Conserved CPEs in the P53 3' Untranslated Region Influence mRNA Stability and Protein Synthesis

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Abstract. *Background: The 3'untranslated region (UTR) of p53 mRNA contains two conserved U-rich sequences resembling cytoplasmic polyadenylation elements (CPE). It is not known if these sequences regulate p53 expression by post-transcriptional mechanisms. Materials and Methods: Stable p53 3'UTR reporter HaCaT skin and MCF-7 breast cancer cell lines were established. Quantitative PCR and an enzymatic assay were used to quantify the reporter mRNA and protein levels, respectively. Proteins binding to the CPEs were identified by RNA-immunoprecipitation (IP) and quantitative mass spectroscopy. Results: The wild-type p53 3'UTR reduced mRNA steady state levels of the reporter gene and point mutations in the CPEs rescued the mRNA steady state levels in the MCF-7 cells, but not in the HaCaT cells. In both cell lines, the CPEs had a significant effect on translation of the reporter and influenced the effect of UV irradiation. Several proteins (including GAPDH, heterogeneous nuclear ribonucleoprotein (hnRNP) D and A/B) were identified from the MCF-7 cytoplasmic extracts that bound specifically to the CPEs. Conclusion: Two conserved CPEs in the p53 3'UTR regulate stability and translation of a reporter mRNA in non-irradiated as well as irradiated cells. GAPDH, hnRNP D and hnRNP A/B bind specifically to the p53 CPEs and could potentially be involved in the post-transcriptional regulation of p53.*

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In normal cells, the tumor suppressor protein p53 inhibits growth or promotes apoptosis in response to intrinsic and extrinsic stress signals. This function is important for the prevention of cancer development and in the majority of human carcinomas the function and/or expression of p53 is compromised either by mutational events or interactions with other proteins (1). Stress stimuli such as DNA damage, hypoxia or nutrient deprivation enhance the expression and activity of p53 (2, 3). In normal unstressed cells, p53 protein levels are usually very low due to a very short half-life of the protein (4, 5), but in response to stress stimuli, the expression of p53 protein is enhanced (6). This up-regulation has mainly been accredited to post-translational modifications that alter the stability, location and activity of the p53 protein (7). Accumulating evidence has indicated that p53 expression is also regulated at the translational level. Reports have shown that inhibitors of protein synthesis block the increase in p53 protein expression after DNA damage (8-10), suggesting that translation is required for efficient up-regulation of p53. Murine p53 inhibits its own expression through binding to its own 5' untranslated region (UTR) (11). The 5'UTR of human p53 is also involved in translational control, but depends on the binding of ribosomal protein L26 and nucleolin (12) and an internal ribosome entry site (IRES) (13), which mediates translational regulation of p53 in response to DNA damage (14). In addition, human p53 3'UTR also influences the level of translation by binding of an unidentified protein (15-17) and increases the stability of the p53 mRNA by binding of the RNA-binding protein HuR (18, 19). The p53 3' UTR is not well conserved among vertebrates (Figure 1A), but does contain two highly conserved U-rich sequences resembling the cytoplasmic polyadenylation element (CPE) located immediately upstream of the polyadenylation sequence (20).

Cytoplasmic polyadenylation regulates translation of CPE-specific mRNAs during oocyte maturation, early development and long-term potentiation (LTP) in mouse neurons (21-24). In the present study, the regulatory role of the conserved CPEs present in human p53 3'UTR were investigated.

Materials and Methods

Plasmid constructions. The full-length wild-type (wt) 3'UTR (including the endogenous p53 poly(A) signal) from human p53 was amplified from genomic DNA (primer sequences available on request) and cloned into the *PinaI/NaeI* sites of the pHRSp-GUS (β-glucuronidase) retroviral vector (25) (Figure 1A). The mutated (mt) 3'UTR construct was generated by site-directed PCR mutagenesis (primer sequences available on request).

Cell culture and SILAC. Unless otherwise specified, all the cells were cultured in DMEM containing 10% FBS (VWR International, Stockholm, Sweden), 1% L-glutamine (Invitrogen, Merelbeke, Belgium) and 1% penicillin/streptomycin (Invitrogen) at 37˚C, 5% CO_2. PG13 cells were cultured in 10% newborn calf serum. For the mRNA stability and UV irradiation experiments, 1×10^6 cells were plated in 8.8 cm^2 dishes, incubated for 48 h and treated with actinomycin D (2 μg/ml) or UV-light (15 J/m^2). Prior to UV-stimulation the cells were washed once in PBS, exposed to UV-irradiation from a Stratalinker 2400 (Stratagene, La Jolla, CA, USA) and the medium was added back. For SILAC (stable isotope labeling by amino acids in cell culture) (26-28), MCF-7 breast cancer cells were cultured in DMEM with 10% dialyzed FBS (Invitrogen). One pool of cells was encoded with isotope-labeled lysine-$^{13}C_6{}^{15}N_2$ and used for RNA pull-down with a biotin-labelled CPE wt probe, whereas an unlabeled control pool of MCF-7 cells was used for RNA pull-down with a CPE mt probe (Figure 5A). After pull-down, the bound proteins were combined, trypsin digested and analysed by nanoscale liquid chromatography-tandem mass spectrometry (LC MS/MS) on an ion trap-Fourier Transform Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) (29). Peptides were assigned to proteins using the Mascot search engine (Matrix Science) and a protein SILAC ratio was calculated with MSQuant (http://msquant.sourceforge.net/). To increase the accuracy of the SILAC ratios, only the proteins with 4 or more peptides mapped were used.

Generation of viral packaging cells and cell transduction. Four μg of plasmid DNA were transfected into PG13 cells using LipofectAMINE Plus™ (Invitrogen) according to the manufacturer's protocol. The selection of stable transfected cells was performed with puromycin (PURO) (2.5 μM) 48 h post-transfection. The virus was harvested from the PG13 cells (grown to confluence in 175 cm^2 flasks and incubated overnight at 32˚C and 5.0% CO_2) and passed through a 0.45 μm sterile filter (Sartorius, Göttingen, Germany). The collected virus was supplemented with polybrene to a final concentration of 8 μg/ml (30) and added to 8.8 cm^2 dishes containing either HaCaT or MCF-7 cells. The cells were incubated for 4 h after which the medium was changed to fresh 10% FBS medium. Stable MCF-7 and HaCaT cells were selected with puromycin (2.5 μM) 48 h post-infection. Three independent pools of HaCaT and MCF-7 cells were generated for each construct.

Quantitative PCR. The total RNA was purified according to the RNeasy protocol (Qiagen, Hilden, Germany) and DNase treated using amplification grade I DNase (Invitrogen). Quantitative PCR (q-PCR) were performed using one-step RT-PCR QuantiTect™ SYBR® Green RT-PCR (Qiagen) and the Opticon MONITOR™ (MJ Research, Waltham, MA, USA). The primers (primer sequences available on request) used in the q-PCR reactions had PCR efficiencies close to 2, for both the target and reference gene. The GUS mRNA levels were normalized to PURO mRNA levels (31), and the relative ratio to the empty vector was calculated using the $2^{-\Delta\Delta CT}$ method (32).

GUS reporter assay. The protein extracts were collected using a lysis buffer; 100 mM potassium phosphate (pH 7.8), 0.2% Triton-X and 0.5 mM dithiotreitol (DTT) (added just before use). The total protein concentrations were determined by Coomassie®Plus Reagent (Pierce, Rockford, IL, USA). The GUS assay was performed with the FluorAce™ β-glucuronidase reporter assay kit (Biorad, Hemel Hempstead, UK) according to the manufacturer's protocol using a VICTOR^{2}™ Multilabel Counter (Wallac, Turku, Finland).

Preparation of cell extracts. The protein extract was prepared from confluent cells by the method outlined by Hesketh and Pryme (33).

Pull-down of RNA-binding proteins and quantitative mass-spectrometry analysis. The extracts prepared from the lysine-$^{13}C_6{}^{15}N_2$-labeled MCF-7 cells were subjected to affinity chromatography with wt CPE RNA, whereas unlabeled extracts were used for the mt CPE RNA coated beads. Single-stranded biotinylated RNA probes containing the wt or mt CPEs were coupled to streptavidin magnetic beads (2.5 pmol/μg) (Dynabeads M-280; Invitrogen Dynal, Oslo, Norway). The wt (5'-CCUCCUUCUCCCUUUUUAUAUCCCAUUUUUAUAUCGAUCUCUGUAGUAGUAGU-3') and mt (5'-CCUCCUUCUCCCUUUUGCUAUCCCAUUUUGCUAUCGAUCUCUGUAGUAGUAGU-3') CPEs were synthesized by DNA Technology (DNA technology, Aarhus, Denmark). The beads were washed twice with 1x B&W buffer (5 mM Tris-HCl pH 7.5, 0.5 mM EDTA, 1M NaCl, 1 U/μl SUPERase•In (Ambion) and once with binding buffer (150 mM KCl, 25 mM HEPES pH 7.6, 15 mM $MgCl_2$, 12.5% glycerol supplemented with 2.5 mM DTT, 1x complete EDTA-free protease inhibitor cocktail (Roche, Mannheim, Germany), 1 U/μl SUPERase•In (Ambion, Huntingdon Cambridgeshire, UK)). The supernatants were removed and the beads were resuspended in 80 μl binding buffer. One hundred and twenty μl binding buffer, 20 μl beads, 10 μl 60mM $MgCl_2$, and 150 μl MCF-7 protein extract was mixed and after incubation for 2 h under gentle rotation at room temperature, the beads were washed four times with 500 μl binding buffer (without RNase inhibitor). The beads were resuspended in 25 μl binding buffer (without RNase inhibitor) and 2.5 μl RNase cocktail (Ambion) were added. wt CPE and mt CPE beads were combined, eluted and the proteins were separated by SDS-PAGE (NuPAGE, Invitrogen, Merelbeke, Belgium) and analyzed LC MS/MS on an ion trap-Fourier Transform Mass Spectrometer (Thermo Fisher Scientific), capable of very high mass accuracy and of sequencing several peptides per second (29). The peptides were assigned to proteins and a SILAC ratio was calculated as the mean of the peptides ratios mapped to that protein.

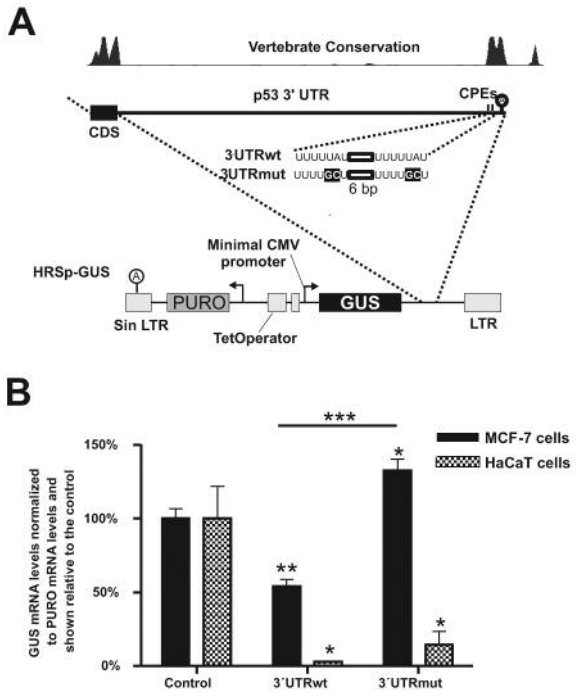

Figure 1. *GUS mRNA steady state levels. (A) Schematic representation of the vertebrate conservation of p53 with coding sequence (CDS), the 3'UTR containing the highly conserved CPEs and polyadenylation signal. Point mutations introduced into the CPEs and schematic representation of the pHRSp-GUS vector (lower section). (B) GUS mRNA steady state levels in pHRSp-GUS wt and mt vector-transfected cells. The GUS mRNA levels were normalized to puromycin (PURO) mRNA levels and the ratio was calculated relative to the data obtained with the control cells (empty vector). Each cell pool was handled separately and the average of the three independent cell pools is shown in addition to the standard deviation and statistical significance (t-test) (*p<0.05; **p<0.01; ***p<0.005).*

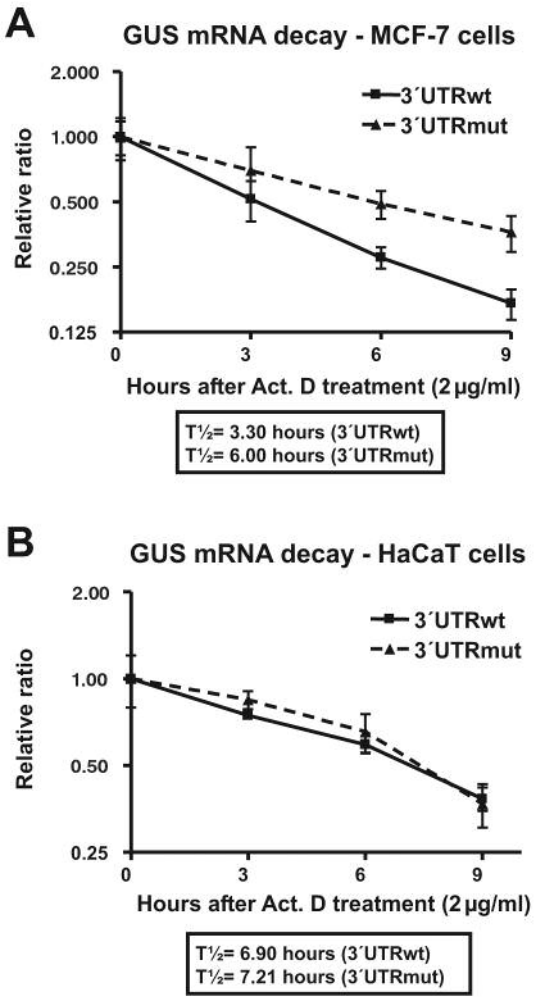

Figure 2. *Reporter mRNA decay. (A) Decay curves for the different constructs in the polyclonal MCF-7 cell pools. (B) Decay curves for the different constructs in the polyclonal HaCaT cell pools. The GUS mRNA levels were normalized to total RNA concentrations and the ratio was calculated relative to the data obtained with the untreated cells. The average of three independent experiments is shown in addition to the standard deviation. The calculated half-lives are shown for each construct.*

Results and Discussion

p53 3 'UTR and reporter expression. The p53 wt 3'UTR (3'UTRwt) repressed the steady state level of GUS reporter mRNA in both the MCF-7 and HaCaT cells (Figure 1B). In the MCF-7 cells, mutations in the CPEs (3'UTRmut) significantly increased the steady state level of GUS mRNA to approximately 130% as compared to the control. In contrast, there was no significant difference between the mRNA level in the HaCaT cells transduced with the 3'UTRwt and 3'UTRmut constructs (Figure 1B). These results indicated that the p53 3'UTR repressed the mRNA steady state level and this repression was dependent on the presence of the CPEs.

CPEs and mRNA stability. To further investigate the mechanisms regulating the mRNA steady state levels, the transduced HaCaT and MCF-7 cells were treated with the unspecific RNA polymerase inhibitor actinomycin D and total RNA was isolated at 3 h intervals after treatment. In the MCF-7 cells (Figure 2A), the half-life of the mRNA with the 3'UTRmut was approximately twice as long ($t_{½}$=6.0 h) as the half-life of 3'UTRwt ($t_{½}$=3.30 h). In the transduced HaCaT cells, no significant difference in the half-lives between the wt and the mt mRNA were observed (Figure 2B). These results showed that the difference in the mRNA steady state levels between the mRNA with the wt and mt 3'UTR (Figure 1B) reflected a difference in the stability of the mRNAs.

CPEs and translational efficiency. The effect of the p53 3'UTR on GUS protein synthesis was also investigated. The steady state level of GUS protein was determined using a GUS reporter assay and to give an estimate of the translation

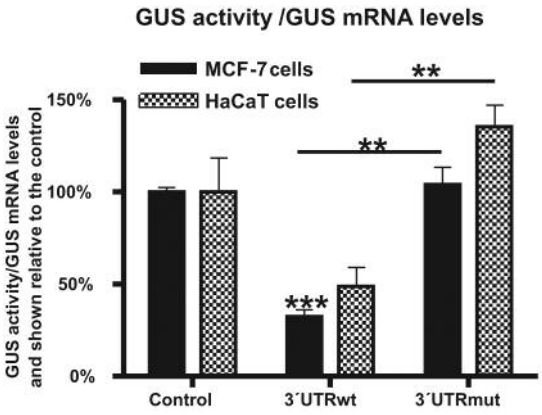

Figure 3. *The GUS activity/GUS mRNA ratio. The GUS activity was normalized to GUS mRNA levels and the ratio measured relative to the data obtained with the control cells (empty vector). Each cell pool was handled separately and the average of the three independent cell pools is shown for each cell line in addition to the standard deviation and statistical significance (t-test) (**p<0.01; ***p<0.005).*

efficiency the GUS activity was normalized to the steady state level of GUS mRNA according to the method described by Spicher *et al.* (31) (Figure 3). The 3'UTRwt had an inhibitory effect on GUS protein synthesis in both cell lines. The level was reduced to approximately 31% in the MCF-7 cells and 50% in the HaCaT cells. The 3'UTRmut restored the GUS activity/GUS mRNA ratio to the level obtained in the controls in both the MCF-7 and HaCaT cells. These results indicated that the CPEs were implicated in the translational repression of the GUS reporter containing the human p53 3'UTR.

UV-irradiation stimulation. It has previously been shown that a low dose of UV-light (15 J/m^2) mediates a translational up-regulation of p53 (18). Irradiation of the transduced HaCaT cells with a similar low dosage of UV-light revealed a significant increase in the GUS mRNA level 6 h after treatment for both the 3'UTR$_{wt}$ and the 3'UTR$_{mut}$ cells. No significant differences were observed between the 3'UTRwt and the 3'UTRmut cells (Figure 4A). The GUS activity/GUS mRNA ratio was not affected by the UV treatment of the control cells carrying the empty vector (Figure 4B), whereas the GUS activity/GUS mRNA ratio of the 3'UTRwt significantly increased to approximately 130% as compared to the untreated cells 2 h hours after UV treatment (Figure 4B). In contrast, the 3'UTRmut showed a small but not statistically significant decrease in the GUS activity/GUS mRNA ratio within the first 2-4 h as compared to the untreated cells. For both the 3'UTRwt and the 3'UTRmut cells, a significant decrease in the GUS activity/GUS mRNA ratio 6 h after treatment was observed, probably reflecting the increase in GUS mRNA

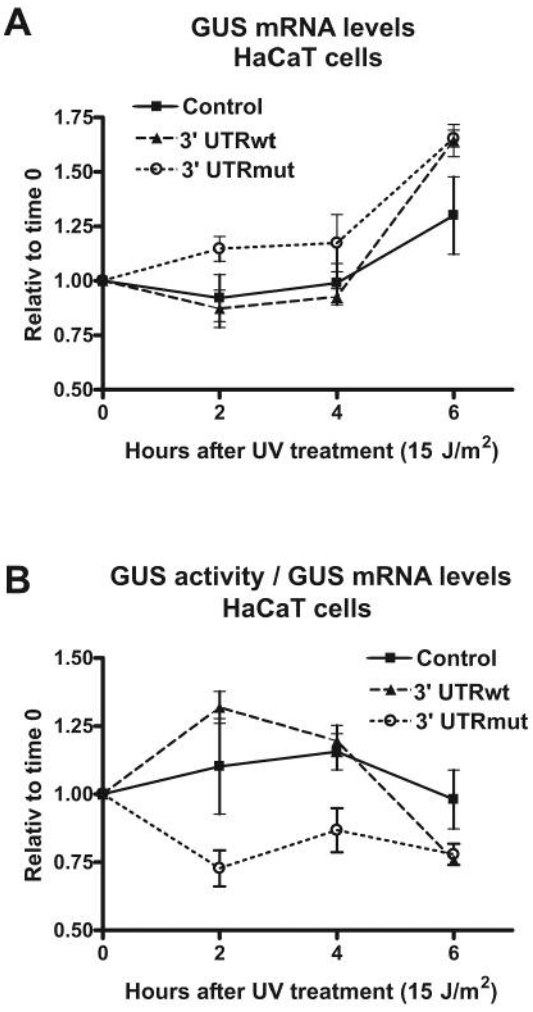

Figure 4. *Effects of UV-irradiation. (A) GUS mRNA steady state levels at different time-points after UV-irradiation. (B) The calculated GUS activity / GUS mRNA ratio. The GUS activity was normalized to GUS mRNA levels and the ratio calculated relative to the data obtained with untreated cells. An average of three independent experiments is shown in addition to the standard derivation.*

levels at this time-point (Figure 4A). These results indicated that the p53 3'UTR mediated a transient increase in the GUS translation after UV-irradiation and this increase was dependent on the integrity of the CPEs.

Identification of proteins binding to the p53 CPEs. To increase the accuracy of the SILAC ratios, only the proteins with 4 or more peptides mapped were used. Using these criteria, 235 nuclear and 151 cytoplasmic proteins were identified and quantified (Figure 5B and C). Proteins binding with a differential affinity to the wt or mt CPE probes had positive or negative $\log_2$ ratios, respectively. In Table I, the top 5 proteins from the cytoplasmic and nuclear extracts that bound preferentially to the wt or mt CPEs are

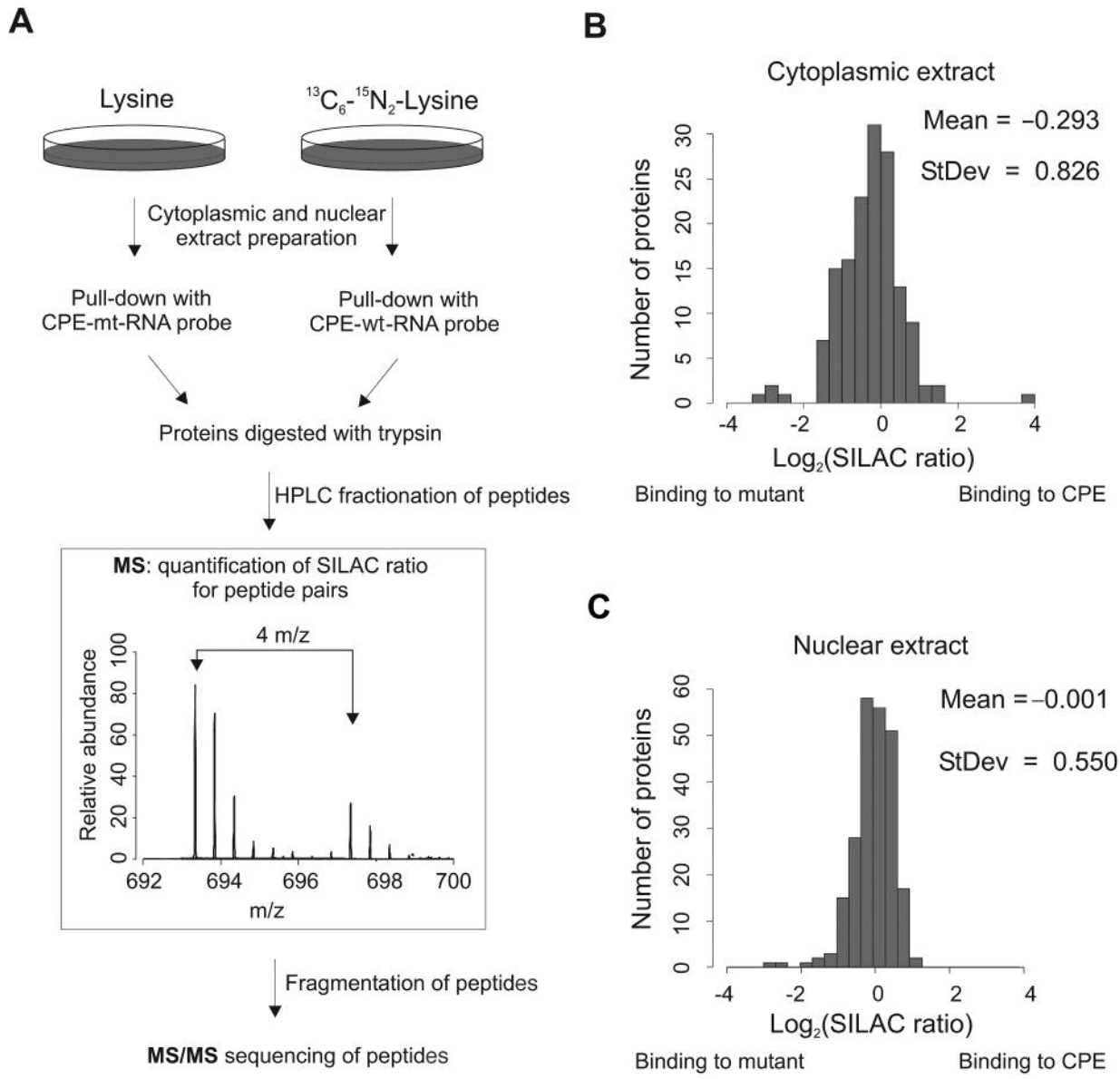

Figure 5. *Identification of CPE-binding proteins with the modified SILAC method. (A) RNA pull-down with biotin-labelled wt and mt CPE probes. One pool of MCF-7 cells was labelled with isotope-labeled lysine (lysine-$^{13}C_6{}^{15}N_2$) and used for RNA pull-down with the wild-type CPE probe, whereas an unlabeled control pool of MCF-7 cells was used for RNA-pull down with the CPE mutated probe. After pull-down, the bound proteins were combined, trypsin digested and analysed on a nanoscale liquid chromatography-tandem mass spectrometry (LC MS/MS) on an ion trap-Fourier Transform Mass Spectrometer. Peptides were mapped to proteins and a SILAC ratio was calculated as the mean of the peptide ratios mapped to that protein. (B) Quantification of wild-type probe to mutant probe binding for 235 cytoplasmic proteins with 4 or more peptides mapped to the protein. A high Log$_2$(SILAC) ratio indicates preferential binding to the wild-type probe. (C) As in (B), but for 151 nuclear proteins with 4 or more peptides mapped to the protein.*

listed (the full list of identified peptides, proteins and corresponding SILAC ratios is available on request). Surprisingly, the cytoplasmic protein with the highest differential binding to the wt CPE motif was GAPDH. However, GAPDH has previously been reported to bind specifically to AU-rich RNA sequences (34) and enhance the stability of mRNA encoding colony-stimulating factor-1 (CSF-1) (35). Interestingly, GAPDH binds to RNA *via* the NAD+ binding domain and RNA binding is inhibited by NAD+, NADH and ATP (34). The human p53 3'UTR has been reported to mediate a translational control either by the binding of an unidentified protein (15-17) or by the

Table I. *A "top 10" list of proteins from cytoplasmic and nuclear extracts that bound differentially to the wt and mt probe. High positive and negative log_2(SILAC ratio) indicates differential binding to the wild-type and the mutant probe, respectively.*

	Symbol	log_2 (SILAC Ratio)
Proteins from cytoplasmic extracts		
Glyceraldehyde-3-phosphate dehydrogenase	GAPDH	3.83
Fusion (involved in t(12;16) in malignant liposarcoma)	FUS	1.45
Heterogeneous nuclear ribonucleo-protein D (AUF1)	HNRPD	1.34
Zinc finger CCCH-type containing 5	ZC3H5	1.20
Heterogeneous nuclear ribonucleoprotein A/B	HNRPAB	1.00
Ribosomal protein, large, P0	RPLP0	–1.59
Polypyrimidine tract-binding protein 2	PTBP2	–1.66
Ribonucleoprotein, PTB-binding 1	RAVER1	–2.67
Poly(rC)-binding protein 2	PCBP2	–2.77
Epiplakin 1	EPPK1	–3.00
Proteins from nuclear extracts		
Heterogeneous nuclear ribonucleoprotein H1 (H)	HNRPH1	1.13
Squamous cell carcinoma antigen recognised by T-cells	SART1	1.06
Eukaryotic translation elongation factor 2	EEF2	0.93
WD repeat domain 57 (U5 snRNP-specific)	WDR57	0.90
PRP31 pre-mRNA processing factor 31 homolog	PRPF31	0.89
DEAD (Asp-Glu-Ala-Asp) box polypeptide 17	DDX17	–1.19
RNA binding motif protein 15	RBM15	–1.26
Heterogeneous nuclear ribonucleoprotein K	HNRPK	–1.82
Matrin 3	MATR3	–2.40
Polypyrimidine tract-binding protein 1	PTBP1	–2.99

binding of human RNA-binding protein (HuR) (18). HuR was not identified in the present study, most likely because of relatively low expression, but another member of the A-rich element (ARE)-binding protein family, heterogeneous nuclear ribonucleoprotein (hnRNP) D (AUF1) (36), bound specifically to the CPEs. It is possible that GAPDH binding to the p53 CPEs is regulated by NAD+, NADH and ATP levels and that GAPDH competes for binding to the CPEs with other proteins, such as hnRNP D and possibly HuR. Another cytoplasmic protein that bound preferentially to the WT CPE motif was hnRNP A/B, which is part of the editosome involved in mRNA editing (37). Although the editosome is located only in the nucleus, hnRNP A/B was found in the pull-down from the cytoplasmic extracts, suggesting that some hnRNP A/B isoforms, like several other hnRNPs, can shuttle between the nucleus and the cytoplasm (38). Among proteins in the nuclear extract, the hnRNP H1 protein was the most specific binder to the wt CPEs. HnRNP H1 is involved in splicing and binds strongly to poly-rG and with less affinity to poly-rU sequences (39).

Conclusion

p53 CPEs play a significant role in controlling both mRNA stability and translation of the GUS reporter carrying the p53 3' UTR both in non-irradiated as well as in irradiated cells. GAPDH, hnRNP D and hnRNP A/B bind specifically to the p53 CPEs and could possibly be involved in post-transcriptional regulation of p53.

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