TRANSLATIONAL CONTROL OF COLLAGEN PROLYL 4-HYDROXYLASE-ALPHA (I) GENE EXPRESSION UNDER HYPOXIA*

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Running title: 5'/3'UTR interaction of C-P4H-alpha (I) mRNA

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Hypoxia is a pro-fibrotic stimulus, which is associated with enhanced collagen synthesis, as well as with augmented collagen-prolyl 4-hydroxylase (C-P4H) activity. C-P4H activity is controlled mainly by regulated expression of the alpha C-P4H subunit. In this study we demonstrate that the increased synthesis of C-P4H-alpha (I) protein in human HT1080 fibroblasts under long term hypoxia (36 h, 1% oxygen) is controlled at the translational level. This is mediated by an interaction of RNA-binding protein nucleolin (~64 kDa form) at the 5'- and 3' untranslated regions (UTR) of the mRNA. The 5'/3'UTR dependent mechanism elevates C-P4H-alpha (I) expression rate 2.3-fold, and participates in a 5.3-fold increased protein level under long term hypoxia. The interaction of nucleolin at the 5'UTR occurs directly and depends on the existence of an AU-rich element. Statistical evaluation of ~64 kDa nucleolin/RNA interaction studies revealed a core binding sequence, corresponding to UAAAUC or AAAUCU. At the 3'UTR, nucleolin assembles indirectly via protein/protein interaction, with the help of another 3'UTR binding protein, presumably annexin A2. The increased protein level of ~64 kDa nucleolin under hypoxia can be attributed to an autocatalytic cleavage of a high molecular weight nucleolin form, without alterations in nucleolin mRNA-concentration. Thus, the alteration of translational efficiency by nucleolin, which occurs through a hypoxia inducible factor (HIF) independent pathway, is an important step in C-P4H-alpha (I) regulation under hypoxia.

Collagen prolyl 4-hydroxylase (C-P4H), an alpha2/ beta2 tetramer, plays a central role in collagen synthesis. 4-hydroxyproline residues are essential for the formation of triple-helical collagen molecules. The quantity and activity of C-P4H affects the composition of the extra cellular matrix (ECM), since collagens constitute the major compound of ECM proteins. The collagen hydroxylation requires iron-ions (Fe2+), 2-oxoglutarate and oxygen (O2) (1). Ascorbate is essential to maintain the iron-ions in their biologically active Fe2+-form. Three different alpha-subunit containing C-P4Hs are known, resulting in the formation of three isoenzymes called C-P4H (I), (II) and (III) (2). The beta-subunit is identical to the enzyme and chaperone protein-disulfide isomerase (1;2), and is required to keep the alpha-subunit in its soluble form (3). The alpha-subunits contain the catalytical domains of the tetramer and are limiting in the formation of active P4Hs. Thus, P4H-activity appears to be mainly regulated by the quantity of the alpha subunit (4). The type (I) enzyme is the most abundant form of enzyme in most cells, except for in chondrocytes and endothelial cells (5). However, enzymatic properties of type I-III isoenzymes are very similar (6;7).

In addition to C-P4Hs, a second class of prolyl hydroxylases exist: the hypoxia inducible factor prolyl hydroxylases (HIF-PHs). They exclusively hydroxylate the transcription factor HIF (8). Both families of PHs depend on oxygen, which is of tangible importance in various physiological (e.g. altitude) and pathophysiological (e.g. ischemia) settings. The mechanisms by which hypoxia induces gene transcription is well established (9). Hypoxia reduces activity of HIF-PHs that hydroxylate specific proline residues in the oxygen-dependent degradation domain of the HIF-1 alpha subunit. As a consequence, HIF-1 alpha accumulates and promotes hypoxic tolerance by activating gene transcription (10). The C-P4H alpha (I) gene is one of the genes, which is transcriptionally activated via HIF (11), but...
additionally, as postulated (12) and as we investigated in detail in this work, it is also controlled posttranscriptionally. There seems to be a certain similarity to the collagens, which are also regulated at the posttranscriptional level (13). This coincidence may constitute a link between collagens and P4H as a basis of their co-regulation in collagen metabolism. Long term or chronic hypoxia is a strong stimulus for collagen synthesis resulting in organ fibrosis, in particular in heart and liver (14;15). The accumulation of collagens in the ECM following hypoxia is mediated by TGF-beta (transforming growth factor) (16-18), often associated with mRNA-specific posttranscriptional control (13). Posttranscriptional regulation, i.e. changes in mRNA-stability or translational efficiency, is mainly attributed to the untranslated regions (UTRs) of mRNAs (19-21).

The alteration of gene expression at the posttranscriptional level under stress conditions (22-24), in particular by hypoxia, has been demonstrated for several genes including collagens (13), vascular endothelial growth factor (22-24), erythropoietin (22-24) or tyrosine hydroxylase (25). Recently it was shown that in response to hypoxia, more genes are regulated at the level of translation than by changes in transcription rates (26). Recent studies revealed that C-P4H-alpha (I) mRNA may belong to a subclass of transcripts which are characterized by an increased translational efficiency under hypoxia (11;12). The aim of this study was to analyze the molecular mechanism in the regulation of C-P4H-alpha (I) expression under hypoxia. In particular, we addressed the role of the 5'- and 3'UTRs of C-P4H-alpha (I) mRNA in this process.

EXPERIMENTAL PROCEDURES

Cell culture and RNA-/ protein isolation
Human fibrosarcoma HT1080 (ATCC, passages 16-21) cells were maintained in Dulbecco’s modified Eagle’s medium (high glucose; PAA Laboratories GmbH), supplemented with 10% heat-inactivated fetal calf serum, 50 U/ml penicillin, 50 µg/ml streptomycin, 15 mM Hepes and 2 mmol/l glutamine, at 37°C, 5% CO2. Before use in experiments, cells were maintained in a medium containing 0.4% fetal calf serum for at least 24 h. Measurements started with the application of fresh medium containing 0.4% fetal calf serum. For hypoxic conditions the cells were incubated in a hypoxic chamber (JOUAN IG750). Oxygen content was reduced to 1% by gas exchange with 95% nitrogen/5% CO2. Control cells were incubated under atmospheric oxygen conditions (21% O2, 5% CO2, 37°C). To inhibit translation, cells were incubated in the presence of cycloheximide (20 µg/ml). For RNA and protein isolation, cells were washed with ice-cold phosphate buffered saline. RNA was prepared using RNA-Bee (Biozol Diagnostica Vertrieb GmbH) according to the manufacturer’s protocol. Protein extracts (10,000 x g supernatants, S10) were isolated using lysis buffer (10 mM Tris, pH 7.5, 140 mM NaCl, 1 mM EDTA, 25% glycerol, 0.1% SDS, 0.5% Nonident P40, 1mM DTT, 1mM PMSF, 1 x complete protease-inhibitor-mix; Roche Diagnostics).

mRNA Quantification
RT-PCR: mRNA levels quantified by RT-PCR were normalized to relative beta-actin levels. Primers were designed to bridge at least one intron. PCR conditions were used as follows: 3 min 95°C, cycles: 30 s 95°C, 30 s annealing, 30 s 72°C, final elongation for 2 min at 72°C; 2.5 mM MgCl2. The primers were as follows: C-P4H-alpha (I), forward, 5'-CCACAGCAGAGGAATTACAG, reverse, 5'-ACACTAGCTCCAACTTCAGG; beta-actin, forward, 5'-TGAAGTGGTACGTGGACATC, reverse, 5'-GTCATAGTCCGCCTAGAAGC; nucleolin, forward, 5'-AGACAGAAGCTGATGCAGAG, reverse, 5'-TGTTGCACTGTAGGAGAGGT.

Differential centrifugation
For investigation of mRNA and protein localization, cells were incubated in the presence...
of cycloheximide (20 µg/ml) for 10 min. Cells were washed in ice-cold phosphate buffered saline supplemented with cycloheximide (20 µg/ml), harvested, and cell extracts were prepared using lysis buffer 2 (20 mM Tris, pH 7.5, 150 mM KCl, 25 mM MgCl₂, 0.25% Nonident P40, 200 U/ml RNaseOUT [Invitrogen], 20 µg/ml cycloheximide, 1 x complete protease inhibitor cocktail [Roche Diagnostics]). After 10 min incubation on ice cells were centrifuged at 1,000 x g, 4°C for 10 min. Supernatants were subjected to ultracentrifugation at 100,000 x g, 4°C, for 2 h. Sediments represent a translationally active fraction. Supernatants represent a polysomes-free fraction. After differential centrifugation, RNA was extracted from sediments and supernatants using RNA-Bee and analyzed by Northern blotting. For determination of protein localization sediments were resolved in equal amounts of lysis buffer 2, and analyzed by Western blotting.

**Western blotting**

Protein extracts (30 µg/sample) were separated by SDS-PAGE. After electrophoresis, proteins were transferred to Hybond™-P membranes (Amersham Pharmacia Biotech) using a Bio-Rad Mini Trans-Blot transfer cell. The membranes were blocked for 1 h with 5% Blot-Quick-Blocker (Chemicon). Following the blocking step, the membranes were incubated in 1% blocking solution containing a primary antibody (anti-P4H-alpha antibody - Acris Antibodies GmbH; anti-nucleolin antibody - Santa Cruz Biotechnology Inc.; anti-annexin A2 antibody – Acris Antibodies GmbH) at room temperature for 1.5 h or overnight at 4°C. The membranes were washed three times with Tris-buffered saline with Tween 20 and incubated with a secondary antibody (anti-mouse - Promega) for 1 h. After additional washing steps bands were detected using the ChemiGlow™-West Detection-Kit (Alpha Innotech Corporation). Membranes were stripped for 5 min with 1% SDS, 50 mM Tris-HCl, pH 6.8, 2% SDS, 50 mM DTT, 1% BSA and re-probed with anti-beta-actin (Chemicon) or anti-GAPDH (Acris antibodies GmbH) antibodies to detect relative beta-actin and GAPDH levels as loading control.

**Molecular cloning and in-vitro transcription**

Partial C-P4H-alpha (I) sequences (GenBank gi:63252885), representing the C-P4H-alpha (I) 5’UTR (133 nt) and 3’UTR (999 nt) were amplified by PCR, cloned and transformed using the TOPO®II TA Cloning® Kit (Invitrogen). Positive clones were confirmed by sequencing. For RNA/protein interaction studies sense-transcripts, representing the 5’- or 3’UTR of C-P4H-alpha (I) mRNA were prepared as described above and transcribed using the T7-polymerase. **In-vitro** transcripts were purified by BD Chroma Spin™-100 (DEPC) columns (BD-Bioscience Clontech).

**UV-crosslinking**

In vitro transcripts representing the 5’- or 3’UTR of C-P4H-alpha (I) mRNA were radioactively labeled using [alpha-32P]uridine-, [alpha-32P]cytosine-, [alpha-32P]adenine-, or [alpha-32P]guanosine-5’-triphosphate (800 Ci/mmol, MP Biomedicals Germany GmbH). UV-crosslinking experiments: 1-2 ng representing 100,000 cpm. [alpha-32P]UTP labeled in-vitro transcripts were incubated with 35 µg cytosolic protein extract for 30 min at room temperature in 10 mM Hepes pH 7.2, 3 mM MgCl₂, 5% glycerol, 1mM DTT, 150 mM KCl and 2 U/µl RNaseOUT (Invitrogen™ life technologies) in the presence of rabbit rRNA (0.5 µg/µl). Then the samples were exposed to UV-light (255 nm, 1.6 Joule, UV-Stratalinker) on ice, treated with RNase-A (30 µg/ml final concentration) and RNase-T1 (750 U/µl final concentration) for 15 min at 37°C and subjected to 12% SDS-PAGE and autoradiography. For competition-assays a 50-fold excess of unlabeled in vitro transcripts was added. For cis-element analyses by separate labeling of the four possible nucleotides the radioactive activity of all nucleotides was adjusted to comparable levels before in vitro transcription. Equal RNA concentrations (2 ng) of the resulting in vitro transcripts were used for the UV-crosslinking assay.

**Mapping the nucleolin binding motif in C-P4H-alpha (I) 5’UTR using a mathematical approach.** Relative amounts \( r_{[A;C;G;U]} \) of UV-crosslinking signals, corresponding to ~64 kDa nucleolin, were scanned and statistically evaluated. The signals result from the label transfer of separate radioactive labeled [alpha-32P]uridine-, [alpha-32P]cytosine-, [alpha-32P]adenine-, or [alpha-32P]guanosine-5’-triphosphate in vitro transcripts to nucleolin. The signal intensity depend on the qualitative and quantitative composition of each nucleotide in the RNA/protein interaction site. To map the protein related intensity pattern of the relative amount \( r_{[A;C;G;U]} \) of the crosslinking experiments to a sequence motif in the 5’UTR of
the P4H-alpha (I) mRNA the following algorithm was applied: A sliding window of 6 nt was shifted over the 5' UTR. For each position \( p \) the relative theoretical amount \( a_{i|A,C,G,U|p} \) of each nucleotide in the window was determined. Our mapping score is the inverse of an error function between the theoretical and the measured value within a sliding window. The error function calculates the sum of the squared differences of the theoretical and measured nucleotide fraction. At each position \( p \) the mapping score was determined according to:

\[
ms(p) = \frac{1}{(r_A - a_{A,p})^2 + (r_C - a_{C,p})^2 + (r_G - a_{G,p})^2 + (r_U - a_{U,p})^2}
\]

A high value of \( ms(p) \) corresponds to a high probability of the motif matching the quantified radioactive labeled pattern. Possible effects of the neighbourhood are considered by the experimental design, i.e. in vitro transcripts were similar in size and sequence. Hence, the influence of neighbouring sequences as well as secondary structure are considered in the assay.

**Affinity chromatography**

For the isolation of mRNA binding proteins, *in vitro* transcripts representing the 5'- or 3'UTR of C-P4H-alpha (I) mRNA were generated in the presence of biotinylated CTP (Invitrogen™ life technologies). Cytosolic extracts (5 mg protein) were incubated with 1 µg *in vitro* labeled transcript for 30 min at room temperature. RNP-(ribonucleoprotein) complexes were isolated using 200 µl streptavidin-agarose/ sample (Sigma-Aldrich Chemie GmbH). Samples without the addition of biotinylated transcripts served as negative control. The agarose-beads were centrifuged 15 sec at 5,000 x g and washed six times (20 mM Tris, pH 7.4, 150 mM KCl, 3 mM MgCl\(_2\), 0.5 mM DTT). The last two washing supernatants were used as control. The RNP-complexes were eluted using 200 µl streptavidin-agarose/ sample (Sigma-Aldrich Chemie GmbH). Samples without the addition of biotinylated transcripts served as negative control. The agose-beads were centrifuged 15 sec at 5,000 x g and washed six times (20 mM Tris, pH 7.4, 150 mM KCl, 3 mM MgCl\(_2\), 0.5 mM DTT). The last two washing supernatants were used as control. The RNP-complexes were eluted using 200 µl streptavidin-agarose/ sample (Sigma-Aldrich Chemie GmbH). Samples without the addition of biotinylated transcripts served as negative control. The agose-beads were centrifuged 15 sec at 5,000 x g and washed six times (20 mM Tris, pH 7.4, 150 mM KCl, 3 mM MgCl\(_2\), 0.5 mM DTT). The last two washing supernatants were used as control. The RNP-complexes were eluted using 200 µl streptavidin-agarose/ sample (Sigma-Aldrich Chemie GmbH). Samples without the addition of biotinylated transcripts served as negative control. The agose-beads were centrifuged 15 sec at 5,000 x g and washed six times (20 mM Tris, pH 7.4, 150 mM KCl, 3 mM MgCl\(_2\), 0.5 mM DTT). The last two washing supernatants were used as control. The RNP-complexes were eluted using 200 µl streptavidin-agarose/ sample (Sigma-Aldrich Chemie GmbH). Samples without the addition of biotinylated transcripts served as negative control. The agose-beads were centrifuged 15 sec at 5,000 x g and washed six times (20 mM Tris, pH 7.4, 150 mM KCl, 3 mM MgCl\(_2\), 0.5 mM DTT). The last two washing supernatants were used as control. The RNP-complexes were eluted using high salt buffer (20 mM Tris, pH 7.4, 2 M KCl, 3 mM MgCl\(_2\), 0.5 mM DTT). Proteins were precipitated, solved and subjected to SDS-PAGE. After coomassie staining protein-signals representing specific RNA-binding factors were excised. Tryptic digestion of proteins was carried out using ZipPlates (Millipore) without reduction or alkylation. Tryptic fragments were analyzed by Reflex IV MALDI-TOF mass spectrometer (Bruker-Daltonics). Mass spectra were analyzed using Mascot software 2.0 with automatic searches in NCBI nonredundant databases. Search parameters allowed for one miscleavage and for oxidation of methionine. Criteria for positive identification of proteins with MS were set according to the scoring algorithm delineated in Mascot (www.matrixscience.com; (28)).

**Reporter gene constructs**

For reporter gene assays the Luciferase vector pGL3-promotor (Promega, constitutive SV40 promotor) was modified. The vector specific 5’ and 3’UTRs of Luciferase mRNA were replaced by the human C-P4H-alpha (I) UTRs. The UTRs were amplified by PCR and restriction sites were added by primer extension. The 5’UTR of P4H-alpha (I) mRNA was cloned using the pGL3p-vector specific Hind III and Neo I restriction sites and the 3’UTR (including the poly-A signal) using the Xba I and BamHI restriction sites. Artificial 3’UTR parts represent the first 500 nt (3’A) and the terminal 522 nt (3’B) of the 3’UTR and were designed to overlap by 20 nt. For the 5’UTR mutations a partial 5’-sequence was amplified and the mutated element was added by primer extension. The quality of processed vectors was confirmed by sequencing. The resulting vector constructs expressed a constitutively transcribed Luciferase transcript with or without the specific C-P4H-alpha (I) UTRs.

**Reporter gene assays**

HT1080 cells were cultured in 96-well plates (µClear Platte 96K, Greiner BIO-ONE GmbH) and co-transfected with the Firefly-luciferase pGL3-promotor vector (Promega), as well as its transformed variants, and the Renilla-luciferase phRL-TK vector (ratio 1:3) using the FuGENE 6 Transfection Reagent (Roche Diagnostics Corporation) according to the manufacturer’s protocol. After 6 h, the transfection medium was removed, and measurements started after adding fresh medium. The luciferase activity was detected using a luminometer (Labsystems Luminoscan RS) programmed with individual software (Luminoscan RII, Ralf Mrowka). The transfection with the Renilla luciferase served as a control.

**Statistical analysis**

Autoradiographic signals were scanned and quantified using the Scion Image software (Scion Corporation). Results appear as means, and error bars represent the standard deviation (S.D.). Data were analyzed using the Student’s t-test, and the null hypothesis was rejected at the 0.05 level.
RESULTS

Posttranscriptional regulation of C-P4H-alpha (I) expression under long term hypoxia

Cell culture experiments, using human fibrosarcoma HT1080 cells, clearly demonstrate that C-P4H-alpha (I) is induced at the mRNA and protein level under hypoxia (1% oxygen) (fig. 1). Interestingly, during the late phase of a time scale up to 36 h C-P4H-alpha (I) protein increases independently of the mRNA concentration, suggesting a posttranscriptional component in the mechanism of expression control. We observed an approximately 2-fold elevated mRNA and protein level after 10 h hypoxia, compared to control. While the mRNA concentration remained relatively constant also under long term hypoxic conditions (up to 36 h) and even dropped slightly, the protein level increased continuously. Under long term conditions C-P4H-alpha (I) protein level increased nearly 6-fold, compared to a less than 3-fold increase at the mRNA-level. The elevated mRNA-level can be attributed to the transcriptional action of HIF (hypoxia inducible factor) (11). Consistently, we did not observe significant alterations of the C-P4H-alpha (I) mRNA stability (fig. 2A, B). The significantly stronger increase seen at the protein level may have two reasons: either protein degradation was inhibited or translational efficiency was increased. Inhibition of translation by cycloheximide prevented the increase of the protein level by hypoxia, indicating that the elevated protein concentration was due to newly synthesized protein (fig.2C, D). Furthermore, the C-P4H-alpha (I) protein level dropped to about a third by cycloheximide under both, hypoxic and atmospheric conditions. This suggests that the rate of protein degradation was not affected by hypoxia.

To determine whether C-P4H-alpha (I) is regulated at the posttranscriptional level, we generated reporter gene constructs. For this purpose 5' and 3' untranslated regions (UTRs) of luciferase mRNA were replaced by specific 5'- and 3'UTRs of C-P4H-alpha (I) mRNA (for a schematic illustration see fig. 3A), and reporter gene assays were performed after transient transfection. The transcription rate of the reporter gene was controlled by a constitutive SV40 promoter. Thus, differences in luciferase activity depended solely on the regulatory capacity mediated by the C-P4H-alpha (I) UTRs. Long term hypoxia (36 h) did not influence the luciferase expression/ activity resulting from the original reporter gene. The replacement of the original 5'UTR by the C-P4H-alpha (I) 5'UTR also showed no significant changes, whereas the replacement of the 3'UTR led to a 1.3-fold increased luciferase activity. Interestingly, the combination of C-P4H-alpha (I) 5'- and 3'UTR potentiated the effect: expression reached a 2.2-fold level versus control (fig. 3B), which correlated well with the discrepancy between mRNA- and protein level under long term hypoxia. Dividing the 3'UTR into two parts (termed 3'A and 3'B) did not result in a comparable activation (fig. 3C). Neither the individual 5' ~500 nt, nor the terminal 3' ~500 nt of the 3'UTR reached the full activity of the complete 3'UTR. Obviously, 3'UTR parts did not represent the properties of the complete 3'UTR. This is in contrast to findings after Fe2+ diminishment, where the regulative properties of 3'UTR part B is dominated by an U-rich element (27). Although the complete 3'UTR showed a weak independent influence (1.3-fold), the 5'/ 3'UTR combination seems to be a prerequisite for an optimal translational efficiency of C-P4H-alpha (I) mRNA under hypoxia.

The data revealed that under long term hypoxia C-P4H-alpha (I) in HT1080 fibroblasts is not only regulated at the transcriptional level (11), but is dependent on a 5'/ 3'UTR interaction in a posttranscriptional process. We did not observe a significant influence on mRNA-stability, and conclude that the posttranscriptional regulation is mainly attributed to the modulation of translational efficiency through 5'/ 3'UTR interaction.

C-P4H-alpha (I) UTR/ protein interaction

Posttranscriptional regulation is mainly established through RNA/protein interaction. We used an avidin/biotin based affinity chromatography approach (13;29) to isolate C-P4H-alpha (I) 5'- and 3'UTR binding proteins as possible candidates for factors involved in posttranscriptional regulation of C-P4H-alpha (I) expression. The results of protein identification by MALDI-TOF-MS analysis are shown in table 1. Nucleolin, RPL7a and EEF1A1 were identified to interact at the 5'UTR. Nucleolin was also found to participate in the 3'UTR RNP assembling, as well as SFPQ (splicing factor proline/glutamine-rich, polypyrimidine tract binding protein associated), HSP-A8, the members of hnRNP-family hnRNP-R, hnRNP-A2/B1, and hnRNP-A3, annexin A2 and BRD3 protein. All identified proteins are known RNA-
binding proteins and potential mediators of posttranscriptional control in C-P4H-alpha (I) gene expression. However, these findings have to be verified by further investigations. Earlier hnRNP-A2/B1 has been shown by us to interact with an U-rich element within the 3'UTR and modulates the C-P4H-alpha (I) mRNA-stability (27).

As a next step, we performed UV-crosslinking assays to detect changes in the binding behavior of trans-factors (RNA-binding proteins) in response to hypoxia. The data indicate multiple quantitative changes in the binding properties of 5'- as well as 3'UTR binding proteins (fig. 4). The most prominent alteration of a UV-crosslinking signal was observed at the 5'UTR. It corresponded to a ~64 kDa RNA-binding protein. From the data obtained from UTR-dependent reporter gene assays it was evident that the 5’UTR did not promote a posttranscriptional control on its own. This result supported the view that the interaction of the ~64 kDa protein with the 5'UTR is involved in a functional 5'/ 3'UTR cross-talk. We therefore focused our further efforts on this ~64 kDa protein.

MALDI-TOF-MS analysis identified this ~64 kDa protein as nucleolin. Hypoxia, however, did not change the nucleolin mRNA-level (fig. 5A). Western blotting analyses revealed that nucleolin may be regulated at the posttranslational level. We observed a marked decrease of a ~100 kDa nucleolin form in favor of an increase in smaller nucleolin fragments (fig. 5B). The increase of a ~64 kDa nucleolin form, which shows mRNA-binding properties, could explain the increased binding to the 5'UTR. Interestingly, this particular nucleolin form was also identified as 3'UTR binding protein by RNA-affinity-chromatography, but we did not observe a distinct nucleolin related UV-crosslinking signal in several independent experiments. UV-crosslinking signals represent proteins, which bind to the RNA by direct RNA/protein interaction, so we conclude that the interaction of nucleolin at the C-P4H-alpha (I) 3'UTR was brought about by a secondary protein/protein interaction between nucleolin and other RNA-binding proteins. One of these possible nucleolin interacting proteins is annexin A2, which was experimentally identified as C-P4H-alpha (I) 3'UTR binding protein (see table 1). In order to test our line of reasoning we performed differential centrifugation assays, investigating separately a translationally active fraction (1,000 – 100,000 x g), which includes rough endoplasmatic reticulum residues, free polysomes and other polysomes-associated aggregates, and a translationally inactive (100,000 x g supernatant) polysomes-free fraction, containing ribonucleoprotein complexes, as well as free RNAs and cytosolic proteins under hypoxia. We observed a nearly 5-fold increase of C-P4H-alpha (I) mRNA in the polysomal fraction (fig. 6A). This increase seen in the translationally active fraction exceeds the overall induced mRNA-level (which is approximately 2.5-fold), and can be explained by a significant decrease of C-P4H-alpha (I) mRNA concentration in the translationally inactive RNP-fraction. These findings indicate a recruitment of C-P4H-alpha (I) mRNAs into polysomes, which supports the view of an enhanced translational control under hypoxia. Furthermore, the C-P4H-alpha (I) mRNA binding proteins nucleolin (~64 kDa form) (fig. 6B) and annexin A2 (fig. 6C) are enriched in the polysomal fraction as a result of hypoxia, which is in line with the finding regarding its bound C-P4H-alpha (I) mRNA. Interestingly, not all nucleolin forms shifted into the translationally active fraction. We observed an elevated presence of the high molecular weight forms (~100 kDa) as well as the ~64 kDa nucleolin fragment in the polysomal fraction, whereas smaller nucleolin fragments are located predominantly in the postpolysomal fraction (fig. 6B).

In summary, UV-crosslinking and affinity chromatography revealed that the ~64 kDa nucleolin form bound directly to the 5'UTR and interacted indirectly with the 3'UTR, causing an elevated recruitment of ribosomes, and consequently, an increased translational efficiency.

Identification of ~64 kDa nucleolin/ C-P4H-alpha (I) 5'UTR interaction site

To analyze the cis-element (RNA-binding motif) of C-P4H-alpha (I) mRNA 5'UTR in detail, which is responsible for the nucleolin interaction, we carried out competition assays. Different parts of the 5'UTR were transcribed in vitro and added in 50-fold molar excess to the UV-crosslinking samples (fig. 7A). The competition assay revealed that the ~64 kDa nucleolin signal could not be suppressed by the 5'-1-110 nt of the 5'UTR. However, transcripts representing the 3'-terminal 23 nt of the 5'UTR effectively suppressed it. Central to this 23 nt region is an UAAAAUUAAAU motif (see fig. 9A). It seems to be involved in binding of
several trans-factors. However, under hypoxic conditions the increased binding capacity of ~64 kDa nucleolin is the most obvious alteration in RNA/protein interaction and may refer to a posttranscriptional effect on gene expression. To confirm the AU-rich element/nucleolin interaction we performed additional UV-crosslinking assays. UV-crosslinking signals, corresponding to RNA-binding proteins, depend on the direct interaction of trans-acting factors with cis-acting elements. The observed signal intensity depends further on the quality and quantity of nucleotides, which are involved in the RNA/protein interaction (30). The separate labeling of 5'UTR transcripts with [alpha-32P]-UTP, -CTP, -ATP or -GTP revealed, that the nucleolin binding site is indeed AU-rich (fig. 7B). To compare RNA-binding properties of 3'UTR-binding proteins we also labeled transcripts representing the 3'UTR separately (fig. 7C). We observed no ~64 kDa UV-crosslinking signal at the 3'UTR with similar binding properties seen by nucleolin at the 5'UTR, which supports independently the view that the mechanism of nucleolin interaction differs between 5' and 3'UTR.

For fine-mapping of the C-P4H-alpha (I) 5'UTR/nucleolin interaction site we used a new mathematical strategy, based on the relative abundance of nucleotides obtained by UV-crosslinking assays. A high score value was observed for the sequences UAAAUC and AAAUCU (fig. 8), which correlated well with the data obtained by competition assays. The data furthermore indicate that nucleolin interacts only with parts of the AU-rich element.

To confirm the functional importance of the C-P4H-alpha (I) 5'UTR/nucleolin interaction in the modulation of translational efficiency of C-P4H-alpha (I) mRNA under hypoxia, we mutated the identified cis-element, as well as the flanking regions and performed additional UTR dependent reporter gene assays. The results show that not only the mutation of the calculated nucleolin interaction site, but also mutations of the flanking regions reduced the hypoxia inducible luciferase activity (fig. 9A). 5'UTR mutations did not abolish the UTR mediated response to hypoxia completely, which can be attributed to the independent influence of the 3'UTR (see fig. 3B). Furthermore, mutations of the hypoxia response relevant part of C-P4H-alpha 5'UTR caused a qualitative change in RNP assembling, seen by UV-crosslinking assays (fig. 9B). This alteration included a loss of the binding ability of nucleolin, important for the 5'UTR cross-talk mediated hypoxic response. The results further support the finding, that the 3' terminal part of the 5'UTR represents an important interaction site, not only for nucleolin. The functional significance of this region is supported by the observation, that the mutations led to a lower gene expression rate, compared to the non-mutated C-P4H-alpha (I) 5'UTR (fig. 9C). This finally demonstrates a crucial role of the 5'UTR in the modulation of C-P4H-alpha (I) translational efficiency, not only under hypoxia.

**DISCUSSION**

Hypoxia is important under variable physiological conditions (e.g. altitude, hibernation, diving mammals, working muscles) and in pathophysiological settings (ischemia, anemia, diffusion barriers). Furthermore, hypoxia is a central issue in tumorigenesis. The first striking cellular response to hypoxia is the suppression of energy consuming processes, such as translation, protein degradation and transcription (31;32). Although the overall metabolic rate is suppressed, several genes show an increased expression rate against the trend, which has been attributed to activation of the transcription factor HIF (hypoxia inducible factor). The alteration of gene expression can be modulated further at posttranscriptional or posttranslational level. Posttranscriptional control involves alteration in mRNA-stability, translational efficiency or processes related to mRNA-localization. In particular, mRNA translation is a highly controlled process that is sensitive to a variety of cellular stressors (22-24). Hitherto, the hypoxic adaptation of mRNA translation was attributed to the phosphorylation of the essential eukaryotic initiation factor eIF2-alpha, or mTOR mediated inactivation of eIF4F (see: (33)).

The induction of fibrosis following hypoxia is of major importance. The enhanced expression and deposition of collagens under hypoxia appears paradoxical, due to the formation of diffusion barriers, which further impair oxygen supply. Obviously the hypoxic induced fibrosis is a conserved pathway and serves the evolutionary importance of wound healing and scarring, since hypoxia constitutes a local condition after wounding. The restoring of blood and oxygen supply may be attributed to HIF, a factor known to induce angiogenesis.

The increased collagen synthesis requires posttranslational modifications, especially the hydroxylation by prolyl 4-
nuclear translocation of S100/A11 in Ca²⁺

Furthermore, nucleolin interacts with ribosomal proteins and transcription factor complexes (36-38), as well as with small ribonucleoproteins (RNP) (39). Nucleolin is also important in nuclear translocation of S100/A11 in Ca²⁺-induced growth inhibition (40). Despite its nuclear function, nucleolin has been shown to be a marker at the cell surface of angiogenic endothelial cells (41), and also plays an important role in the cytoplasm (42). Cytoplasmic nucleolin was regarded as an mRNA stabilization factor (29;43-45) and is involved in translational control (46-48).

Interestingly, nucleolin can undergo an autocatalytic cleavage into distinct fragments (49). Singh et al. (45) showed at least 8 different nucleolin forms, which may have different properties and functions. In the present work we demonstrate that a ~64 kDa nucleolin fragment has mRNA-binding properties and is associated with an enhanced translational efficiency of the C-P4H-alpha (I) mRNA. Recently we showed, that in the absence of Fe²⁺-ions, this ~64 kDa nucleolin form binds increasingly at the 3'UTR of matrix-metalloproteinase (MMP)-9 mRNA, and causes an enhancement in translation (48). Fe²⁺ depletion also affects the posttranscriptional control of C-P4H-alpha (I) expression by nucleolin mediated 5'/3'UTR interaction. In contrast to hypoxia, treatment by an iron chelator also affects the 5'UTR binding properties of other proteins (M. Fähling, unpublished results), resulting in a less importance of nucleolin mediated control up to 18 h (27). However, under hypoxia (36 h) the ~64 kDa nucleolin mediated translational control is the dominant posttranscriptional influence controlling C-P4H-alpha (I) expression. Nucleolin binds directly at the C-P4H-alpha (I) 5'UTR and, probably mediated through other RNA-binding proteins, indirectly at the 3'UTR. The identification of C-P4H-alpha (I) 3'UTR binding proteins revealed that annexin A2, an already known nucleolin binding protein (50), participates in the 3'UTR/protein assembling. The constitutive presence of this or other 3'UTR binding proteins may be crucial for the 5'/3'UTR interaction, because they present a link to the 5'UTR by nucleolin interaction.

The RNA-binding property of the distinct ~64 kDa nucleolin fragment is different to the known rRNA related binding element (U/G)CCCG(A/G) (51;52) of the high molecular weight form. The C-P4H-alpha (I) 5'UTR recognition motif, interacting with nucleolin, involves an AU-rich element and corresponds to UAAAUC or AAAUCU. We calculated this core sequence for nucleolin binding using a new mathematical approach, based on the experimentally confirmed qualitative and quantitative involvement of each possible...
nucleotide in the nucleolin binding motif by UV-crosslinking assays. As mentioned above we recently also identified this ~64 kDa nucleolin form as RNA-binding protein, interacting with the 3'UTR of MMP-9 mRNA. We favor the calculated UAAAUC sequence as core binding motif for ~64 kDa nucleolin, because this motif is also present in the 3'UTR of MMP-9 mRNA. However, functional UTR dependent reporter gene assays revealed that not only the direct interaction motif is responsible for the nucleolin interaction. Mutations of the flanking regions also inhibit the 5'/3'UTR cross-talk mediated hypoxic response and the nucleolin binding seen in UV-crosslinking assays. These observations may be explained by the importance of mRNA secondary structures and/or other proteins in the recruitment of RNA-binding factors and the assembling of RNP-complexes. Consistently, nucleolin was described to optimize access of other RNA-binding proteins to its functional binding sites (53). The involvement of an AU-rich element in the RNA/nucleolin interaction was previously described by Sengupta et al. (54), who showed that ~100 kDa and ~70 kDa nucleolin forms affect the mRNA stability by interacting with a classical ARE within the 3'UTR of bcl-2 mRNA. These findings support the view, that nucleolin and its distinct fragments are multifunctional and influence gene expression at different levels.

Furthermore, we observed that the 3' terminal part of the C-P4H-alpha 5'UTR, including the AU-rich element, also affects the binding properties of several other 5'UTR binding proteins shown by competition and UV-crosslinking assays. Mutation of this region led to a qualitative change of binding properties of 5'UTR interacting proteins and decreased the basal expression rate in UTR dependent reporter gene assays. Under hypoxic conditions the absence of nucleolin interaction appears crucial, and caused a loss in the ability of 5'/3'UTR interaction mediated elevated expression rate. The results clearly show the importance of the 5'UTR in the modulation of C-P4H-alpha (I) gene expression, which was postulated earlier (55).

Apart from nucleolin, we identified other putative regulatory factors interacting with the C-P4H-alpha (I) UTRs by MALDI-TOF-MS analysis. All identified proteins (see tab. 1) represent known RNA-binding factors and may be necessary in the posttranscriptional regulation of C-P4H-alpha (I) expression under different environmental conditions, in different developmental stages or cell types. UV-crosslinking assays indicate that more UTR-binding proteins are yet to be identified. In this study, we focused on the RNA-binding factor nucleolin, since under hypoxic conditions the nucleolin mediated influence in the posttranscriptional alteration of C-P4H-alpha (I) expression appeared dominant. The importance of the other candidates has to be confirmed by additional experiments under different physiological conditions.

Finally, we hypothesize that the posttranslational regulation of nucleolin by autocatalytic cleavage into distinct fragments may be a key step in the enhanced translation rate of a set of specific mRNAs under hypoxia. We observed no alteration at the nucleolin mRNA level following hypoxia, indicating a HIF independent response. Nucleolin was shown to be a Ca\(^{2+}\) binding protein (56). Hypoxia is a stimulus which modulates Ca\(^{2+}\) signaling, mediated by voltage-gated calcium entry through oxygen sensitive potassium channels (57-59), calcium-sensitive potassium (BK) channels regulated by hemoxygenase-2 (60), as well as liberation of Ca\(^{2+}\) from the endoplasmatic reticulum (ER) through regulation of reactive oxygen species and via ryanodine receptors (61). The HIF independent response at the level of translation may therefore be linked to the Ca\(^{2+}\) signaling, affecting posttranslational control of nucleolin and translational efficiency of certain mRNAs. Recently, the ~100 kDa form of nucleolin was identified as a putative translational repressor of p53 mRNA translation (47). However, the dramatic decrease of this high molecular weight nucleolin form in favor of distinct fragments as a result of hypoxia, may cause a switch from translational inhibition to a enhanced rate of translation of a specific set of mRNAs. Furthermore, the multiple functions of nucleolin, as mentioned above, may affect the metabolic regulation under hypoxia in a broad manner, including ribosomal biogenesis, RNA/protein shuttling, RNP configurations, protein/protein interaction, as well as translational control. We hypothesize that nucleolin may participate in the recruitment of specific mRNA transcripts into stress granules (SGs), an important step in the stress induced alteration of mRNA-stability and translational properties (reviewed in: (62)).

In summary, enhanced C-P4H-alpha (I) expression is not only regulated at transcriptional level in response to hypoxia. Under conditions where energy-consuming processes are...
suppressed, the synthesis of C-P4H-alpha (I) protein is enhanced by posttranscriptional control. The increase in the concentration of the limiting alpha (I) subunit (necessary to form an active C-P4H tetramer) can partially be attributed to a 5’/3’UTR interaction, leading to an enhanced translational efficiency under long term hypoxia. The posttranscriptional control of C-P4H-alpha (I) expression is strongly associated with the increased binding of a ~64 kDa nucleolin form at the 5’- as well as 3’UTR. The interaction of nucleolin appears to be attributed to the 3’ terminal part of the 5’UTR, and involves an UAAAUCC motif as the direct contact site. Interaction of nucleolin at the 3’UTR requires additional RNA-binding factors, possibly annexin A2.

REFERENCES
FOOTNOTES
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Figures/ Table legends

**Table 1:** RNA-binding proteins, interacting with the 5’- and 3’UTR of C-P4H-alpha (I) mRNA. RNA binding proteins were purified by affinity chromatography using biotinylated *in vitro* transcripts, which represent the 5’- or 3’UTR of P4H-alpha (I) mRNA. Proteins were identified by MALDI-TOF-MS analysis.

**Figure 1:** Time dependent expression of C-P4H-alpha (I) mRNA and protein in response to hypoxia (1% oxygen). Human fibrosarcoma cells (HT1080) were cultivated up to 36 h under control (C) or hypoxic (Hy) conditions. A: C-P4H-alpha (I) mRNA-levels were determined by RT-PCR, protein levels by western blotting. Beta-actin served as a control. Shown are pools of six independent experiments. B: Statistical analyses. Relative values are normalized to beta-actin, the numerical values are means, error bars represent the standard deviation (n=6).

**Figure 2:** C-P4H-alpha (I) mRNA- and protein stability under hypoxia. HT1080 cells were incubated under control or hypoxic conditions for 24 h. A: Cytosolic extracts were isolated as described in (27) and incubated at room temperature up to 4 h. mRNA levels were estimated by RT-PCR. The time dependent mRNA decay is indicated by the decreased mRNA level. A pool of six independent samples is shown. Beta-actin mRNA levels (half-life time about 4 h) are shown as a comparison and were not changed significantly. B: Graphic display of mRNA decay of C-P4H-alpha (I) mRNA under control conditions (half-life time 1.82 h ± 0.41 S.D.), compared to hypoxia (half-life time 2.1 h ± 0.28 S.D.). n=6. There is no significant alteration in the C-P4H-alpha (I) mRNA stability under hypoxia. C, D: Cycloheximide [20 µg/ ml] was used to inhibit the translation of proteins. C-P4H-alpha (I), beta-actin and GAPDH protein levels were visualized by western blotting technique. A representative set of data is shown and was statistically evaluated (n=6). 24 h cycloheximide treatment reduced the C-P4H-alpha (I) level to a third, which is similar under both, control and hypoxic conditions.

**Figure 3:** Influence of C-P4H-alpha (I) mRNA untranslated regions (UTRs) on luciferase expression. HT1080 cells were transfected using the pGL3-promoter vector (SV40 promoter) and transgenic variants, in which vector specific 5’ and 3’UTRs of luciferase mRNA were replaced by C-P4H-alpha (I) UTRs. The influence of hypoxia (1% oxygen, 36 h) on luciferase activity of the original vector (pGL3p) and vector constructs with C-P4H-alpha (I) 5’ and/or 3’UTR sequences are shown as black bars. A: Schematic illustration of reporter gene constructs. The dotted lines represent the original UTRs of luciferase mRNA. Boxes termed “5’UTR” or “3’UTR” represent the specific C-P4H-alpha (I) UTRs. The 3’UTR was experimentally divided into two parts, termed A and B, to test individual influences. Boxes termed “Luciferase” represent the coding sequence of luciferase transcript. B: Statistical analyses of results, regarding to original luciferase transcript and native C-P4H-alpha (I) UTRs. (n = 12, *: p < 0.05, **: p < 0.01). Shown are relative values compared to control levels. The combination of C-P4H-alpha (I) 5’- and 3’UTR significantly enhances the UTR-mediated luciferase activity under hypoxia, compared to the hypoxic response of separate 5’- or 3’UTRs. C: Impact of hypoxia (36 h) mediated 5’/ 3’UTR interaction on luciferase activity by combination of C-P4H-alpha (I) 5’UTR with artificial 3’UTR parts. 3’A represent the 5’ localized 500 nt and 3’B represent the 3’ terminal 522 nt of the complete 3’UTR as described in (27). Shown are relative values. The results show that 3’UTR parts do not mediate a 5’/ 3’UTR cross-talk, and therefore do not reflect the feature of the complete 3’UTR.

**Figure 4:** Influence of hypoxia on interaction of cytosolic proteins with C-P4H-alpha (I) mRNA UTRs, analyzed by UV-crosslinking. 32P-UTP labeled *in vitro* transcripts, which represent the 5’- or 3’UTR of C-P4H-alpha (I) mRNA, were incubated with cytosolic extracts, isolated from cells exposed to control (C) or hypoxic (Hy) conditions. The arrow head marks a ~64 kDa protein (nucleolin), which shows an increased binding capacity in response to hypoxia.

**Figure 5:** Formation of nucleolin subfragments by hypoxia. Human fibrosarcoma cells (HT1080) were cultivated up to 36 h under control or hypoxic conditions. A: RT-PCR was performed to detect
relative mRNA-levels. Hypoxia did not influence the nucleolin mRNA concentration. B: Western blotting analysis shows relative nucleolin protein levels. A pool of six independent samples is shown. Beta-actin served as loading control. The formation of ~64 kDa nucleolin, active in translational control, is increased as a result of hypoxia.

**Figure 6**: Localization of C-P4H-alpha (I) mRNA, as well as nucleolin and annexin A2 proteins in translational active or inactive fractions.

HT1080 cells were cultivated under control or hypoxic conditions for 24 h. Cellular extracts were separated by ultra-centrifugation in a polysomal fraction (1,000 – 100,000 x g), which contained rough endoplasmatic reticulum residues, free polyosomes and other polysomal aggregates, and a 100,000 x g supernatant (RNP fraction), which contained translationally inactive ribonucleoprotein (RNP) complexes, free RNAs and cytosolic proteins. A: Representative Northern blotting analysis of C-P4H-alpha (I) mRNA level. Three independent samples were statistically evaluated. The hypoxic induction of C-P4H-alpha (I) mRNA level under these methodical conditions (total RNA) served as a comparison, and is in line with the data obtained by RT-PCR. The C-P4H-alpha (I) mRNA level in the translationally inactive (RNP) fraction is decreased in favor of an increase in the polysomal fraction. B, C: Western blotting analysis to detect the localization of the C-P4H-alpha (I) 5’UTR binding protein nucleolin (~64 kDa fragment) (B), and the 3’UTR interacting protein annexin A2 (C). The ~64 kDa nucleolin form, as well as the phosphorylated form of annexin A2 (arrow head) are shifted from the RNP compartment into the translationally active fraction as a result of hypoxia. Pools of three independent samples are shown. Detection of beta-actin served as a control.

**Figure 7**: Characterization of C-P4H-alpha (I) 5’UTR/ nucleolin interaction. 32P- labeled in vitro transcripts, which represent the 5’UTR of C-P4H-alpha (I) mRNA, were incubated with cytosolic extracts. A: Mapping of C-P4H-alpha (I) 5’UTR/ protein interaction by competition. Non labeled in vitro transcripts, representing partial 5’UTR sequences, were added in an ~50-fold molar excess to the radioactively labeled (*) 5’UTR (133 nt in size) as competitor. The suppression of signals, corresponding to 5’UTR binding proteins, indicate RNA/protein interaction sites. An unspecific vector transcript served as control. fP – free probe. B, C: Label transfer to RNA-binding proteins of C-P4H-alpha (I) 5’UTR (B) or 3’UTR (C) using [alpha-32P]uridine-, [alpha-32P]cytosine-, [alpha-32P]adenine-, or [alpha-32P]guanosine-5’-triphosphate labeled transcripts was performed by UV-crosslinking technique. The signal intensity indicates the qualitative and quantitative involvement of each nucleotide in the RNA/protein interaction. The separate labeling of C-P4H-alpha (I) 3’UTR is shown as a comparison.

**Figure 8**: Mapping of the motif position of ~64 kDa nucleolin interaction site in the 5’UTR of C-P4H-alpha (I) mRNA. Relative amounts r[A;C;G;U] of UV-crosslinking signals at the molecular weight of nucleolin (~64 kDa) were statistically evaluated (n=4), resulting in an experimentally obtained nucleotide abundance (inset) of nucleolin binding motif in UV-crosslinking assays (see fig. 7B). A sliding window of 6 nt was shifted over the 5’ UTR. The calculated mapping score is a function of motif position. High values of the score correspond to a good match with the obtained nucleotide abundance. The results show, that the nucleolin interaction site fits best either to UAAAUC or to AAAUCU.

**Figure 9**: Influence of 5’UTR mutations on luciferase expression under long term hypoxia. HT1080 cells were transfected with original pGL3-promotor (pGL3p) or transgenic variants, where the original luciferase UTRs were replaced by C-P4H-alpha (I) UTRs, and incubated under control or hypoxic conditions (36 h). A: The mutation of the 5’UTR localized nucleolin binding site, as well as flanking regions (mut1, mut2, mut3, mut4) as indicated at the right, significantly suppresses the 5’/ 3’UTR interaction mediated enhancement of luciferase activity as a result of hypoxia. n=8, ** - p < 0.01. B: UV-crosslinking assays show that these mutations inhibit the binding of nucleolin. Furthermore, the mutations led to a qualitative alteration of the pattern of 5’UTR interacting proteins. C: In reporter gene assays the basal luciferase activity (control conditions) is decreased by 5’UTR mutations, indicating an alteration in the 5’UTR/ protein assembling.
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Figure 1
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Figure 5

A 5' 3'UTR cross-talk of C-P4H-alpha (I) mRNA

nucleolin mRNA-level

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B nucleolin protein-level

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5′ 3′UTR cross-talk of C-P4H-alpha (I) mRNA

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< C-P4H-alpha (I)

< 28S rRNA

< 18S rRNA

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**Figure 6**

**A**

**B**

**C**

< ~64 kDa nucleolin

< beta-actin

< annexin A2

< beta-actin
Figure 7

A

B

C

5' 3' UTR cross-talk of C-P4H-alpha (I) mRNA
Figure 8
5′ 3′UTR cross-talk of C-P4H-alpha (I) mRNA

Figure 9