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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL RESULTS

HuR does not measurably affect SIRT1 mRNA translation

HuR has been shown to influence the translation of several target mRNAs (Kullmann et al., 2002; Mazan-Mamczarz et al., 2003; Lal et al., 2005; Meng et al., 2005). To test if HuR also directly modulated the rate of SIRT1 mRNA translation, nascent SIRT1 production was monitored following a brief (20-min long) incubation with L-[^{35}S]methionine and L-[^{35}S]cysteine in cells expressing either control or silenced HuR levels (Suppl. Fig. S1, panel A). This assay revealed that the reduced *de novo* translation of SIRT1 in HuR-silenced cells (one third of the translation levels seen in control cells) was comparable to the reduction in SIRT1 mRNA levels (also one third of control cells), suggesting that the reduced translation rate was simply a reflection of the reduced SIRT1 mRNA abundance (Fig. 2B). Of note, HuR-silenced populations exhibit a reduction in total Sirt1 that is significantly greater than the reduction in mRNA levels or translation rate (compare Fig. 2A with Fig. 2B and with Fig. S1), suggesting that additional regulatory levels, possibly at the level of proteolysis, may participate in dictating SIRT1 abundance. To further assess a possible influence of HuR on the rate of SIRT1 translation, the relative distribution of the SIRT1 mRNA on polysome gradients was examined (Suppl. Fig. S1, panel B). By this approach, increases in the abundance of the SIRT1 mRNA in the heavy fractions of the gradient would indicate an increased association of the SIRT1 mRNA with the translational machinery and hence an elevation in SIRT1 protein biosynthesis. However, as shown in Fig. S1B, the relative distribution of the SIRT1 mRNA along the polysome gradients prepared from control cultures was virtually indistinguishable from that obtained using HuR siRNA cultures, further supporting the notion that HuR does not influence the translational status of the SIRT1 mRNA. In sum, HuR enhances SIRT1 mRNA stability but does not seem to influence its translation rate.

SUPPLEMENTAL DISCUSSION

HuR-mediated mRNA stabilization and translational regulation

Our data indicate that HuR regulates the stability of SIRT1 mRNA in both HeLa and WI-38 cells (Figs. 2 and 3), but does not overtly affect its translation rate (Suppl. Figs. S1 and S2). With a growing number of HuR targets described in detail, it is somewhat puzzling that HuR regulates the stability of a subset of target mRNAs such as those encoding p21, cyclin A, cyclin B1, cyclin D1, c-fos (Wang et al., 2000a, 2000b, 2001;

Lal et al., 2004) and SIRT1, as described here, while it functions as a translational regulator of a different subset of target mRNAs [such as those encoding p53, ProTα, p27, IGF-IR (Kullmann et al., 2002; Mazan-Mamczarz et al., 2003; Lal et al., 2005; Meng et al., 2005)]. The reasons for these differential effects have not been examined systematically, but they could be linked to the subcellular localization of the particular HuR-containing RNP: a nuclear RNP might be more stable than a cytosolic RNP, an RNP being recruited to polysomes would be associated with higher translation than one being recruited to stress granules, etc. Another possibility is that HuR displaces or competes for binding with other ARE-RBPs. According to this regulatory scheme, if HuR binding causes the dissociation of a translational repressor (e.g., TIA proteins) on a shared mRNA, the likely outcome would be an enhancement in the translation of the mRNA; if HuR binding to an mRNA displaces an RBP that promotes decay (e.g., AUF1, TTP, KSRP, BRF1, etc), then the net effect would be mRNA stabilization. While many ARE-RBP have indeed been found to share in binding to HuR target mRNAs (Cok et al., 2003; Lal et al., 2004; Tran et al., 2004; Kawai et al., 2006), the interplay between these RBPs remains largely unexplored.

SUPPLEMENTAL METHODS

Cell fractionation

For the preparation of cytosolic fractions, cells were scraped in 200 μl of lysis buffer (10 mM Tris–HCl (pH 7.5), 100 mM NaCl, 2.5 mM $MgCl_2$, and 40 mg/ml digitonin). The lysate was incubated on ice for 5 min and centrifuged (2060 × *g*, 8 min, 4°C), and the supernatant was designated as the soluble cytosolic fraction. The pellet was washed twice with the same buffer before adding RIPA lysis buffer (10 mM Tris-HCl [pH 7.4], 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.1% SDS, and 1 mM DTT), incubated on ice for 5 min and centrifuged (21,000 × *g*, 8 min, 4°C), to obtain the nuclear fraction. Whole-cell lysates were prepared using RIPA buffer, as described (Lal et al*.,* 2004).

Primers used for qPCR detection of mRNA after immunoprecipitation of RNP complexes and reverse transcription. The RNA isolated from IP material was reverse-transcribed using random hexamers, oligo-dT primer, and SSII Reverse Transcriptase (Invitrogen). The oligomers pairs (each forward and reverse) used for the amplification of PCR products were:
CAGGTTGCGGGAATCCAAAG and GCTGGGCACCTAGGACATCG for SIRT1,
GAGGCCAGGACAACAGAGAG and TAGAGATTTGCTGGGGTTGG for SIRT2,
CATGAGCTGCAGTGACTGGT and GAGCTTGCCGTTCAACTAGG for SIRT3,
CAGCAAGTCCTCCTCTGGAC and CCAGCCTACGAAGTTTCTCG for SIRT4,

TTCAAAGGCAGAAACCAACC and CTAATGCAAAAGCAGCCACA for SIRT5, CCAAGTTCGACACCACCTTT and CGGACGTACTGCGTCTTACA for SIRT6, CGCCAAATACTTGGTCGTCT and GTGATGCTCATGTGGGTGAG for SIRT7, CCTGAAAGCAGAAACCGGTC and CCTCATACCAGGCTTCCAGC for GADD153, TGCACCACCAACTGCTTAGC and GGCATGGACTGTGGTCATGAG for GAPDH, GGGGAAGGGACACACAAGAAGA and AATGAACTGGGGAGGGATGG for p21, ATTTGGGTCGCGGTTCTTG and TGCCTTGACATTCTCGATGGT for UBC, CAGAACACGGCTCACGCTTAC and CTTGCCCCATCACGACAGAC for cyclin D1, AAGAAGCCAGCTGAATCTCAAA and GGTCCAGGTAAACTAATGGCTGAA for cyclin A, CCCTGCTGCAACCTCCAA and TGTTCACTGACTTTGTTACCAATGTC for cyclin B1, CAACTTTTCACAAAGATGGTGAGTG and GAGGCAAATGAACATGAACACAA for cytochrome *c*, CCAACCCAAACCATGAGAA and GGTCACACCACAAGTAAAGTCAG for ProTα, and CCCTATCAACTTTCGATGGTAGTCG and CCAATGGATCCTCGTTAAAGGATTT for 18S.

Analysis of newly translated protein. New synthesis of SIRT1 was measured by incubating HeLa cells with 1 mCi L-[^{35}S]methionine and L-[^{35}S]cysteine (Easy Tag^{TM}EXPRESS, NEN/Perkin Elmer, Boston, MA) per 60-mm plate for 20 min, whereupon cells were lysed using RIPA buffer. Immunoprecipitations were carried out for 1 h at 4 ºC using either a polyclonal antibody recognizing SIRT1, GAPDH (Santa Cruz Biotech.) or IgG1 (BD Pharmingen). Following extensive washes in TNN buffer (50 mM Tris-HCl [pH 7.5], 250 mM NaCl, 5 mM EDTA, 0.5% NP-40), the immunoprecipitated material was resolved by 10% SDS-PAGE, transferred onto PVDF filters, and visualized with a PhosphorImager (Molecular Dynamics).

Assessment of DNA synthesis by monitoring [^{3}H]-Thymidine incorporation. [Methyl-^{3}H] thymidine (85 Ci/mmol, Amersham) incorporation into DNA was monitored after incubation of cells with 2 μCi [^{3}H] thymidine per ml for 16 h, after which they were washed twice with PBS, immersed in 1 ml of cold 5% trichloroacetic acid (TCA) for 30 min at 4 °C, washed twice with PBS and then lysed in 1 ml of 0.5 N NaOH, 0.5% SDS (37 °C, 30 min). The lysate (1 ml) was then mixed with 4 ml of liquid scintillation cocktail (Beckman), and radioactivity was measured by liquid scintillation counting. Data are shown as cpm incorporated per mg of whole-cell protein lysate.

Polysome analysis. HeLa cells (5×10^6 per sample) at ~80% confluence were incubated for 15 min in 0.1 mg/ml cycloheximide, then lifted by scraping in 1 ml PEB lysis buffer (0.3 M NaCl, 15 mM $MgCl_2$, 15 mM Tris-HCl, pH 7.6, 1% Triton X-100, 1 mg/ml heparin, and 0.1 mg/ml cycloheximide) and lysed on ice for 10 min. Nuclei were pelleted (10,000 × *g*, 10 min) and the resulting supernatant was fractionated through a 10-

50% linear sucrose gradient, as described (Galban et al., 2003; Lal et al., 2004). The eluted fractions were prepared with a fraction collector (Brandel) and their quality monitored at 254 nm using a UV-6 detector (ISCO). RNA in each fraction was extracted with 8 M guanidine-HCl.

Yeast two-hybrid screen. Human Chk2 was fused to the Gal4 DNA-binding domain in plasmid pGBkT7 and transformed into yeast strain AH109. A two-hybrid screen was then performed by mating AH109-pGBkT7-Chk2 cells with yeast strain Y187 expressing a Clontech Matchmaker Hela cDNA library from plasmid pGADT7. A total of 5.3×10^6 diploid AH109/Y187 colony forming units were plated on quadruple drop-out (QDO; -His, -Ade, -Leu, -Trp) medium and surviving colonies screened for β-galactosidase reporter activity. pGADT7 plasmids encoding potential interacting cDNAs from β-galactosidase-positive colonies were recovered, re-transformed into Y187 and retested by plate mating against AH109 bearing pGBkT7-Chk2 together with appropriate positive and negative controls. Of 12 Chk2-interacting cDNAs isolated in this screen, one corresponded to human HuR. The outcome of this screen will be described in detail elsewhere.

Construction and purification of baterially expressed HuR point mutants. HuR S88, S100, and T118 amino acid residues were changed individually to alanine by site-directed mutagenesis in plasmid pGEX2T-HuR (Ma et al., 1996). Wild-type GST-HuR and mutant derivatives were expressed in *E. coli* strain BL21, purified by chromatography on glutathione-agarose beads, and incubated with purified recombinant Chk1 and Chk2 (Upstate) in a kinase reaction containing 20 mM HEPES, 50 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.5 mM DTT, 40 μM ATP and μ15 Ci [γ-^{32}ATP] for 30 min at 37°C. GST-Cdc25C was used as a positive control. Kinase reactions were resolved on 10% SDS-PAGE after which the gels were dried and subjected to autoradiography.

Pro-Q Diamond and SYPRO Ruby staining. Recombinant purified GST-HuR (0.4 mg) (Wang et al., 2005) was incubated with 4 ng Chk2 active enzyme (Upstate) and 0.16 mM ATP (10 min, 30°C). The reactions were stopped by adding 2× SDS-PAGE buffer, and the products size-separated by SDS-PAGE. Total and phosphorylated protein were detected as instructed by the manufacturer (Molecular Probes). To detect phosphorylated HuR, gels were fixed (50% methanol, 10% acetic acid) for 16 h, stained for 2 h using Pro-Q®Diamond (Molecular Probes), destained, rinsed, and visualized using a Typhoon 9410 (Amersham Biosciences) at excitation 532 nm and emission 560 nm. To detect total protein, gels were incubated with SYPRO®Ruby (Molecular Probes) for 16 h, washed (10% methanol, 7% acetic acid), and rinsed. Stained gels were visualized at 450 nm (excitiation) and 610 nm (emission).

Immunoprecipitation of RNP complexes and RT-PCR. Immunoprecipitation (IP) of endogenous RNA-protein complexes was previously described (Lal et al., 2004). Briefly, cytoplasmic lysates, prepared from either untreated or H_2O_2-treated cells, were divided into two equal parts and incubated (1 h, 4°C) with 100 µl of a 50% (v/v) suspension of Protein-A Sepharose beads precoated with 30 µg each of mouse IgG1 (BD Pharmingen) or anti-HuR (Santa Cruz Biotech.). The beads were washed five times with NT2 buffer. For RNA analysis, the beads were incubated with 100 µl NT2 buffer containing 20 units of RNase-free DNase I (15 min, 30°C), washed twice with 1 ml NT2 buffer, and further incubated in 100 µl NT2 buffer containing 0.1% SDS and 0.5 mg/ml Proteinase K (15 min, 55°C) to digest the proteins bound to the beads. Before adding Proteinase K, 10-µl aliquots were taken for Western blot analysis. RNA was extracted using phenol and chloroform, and precipitated in the presence of glycoblue.

Synthesis of biotinylated transcripts and analysis of RNP bound to biotinylated RNA. For *in vitro* synthesis of biotinylated transcripts, reverse-transcribed total RNA was used as the template for PCR amplification using 5' oligonucleotides that contained the T7 RNA polymerase promoter sequence (T_7, CCAAGCTTCTAATACGACTCACTATAGGGAGA). Oligonucleotide pairs (sense and antisense) used to synthesize DNA templates for the production of biotinylated transcripts were as follows: for the coding region of SIRT1, (T_7)TTCACCACCAGATTCTTCAGTG and TCTCTGGAACATCAGGCTCA; for the 3'UTR of SIRT1, (T7)CCCTGATTATACAGTTCCAAAGTAA and AAACAGTCTACAAAACATATG CCAGT; for the 3'UTR of GAPDH, (T_7)CCTCAA CGACCACTTTGTCA and GGTTGAGCACAGGGTA CTTTATT. PCR-amplified products were used as templates for the synthesis of the corresponding biotinylated RNAs using T7 RNA polymerase and biotin-CTP. Biotin pulldown assays were carried out by incubating either whole-cell lysates with purified biotinylated transcripts (25 µg lysate, 1 µg RNA) for 1 h at 25 °C. Complexes were isolated with paramagnetic streptavidin-conjugated Dynabeads (Dynal, Oslo, Norway), and bound proteins in the pulldown material were analyzed by Western blotting using monoclonal antibodies that recognized either HuR, NF90 or TIA-1 (Santa Cruz Biotech.).

Abbreviations: CR, coding region; IP, immunoprecipitation; RBP, RNA-binding protein; RNP, ribonucleoprotein complex; SIRT, silencer of transcription; UTR, untranslated region

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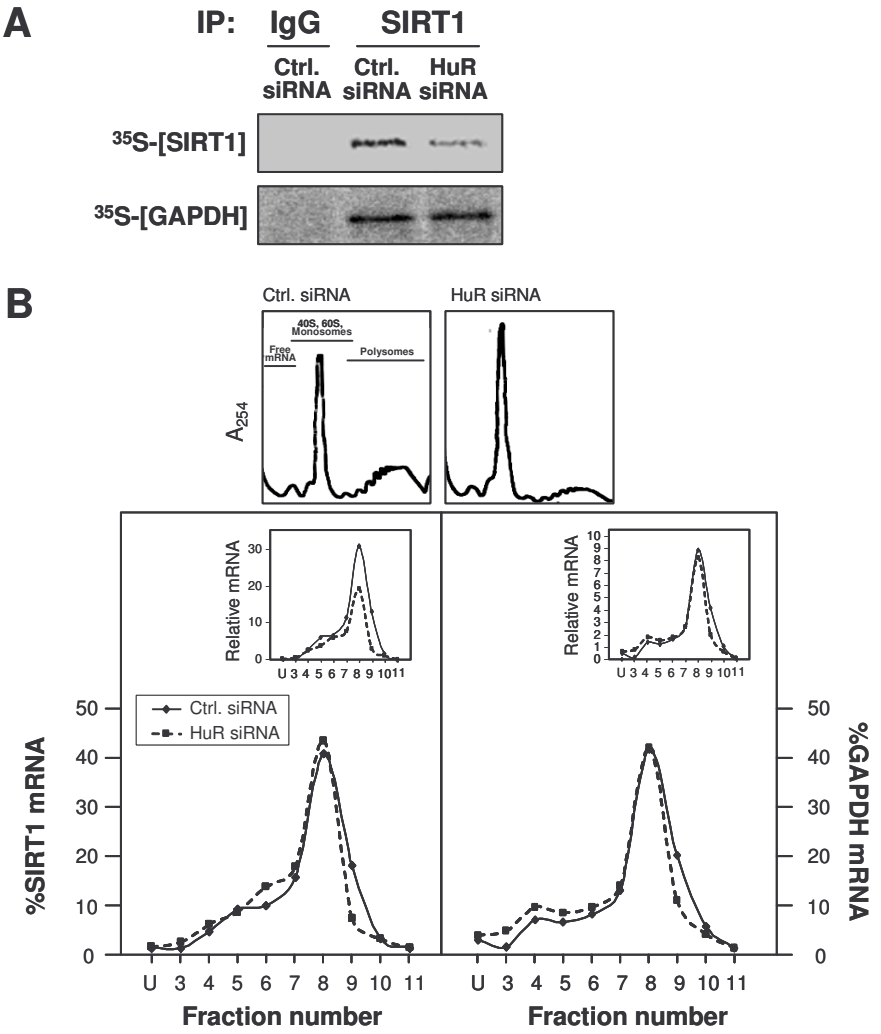
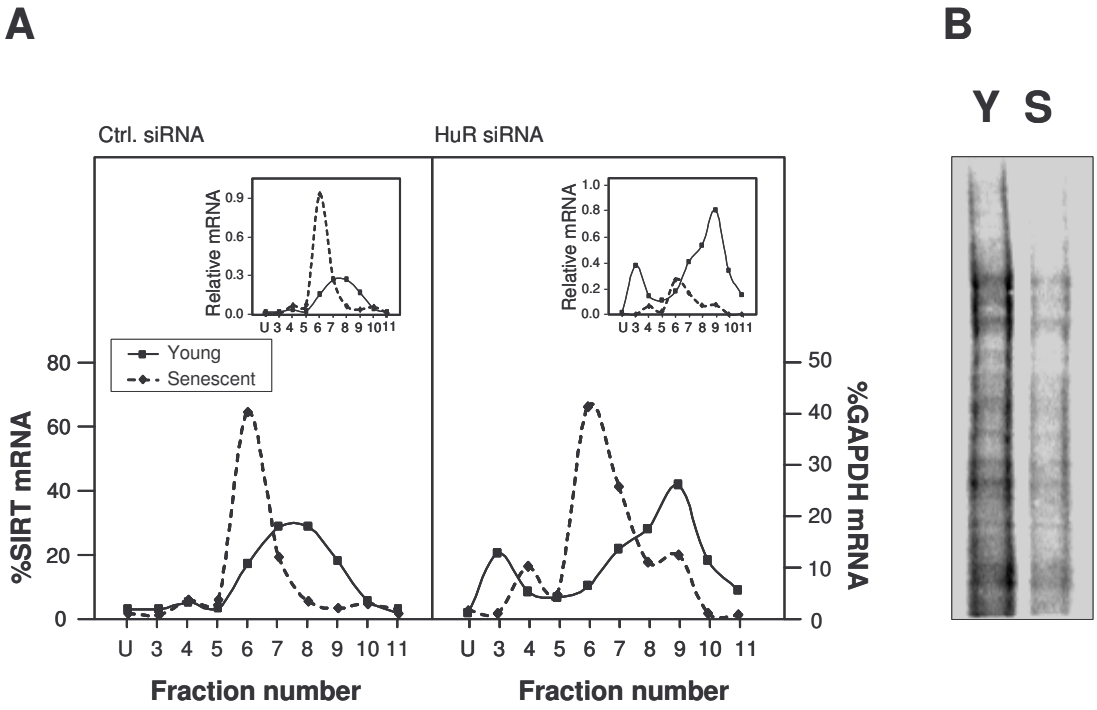


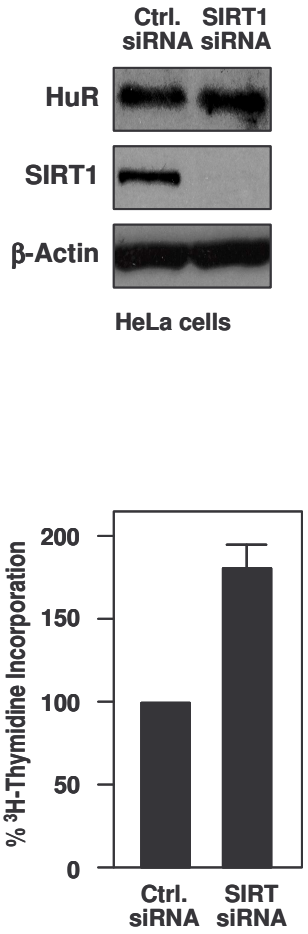
Supplemental Figure S1. SIRT1 mRNA translation is not influenced by HuR. (A) HeLa cells transfected with the indicated siRNAs (as in Fig. 2) were pulse-labeled with L-[^{35}S]methionine and L-[^{35}S]cysteine, and nascent SIRT1 detected by IP (Suppl. Experimental Procedures). IgG was used as a negative control IP, and anti-GAPDH IP was included to control the input material. IP samples were resolved by SDS-PAGE (7% polyacrylamide), transferred onto filters, and visualized with a PhosphorImager. **(B)** Cytoplasmic lysates of cells transfected as in Fig. 2 were size-separated through linear sucrose gradients. RNA was isolated from the resulting fractions (11 in total; fractions 1+2 were combined and termed the 'unbound' fraction, 'U') and SIRT1 and GAPDH mRNAs detected in each fraction by using RT-qPCR. Data are represented as the percentage of the total mRNA in each fraction of each treatment group; inset, relative mRNA abundance in each fraction of each group (SIRT1 mRNA was higher overall in Ctrl. siRNA cells).

These data indicate that nascent SIRT1 translation was reduced by ~one-third after HuR silencing, suggesting that HuR would be directly necessary for SIRT1 translation. However, HuR silencing reduced SIRT1 mRNA expression levels down to <u>also</u> ~one-third, while HuR silencing did not cause a shift (a leftward shift, towards lower molecular weight polysomes) in the relative distribution of the SIRT1 mRNA on sucrose gradients, which would also support the view that translation was repressed. Taken all of the available data together, the most adequate interpretation at this point is that HuR reduces only the stability and steady-state levels of the SIRT1 mRNA and this, in turn, reflects in a reduction of comparable magnitude in SIRT1 translation.

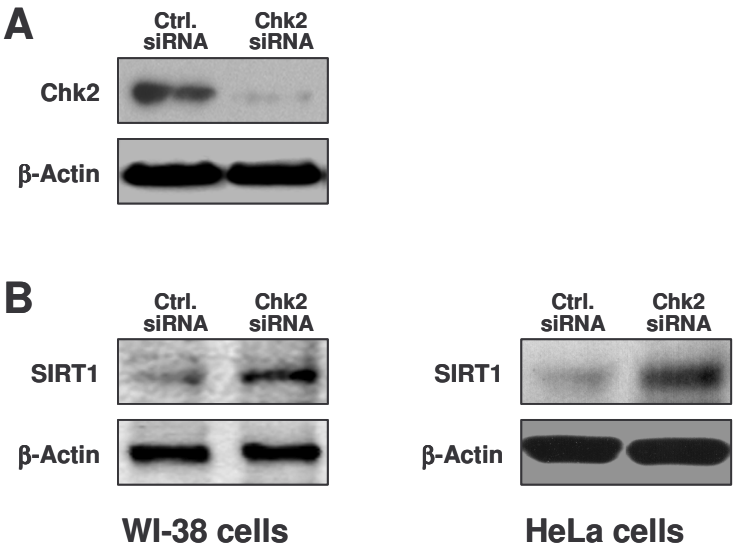


Supplemental Figure S2. Translational status of SIRT1 mRNA in Young and Senescent WI-38 cells. (A) Cytoplasmic lysates from either Young (Y) or Senescent (S) WI-38 cells were size-separated through linear sucrose gradients. RNA was isolated from the resulting fractions (11 in total; fractions 1+2 were combined and termed the 'unbound' fraction, 'U') and the SIRT1 and GAPDH mRNAs detected in each fraction by using RT-qPCR. Data are represented as a the percentage of the total mRNA detected in each fraction of each treatment group; inset graph, relative mRNA abundance in each fraction of each group (SIRT1 mRNA was higher overall in Ctrl. siRNA cells). **(B)** To assess differences in global translation profiles in Young (Y) and senescent (S) WI-38 cells, cultures were labeled with L-[^{35}S]methionine and L-[^{35}S]cysteine for 30 min and 5-μg aliquots were resolved by SDS-PAGE (7% polyacrylamide), transferred onto filters, and visualized using a PhosphorImager.

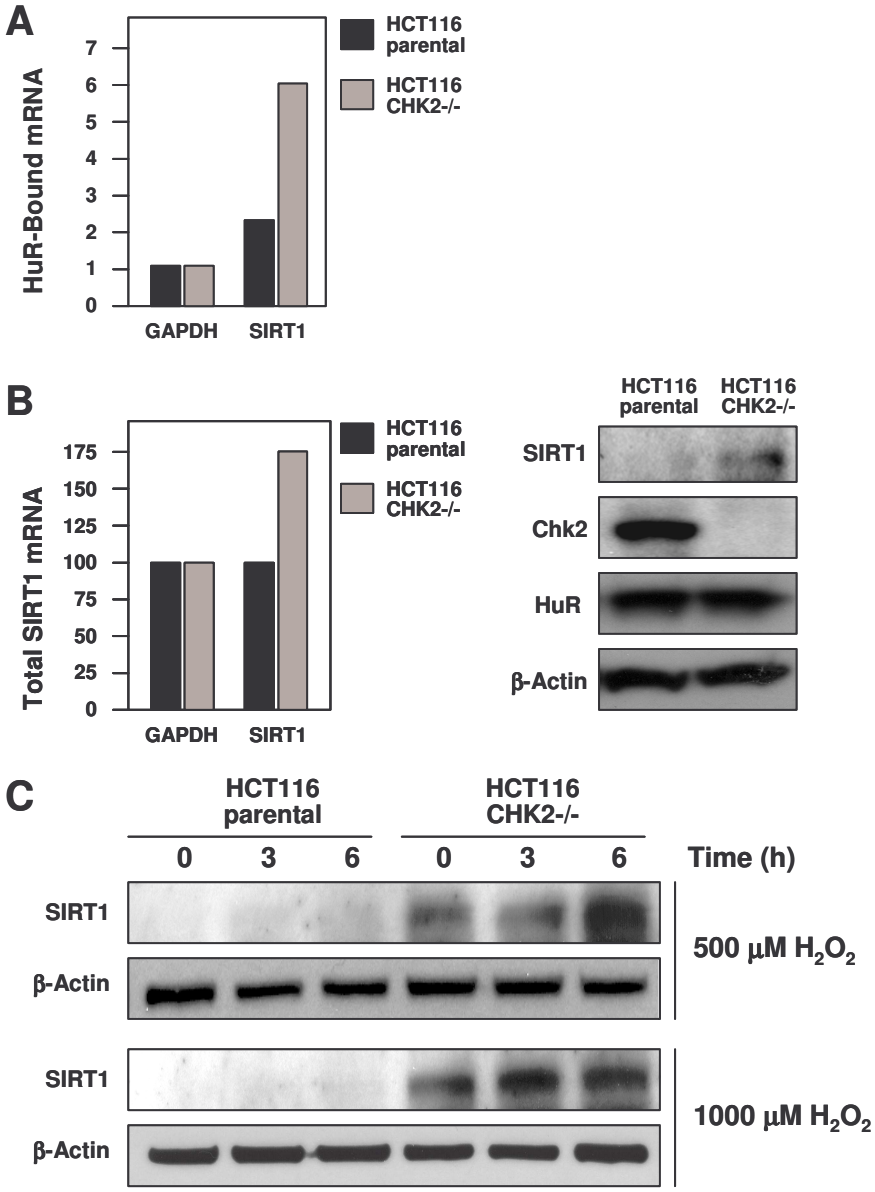
These results suggest that the SIRT1 mRNA is more actively translated in Young cells than in Senescent cells, since Young cells display a greater degree of association of the SIRT1 mRNA with the translational machinery (heavier polysomal fractions). However, this relative difference is comparable in magnitude to that seen for GAPDH mRNA (panel A), and is in keeping with the relatively higher translation rate seen in Young cells (panel B), so the resulting differences in translation appear not to be specific to SIRT1.



Supplemental Figure S3. Effect of silencing SIRT1 on HuR expression levels and cell proliferation. (A) Following transfection of HeLa cells with either control (Ctrl.) or SIRT1-targeting siRNAs, the expression of SIRT1, HuR, and loading control β-Actin was monitored by Western blot analysis. Ten μg of whole-cell lysate was loaded per lane. **(B)** Incorporation of ^{3}H-Thymidine increased after silencing SIRT1. Forty-eight h after transfection of WI-38 cells with either control or SIRT1-targeting siRNAs, cell proliferation was assessed by monitoring ^{3}H-thymidine incorporation (as described in Experimental Procedures).

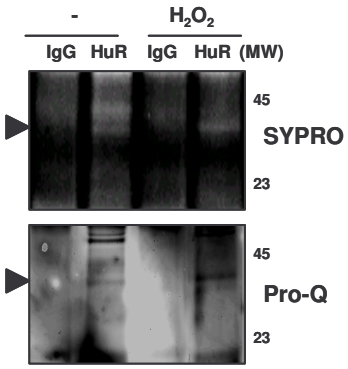


Supplemental Figure S4. Silencing Chk2 elevates SIRT1 expression levels. (A) Western blot analysis of Chk2 expression levels in WI-38 whole-cell lysates prepared from siRNA transfection groups that were treated as described in Fig. 6B; similar reductions in Chk2 were achieved in HeLa cultures (not shown). **(B)** Western blot analysis of SIRT1 expression levels in whole-cell lysates prepared from each siRNA transfection group. WI-38 cells (left) or HeLa cells (right) were processed as described in panel A and in Fig. 6B. The levels of β-Actin served to monitor differences in sample loading.

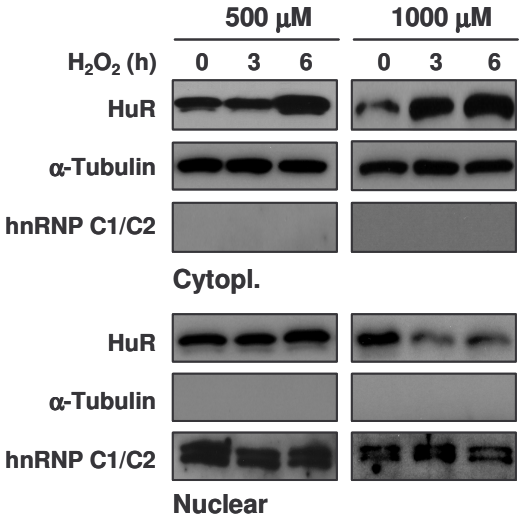


Supplemental Figure S5. SIRT1 expression in a model of genetic CHK2 inactivation. Colon cancer cells *HCT116 parental* as well as HCT116 cells deficient in CHK2 due to somatic deletion of both CHK2 alleles [*HCT116 CHK2-/-* cells, kindly provided by F. Bunz, Johns Hopkins University, Baltimore, MD (Jallepalli et al., 2003)] were used to measure HuR-bound SIRT1 mRNA by RNP IP analysis **(A)**, and steady-state SIRT1 mRNA and protein levels, as well as the levels of other mRNAs and proteins **(B)**. **(C)** Western blot analysis of SIRT1 expression at the doses and time periods shown after addition of H_2O_2. Data are representative of 2-3 independent experiments yielding similar results.

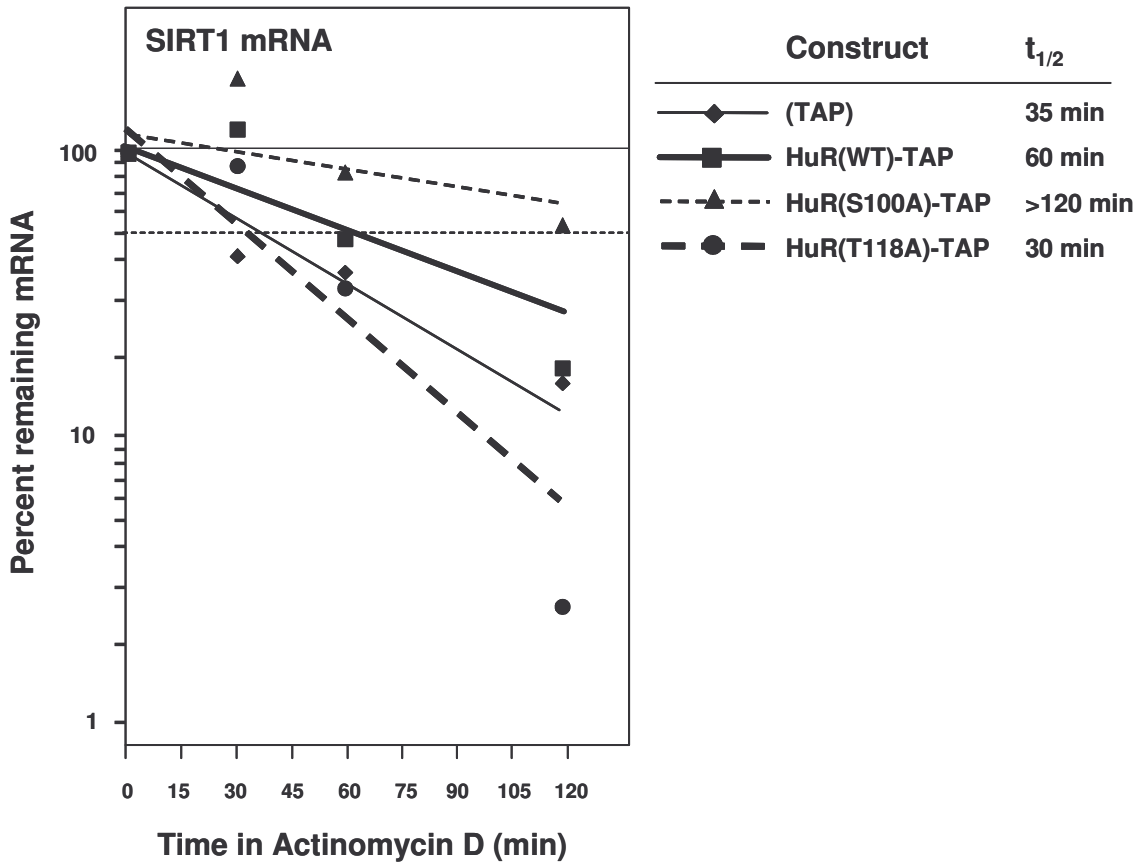
These results indicate that SIRT1 expression is constitutively elevated in HCT116 cells lacking CHK2 (HCT116 CHK2-/-), suggesting that CHK2 contributes to keeping SIRT1 expression levels down. The levels of SIRT1 in the parental HCT116 line were too low to study changes associated with H_2O_2 treatment.



Supplemental Figure S6. HuR phosphorylation as assessed by Pro-Q and SYPRO staining. HeLa cells were either treated with H_2O_2 (1000 μM, 3 h) or left untreated (-), whereupon whole-cell lysates were prepared, subjected to IP using IgG or anti-HuR antibody, size-fractionated by SDS-PAGE, and stained using Pro-Q Diamond or SYPRO Ruby. (MW), molecular weight marker (kDa).



Supplemental Figure S7. Increased HuR abundance in the cytoplasm of WI-38 cells following H_2O_2 treatment. Despite the lack of changes in whole-cell (WI-38) HuR abundance after H_2O_2 treatment (Fig. 4), these cells exhibited a significant increase in cytoplasmic HuR, in agreement with earlier reports (Wang et al, 2000b); the increase in cytoplasmic HuR levels was time- and dose-dependent. Nuclear HuR either remained unchanged (500 μM) or was modestly reduced (1000 μM). The levels of hnRNP C1/C2 (a predominantly nuclear protein) and α-Tubulin (a cytoplasmic protein) were used to monitor the quality of the fractionation and the even loading of samples.



Supplemental Figure S8. SIRT1 mRNA stability in each of the HuR-TAP transfection groups. HeLa cells were transfected with both *HuRU1 siRNA* (as described in Fig. 2B) to silence endogenous HuR selectively (since it targets the HuR 3'UTR) and with one of the plasmid vectors shown [to express TAP, HuR(WT)-TAP, HuR(S100A)-TAP, or HuR(T118A)-TAP] using Lipofectamine 2000 (Invitrogen). Forty-eight h later, cells were treated with Actinomycin D (2 μg/ml) and the levels of SIRT1 mRNA in each transfection group were measured from total RNA that was prepared at the times shown following the addition of Actinomycin D. SIRT1 mRNA levels were normalized to 18S rRNA levels and plotted as the percentage of mRNA remaining compared with the levels at time 0. The SIRT1 mRNA half-life in each transfection group was calculated as the time required to achieve 50% of its initial abundance in each population. The data represent the average of two independent experiments, each showing similar results.

Testing of the same transfection groups in the presence of *Ctrl. siRNA* (instead of *HuRU1 siRNA*) revealed that the SIRT1 mRNA stability was comparable in all transfection groups (not shown). These results indicated that silencing of endogenous HuR was necessary in order to study the effects of the HuR-TAP mutants.