid Anti-miR miRNA inhibitor for inhibition of endogenous miR-34a activity and a control inhibitor (Anti-miR miRNA inhibitor, negative control #1) were obtained from Ambion (Applied Biosystems) and used at a concentration of 150 nmol/L. Transfer of plasmids, miRNA mimics and miRNA inhibitors was carried out with the transfection reagents Lipofectamine or Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. The MDM2

inhibitor Nutlin-3a (Cayman Chemical) was added to the culture medium at indicated concentrations, control cells were treated with the solvent only.

Luciferase assay

HEK293 cells, seeded into 6-well plates, were cotransfected with 1 μ g of the reporter gene constructs (pULBP2-3.1, pULBP2-3.2, pULBP2-3.3, pULBP2-3.1 Mut) and 2.5 nmol/L of miScript miRNA mimics. After 48 hours, cells were lysed and analyzed for *Renilla* and *Firefly* luciferase activity using the Dual-Glo luciferase assay system (Promega). Each transfectant was assayed in triplicates. Activity of *Renilla* luciferase was normalized to *Firefly* luciferase.

Quantitative real-time RT-PCR

Total miRNA and mRNA were isolated from tumor cells using the TRIzol reagent (Invitrogen), according to the protocol of the manufacturer. Reverse transcription of miRNA was carried out with the Taqman MicroRNA Assay kit, mRNA was reverse transcribed into cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Real-time PCR was carried out using specific TaqMan Gene Expression assays or miRNA assays in combination with the StepOnePlus Real-Time PCR system (Applied Biosystems). Relative RNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method after normalizing expression levels of ULBP2 mRNA to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA and miRNA to RNU6B.

Western blot

Tumor cells were lysed in RIPA buffer. Nuclear proteins were isolated using the Nuclear Extract Kit (Active Motif). Proteins were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes and probed with the following primary anti-human antibodies: ULBP2 (AF1298, R&D Systems); p53 (DO-1 sc-126), p21 (F-5 sc-6246), and Sp1 (1C6 sc-420) all from Santa Cruz, β -tubulin (#2146) from Cell Signaling. After washing, membranes were probed with the appropriate secondary antibodies linked to horseradish peroxidase. Antibody binding was visualized using the enhanced chemiluminescence system. Band intensities were quantified by using ImageJ software.

DNA binding ELISA for activated p53

Nuclear protein extracts from different cell lines were assayed for the DNA binding capacity of p53 using the TransAM p53 Kit according to the manufacturer's protocol (Active Motif).

Flow cytometry

For analysis of MICA and ULBP2 surface expression, melanoma cells were incubated with anti-MICA mAb AMO1 and anti-ULBP2 mAb BUMO1 (18), followed by staining with a Cy5-conjugated goat anti-mouse F(ab')₂ (Dianova). Background fluorescence was determined by staining with the secondary antibody only. After fixation, cells were analyzed employing the Gallios (Beckman Coulter) and the FlowJo software. ULBP2 expression was determined as mean fluorescence intensity (MFI) and normalized to the background MFI (secondary antibody only).

Enrichment of primary polyclonal NK cells

CD3⁻CD56⁺ NK cells were enriched from human peripheral blood mononuclear cells (PBMC) of healthy donors, using the MACS technology (Miltenyi Biotec). PBMCs were first depleted of CD3⁺ cells then positive selection of CD56⁺ cells was carried out according to manufacturer's protocol. Enrichment of CD3⁻CD56⁺ NK cells was confirmed by flow cytometry, using the anti-human mAb anti-CD3-PE-Cy-7 and anti-CD56-APC (BD Bioscience) and ranged between 60% to 90%. NK cells were cultured for 48 hours in the

presence of 250 to 500 IU/mL IL-2 (Chiron Cooperation) before assaying.

CD107a degranulation assay

miRNA-transfected melanoma cells were coincubated with enriched NK cells at an effector/target ratio of 1:1 for 1 hour in the presence of PE-conjugated CD107a mAb (Clone H4A3; BD Bioscience) or the corresponding isotype control. Mouse anti-human NKG2D mAb (MAB139; R&D Systems) or mouse IgG1 antibody (MAB002; R&D Systems)

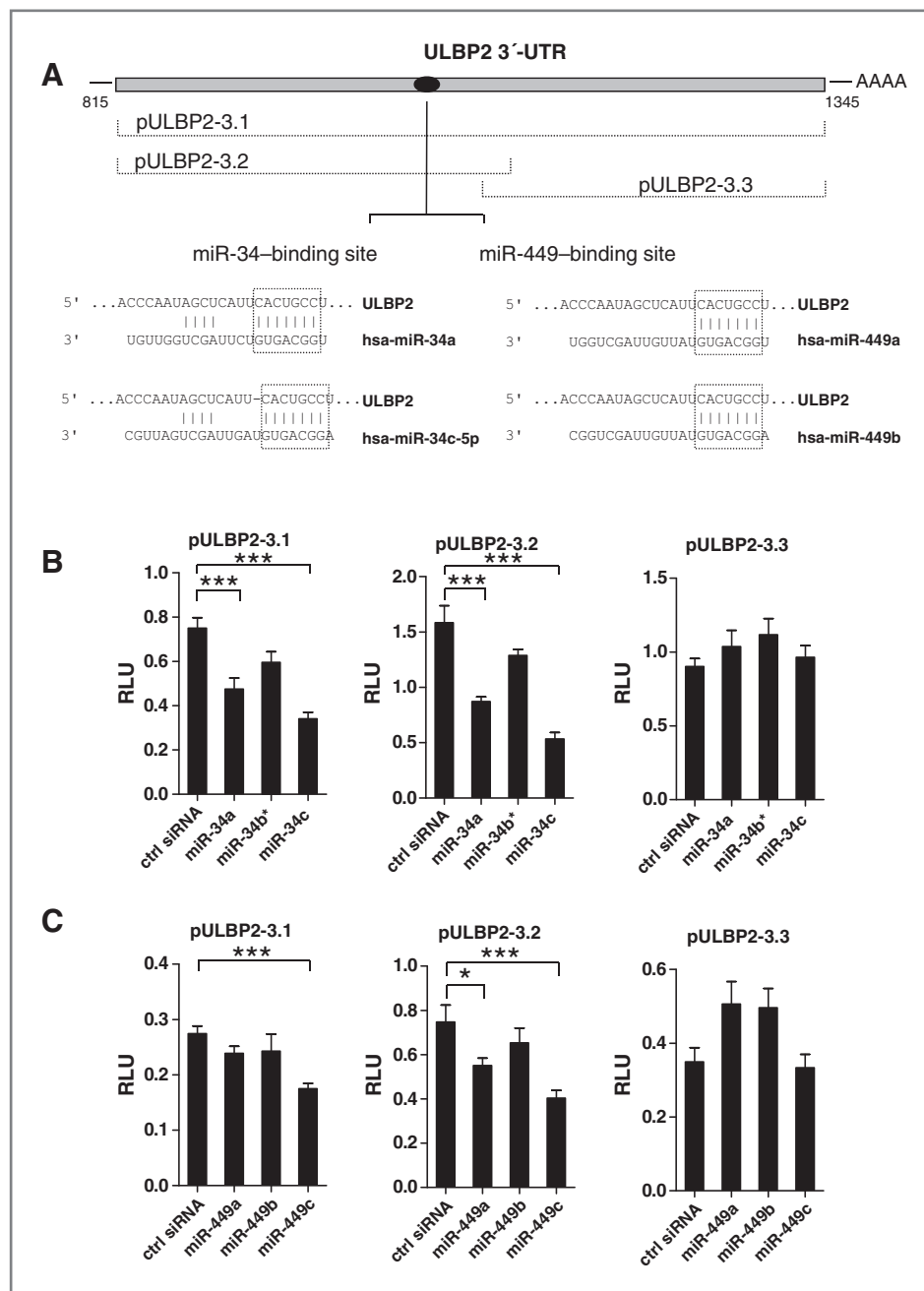


Figure 1. Binding of miR-34 and miR-449 family members to the 3'-UTR of ULBP2. **A**, scheme of the ULBP2 3'-UTR containing luciferase constructs (pULBP2-3.1, pULBP2-3.2, and pULBP2-3.3). The gray horizontal bar represents the ULBP2 3'-UTR, dashed lines indicate different 3'-UTR fragments fused to *Renilla* luciferase in psiCHECK2. The black dot indicates the location of the predicted miR-34/miR-449-binding site. **B** and **C**, plasmid reporter gene constructs were cotransfected with the indicated miRNA mimics or control siRNA into HEK293 cells. After 48 hours, lysates from transfectants were analyzed for luciferase activity. miR-34a and miR-34c (**B**) as well as miR-449a and miR-449c (**C**) induced a significant reduction in luciferase activity driven by pULBP2-3.1 and pULBP2-3.2 containing the specific miRNA-binding site. Data of normalized *Renilla* luciferase activity (RLU) represent means (\pm SEM) of $n = 3$ experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

was added to the NK cells 30 minutes before cocultivation with target cells. After coculture for 1 hour, cells were incubated in the presence of 2 mmol/L Monensin (Sigma-Aldrich) for additional 3 hours to inhibit CD107a internalization. Thereafter cells were washed and stained with additional fluorescence labeled mAbs. After fixation, cells were analyzed by flow cytometry employing the Kaluza (Beckman Coulter) and FlowJo software. The gating strategy used for analysis is indicated in Supplementary Fig. S1.

Cytotoxicity assay

Killing of melanoma cells was determined in a flow cytometry assay. Therefore, melanoma cells (1×10^6 cells/mL) were labeled with 20 nmol/L CFSE (Invitrogen). After 10 minutes, labeling was stopped by the addition of FCS. In parallel, IL-2 activated enriched CD3⁻CD56⁺ NK cells were incubated for 30 minutes with either mouse IgG1 or mouse anti-human NKG2D antibody. Then NK cells were added to 5×10^4 CFSE-labeled melanoma cells at the indicated effector to target ratios. After 3 hours of coculture 7-AAD (BD Bioscience) was added to each sample at a final concentration of 1 μ g/mL. Probes were measured directly by flow cytometry. The gating strategy used for analysis is indicated in Supplementary Fig. S2.

Statistical analysis

Quantitative data from luciferase assays, Quantitative real-time RT-PCR (qRT-PCR), Western blot and flow cytometry were plotted as mean \pm SEM. For comparison between experimental groups the 2-tailed Student *t* test was carried out using the GraphPad Prism 5.03 software (GraphPad). We report Spearman correlations as measurement of correlations between ULBP2 cell surface expression and miRNA levels, using SPSS v.17. Experimental groups were considered to be significantly different at levels α of <5% ($P < 0.05$, indicated by *), of <1% ($P < 0.01$, indicated by **), and of <0.5% ($P < 0.005$, marked by ***).

Results

miR-34a/miR-34c and miR-449a/miR-449c bind to the 3'-UTR of ULBP2 mRNA

To address whether cellular miRNAs are involved in the regulation of ULBP2 expression, we screened the 3'-UTR of ULBP2 mRNA for conserved miRNA-binding sites. The TargetScan database predicted binding of miR-34 family members, miR-34a and miR-34c-5p, to the 3'-UTR of ULBP2 (Fig. 1A). This miRNA family, consisting of miR-34a, miR-34b, and miR-34c, is known for its tumor-suppressive activity by inducing cell-cycle arrest, senescence or apoptosis upon ectopic expression in malignant cells (25). To evaluate the role of miR-34 in the regulation of ULBP2 expression, we first generated reporter gene constructs, fusing the ULBP2 3'-UTR downstream to a luciferase reporter gene, yielding the construct pULBP2-3.1 (Fig. 1A). Furthermore, 2 deletion variants of pULBP2-3.1 were generated, one encompassing the proximal half of

the 3'-UTR (pULBP2-3.2) also including the predicted miR-34-binding site, and a second overlapping construct containing the distal half without the miR-34-binding site (pULBP2-3.3; Fig. 1A).

These reporter constructs were then transfected into HEK293 cells together with the various synthetic miR-34 mimics. Though not highly predicted, we also included miR-34b (miR-34b*) in our studies, to test all miR-34 family members. Transfection of pULBP2-3.1 in combination with the miRNA mimics revealed a clear reduction of luciferase activity for miR-34a and miR-34c, whereas miR-34b* only slightly reduced reporter gene expression (Fig. 1B). In case of pULBP2-3.2, the negative effects of miR-34a and miR-34c on reporter gene expression were even more pronounced. In contrast, the specific miRNAs did not negatively influence the activity of the pULBP2-3.3 construct lacking the predicted miR-34-binding site (Fig. 1B).

Besides the miR-34 family, the TargetScan database predicted also binding of different members of the miR-449 family to the 3'-UTR of ULBP2 (Fig. 1A). Interestingly, the miR-34 and miR-449 families are structurally related and share the same seed sequence and like miR-34 also miR-449 has been described to be involved in the control of cell proliferation (26). Although the TargetScan program predicted only binding of miR-449a and miR-449b, we additionally included miR-449c in our studies. Transfection experiments revealed that miR-449a and miR-449c

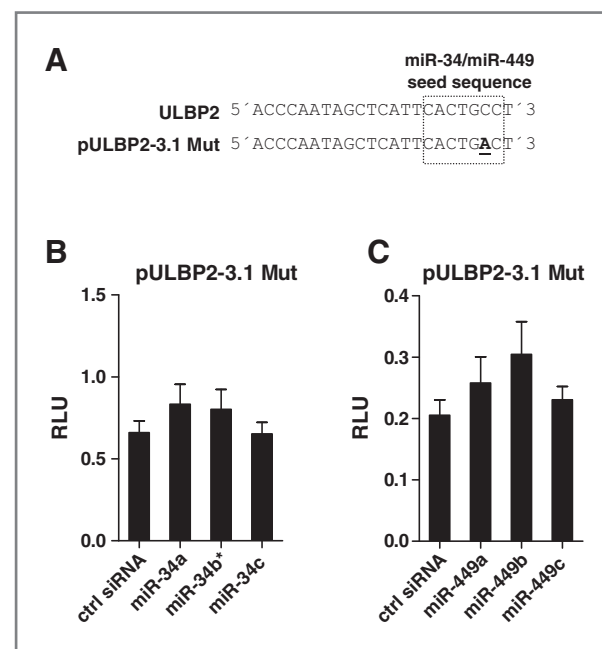


Figure 2. Specific mutation of the ULBP2 3'-UTR relieves from miR-34/miR-449-mediated repression. A, a point mutation was introduced into the ULBP2 3'-UTR of pULBP2-3.1 to destroy the predicted miR-34/miR-449-binding motif. The mutated plasmid was designated pULBP2-3.1 Mut. B and C, luciferase activity of pULBP2-3.1 Mut is not affected by transfection of miR-34 and miR-449 mimics. Data of normalized *Renilla* luciferase activity (RLU) represent means (\pm SEM) of $n = 3$ experiments.

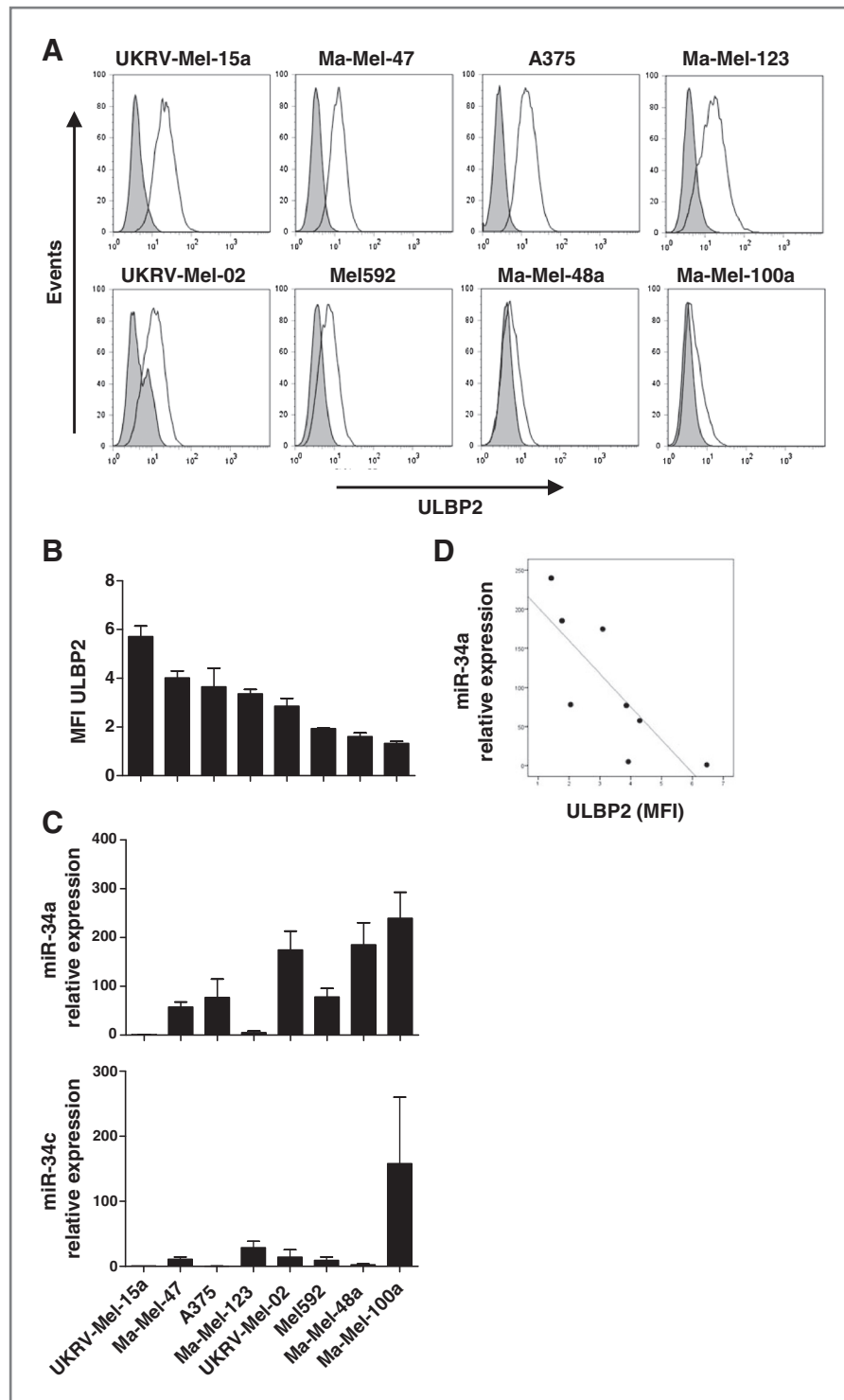


Figure 3. Expression of miR-34a and ULBP2 surface molecules inversely correlate. A and B, ULBP2 surface expression on different melanoma cell lines ($n = 8$) was measured by flow cytometry. A, representative histograms of ULBP2 expression on melanoma cells. B, data are shown as mean MFI (+ SEM) of $n = 3$ experiments. C, cells were analyzed for miR-34a and miR-34c expression levels by qRT-PCR. miRNA expression was normalized to endogenous RNU6B. Expression levels, given as means (+ SEM) of $n = 3$ experiments, are indicated relative to the expression in UKRV-Mel-15a cells. D, statistical analysis revealed a significant negative correlation between expression levels of miR-34a and ULBP2 surface molecules (Spearman, $P < 0.01$).

have the capability to downregulate expression of the luciferase reporter encoded by pULBP2-3.1 and pULBP2-3.2 while no effects were seen for pULBP2-3.3 (Fig. 1C). In summary, these results suggested that specific miR-34 and miR-449 family members are involved in the control of ULBP2 expression by binding to the 3'-UTR of its mRNA.

To confirm the functional significance of the specific miRNA-binding motif, a nucleotide substitution was introduced into the miR-34/miR-449 seed sequence-binding site of pULBP2-3.1, resulting in pULBP2-3.1 Mut (Fig. 2A). Indeed, this mutation abrogated the negative effect of miR-34a/miR-34c and miR-449a/miR-449c mimics on reporter gene expression (Fig. 2B and C).

Inverse correlation between expression levels of miR-34a and ULBP2 surface molecules

To assess a role of endogenous miR-34a/miR-34c and miR-449a/miR-449c in the regulation of ULBP2 expression, different melanoma cell lines were studied in parallel for their expression levels of ULBP2 surface molecules and specific miRNAs. All cell lines showed significant but variable surface expression of ULBP2 (Fig. 3A and B). Although miR-449a and miR-449c were not detectable (data not shown), several melanoma cell lines expressed miR-34a but only 1 out of the 8 contained miR-34c (Fig. 3C). Statistical analysis revealed a significant inverse correlation between expression levels of endogenous miR-34a and ULBP2 surface molecules ($r_s = -0.95$; **, $P < 0.01$; Fig. 3D), again pointing toward an involvement of miR-34 in ULBP2 regulation.

miR-34a and miR-34c control ULBP2 expression

Focusing on the regulation of ULBP2 by miR-34, we first transfected specific miRNA mimics into UKRV-Mel-02 cells. Transfection efficiency was verified by qRT-PCR (Supplementary Fig. S3). Western blot analysis of total cell lysates from transfectants showed that both miRNAs, in contrast to control siRNA, downregulated ULBP2 protein expression (Fig. 4A). This downregulation was associated with a reduction in ULBP2 mRNA levels, suggesting that transfected miR-34a and miR-34c mimics induced degradation of the specific mRNA, though an impact on translation could not be excluded (Fig. 4B). The

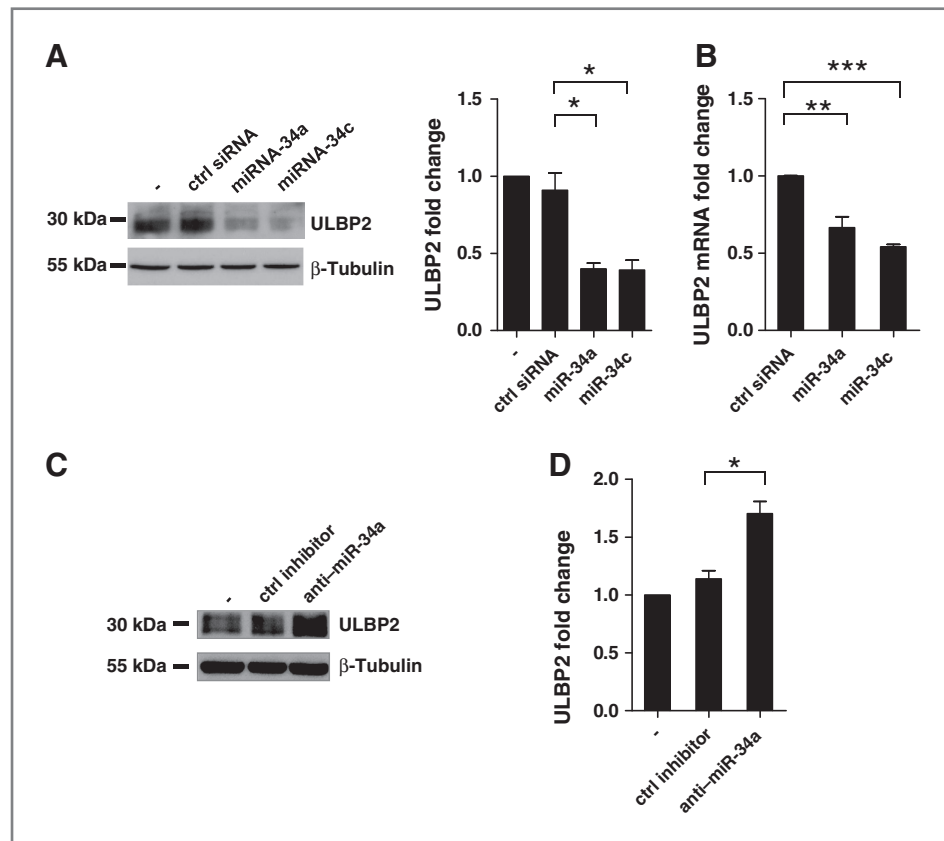
strongest inhibitory effect on target expression was exerted by miR-34c, as already observed in reporter gene assays (Fig. 1B).

Next, we asked for the impact of endogenous miR-34a on the expression level of the specific NKG2DL in melanoma cells. To decrease endogenous miR-34a levels, Ma-Mel-47 cells were transfected with a specific miR-34a inhibitor (anti-miR-34a) in comparison with a control inhibitor. As shown in Fig. 4C and D, downregulation of cellular miR-34a in Ma-Mel-47 cells clearly increased the expression of the ULBP2 protein.

Diminished NK cell recognition of miR-34-transfected melanoma cells

In the following, we determined the influence of ULBP2 downregulation on the recognition of melanoma cells by NK cells. These functional analyses were carried out on the MICA-negative UKRV-Mel-15a cells (Supplementary Fig. S4) characterized by the lowest endogenous miR-34 levels of all melanoma cell lines tested. Upon transfection of the miR-34c mimic into UKRV-Mel-15a, a reduction of more than 50% in ULBP2 surface expression was observed in comparison with control siRNA-treated cells (Fig. 5A). The transfected tumor cells were then analyzed for their capacity to stimulate NK cells. Activation of NK cells was measured by surface expression of the degranulation marker CD107a. As shown in Fig. 5B, maximal NK degranulation was observed in the presence of control siRNA-transfected target cells.

Figure 4. miR-34 controls ULBP2 expression in melanoma cells. A, left, transfection of miR-34 mimics downregulates ULBP2 expression in melanoma cells. UKRV-Mel-02 cells were transfected with miRNA mimics or control siRNA. After 48 hours, lysates of cells were analyzed for ULBP2 expression by Western blot. β -Tubulin served as loading control and for normalization. A, right, fold change in ULBP2 expression was calculated from $n = 3$ experiments. B, the influence of miR-34 mimic transfection on ULBP2 mRNA levels was determined by qRT-PCR. Expression of ULBP2 mRNA was normalized to endogenous GAPDH mRNA. Mean expression levels (\pm SEM) of $n = 3$ experiments are presented relative to control siRNA-transfected cells. C, endogenous miR-34a reduces ULBP2 expression in melanoma cells. Ma-Mel-47 cells were transfected with either anti-miR-34a or control anti-miRNA. After 48 hours, cells were lysed and ULBP2 expression levels were determined by Western blot. β -Tubulin served as loading control and for normalization. D, the fold change in ULBP2 expression was calculated from $n = 3$ experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.



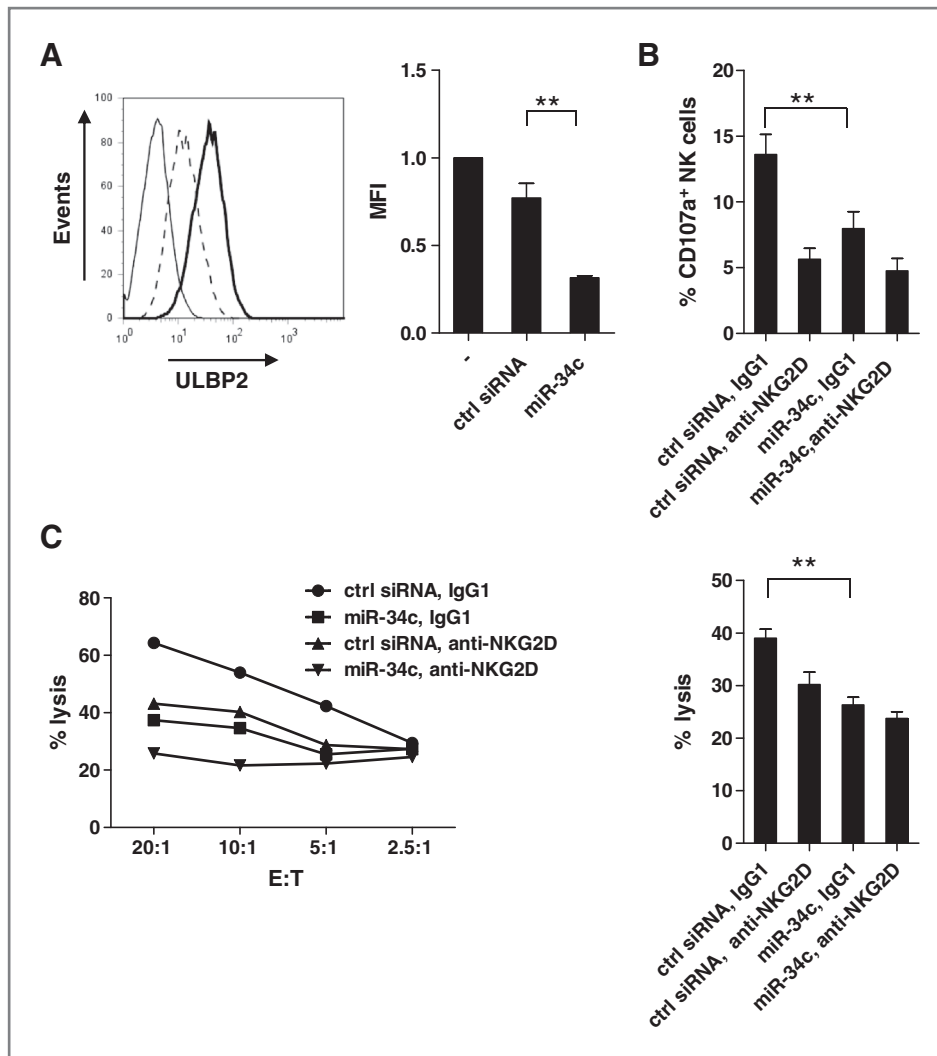


Figure 5. Impaired NK cell recognition of melanoma cells upon transfection with miR-34. **A**, expression of ULBP2 on UKRV-Mel-15a cells upon transfection of miR-34c mimics or control siRNA was determined by flow cytometry. Left, representative histogram of 1 experiment: heavy line, control siRNA-treated cells; dashed line, miR-34c-transfected cells; thin line, background fluorescence. Right, mean MFI values (\pm SEM) of $n = 3$ experiments are indicated. **B**, activation of CD3⁻CD56⁺ NK cells by miR-34c mimic or control siRNA-transfected UKRV-Mel-15a cells was determined as degranulation by surface staining for CD107a. To block NKG2D-dependent signaling, NK cells were incubated with an anti-NKG2D mAb, controls were treated with IgG1 mAb. Results are given as mean of $n = 4$ experiments (\pm SEM). **C**, lysis of miR-34c mimic or control siRNA-transfected UKRV-Mel-15a cells by enriched CD3⁻CD56⁺ NK cells was determined by CFSE/7-AAD cytotoxicity assay. Similar to the CD107a degranulation assay, lysis of miR-34c mimic-transfected cells was significantly reduced compared with control siRNA-transfected cells (Fig. 5C). In summary, these data clearly showed the functional significance of ULBP2 downregulation by miR-34.

This stimulation was strongly reduced in the presence of an anti-NKG2D blocking antibody, indicating the dependency of efficient NK cell activation from NKG2D receptor signaling. Tumor cells transfected with miR-34c mimics showed significantly lower NK cell stimulatory capacity than control cells. The low residual expression of ULBP2 on miR-34c-transfected melanoma cells only slightly stimulated NK cells as indicated by NKG2D receptor blockade. The downregulation of ULBP2 also influenced the cytolytic activity NK cells as showed in a flow cytometry-based CFSE/7-AAD cytotoxicity assay. Similar to the CD107a degranulation assay, lysis of miR-34c mimic-transfected cells was significantly reduced compared with control siRNA-transfected cells (Fig. 5C). In summary, these data clearly showed the functional significance of ULBP2 downregulation by miR-34.

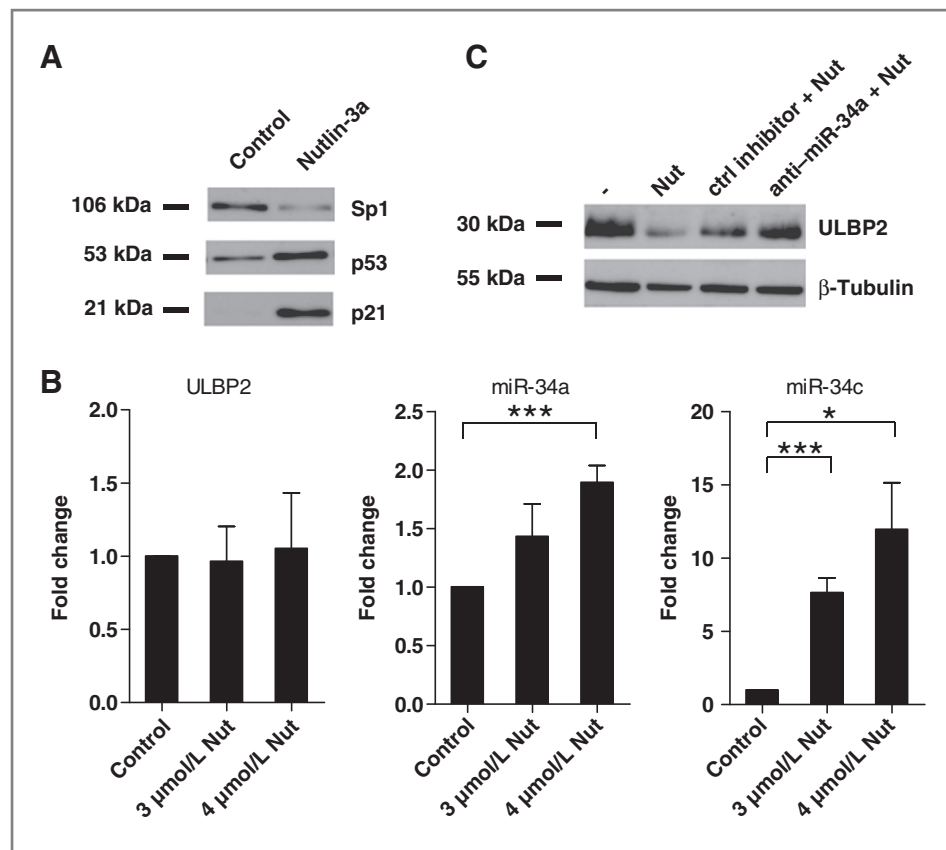
miR-34 contributes to the downregulation of ULBP2 induced by Nutlin-3a

Next, we asked how an induction of endogenous miR-34 levels would influence ULBP2 expression in melanoma cells.

Therefore, Ma-Mel-47 cells were treated with the small molecule inhibitor Nutlin-3a. As an inhibitor of MDM2, Nutlin-3a has been shown to activate p53 (27) that in turn induces transcription of the miR-34 genes (28). We confirmed activation of p53 in Nutlin-3a treated Ma-Mel-47 cells by showing an induction of the p53 target gene p21 and measuring the DNA binding capacity of p53 (Fig. 6A and Supplementary Fig. S5). As expected, in response to Nutlin-3a, Ma-Mel-47 cells increased the expression of miR-34a and miR-34c (Fig. 6B). Although the calculated increase was much higher for miR-34c, its absolute expression level was still far below that of miR-34a.

As shown in Fig. 6C, Nutlin-3a treatment reduced ULBP2 protein expression in melanoma cells. To show an involvement of miR-34a in this process, Ma-Mel-47 cells were treated in parallel with Nutlin-3a and anti-miR-34a. Indeed, anti-miR-34a transfection increased ULBP2 protein expression in Nutlin-3a-treated tumor cells, indicating that miR-34 still acts on the ULBP2 3'-UTR under these conditions. However, transfection of anti-miR-34a into Nutlin-3a-treated tumor cells could not restore ULBP2 expression to levels of untreated control cells

Figure 6. Tumor cells downregulate ULBP2 expression in response to Nutlin-3a treatment. **A**, nuclear protein extracts from Nutlin-3a (3 $\mu\text{mol/L}$) or solvent-treated Ma-Mel-47 cells were analyzed for p53 and p21 expression levels by Western blot. Sp1 served as loading control. **B**, the influence of Nutlin-3a treatment on ULBP2 mRNA, miR-34a, and miR-34c expression in Ma-Mel-47 cells was determined by qRT-PCR. Expression of ULBP2 mRNA was normalized to endogenous GAPDH mRNA; miR-34a and miR-34c levels were normalized to endogenous RNU6B. Mean expression levels (\pm SEM) of $n = 3$ experiments are presented relative to solvent-treated control cells. **C**, Ma-Mel-47 cells treated with 3 $\mu\text{mol/L}$ Nutlin-3a (Nut) were in addition transfected with anti-miR-34a or control anti-miRNA. After 48 hours, cells were lysed and ULBP2 expression levels were determined by Western blot. β -Tubulin served as loading control and for normalization. *, $P < 0.05$; ***, $P < 0.005$.



(no transfection, no Nutlin-3a), suggesting that Nutlin-3a downregulated ULBP2 protein expression by additional regulatory mechanisms.

Interestingly, in Nutlin-3a-treated melanoma cells ULBP2 mRNA levels remained unchanged in comparison with control cells, despite the induction of miR-34. This contrasts the results obtained upon transfection of miR-34 mimics into melanoma cells, where downregulation of ULBP2 protein expression was associated with a decrease in its mRNA levels (Fig. 4B). One cannot exclude a concentration-dependent effect, as specific miRNA levels in transfected cells were much higher than in Nutlin-3a-treated cells. However, the discrepancy might also be explained by different regulatory mechanisms that act on ULBP2 mRNA in the presence of Nutlin-3a.

p53 activity is essential for ULBP2 downregulation in Nutlin-3a-treated tumor cells

To clarify the role of p53 in Nutlin-3a mediated downregulation of ULBP2, we took advantage of the colon carcinoma HCT116 p53^{+/+} and HCT116 p53^{-/-} cell system. Activation of p53 upon Nutlin-3a treatment of HCT116 p53^{+/+} cells was again confirmed by p21 expression and measurement of p53 DNA capacity (Supplementary Fig. S5 and S6). Indeed, only HCT116 p53^{+/+} cells showed a downregulation of ULBP2, detectable at the level of total cellular protein (Fig. 7A and B) and cell surface protein (Fig. 7D

and E). As expected, Nutlin-3a treated HCT116 p53^{+/+} cells contained strongly enhanced levels of miR-34a and miR-34c in comparison with HCT116 p53^{-/-} cells (Fig. 7C). Again, we measured constant mRNA levels for ULBP2 under Nutlin-3a treatment (Fig. 7C).

Discussion

The NKG2D receptor allows cytotoxic lymphocytes to detect cellular stress associated with malignant transformation, which in turn can lead to tumor rejection as showed in several mouse studies (2–7). Interestingly, the human NKG2DL ULBP2 was identified as a marker of remarkable prognostic relevance in different tumor entities (12, 29). We found elevated levels of shed soluble ULBP2 in sera from melanoma patients to be associated with poor survival, as this was in the following also described for B-cell chronic lymphocytic leukemia patients (12, 29). Ligand shedding protects malignant cells from NKG2D-mediated immune surveillance (13–15), which in turn could favor tumor progression. This observation prompted us to ask for the regulation of ULBP2 in malignant cells. Although it has been shown that DNA damaging agents and proteasome inhibitors induce ULBP2 expression (19, 30), the underlying molecular mechanisms and in particular the role of tumor suppressors in the regulation of this specific NKG2DL remained elusive so far.

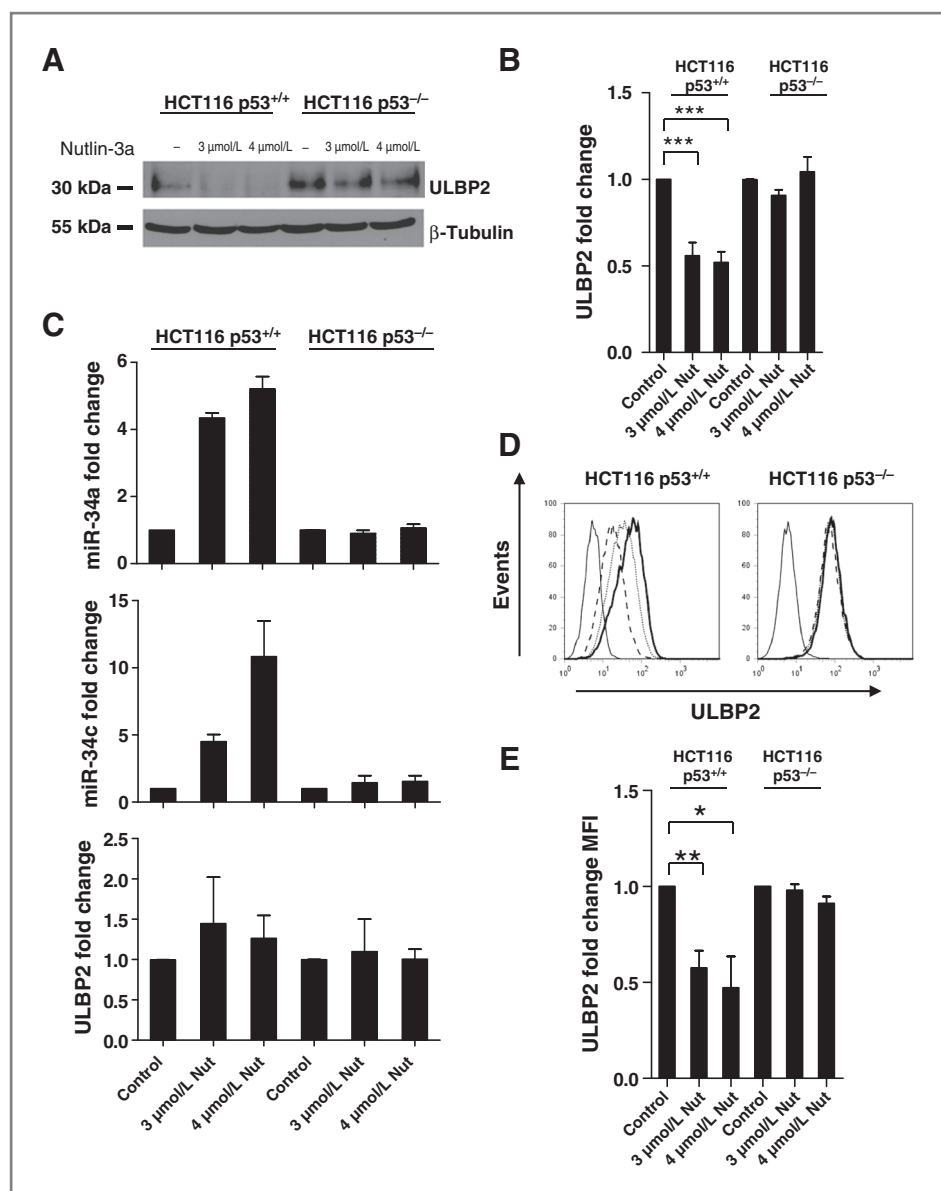


Figure 7. Nutlin-3a-mediated activation of p53 induces ULBP2 downregulation. A, HCT116 p53^{+/+} and HCT116 p53^{-/-} cells were treated with Nutlin-3a or solvent (dimethyl sulfoxide). After 48 hours, cells were lysed and analyzed for ULBP2 expression by Western blot. β -Tubulin served as loading control and for normalization. B, calculated fold change in ULBP2 protein expression from $n = 3$ experiments. C, the influence of Nutlin-3a on ULBP2 mRNA, miR-34a, and miR-34c expression in HCT116 p53^{+/+} and HCT116 p53^{-/-} cells was determined by qRT-PCR. Expression of ULBP2 mRNA was normalized to endogenous GAPDH mRNA; miR-34a and miR-34c levels were normalized to endogenous RNU6B. Mean expression levels (\pm SEM) of $n = 3$ experiments are indicated relative to solvent-treated control cells. D and E, ULBP2 cell surface expression on Nutlin-3a-treated HCT116 p53^{+/+} and HCT116 p53^{-/-} cells in comparison with solvent-treated control cells was determined by flow cytometry. D, histograms of 1 representative experiment: bold black line, solvent-treated cells; dotted line, cells treated with 3 μ mol/L Nutlin-3a; dashed line, cells treated with 4 μ mol/L Nutlin-3a; thin gray line, background fluorescence. E, mean MFI values (\pm SEM) of $n = 3$ experiments are presented. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.005$.

In this study, we provide evidence that members of the miR-34 family, miR-34a and miR-34c, repress ULBP2 expression by directly binding to the 3'-UTR of the specific mRNA. A different subset of cellular miRNAs has already been shown to control expression of the MIC molecules suggesting that miRNAs are important players in NKG2DL regulation (31–33). Interestingly, via miR-34 we link the control of ULBP2 expression to a miRNA with tumor-suppressive activity. It has been shown that ectopic expression of miR-34 family members in malignant cells promotes cell-cycle arrest, senescence or apoptosis (25). According to its tumor-suppressive function, several proto-oncogenes have been identified as direct targets of miR-34a and miR-34c, such as c-Myc and c-Met (34–36). In addition, recent studies indicated that miR-34a and miR-34c are upregulated during cellular differentiation (37–39). Besides miR-

34a and miR-34c, we showed binding also of miR-449a and miR-449c to the 3'-UTR of ULBP2. The miR-34 and miR-449 families share the same seed sequence and notably, similar to miR-34a, miR-449a has been described to mediate antiproliferative effects and to be involved in cellular differentiation (26, 40, 41).

In our cohort of metastatic melanoma cell lines, miR-449a and miR-449c were not detectable and thus we did not follow up on their role in ULBP2 regulation. Also expression of miR-34c was rare according to its detection in only a few normal tissues such as lung, testis (39, 42), but several melanoma cell lines contained significant amounts of miR-34a. Notably, we observed an inverse correlation between levels of miR-34a and ULBP2 surface molecules. Recently, Lodygin and colleagues described a downregulation of miR-34a by epigenetic silencing

mechanisms in melanoma and also in other tumor entities (43). In comparison to melanocytes the authors found miR-34a expression to be silenced in 20 of 32 primary melanoma specimens and 19 of 44 melanoma cell lines and suggested this to be a mechanism of tumors to prevent cell-cycle arrest and apoptosis (43). Importantly, miR-34-low melanoma cells, upon ectopic expression of miR-34a or miR-34c, strongly downregulated ULBP2. This led to an impairment of tumor cell recognition by NK cells emphasizing the functional significance of the regulatory mechanism. The decrease of ULBP2 in response to transfection of miR-34 mimics was detectable at the specific protein and mRNA level. This suggests that miR-34a and miR-34c by binding to the 3'-UTR of ULBP2 mRNA enhance its degradation, though we cannot absolutely exclude a contribution of indirect miR-34 effects on ULBP2 mRNA levels, for example, by interference with ULBP2 promoter activity.

Besides ectopic miR-34 expression, treatment of melanoma cells with Nutlin-3a downregulated ULBP2 in a p53-dependent manner. Nutlin-3a, by binding to MDM2, blocks ubiquitination of the tumor suppressor p53, which leads to p53 accumulation and activation in the absence of genotoxic stress (27, 28, 44, 45). Although our results point to p53 as negative regulator of ULBP2, its role seems to be much more complex. Previously, Gasser and colleagues showed an induction of ULBP2 expression in response to DNA damage in HCT116 p53^{+/+} and HCT116 p53^{-/-} cells indicating that under these conditions activated p53 neither downregulated ULBP2 nor was it required for NKG2DL induction (19). But a recent study by Textor and colleagues showed that an inducible overexpression of p53 in genetically modified cells activates ULBP2 transcription (46). Thus, we assume that the outcome of p53 activation on ULBP2 expression depends on the context of its activation, pointing to a multifaceted role of p53 that awaits further investigation.

p53 has been described as an important activator of miR-34a and miR-34b/c gene expression in several studies (28, 42, 47, 48). Accordingly, we observed a p53-dependent induction of cellular miR-34 levels in response to Nutlin-3a. The miRNA increase contributed to the downregulation of ULBP2 that could be partially counteracted by transfection of a miR-34a-specific inhibitor. Unexpectedly, despite the increase of cellular miR-34 levels, the amount of ULBP2 mRNA remained constant suggesting that under these conditions binding of miR-34 to the 3'-UTR of ULBP2 mRNA mainly affected the process of translation. Indeed, 1 miRNA can control 1 specific target mRNA by repression

of translation and degradation, however, the question on the relative contribution of the different modes of target regulation is still under intense investigation (23). Very recently the group of Hermeking showed that miR-34a affects the majority of its target mRNAs by degradation and translational repression, with a few targets controlled at the level of translation only (49). Thus, miR-34a most likely affects both, ULBP2 mRNA translation and degradation. Why in Nutlin-3a-treated tumor cells, despite the induction of miR-34, ULBP2 mRNA levels remained constant is unclear. It is tempting to speculate that Nutlin-3a by blockade of MDM2 not only activates p53 but also additional regulators that might contribute to the control of ULBP2 mRNA levels. Interestingly, it has already been shown that the repressive activity of miRNA can be modulated by RNA-binding proteins (50).

On the basis of our data and the current knowledge about the miR-34 family, we assume that high levels of miR-34, present in differentiated tissues, interfere with ULBP2 protein expression, whereas loss of miR-34a/miR-34c expression, as it frequently occurs in cancer, might assist tumor immune surveillance by enhancing ULBP2 expression. In summary, our data show that different tumor suppressors play a central role in ULBP2 regulation, strengthening the model of the NKG2D receptor-ligand system as a barrier against tumor development.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Tumor Suppressive MicroRNAs miR-34a/c Control Cancer Cell Expression of ULBP2, a Stress-Induced Ligand of the Natural Killer Cell Receptor NKG2D

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