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HuR-dependent loading of miRNA RISC to the mRNA encoding the Ras-related small GTPase RhoB controls its translation during UV-induced apoptosis

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Of critical importance in the stress response is the post-transcriptional control of the expression of important genes involved in the control of cell survival and apoptosis. Here we report that miR-19, an oncogenic component of the miR-17-92/Oncomir-1 microRNA polycistron, regulates the expression of Ras homolog B (RhoB) in keratinocytes upon exposure to ultraviolet (UV) radiation. Strikingly, we could not find any evidence for deregulated expression of miR-19 during UV treatment. However, we show that miR-19-mediated regulation of antiapoptotic RhoB expression requires the binding of human antigen R (HuR), an AU-rich element binding protein, to the 3′-untranslated region of the *rhoB* mRNA. We propose that the loss of the interdependent binding between HuR and miR-19 to the *rhoB* mRNA upon UV exposure relieves this mRNA from miR-19-dependent inhibition of translation and contributes to the apoptotic response.
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When confronted with environmental stress, cells change their gene expression patterns and activate defense mechanisms to either promote survival or initiate apoptosis. The initiation phase of mRNA translation has gained increasing importance for its role in the establishment of correct gene expression patterns during diverse forms of stress.[1]

Uncovering the mechanisms whereby the translation machinery permits cells to respond to stress in the context of global translation inhibition represents a major breakthrough. Several studies have already defined molecular mechanisms of stress-induced translational control. Although eukaryotic initiation factor 2 (eIF2) phosphorylation leads to a general defect in translation initiation, it also induces translation of specific mRNAs, such as the one encoding the transcriptional regulator ATF4 (activating transcription factor 4).[2] Specific features of the *ATF4* mRNA 5′-untranslated region (UTR), which contains a particular arrangement of short upstream open reading frames (uORF), facilitate translation of this mRNA regardless of the 43S preinitiation complex.[3] Interestingly, there is also a prevalence of uORFs in the 5′-UTRs of several mRNAs that encode DNA damage response proteins that are, in fact, translated during ultraviolet (UV) radiation.[4] In these cases, regulation may not strictly depend on the availability of the 43S preinitiation complex, but rather on still unknown *trans*-acting factors that may specifically interact with uORF-containing mRNA sequences.

Indeed, several *trans*-acting mRNA binding proteins (RBPs) have been shown to regulate translation of specific mRNAs during stress. Human antigen R (HuR) is a ubiquitous RBP that regulates several aspects of the post-transcriptional control of gene expression. Numerous mRNAs that encode stress response proteins involved in apoptotic control are regulated at the translational level through HuR binding at specific sequences in their 3′-UTRs.[5–7] Other RBPs, such as RNA binding motif protein 4 (RBM4) and several members of the hnRNP (RiboNucleoProtein) family, regulate internal ribosome entry site-mediated translation during stress through their direct binding to specific 5′-UTRs.[8–11]

Emerging evidence has shown an important role for microRNAs (miRNAs) in the post-transcriptional regulation of gene products during stress.[12] MiRNAs are a class of short, non-coding, single-stranded RNAs that when assembled with argonaute (Ago) proteins form the miRNA-induced silencing complex (miRISC), which base pairs with target mRNAs and represses their expression.[13] Aberrant miRNA expression has been frequently observed during stress[14,15] and is linked to both the post-transcriptional regulation of stress response genes and to the stress response itself. For example, several miRNAs, regulated by HIF1α, enhance the survival of neoplastic cells during hypoxia.[14] Upon DNA damage or oncogenic stress, miR-34a, whose expression is regulated by p53, controls the expression of cell cycle and DNA damage response genes.[16] More recently, the UV-regulated miR-16

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Abbreviations: Ago, argonaute; ARE, AU-rich element; ATF4, activating transcription factor 4; eiF, eukaryotic initiation factor; FLuc, Firefly luciferase; GTP, guanosine triphosphate; HuR, human antigen R; IP, immunoprecipitation; miRNA, microRNA; miRISC, miRNA-induced silencing complex; NP, non-polysome; nt, nucleotide; PARP, poly (ADP-ribose) polymerase; P, polysome; RBM4, RNA binding motif protein 4; RBP, mRNA binding protein; RhoB, Ras homolog B; RLuc, *Renilla* luciferase; RNP, RiboNucleoProtein; ser, serine; uORF, upstream open reading frame; UTR, untranslated region; UV, Ultraviolet
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has been shown to control the checkpoint gene *CDC25a* to regulate cell proliferation.[15] Strikingly, miRNAs can regulate gene expression during stress without any apparent change in their expression.[17,18] In that case, auxiliary factors, such as RBPs, may regulate the action of miRNAs. In this context, it has been shown that HuR plays a negative role on miRNA action as HuR alters the potential of miR-122 to repress gene expression upon amino-acid starvation.[17]

To study stress-dependent regulation of translation, we focused on the immediate-early gene *rhoB* (Ras homolog B), a member of the Rho family of small GTPases that includes Cdc42, Rac, RhoA and RhoC.[19] RhoB is essential for the stress response in keratinocytes.[20] Although downregulation of RhoB potentiates UV-induced apoptosis, RhoB overexpression protects human keratinocytes against UV-induced apoptosis, an essential process that maintains epidermal integrity and skin barrier function upon chronic UV exposure. Expression of the intronless *rhoB* gene is induced by UV radiation at the transcriptional level[21] and through an increase in mRNA stability in both normal human keratinocytes[22] and immortalized HaCat keratinocytes.[20] HuR and several miRNAs have been shown to interact with the 1.4-kb-long *rhoB* 3′-UTR.[22–24] The *rhoB* mRNA has also been found to associate with both HuR and Ago2 through procedures where RNA immunoprecipitates are hybridized to microarrays.[25,26] However, the functional consequences of these interactions involving HuR and miRNAs/Ago2 following UV exposure are unknown.

Here we have uncovered a novel mode of stress-dependent miRNA-mediated translational regulation. In contrast to the Bhattacharyya *et al*. study,[17] we have found that HuR acts as a positive regulator of miRNA action. Upon UV treatment, the interaction between both HuR and miR-19 with the *rhoB* mRNA is lost, thereby relieving *rhoB* from translation repression. Importantly, this regulation is linked to RhoB's function as a protector against keratinocyte apoptosis.

Results

The *rhoB* 3′-UTR is involved in UV-dependent translational regulation of RhoB expression. The expression of RhoB increased significantly upon UV irradiation of HaCat cells, reaching a maximal fourfold stimulation at a dose of 60 J/m^2 (Figure 1a). Induction was accompanied by a threefold increase in the steady-state level of the *rhoB* mRNA (Supplementary Figure S1), consistent with previous results.[20] To investigate how *rhoB* translation was maintained under conditions of compromised translation, as revealed by an increase in eIF2α phosphorylation (Figure 1a), we examined *rhoB* mRNA polysomal association, using *p53* as a control known to be resistant to global translation inhibition.[27] Polysome gradients (Figure 1b (i)) were prepared from untreated cells or HaCat cells irradiated with an UV dose of 60 J/m^2. Real-time quantitative RT-PCR analysis (qRT-PCR) showed an UV-dependent decrease in the presence of *actin* and *gapdh* mRNAs in fractions containing translationally engaged polyribosomes ('P') as compared with fractions containing free ribosomal subunits and ribonucleoprotein (RNP) complexes (non-polysome 'NP') (Figure 1b (ii)), consistent with general translational repression. In contrast, the distribution of both the *rhoB* and the *p53* mRNAs were unchanged, showing that the translation of the *rhoB* mRNA resisted to UV-dependent translational inhibition (Figure 1b (ii) and Supplementary Figure S2).

Recently, miRNAs have been shown to regulate the UV-induced DNA damage response[15] and are known to mainly target 3′-UTRs. To explore the mechanisms by which the translation of the *rhoB* mRNA is maintained upon UV exposure, we focused on the role of the 1381-nucleotide (nt)-long *rhoB* 3′-UTR in modulating translation efficiency by transfecting cells with a reporter construct containing the *Renilla* luciferase (*RLuc*) gene flanked by the *rhoB* 3′-UTR. RLuc activity was normalized by co-transfection with a *Firefly* luciferase (FLuc) reporter construct. Transfection of these constructs into HaCat cells showed that the *rhoB* 3′-UTR repressed expression of reporter transcripts in comparison with vector-derived UTRs (Figure 1c). By representing the effect of UV treatment on luciferase expression as the fold-change over untreated cells, we found that the expression of reporter constructs containing either the *rhoB* or the *p53* 3′-UTR used as a control is less affected by UV treatment than the control reporter containing the vector-derived UTR in HaCat cells (Figure 1d). These effects were also found when we transfected the reporter constructs in cultured human primary keratinocytes (Supplementary Figure S3), highlighting the physiological importance of this regulation. To distinguish translational control from mRNA turnover and thus obtain the translational efficiency, we normalized luciferase activity to luciferase reporter RNA levels[28] and observed again that the expression of the reporter constructs containing the *rhoB* or *p53* 3′-UTR was less affected by UV treatment, as compared with the control, vector-derived UTR reporter (Figure 1e). Furthermore, analysis of the polysome distribution of reporter constructs revealed that the amount of reporter mRNAs containing the *rhoB* or *p53* 3′-UTR in polysomes is less affected by UV treatment than the vector-derived UTR-containing mRNA (Figure 1f). In conclusion, the *rhoB* 3′-UTR, as well as the *p53* 3′-UTR, contains elements that regulate its translation upon UV treatment.

UV impairs the binding and/or the expression of an miRNA RISC component to the *rhoB* 3′-UTR. To address the possible involvement of miRNAs in the translational control of RhoB, we asked whether some miRNAs indeed bound the *rhoB* mRNA. As miRNAs guide Ago-containing miRNP effector complexes to target mRNAs,[26] we immunoprecipitated endogenous Ago2 from UV-exposed HaCat cells or control cells (Figure 2a, top). Co-immunoprecipitated mRNAs were extracted and the presence of the *rhoB* mRNA was investigated by qRT-PCR. The endogenous *rhoB* mRNA was strongly enriched in Ago2 immunoprecipitates as compared with control Igg1 IP, indicating that it may indeed be bound by miRNAs (Figure 2a, middle). The amount of Ago2-associated *rhoB* mRNA was strongly reduced in extracts of UV-treated cells (Figure 2a, middle). In contrast, *p53* mRNA was equally present in Ago2 IPs, regardless of UV treatment (Figure 2a, bottom).

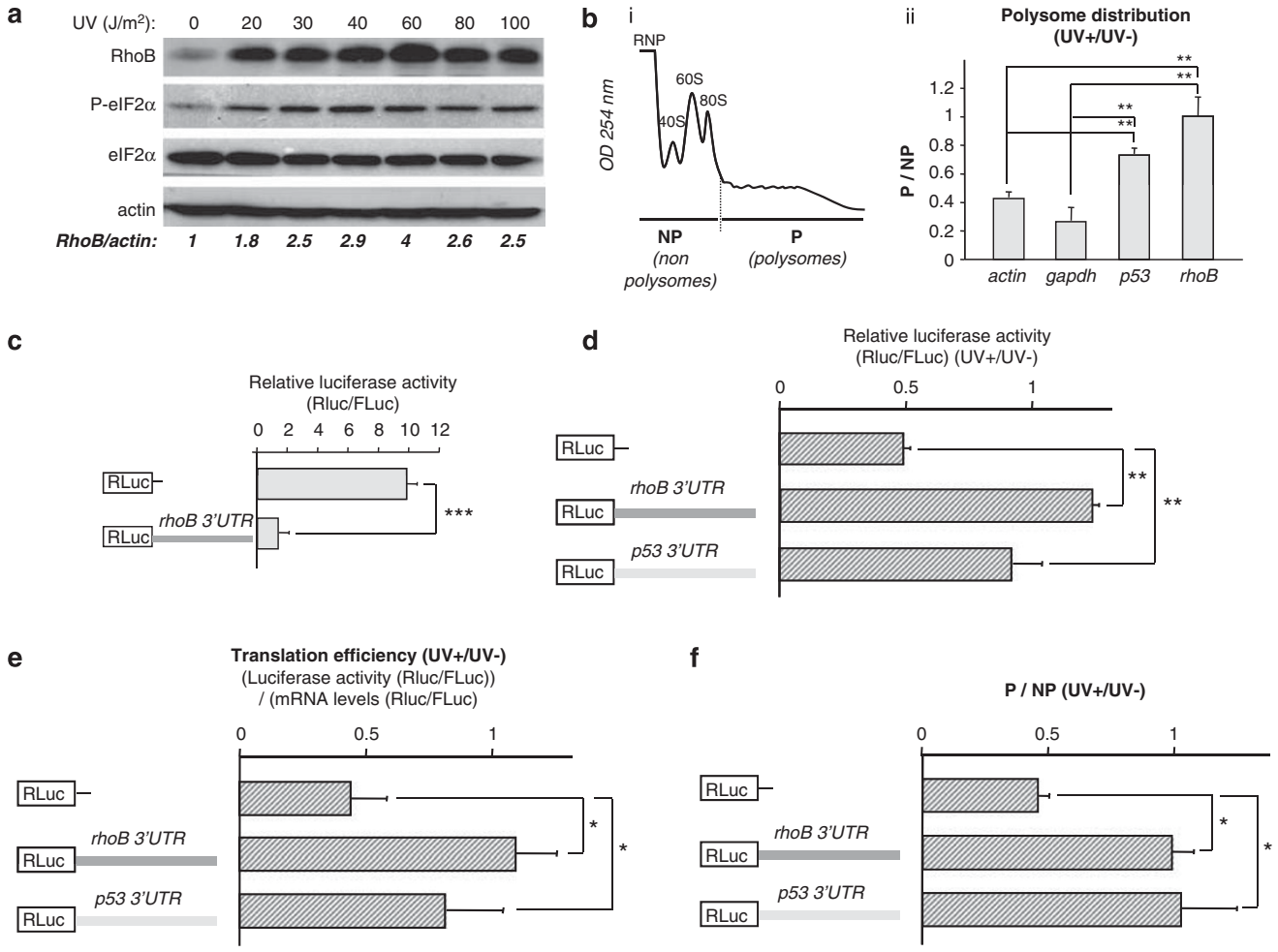

Figure 1 The *rhoB* 3′-untranslated region (3′-UTR) is involved in ultraviolet (UV)-dependent translation regulation of Ras homolog B (RhoB) expression. (**a**) HaCat cells were irradiated with increasing UV doses (J/m^2). After 8 h, proteins were extracted and immunoblotted with anti-RhoB, anti-P-eukaryotic initiation factor 2α (eIF2α), anti-eIF2α and anti-actin antibodies. (**b**) (i) Representative polysome distribution profiles obtained after centrifugation of cytoplasmic lysates over sucrose gradients. OD: optical density; RNP: RiboNucleoProtein complexes. (ii) Lysates were prepared from either irradiated cells (UV+: 8 h after receiving 60 J/m^2 UVC) or non-irradiated cells (UV−). The ratio of polysome distribution (P/NP) in 'polysome' (P) fraction *versus* 'non-polysome' (NP) fraction in UV-treated as compared with -untreated cells for each indicated mRNA was measured by real time quantitative RT-PCR analysis (qRT-PCR). (**c**) HaCat cells were transfected with the indicated RLuc reporter constructs. After 16 h, *Firefly* and *Renilla* luciferase activities were assayed. The values are averages from at least three transfections ± S.D. (**d**) HaCat cells were transfected with the *Renilla* luciferase reporter constructs containing either the vector-derived 3′-UTR ('RLuc'), the *rhoB* 3′-UTR ('RLuc-*rhoB* 3′-UTR') or the *p53* 3′-UTR ('RLuc-*p53* 3′-UTR'). After 16 h, cells were either UV irradiated or left untreated. After 8 h, *Firefly* and *Renilla* luciferase activities were assayed. (**e**) Luciferase values (**d**) were normalized to the mRNA levels (determined by qRT-PCR) to obtain translation efficiencies. (**f**) The ratio of polysome distribution (P/NP) in 'polysome' (P) fraction *versus* 'non-polysome' (NP) fraction in UV-treated as compared with -untreated transfected cells was measured by qRT-PCR for each transfected construct. The values in all panels (c)–(f) are averages from at least three transfections ± S.D. *$P<0.05$; **$P<0.01$; ***$P<0.005$

To explore the role of the *rhoB* 3′-UTR in mediating UV-dependent miRNA-related effects, we transfected luciferase reporter constructs with or without the *rhoB* 3′-UTR into UV-treated or control HaCat cells. We found a *rhoB* 3′-UTR-dependent enrichment of reporters in anti-Ago2 IP and that this enrichment was lost in UV-treated cells (Figure 2b), suggesting that UV impairs binding and/or expression of miRNAs that target the *rhoB* 3′-UTR.

A region containing a putative miR-19 binding site is required to sustain UV-dependent regulation of the *rhoB* 3′-UTR. To find the region of the *rhoB* 3′-UTR that might contain an UV-responsive miRNA binding site, we determined the *cis*-acting elements in the *rhoB* 3′-UTR that were required for UV-dependent expression. Deletions were made in the 3′-UTR. Each resulting reporter construct was transfected in UV-treated or control HaCat cells and luciferase activity was determined. The results determined that a 98 base pair sequence element, spanning bases 779–877 of the *rhoB* 3′-UTR, was required for UV regulation (Figure 3a). This region also comprised a conserved canonical ARE (AU-rich element), for which an interaction with HuR had been shown in rat cells.[22] TargetScan (http://www.targetscan.org) was used to search for potential miRNAs that might directly target this UV-responsive region of the *rhoB* 3′-UTR and found a putative miR-19 binding site (Figure 3b). This site has an eight base sequence UUUGCACA, which putatively binds to the 5′ end (bases

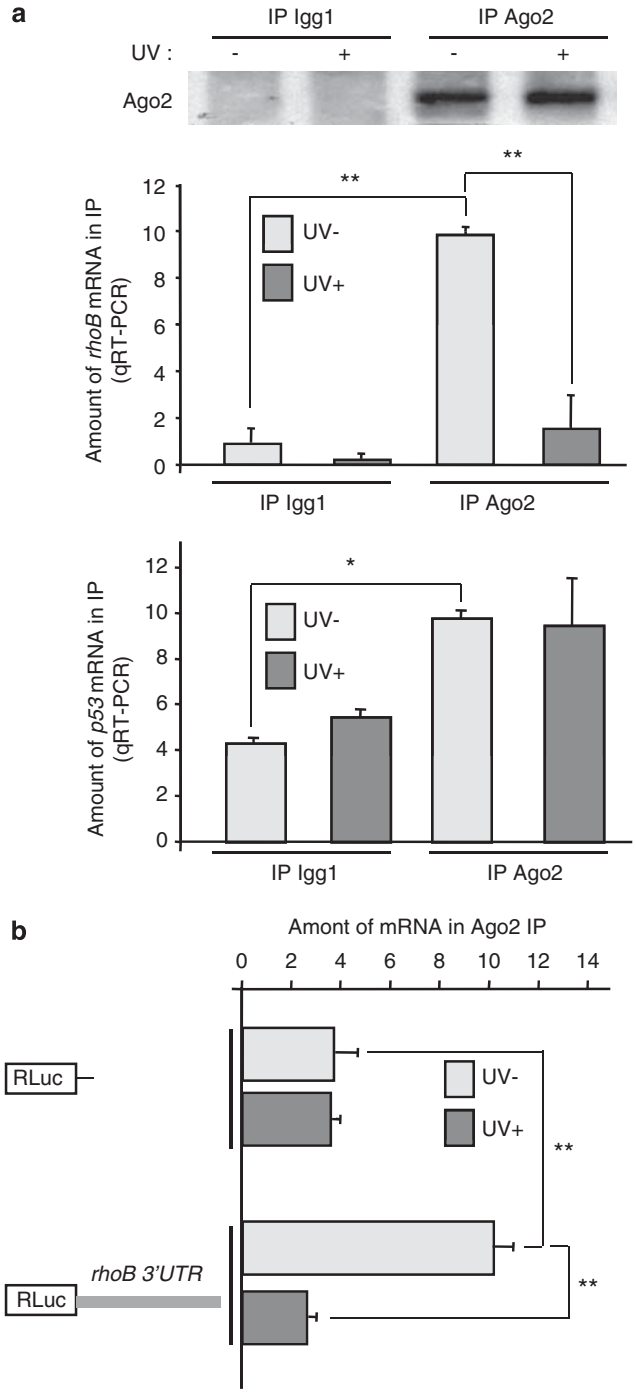

Figure 2 Argonaute2 (Ago2) recruitment to the *rhoB* 3′-untranslated region (3′-UTR), but not to the *p53* 3′-UTR, is impaired upon ultraviolet (UV) treatment. (**a**) Ago2 was immunoprecipitated from UV-treated or control HaCat cells before western blot (WB) analysis (upper panel). Igg1-loaded beads were used as a control. The levels of Ago2-associated *rhoB* and *p53* mRNAs in Ago2 immunoprecipitates (IP) were quantified by real time quantitative RT-PCR analysis (qRT-PCR) relative to *gapdh* mRNA. For each Ago2 IP sample, relative mRNA levels were normalized to the corresponding input samples. (**b**) HaCat cells were transfected with the RLuc or RLuc-*rhoB* 3′-UTR reporter construct and UV irradiated 16 h following transfection. After 8 h, Ago2 was immunoprecipitated from UV-treated or control HaCat cells. The levels of Ago2-associated mRNAs were quantified by qRT-PCR and normalized to the corresponding input samples. $^{*}P<0.05$; $^{**}P<0.01$

2–9) of miR-19 and is perfectly conserved in 14 out of 15 of the organisms aligned by TargetScan. miR-19a and/or miR-19b, which differ by only a single base at position 11, may be involved in UV-dependent translation control of the *rhoB* mRNA.

Strikingly, a 52-nucleotide-long sequence of the *rhoB* 3′-UTR spanning bases 818–870 and containing the miR-19 binding site and the HuR binding site was sufficient for UV regulation (Figure 3c).

UV regulates interaction between miR-19 and the *rhoB* mRNA, but not miR-19 expression. To ask whether miR-19 regulated endogenous RhoB expression, we transfected an 'antagomir' against miR-19b and found a concomitant increase in RhoB expression in untreated HaCat cells (Figure 4a). Conversely, increasing endogenous levels of miR-19b by transfecting a chemically synthesized precursor duplex RNA (Supplementary Figure S4) reduced RhoB expression in both UV-treated and control cells (Figure 4b). This was accompanied by an increase in association between the endogenous *rhoB* mRNA and Ago2 complexes in miR-19b-transfected cells (Figure 4c), indicating that miR-19 might indeed bind to the *rhoB* mRNA. To show the direct involvement of miR-19 in RhoB expression, we generated a luciferase reporter construct containing the *rhoB* 3′-UTR mutated in the putative miR-19 binding site. This mutation led to a loss of association of the mutated mRNA in anti-Ago2 IP (Figure 4d), showing that miR-19 directly regulated RhoB expression through its binding to the *rhoB* 3′-UTR. Furthermore, whereas the association of the reporter mRNA containing the whole 3′-UTR in anti-Ago2 IP was reduced in extracts of UV-treated cells, this was not the case for the reporter containing the mutated miR-19 binding site in the 3′-UTR (Figure 4d). Thus, loss of an miR-19 interaction with the *rhoB* 3′-UTR was required for UV regulation of RhoB expression. We next investigated whether or not the expression of miR-19 might be downregulated upon UV exposure. We measured the expression of miR-19a and miR-19b in UV-treated and control HaCat cells and could not find any evidence for UV-regulated expression of these miRNAs by miRNA microarrays (Figure 5a) or qRT-PCR (Figure 5b). Of note, UV treatment was not able to regulate the expression of miR-18a and miR-92 (Supplementary Figure S5), members of the miR-17-92 genomic cluster that also contains miR-19a/b. To ensure that UV-induced regulation of RhoB expression did not involve other miRNAs, we examined the UV-dependent regulation of miR-21, miR-30, miR-223 and miR-183, which were predicted by TargetScan to target the *rhoB* mRNA and/or known to regulate RhoB expression.[23,24] No evidence of UV-dependent regulation of these miRNAs was found with the microarray or RT-qPCR (Supplementary Figure S5). Altogether, these data indicated that, although the expression of miR-19 or other miRNAs targeting the *rhoB* mRNA were not regulated by UV, the binding of miR-19 to the UV-responsive region of the *rhoB* mRNA was regulated by UV.

miR-19 and HuR bind to the *rhoB* 3′-UTR in an interdependent manner to control RhoB expression. The presence of a potential HuR binding site, proximal to the miR-19 site (Figure 3b), prompted us to investigate if HuR

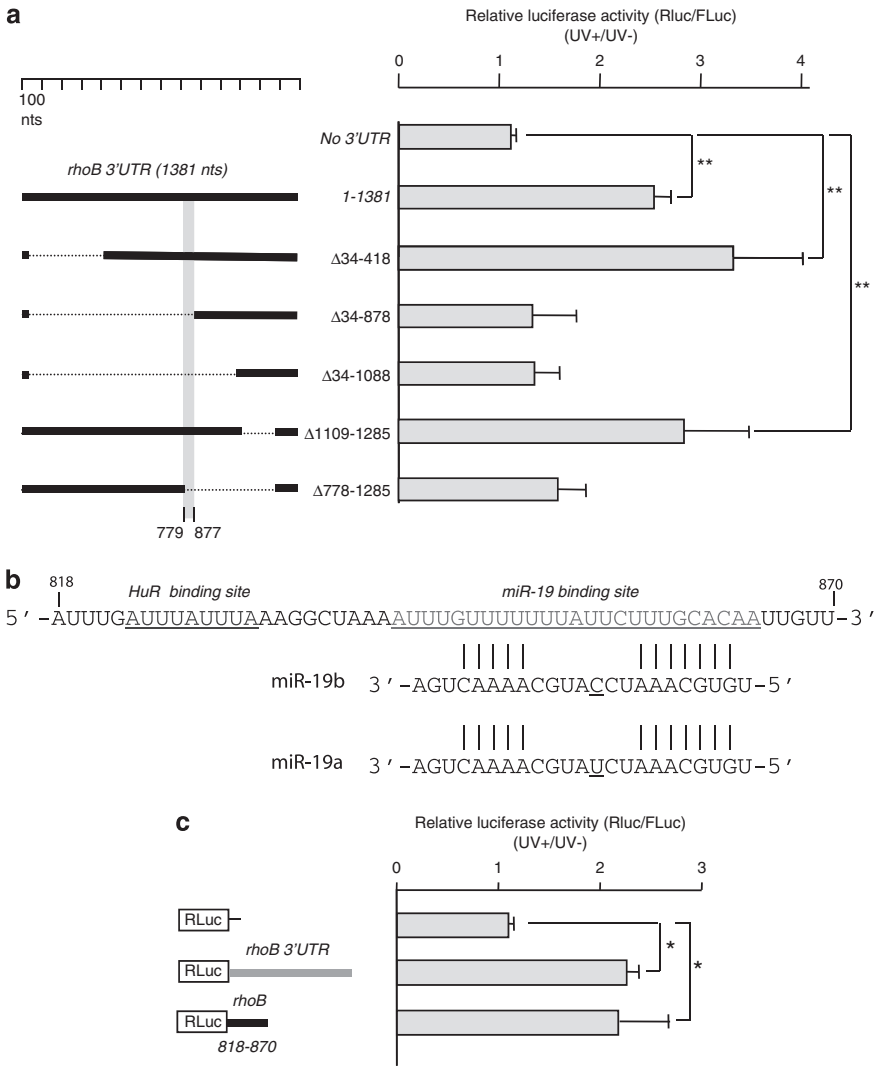

Figure 3 Identification of a 52-nucleotide (nt)-long sequence (from nt 818 to 870 of the *rhoB* 3′-untranslated region (3′-UTR) involved in ultraviolet (UV)-dependent regulation of the *rhoB* mRNA. (**a**) The RLuc activities of the constructs containing the indicated deletions in the *rhoB* 3′-UTR, normalized to the co-transfected Firefly luciferase (FLuc) activities are shown. The values are averages from at least three transfections ± standard deviation (S.D.). (**b**) Putative human antigen R (HuR) and miR-19 binding sites are shown in the sequence located between nts 818 and 870. (**c**) The RLuc activities of the indicated constructs normalized to the co-transfected FLuc activities are shown. The values are averages from at least three transfections ± S.D. $^{*}P<0.05$; $^{**}P<0.01$

interacts with the *rhoB* mRNA. We immunoprecipitated endogenous HuR from UV-exposed HaCat cells or control cells (Figure 6a, top). The endogenous *rhoB* mRNA was strongly enriched in HuR IP as compared with control Igg1 IP and the amount of HuR-associated *rhoB* mRNA was strongly reduced in extracts of UV-treated cells (Figure 6a, bottom). The same was observed when we performed the IP from extracts of cells having been UV irradiated to crosslink RNA–protein interactions before lysis (Supplementary Figure S6). As HuR relocates from the nucleus to the cytoplasm in several cell lines including HaCat cells (Supplementary Figure S7), we performed HuR IP from cytoplasmic extracts and found that the amount of HuR-associated *rhoB* mRNA was strongly reduced in cytoplasmic extracts of UV-treated cells (Supplementary Figure S8).

Altogether, these data indicated that the binding of HuR to the *rhoB* mRNA was lost upon UV exposure. Of note, the binding of HuR to the *rhoB* mRNA was shown to be increased in UV-treated NIH-3T3 cells.[22] This may reflect the ability of HuR to bind and regulate different mRNAs in different cell types, as shown recently.[25]

We next analyzed the influence of HuR on miRNA binding to the *rhoB* 3′-UTR, as HuR might be involved in the recruitment of miRNAs to mRNAs as seen with *c-Myc* and suggested by Kim *et al.*[29] We found that the association of a luciferase reporter mRNA containing the *rhoB* 3′-UTR with a mutated HuR binding site was reduced in the anti-HuR IP, as compared with the vector-derived 3′-UTR, showing that HuR bound to the putative HuR site (Figure 6b). In addition, the luciferase reporter mRNA containing the *rhoB* 3′-UTR with the mutated HuR site was poorly recovered in the anti-Ago2 IP, as compared with the wild-type UTR (Figure 6b). Conversely, the luciferase reporter mRNA containing the *rhoB* 3′-UTR with a mutated miR-19 site was poorly recovered in the anti-HuR IP

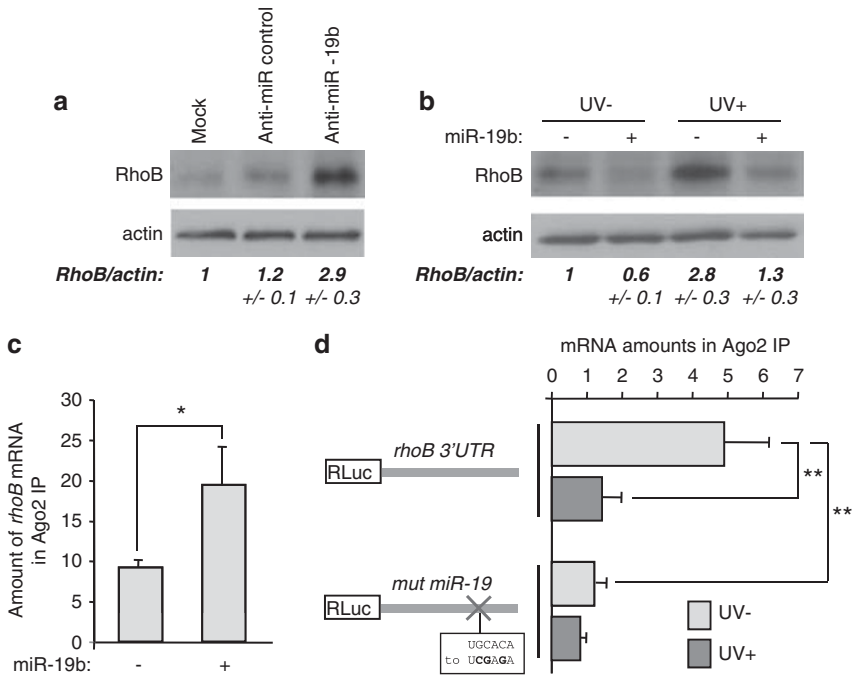

Figure 4 miR-19 is involved in ultraviolet (UV)-regulated Ras homolog B (RhoB) expression. HaCat cells were transfected with 50 nM of an anti miR-19 'antagomir' RNA ('anti-miR-19b') or a control anti-miR ('anti-miR-control') (**a**) or 2 nM of an miR-19b synthetic oligonucleotide (**b**) and either UV treated (UV+: 8 h, 60 J/m^2) or left untreated (UV−). After 48 h, proteins were extracted and immunoblotted with anti-RhoB or anti-actin antibodies. (**c**) HaCats were transfected with an miR-19b synthetic oligonucleotide or mock transfected; RNAs extracted from anti-argonaute2 (Ago2) immunoprecipitates were subjected to real time quantitative RT-PCR analysis (qRT-PCR). *rhoB* mRNA association with Ago2 was calculated relative to the input sample and to *gapdh* mRNA. (**d**) HaCat cells, UV treated or left untreated, were transfected with *Renilla* luciferase reporter construct RLuc-*rhoB* 3′-untranslated region (3′-UTR) or RLuc-*rhoB* 3′-UTR mutated in the miR-19 binding site. The presence of *RLuc* mRNAs in Ago2 IP was measured by qRT-PCR and quantified relative to the input sample and to the Firefly luciferase (*FLuc*) mRNA. $^*P<0.05$; $^{**}P<0.01$

(Figure 6b). These data showed that HuR and miR-19 associated to the *rhoB* 3′-UTR in an interdependent manner.

These results were extended by showing an interdependent action between HuR and miR-19 in the regulation of RhoB expression. Indeed, mutation of both the HuR and miR-19 binding sites led to the same relief in *rhoB* 3′-UTR-mediated repression of translation as each mutation taken individually (Figure 6c). The role of HuR as a negative regulator of RhoB expression was confirmed through siRNA-mediated silencing of HuR, which led to a 3.5-fold increase in RhoB expression as judged by western blot (Figure 6d and Supplementary Figure S9). However, in UV-treated cells, HuR silencing had a weaker effect (1.3-fold) on RhoB upregulation (Figure 6d). HuR was required for miR-19 effects as transfection of miR-19b oligonucleotides did not lead to decreased RhoB expression in HuR-silenced cells (Figure 6d, lanes 7–8). Thus, both HuR and miR-19 are required for the UV-dependent regulation of RhoB expression. Of note, in UV-treated cells in which HuR has the tendency to dissociate from the *rhoB* mRNA (Figure 6a), increasing endogenous levels of miR-19b led to an increase in the association between the endogenous *rhoB* mRNA and HuR (as revealed by HuR IP in miR-19b-transfected cells; Supplementary Figure S10) explaining the inhibitory effect of miR-19 on rhoB expression (Figure 6d, lanes 5–6).

miR-19 increases UV-induced apoptosis in an HuR-dependent manner. RhoB protects HaCat cells from UV-induced apoptosis.[20] To assess the role of miR-19 on the aforementioned function of RhoB, HaCat cells were transfected with miR-19b oligonucleotides, followed by UV treatment. Apoptosis was monitored by the analysis of poly (ADP-ribose) polymerase (PARP) cleavage, as measured by the appearance of the 86 kDa caspase-3 cleavage product. UV treatment enhanced PARP cleavage in miR-19b oligonucleotide-transfected cells, but not in control cells (transfected with miR-24), under conditions with an observed decrease in RhoB expression (Figure 7a). Thus, miR-19 increased UV-mediated apoptosis of HaCat cells (also confirmed by TUNEL staining; Supplementary Figure S11), in parallel with a decrease in RhoB expression. The extent of PARP cleavage upon miR-19b transfection was similar to the one observed after siRNA-mediated depletion of RhoB (Figure 7b). miR-19-dependent induction of PARP cleavage was abolished in HuR-depleted cells (Figure 7b), showing that the action of miR-19 in the induction of apoptosis is linked to HuR.

Discussion

We have identified a novel mechanism of stress-induced translational control involving an intricate association between an RBP and an miRNA. We show that the action of miR-19 in the regulation of the post-transcriptional control of RhoB expression during UV exposure is strictly dependent on the binding of HuR to the *rhoB* mRNA. Although the notion that some RBP modulate the action of miRNAs already exists,[17,28–30] our study extends this concept in two ways.

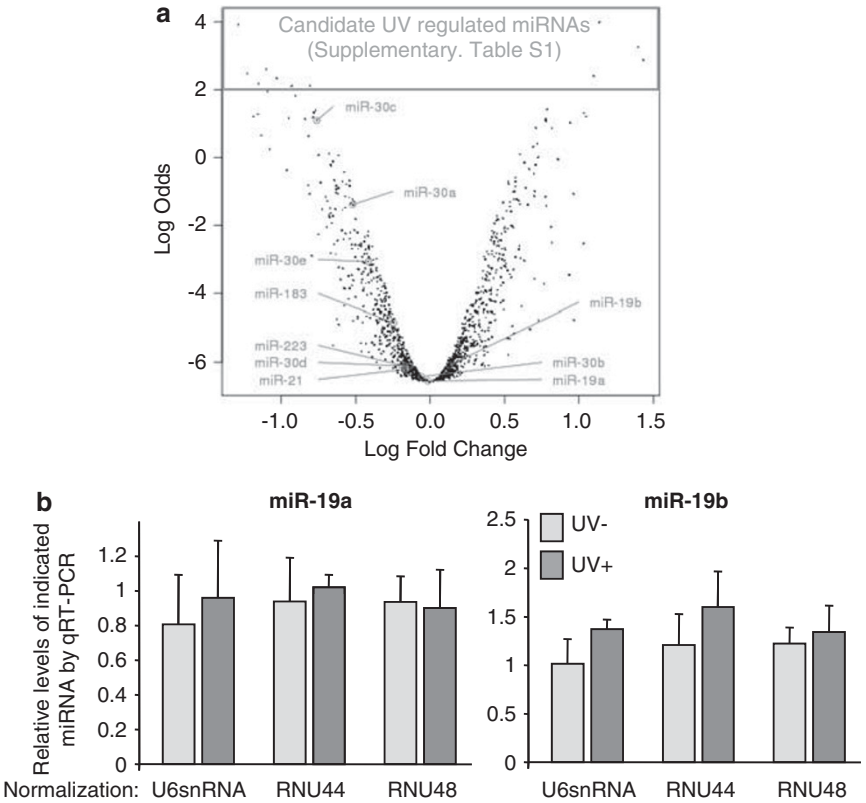

Figure 5 The expression of miRNAs that potentially target the *rhoB* mRNA is not regulated by ultraviolet (UV) treatment. (**a**) A volcano plot displaying miRNA log-fold changes *versus* Log Odds as a statistical significance of the changes. Each point represents the data for one miRNA. miRNAs whose expression is regulated with a Log Odd >2 are represented in the green rectangle. The list of these miRNAS can be found in Supplementary Table 1. (**b**) Relative expression of miR-19a and miR-19b was determined by real time quantitative RT-PCR analysis (qRT-PCR) 8 h after UV treatment. Data are presented as mean $\pm$ S.D. ($n=3$)

First, we show that the RBP-dependent action of miRNAs is regulated upon stress in an unprecedented manner. UV treatment relieves the *rhoB* mRNA from the concerted HuR/miR-19 expression inhibition. Whereas HuR prevents the action of miR-222 during stress through mutually exclusive binding on the *CAT-1* mRNA,[17] HuR can act in an opposite manner, as we show here that HuR binding to the *rhoB* mRNA is required for miR-19 action in repressing RhoB expression in unstressed cells. Second, we provide *in vivo* evidence that the direct binding of the RBP to the miRNA-targeted transcript is required to exert the inhibitory effect on gene expression. We show, using targeted mutations in the *rhoB* mRNA and RNA immunoprecipitation (IP), that the direct binding of HuR to the mRNA in the vicinity of the miR-19 binding site is required for the *in vivo* RBP-dependent repressive action of the miRNA. This extends previously published studies in which only *in vitro* experiments with reconstituted components were used.[30] Furthermore, although HuR was shown, through siRNA-mediated depletion of HuR in HeLa cells, to be essential for let-7-dependent repression of the *c-Myc* mRNA, no experiments directly investigated the requirement of HuR binding to the *c-Myc* mRNA.[29]

Aberrant expression of miRNAs has been observed in many critical biological processes. However, we have found here that the expression of miR-19 remains unchanged upon UV exposure, although miR-19 has a role in protecting keratinocytes from UV-induced apoptosis. This is reminiscent of another recent study showing that miR-208 is involved in remodeling the overloaded heart to augment cardiac output without any observed changes in miR-208 expression.[18] Such observations illustrate the need to consider the role of miRNAs regardless of whether or not changes in expression levels can be detected.

Several possibilities exist to explain the interdependent binding of HuR and miR-19 to the *rhoB* mRNA, including changes in the local RNA conformation upon binding of either HuR or miR-19 or indirect interactions between HuR and components of the miRNP machinery. It is unclear as to how the tripartite complex between HuR, the miRNP and the *rhoB* mRNA may be disrupted upon UV exposure. First, HuR binding to mRNAs may be regulated (Figure 8 (i)) through post-translational modifications. Several studies have indeed shown that phosphorylation or methylation of HuR has a critical role in HuR function.[31] Highly relevant for this study, the association of HuR to mRNAs is regulated by its phosphorylation.[32,33] Interestingly, the spectra of HuR-bound mRNAs depends on the cell context,[25] and this may be explained by phosphorylation-specific modifications of HuR that may confer target specificity in HuR binding towards a certain class of ARE.[33] It is also possible that some components of an HuR-containing complex are regulated. For example, the Akt2-mediated phosphorylation of Pitx2, part of an mRNA-stabilizing complex that, together with HuR, regulates its binding to the *cyclin D1* mRNA.[34] Second, the

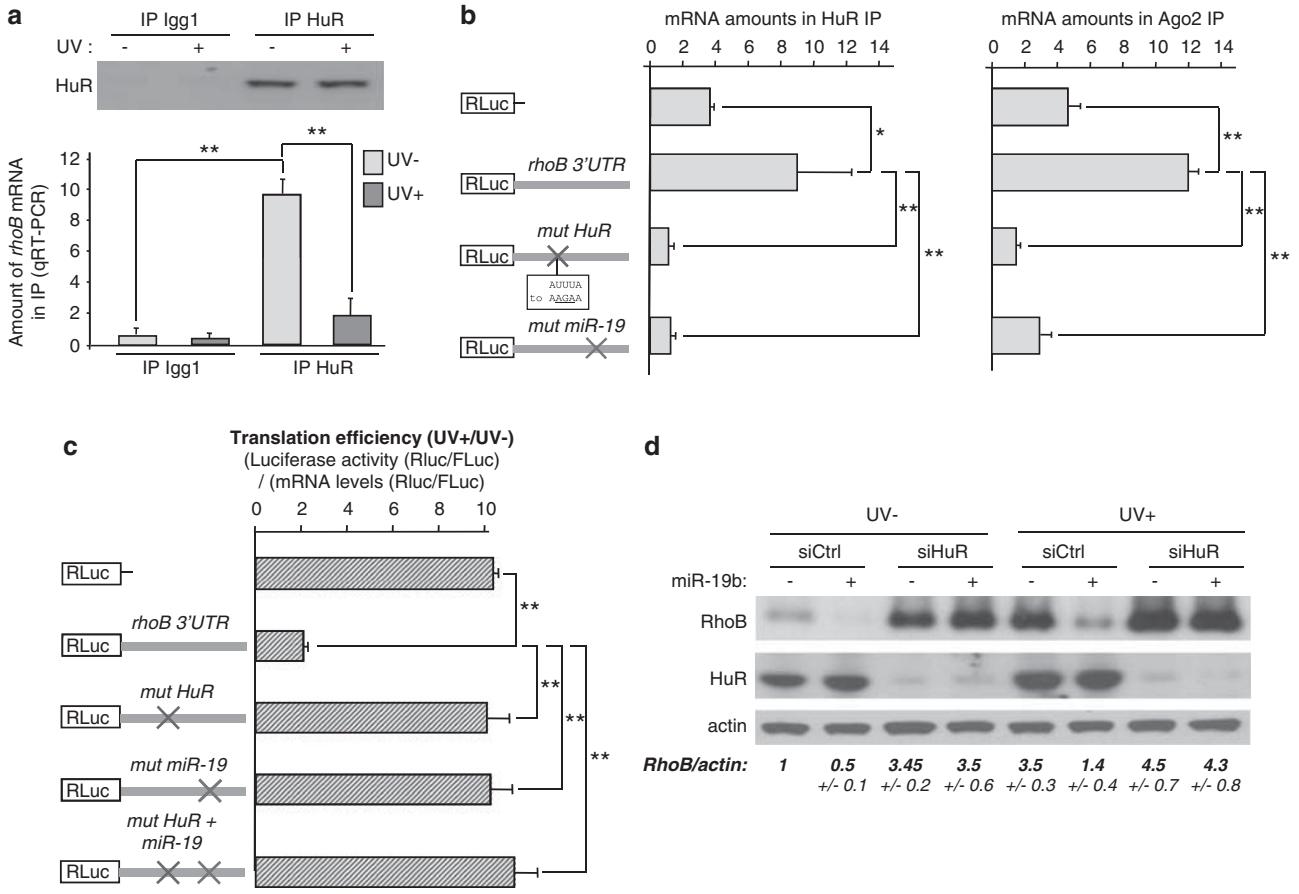

Figure 6 Human antigen R (HuR) and miR-19 control ultraviolet (UV) regulated Ras homolog B (RhoB) expression in an interdependent manner. (**a**) HuR was immunoprecipitated from UV-treated or control HaCat cells before western blot (WB) analysis (upper panel). Igg1-loaded beads were used as a control. The endogenous *rhoB* mRNAs was isolated from HuR immunoprecipitates (IP) and reverse transcribed. The levels of HuR-associated mRNAs were quantified by real time quantitative RT-PCR analysis (qRT-PCR) relative to *gapdh* mRNA. For each HuR IP sample, relative mRNA levels were normalized to the corresponding input samples. (**b**) The presence of each indicated *RLuc* mRNA in either HuR IP (left) or argonaute2 (Ago2) (right) IP was measured by qRT-PCR and quantified relative to the input sample and to the Firefly luciferase (*FLuc*) mRNA. (**c**) Luciferase values were normalized to luciferase mRNA levels (determined by qRT-PCR) to obtain translation efficiencies. The values are averages from at least three transfections ± S.D. (**d**) HaCat cells were transfected with an miR-19b synthetic oligonucleotide and/or with an HuR small interfering (siRNA) (or a control siRNA). After 48 h, HaCat cells were UV irradiated, and 8 h later, proteins were extracted and immunoblotted with anti-RhoB, anti-HuR or anti-actin antibodies. $^{*}P<0.05$; $^{**}P<0.01$

activity of Ago2 may be regulated by post-translational modifications during UV exposure[35,36] (Figure 8 (ii)). Finally, an unknown component may mask the various interactions between the mRNA, the RBP and the miRISC complex (Figure 8 (iii)).

Given the central role of HuR and other RBPs that interact with 3′-UTRs and regulate mRNA translation, we anticipate that RBP-dependent regulatory modes like the one described here will be widely found as mechanisms for post-transcriptional control of gene expression during stress and other stimuli.

Materials and Methods

Cell culture/UV irradiation. Immortalized human keratinocyte cells (HaCaT) were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum at 37 °C in a 5% CO_2 atmosphere with 95% humidity. For UV irradiation, HaCat cells, serum starved for 24 h, were irradiated at 254 nm using a UV Stratalinker (Stratagene, La Jolla, CA, USA). For all experiments, except where indicated, cells were exposed to a dose of 60 J/m^2 and collected 8 h later.

Western blot. Fifty micrograms of cell extract were separated on SDS-PAGE and electrotransferred onto polyvinylidene difluoride membranes. Blots were probed with the following antibodies: a rabbit polyclonal anti-RhoB (119; Santa Cruz Biotechnology, Santa Cruz, CA, USA), a mouse monoclonal anti-HuR (3A2; Santa Cruz Biotechnology), a rabbit polyclonal anti-PARP (Cell Signaling, Danvers, MA, USA), a rabbit polyclonal anti-eiF2α (Cell Signaling), a rabbit polyclonal anti-phosphorylated eiF2α (Cell signaling) and a mouse monoclonal anti-actin (Chemicon, Temecula, CA, USA). Detection was performed using peroxidase-conjugated secondary antibodies and chemiluminescence detection kit (Pierce, Rockford, IL, USA). Signals obtained from linear-scanned autoradiographies were quantified with Image J software.

Plasmid constructs. The RLuc-*p53* 3′-UTR and RLuc-*rhoB* 3′-UTR plasmids were constructed by inserting the *p53* 3′-UTR or the *rhoB* 3′-UTR into the *Xba*I/*Bam*HI restriction sites of the intronless pRL-CMV (Promega, Madison, WI, USA). The RLuc-*rhoB* 818–870 plasmid was constructed by inserting the sequence of the *rhoB* 3′-UTR spanning nucleotides 818–870 into the *Xba*I/*Bam*HI restriction sites of pRL-CMV. Deletions in the *rhoB* 3′-UTR were obtained by PCR using primers containing *Xho*I restriction sites. Primers used for PCR are described in Supplementary Table S2. Mutations in miR-19b or HuR binding sites were introduced with the QuickChange site-directed mutagenesis kit II (Stratagene) according to the manufacturer's instructions. Mutations were confirmed by sequencing.

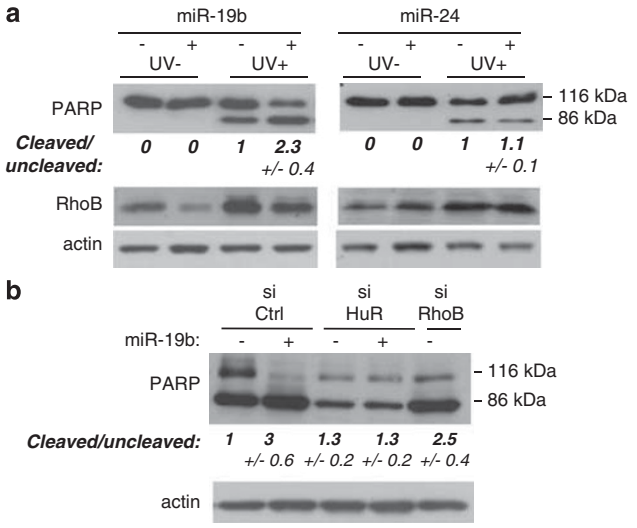

Figure 7 miR-19 increases ultraviolet (UV)-induced apoptosis in a human antigen R (HuR)-dependent manner. (**a** and **b**) HaCat cells were transfected with an miR-19b synthetic oligonucleotide or a miR-24 synthetic oligonucleotide and/or small interfering RNA (siRNA) against either HuR, Ras homolog B (RhoB) or a control siRNA. After 48 h, cells were UV irradiated, and 8 h later, proteins were extracted and immunoblotted with anti-RhoB, anti-PARP or anti-actin antibodies. The cleaved/uncleaved ratio was set to 1 for the UV + condition in the left panel

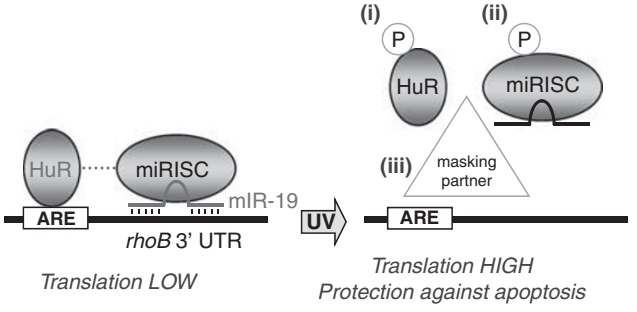

Figure 8 A scheme representing the proposed model of the regulation of the concerted binding of human antigen R (HuR) and an miRNA-induced silencing complex (miRISC) complex to *rhoB* 3′-untranslated region (3′-UTR)-containing mRNA. Disruption of the tripartite complex between the mRNA, HuR and miRISC may be regulated by a post-translational modification (P) in HuR (i), in the miRISC (ii) or by a masking component (iii)

Transfections and reporter gene assays. Plasmid constructs (1.5 μg) were transiently transfected in cells plated on 3.5 cm Petri dishes using jetPEI (Polyplus transfection). Cells were harvested 48 h after transfection and luciferase activities were measured using the Dual Luciferase Assay system (Promega). Lipofectamine RNAimax reagent (Invitrogen, Carlsbad, CA, USA) was used to transfect siRNAs (synthesized by Eurogentec (Angers, France); sequences in Supplementary Table S3) at a concentration of 20 nM, miRNA oligonucleotides (Qiagen, Hilden, Germany) at a concentration of 2 nM and anti-miRNAs (Qiagen) at a concentration of 50 nM.

Polysomal fractionation analysis. Experiments were carried out essentially as in Gherzi *et al.*[34] Briefly, HaCat cells (30 million) were treated with 0.1 mg/ml cycloheximide (CHX) for 15 min at 37 °C, washed twice with ice-cold PBS/CHX and scraped in PBS/CHX. After centrifugation, the cell pellet was resuspended in 400 μl of LSB buffer (20 mM Tris-HCl, pH = 7.5, 100 mM NaCl, 3 mM $MgCl_2$ and 100 U/ml RNAsine). After 10 strokes of Dounce homogenization, 400 μl of LSB containing 0.2% Triton X-100 and 0.25 M sucrose was added. Cellular debris was removed by centrifugation. The lysate was layered on an 11.3 ml continuous sucrose gradient (15–50% sucrose in LSB buffer). After 120 min of centrifugation at 38 000 r.p.m. in SW41-ti rotor at 4 °C, the fractions were collected with an ISCO (Lincoln, NE, USA) density gradient fractionation system (Foxy Jr fraction collector coupled to UA-6 UV detector). The settings were as follows: pump speed 0.75 ml/min, fraction time 1.2 min/fraction, chart speed 150 cm/h and sensitivity of OD254 recorder to 1. The absorbance at 254 nm was measured continuously as a function of gradient depth. Seventeen fractions of 0.9 ml were collected. RNAs from polysomal fractions were extracted by TRIzol LS Reagent (Invitrogen).

RNA extraction. Total RNA from cultured cells were isolated using TRIzol Reagent (Invitrogen). RNA quality was ascertained using a spectrophotometer and visualized on agarose gel.

qRT-PCR quantification of mRNAs. Total RNA (1 μg) was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. qRT-PCR was performed with an iCycler iQreal-time PCR detection system (Bio-Rad) using iQ SYBR Green Supermix (Bio-Rad) and the primers described in Supplementary Table S4.

Quantification of miRNAs. qRT-PCR was carried out using TaqMAn MicroRNA Assays Kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. MiRNA microarrays (Exiqon, Vedbaek, Denmark) were performed.[37]

Ribonucleoprotein immunoprecipitation. Thirty million cells were used per condition. Cells were washed twice with PBS and lysed in 750 μl cold lysis buffer (25 mM Tris-HCl, pH = 7.4; 150 mM KCl; 0.5% NP40; 2 mM EDTA; 1 mM NaF; 0.5 mM DTT; 0.2 U RNAsin (Promega)) for 30 min at 4 °C. Lysate supernatants were precleared for 30 min at 4 °C by using 20 μl of Protein A/G + agarose beads (Sigma, St. Louis, MO, USA) that had been previously blocked in washing buffer (300 mM KCl; 50 mM Tris-HCl, pH = 7.4; 1 mM $MgCl_2$; 0.1% NP40) containing yeast tRNA (Invitrogen) for 5 h at 4 °C with rotation. Beads (20 μl) were incubated (16 h at 4 °C) with 15 μg of anti-Ago2 (clone 2E12-1C9; Abnova, Heidelberg, Germany) antibody or anti-HuR (3A2; Santa Cruz Biotechnoloy) antibody and next overnight at 4 °C with 1 mg of cell lysate. After extensive washes, the extract was digested with proteinase K and RNAs were extracted using TRIzol LS Reagent (Invitrogen). RNAs isolated from ribonucleoprotein immunoprecipitation material were treated with turboDNAse (Ambion, Austin, TX, USA) and were subsequently used to perform qRT-PCR.

miRNAs microarrays. Total RNA (5 μg) was ligated to RNA-linker-Cy3-dye using T4 RNA ligase (Ambion) overnight at 4 °C, followed by ethanol precipitation. After overnight hybridization at 54 °C of labeled RNA on LNA-modified microarray slides (Exiqon) and extensive washing, slides were scanned using an Axon 4200 AL scanner where the photomultiplier settings were automatically adjusted. Microarray images were analyzed using the GenePix pro 6.0 software (Axon Instruments, Sunnyvale, CA, USA).

Statistical analysis. The mean ± S.D. was calculated for each data point. Differences between groups were analyzed by the Student's *t*-test or ANOVA (Dunnett's multiple comparison test).

Conflict of Interest

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)