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CYTOPLASMIC POLYADENYLATION ELEMENT BINDING PROTEIN (CPEB)1 AND 2 BIND TO THE HIF-1 α mRNA 3' UTR AND MODULATE HIF-1 α PROTEIN EXPRESSION

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Running head: CPEBs bind to the HIF-1 α mRNA 3'-UTR

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The heterodimeric hypoxia-inducible factor (HIF)-1 is a transcriptional master regulator of several genes involved in mammalian oxygen homeostasis. Besides a well described regulation of the HIF α -subunit via hydroxylation-mediated protein stability in hypoxia, there are several indications for an additional translational control of the HIF-1 α mRNA especially after growth factor stimulation. We identified an interaction of the cytoplasmic polyadenylation element binding protein (CPEB) 1 and 2 with the 3'-UTR of the HIF-1 α mRNA. Overexpression of CPEB1 and 2 affected HIF-1 α protein levels mediated by its mRNA 3'-UTR. Stimulation of neuroblastoma SK-N-MC cells with insulin and thus activation of endogenous CPEBs increased the expression of a luciferase reporter gene that was fused to the 3'-UTR of HIF-1 α as well as endogenous HIF-1 α protein levels. This could be abrogated by treating the cells with CPEB1 or CPEB2 siRNAs. Injection of HIF-1 α cRNA in *Xenopus* oocytes verified the elongation of the polyA tail by cytoplasmic polyadenylation. Thus, CPEB1 and 2 are involved in the regulation of HIF-1 α following insulin stimulation.

Key words: oxygen, polyadenylation, HIF, translation, insulin

Abbreviations used: HIF, hypoxia-inducible factor; CAIX, carbonic anhydrase IX ; CPEB, cytoplasmic polyadenylation element binding protein; pVHL, von Hippel-Lindau protein

Introduction

Adaptation of cells towards a decreased oxygen supply involves hypoxia-mediated gene expression which is mainly initiated by the hypoxia-inducible factor (HIF)-1. The hypoxic stabilization and activation of the HIF-1 α transcription factor is tightly regulated by oxygen [1, 2]. Under normoxic conditions, the HIF-1 α protein is hydroxylated under consumption of molecular oxygen by three described prolyl-4-hydroxylase domain containing enzymes [3, 4]. In its hydroxylated form, HIF-1 α is ubiquitinated by the von Hippel-Lindau protein (pVHL) and then degraded in the proteasome [5, 6]. Since under hypoxic conditions oxygen-dependent hydroxylation is alleviated, HIF-1 α is stabilized, heterodimerizes with HIF-1 β and activates HIF-1-dependent target genes, including genes required for oxygen transport and supply, metabolic adaptation as well as pH regulation [1].

Besides these well described hypoxia-induced features at the level of protein stability and transcriptional activation, additional modulations in gene expression based on translational changes have been described under severe hypoxic/anoxic conditions [7-10]. The inhibition of overall mRNA translation under hypoxia and anoxia paradoxically goes along with the privileged translation of certain mRNAs [11, 12]. Blais et al. identified several genes with a translational control during severe hypoxia/anoxia including VEGF, ATF-3 and ATF-4 [13]. Interestingly, a translational regulation has been described for HIF-1 α , too. The most prominent effect on HIF-1 α translation has been demonstrated so far after alterations in the pathways, which control mTOR activity or the AMP protein kinase [8, 14, 15]. Furthermore, a recent study has identified HuR and PTB as two HIF-1 α mRNA-binding proteins that function jointly to stimulate HIF-1 α translation in response to CoCl₂ treatment [16]. A functional IRES element, which facilitates translation under stress conditions, when protein synthesis via cap-dependent translation is reduced, has also been identified [17]. In subsequent studies, however, it has been demonstrated that the IRES activity is minimal and can not account for changes in translation after hypoxic conditions [18].

In the 3'-UTR of the HIF-1 α mRNA adenosine- and uridine (AU)-rich elements can be found. Those elements have been shown to control translation by promoting assembly of RNA binding proteins.

Here we provide evidence that the 3'-UTR of the HIF-1 α mRNA has regulatory functions by binding the cytoplasmic polyadenylation binding protein (CPEB) 1 and 2.

EXPERIMENTAL

Plasmids

All cloning work was carried out using Gateway technology (Invitrogen, Karlsruhe, Germany). The CPEB1 entry vector was generated by cloning a PCR fragment into the *NcoI/EcoRV*-digested pENTR4 vector. The coding region of the CPEB1 zinc finger (aa 514-544) was deleted in the entry vector by site directed mutagenesis. pDONR221 containing full-length CPEB2 (Invitrogen) was used to perform a BP clonase reaction to obtain a CPEB2-containing entry vector. The inserts of both entry vectors were verified by DNA sequencing. To generate plasmids expressing fusion proteins, DNA inserts were transferred from entry clones to destination vectors using LR Clonase recombination enzyme mix (Invitrogen). Destination vector pcDNA3.1/nV5-Dest was used to express N-terminal V5-tagged proteins in mammalian cells. Vector pMal-c2x (NewEngland Biolabs, Frankfurt, Germany) was converted to a destination vector by ligation of the gateway vector conversion cassette B (Invitrogen) into the *EcoRI* site (blunt ended by Klenow fill-in) of pMal-c2x, allowing to express MBP-fusion proteins in *E. coli* after recombination. The green fluorescent protein EGFP was amplified by PCR and cloned into the gateway compatible *HindIII/EcoRV* digested pcDNA3.1nV5DEST vector (Invitrogen). This vector was used to perform LR Clonase recombinations to obtain fluorescence-coupled expression of CPEB1 or CPEB2. HIF 3'- and 5'-UTR plasmids (kind gifts of R.H. Wenger, University of Zürich) were described previously [19]. Site-directed mutagenesis and deletion variants of the pGlmHIF3'-UTR were performed using the QuickChange kit (Stratagene, Amsterdam, Netherlands). The non-consensus CPE (NC-CPE; TTTTCAT) was mutated to the non-CPE-related sequence TTGGTAC, whereas the classical cytoplasmic polyadenylation element (CPE; TTTTAT) was deleted in these constructs. The mouse cyclin B1 3'-UTR that contains two CPEs was amplified from a mouse testis cDNA, subcloned into pENTR4 and transferred by LR clonase reaction to the luciferase reporter gene plasmid pcDNA3.1Luc. The pCRIIrat HIF-1 α 3'-UTR, which was used for *in vitro* transcription of the HIF-1 α mRNA 3'-UTR was kindly

provided by S.W. Spaulding (Buffalo Veterans Affairs Medical Center, New York) [20]. The CPE and the two NC-CPEs were deleted by site directed mutagenesis using the Quick change kit (Stratagene, Amsterdam, Netherlands).

Cell culture

HeLa cells were obtained from ATCC. The neuroblastoma cell line SK-N-MC and the neuroglioma cell line H4 were obtained from R.H. Wenger (University of Zürich). Cells were cultured in high glucose Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 50 U/ml penicillin G and 50 μ g/ml streptomycin (Invitrogen) in a humidified 5% CO₂, 95% air atmosphere at 37°C. For hypoxic conditions, O₂ levels were decreased to 1% O₂ with N₂ in an oxygen-controlled incubator (In vivo, Ruskinn, England).

Immunofluorescence

For immunofluorescence analyses, cells were fixed with freshly prepared 3.7% formaldehyde in PBS (pH 7.4) for 10 minutes, washed with PBS, permeabilized with 0.5% Triton X-100 for 5 minutes, and rinsed again with PBS. After blocking non-specific binding sites with 3% bovine serum albumin in PBS for 30 minutes, the cells were incubated for 1 hour with anti-HIF-1 α (Transduction Laboratories, Heidelberg, Germany) diluted 1:100 in 3% bovine serum albumin in PBS, followed by a tetramethyl rhodamine isothiocyanate-coupled secondary anti-mouse antibody (Dako, Hamburg, Germany) diluted 1:100 with 3% bovine serum albumin in PBS. After extensive washings with PBS, the slides were mounted and analyzed by fluorescence microscopy (Axioplan 2000, Zeiss, Göttingen, Germany).

Transient transfections

HeLa or SK-N-MC cells were transiently transfected with luciferase reporter gene constructs, whose 3'-UTR or 5'-UTR of HIF-1 α or cyclin B1 were fused to the firefly luciferase cDNA or with the luciferase HIF reporter gene plasmid pH3SVL. In this plasmid the luciferase reporter gene is driven by a simian virus 40 (SV40) promoter and contains a total of 6 HIF DNA-binding sites derived from the transferrin gene (kind gift of R.H. Wenger). Transfections were performed by the calcium phosphate co-precipitation method or by lipofection using Rotifect according to the manufacturer's instructions (Roth, Karlsruhe,

Germany), respectively. Cells were seeded in 24-well plates at 5×10^4 cells/well. One day after seeding, cells were co-transfected with 1.0 μ g reporter gene construct together with 0.5 μ g pcDNA3.1 and 0.5 μ g pcDNA3.1/nV5-Dest containing full-length CPEB1 or CPEB2 and 0.002 μ g renilla luciferase control plasmid pRL-SV40 (Promega, Mannheim, Germany). Cells were subsequently exposed to normoxic (21% O₂) or hypoxic (1% O₂) conditions for 24 hours. Luciferase activities were determined using the dual-luciferase assay kit (Promega). Results were normalized to the pcDNA3.1-transfected control values, which were arbitrarily defined as 1. In case of the UTR-hybrid vectors the normalization was chosen based on in general higher firefly luciferase counts obtained with the pGLmHIF1.2 3'-UTR containing the HIF-1 α 5'-UTR plus the HIF-1 α 3'-UTR (231.092 ± 11.114 FL counts without co-transfection of CPEBs) plasmid compared to the plasmids containing solely the HIF-1 α 3'-UTR (65.462 ± 11.457 FL counts without co-transfection of CPEBs) or the HIF1 α 5'-UTR (45.095 ± 3.250 FL counts without co-transfection of CPEBs).

Tissue samples

Exposure of mice to inspiratory 0.1% carbon monoxide has been published previously [21, 22]. Total RNA from various tissues was purified as described before [23]. RNA concentrations were determined spectrophotometrically, and RNA integrity was monitored by denaturing formaldehyde/agarose gel electrophoresis.

siRNA transfection

HeLa cells were transfected with 20 nM siRNAs (including two siRNA sequences for each gene) targeting CPEB1, CPEB2 or a non target control using lipofectamin (Invitrogen) according to the manufacturer's instructions. The following siRNA sequences were used: CPEB1 siRNA for 5'- ACCCACUGGGAAAUGUCCUAGGAA-3'; CPEB1 siRNA for 5'- ACCCACUGGGAAAUGUCCUAGGAA-3'; CPEB2 siRNA for 5'- GAGCUCAGUUCAGG CACUCAUUGAU-3'; CPEB2 siRNA 5'- for GAGCUCAGUUCAGGCACUCAUUGAU-3'. All Star control siRNA (Qiagen) was used as a non targeting control.

Protein analysis

Protein samples were analyzed for expression of HIF-1 α , V5 and β -actin. Protein concentrations were determined by the Bradford method using BSA as a standard. For immunoblot analysis, cellular protein (50 μ g) was electrophoresed through 7.5% SDS-polyacrylamide gels and electrotransferred onto nitrocellulose membranes (Amersham, Freiburg, Germany) by semi-dry blotting (PeqLab, Erlangen, Germany). HIF-1 α (Transduction Laboratories), V5 (Invitrogen) and β -actin (Sigma) were detected using the primary antibodies indicated above followed by HRP-labeled anti-mouse antibodies raised in goats (Santa Cruz). Chemiluminescence detection of horseradish peroxidase was performed by incubation of the membranes with 100 mM Tris-HCl, pH 8.5, 2.65 mM H₂O₂, 0.45 mM luminol and 0.625 mM coumaric acid for 1 minute and analyzed with a chemiluminescence camera (LAS3000, Fujifilm, Düsseldorf, Germany).

Protein expression and purification

MBP-fusion proteins were expressed in the *E. coli* strain TB1 (NewEngland Biolabs) transfected with pMal-c2x-DestCPEB1 or pMal-c2x-DestCPEB2 and purified using the pMal purification system according to the manufacturer's instructions (NewEngland Biolabs).

RNA binding assays

Filter binding assays were carried out essentially as described by others [24]. For determination of the binding constants, 10 fmoles of radioactively labeled RNA were incubated with increasing amounts of MBP-CPEB1 or MBP-CPEB2 in 40 μ l of RNA binding buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 0.2 mg/ml methylated bovine serum albumin, 0.01% Nonidet P-50, 1 mM EDTA, 1 mM dithiothreitol, 100 mM KCl). After 30 minutes of incubation at room temperature, 35 μ l of each reaction were applied to nitrocellulose filters (Schleicher & Schuell) pre-treated with 1 ml of wash buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl) containing 5 μ g/ml rRNA. After rinsing with 5 ml of ice-cold wash buffer, the filter-bound radioactivity was measured by scintillation counting. Apparent K_D values were determined both from direct and double-reciprocal plots.

Xenopus oocyte polyadenylation assays

*Hind*III linearized pCRIIrat HIF-1 α 3'-UTR or the indicated mutant, in which the CPE and the two NC-CPEs were deleted, were amplified in the presence of α -³²P-UTP by using the T7 mMessage Machine Kit (Ambion, Darmstadt, Germany). Radioactively labelled RNA was injected in stage VI xenopus oocytes (1 fmole/oocyte). Subsequently, oocytes were incubated at 16°C in 0.5x L15 medium overnight. Oocytes which have undergone germinal vesicle breakdown and therefore presented a white spot at the animal pole were separated. RNA was extracted from the matured oocytes by phenol/chloroform precipitation. One part of the isolated RNA was digested with RNase H (NewEngland Biolabs) in the presence of a polyT18 primer to remove the polyA tail. Non-digested and RNase H-digested RNA was separated on a denaturing 4% polyacrylamide/6M urea gel. Radioactive bands were visualized using a phosphoimager system (BioRad, München, Germany).

RNA extraction and real-time RT-PCR

Total RNA from mouse tissue or cultured cells was extracted as described previously [23]. First strand cDNA synthesis was performed with 1 μ g of RNA using the first strand synthesis kit from Fermentas (Fermentas, St. Leon-Rot, Germany). Subsequently, mRNA expression levels for mCPEB1, mCPEB2, hCPEB1, hCPEB2, hCAIX and hHIF-1 α were quantified with 1 μ l of cDNA reaction by real-time RT-qPCR using a SybrGreen Q-PCR reagent kit (Invitrogen) in combination with the MX3000P light cycler (Stratagene). Initial template concentrations of each sample were calculated by comparison with serial dilutions of a calibrated standard. To verify mRNA integrity and equal input levels, ribosomal human L28 or mouse S12 mRNA was determined and data expressed as ratios relative to L28 or S12 levels. mCPEB1

5'-AGAAGTCCGTCCGGGCCT-3',	5'-GGTTGTGAGCTGCAGATAAG-3';	hCPEB1	5'-
GTGATCCCCCTGGGTATTAGC-3',	5'-CAGATATGACACAGAGAATCTT-3';	m/hCPEB2	5'-
GCACTCTCTGGAAAATTCCC-3',	5'-AGAGCTTTCCATCTTCTTCAAT-3';	mS12	5'-
GAAGCTGCCAAGGCCTTAGA-3',	5'-AACTGCAACCAACCACCTTC-3';	hHIF-1 α	5'-
TGCATCTCCATCTCCTACCC-3',	5'-GTAGCTGCATGATCGTCTGG-3';	hCAIX	5'-

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GGGTGTCATCTGGACTGTGTT-3', 5'-CTTCTGTGCTGCCTTCTCATC-3'; hL28 5'-
GCAATTCCTCCGCTACAAC-3', hL28 5'-TGTTCTTGCGGATCATGTGT-3'.

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RESULTS

CPEB1 and 2 bind to the HIF-1 α 3'-UTR

CPEBs are the key factors for regulated mRNA translation via defined changes in the poly(A) tail length. Four different CPEBs have been described in mammalian cells [25, 26]. For CPEB1 and CPEB2 a polyadenylation-dependent function has been specified, whereas CPEB3 and CPEB4 seem to regulate translation of specific mRNAs independent of polyadenylation [27]. To determine the tissue expression profile of CPEB1 and CPEB2, RT-PCRs were performed with samples derived from various mouse tissues. Whereas high levels of CPEB2 mRNA were detected in testis only, high expression levels of CPEB1 mRNA were additionally found in brain and to a lower extent in kidney, heart, skeletal muscle and ovary (Fig. 1A and B). Exposure of mice to inspiratory 0.1% carbon monoxide for 4 hours did not change the expression of CPEB1 and 2 (Fig. 1C and 1D). Testing different tumor cell lines (including H4, SK-N-MC, PC12, HepG2 and HeLa), we found high endogenous CPEB1 and CPEB2 mRNA levels in the neuroblastoma SK-N-MC and the neuroglioma H4 cell lines. Hypoxic incubation (1% O₂ for 24 hrs) of the SK-N-MC and H4 cells did not affect CPEB1 or CPEB2 mRNA levels (Fig. 2). Additionally we tested a shorter incubation time, i.e. 1% O₂ for 4 hrs, which likewise did not affect CPEB1 or CPEB2 mRNA levels (data not shown). The RNA expression of the well described HIF-target gene carbonic anhydrase IX (CAIX) served as a positive control for the hypoxia-induced gene expression in these experiments.

Target mRNAs of CPEBs in mammalian cells include Cdk1, cyclin B1, α CaMKII, δ CaMKII, tPA, ABP, RCM3/calmodulin, Map2 and β -casein [28-33]. Comparable to these mRNAs, the mouse, human and rat 3'-UTRs of HIF-1 α mRNAs contain one CPE element (TTTTAT; 952-958 nt, 969-975 nt and 964-970 nt after the stop codons of human, mouse and rat HIF-1 α mRNAs, respectively) nearby the hexanucleotide polyadenylation signal (Fig. 3A). In addition, one or two NC-CPE elements (TTTTTCAT) can be found in mouse, human and rat HIF-1 α 3'-UTR, respectively. We tested, if CPEB1 and 2 are able to bind the CPE and NC-CPE in the mouse HIF-1 α 3'-UTR. Therefore, we first characterized the binding affinity of CPEB1 and CPEB2 for a fragment containing the mouse 3'-UTR which contains the CPE (Fig.

3B). CPEB1 and 2 did bind the mRNA fragment with a K_d of $7.8 \text{ nM} \pm 1.5$ and $4 \text{ nM} \pm 0.6$, respectively. Specific binding of CPEB2 to the CPE was verified by lack of binding to an mRNA fragment without CPE or NC-CPE element characteristics (777-950 nt post stop codon) as well as competition of CPEB2 binding to the CPE and NC-CPE containing fragment 950-1098 nt post stop codon by poly U but not by poly A or poly C (Fig. 3C).

CPEB1 and 2 affect the translational efficiency of HIF-1 α via its 3'-UTR

Recently Pique et al described a combinatorial code for the CPE-mediated translational control [34]. In this report, it has been demonstrated that the relative position of CPE elements, NC-CPE elements and the hexanucleotide polyadenylation signal AATAAA determines whether an mRNA will be translationally repressed by CPEB as well as the extent and time of cytoplasmic polyadenylation-dependent translational activation. Performing a software-assisted analysis, which has been developed based on the recently published combinatorial code (<http://genome.imim.es/CPE/server.html>), a CPEB-mediated translational repression was predicted, when CPEB is not stimulated.

To test if non-stimulated CPEBs indeed down-regulate HIF-1 α protein levels, we overexpressed GFP, GFP-CPEB1 or GFP-CPEB2 in HeLa cells. Subsequently, cells were exposed to 1% O₂ for four hours. After hypoxic incubation, cells were fixed and HIF-1 α protein expression was analyzed by immunofluorescence analysis. Cells overexpressing CPEB1 or CPEB2 demonstrated a significantly diminished hypoxic accumulation of HIF-1 α compared to non-transfected cells or GFP-transfected control cells (Fig. 4A and B). Similar results were obtained by testing the influence of increasing amounts of V5-CPEB2 on the expression of HIF-1 α as determined by immunoblot analysis (Fig. 4C). Additionally, CPEB1 and CPEB2 significantly diminished HIF-1 activity, which was analyzed in a reporter gene assay (Fig. 4E). Moreover, a significant decrease of hypoxia-induced CAIX RNA expression was detected as a result of CPEB1 or CPEB2 overexpression (Fig. 4F). Diminished HIF-1 α protein levels and HIF-1 activity were not due to changes in gene expression or mRNA stability, since overexpression of CPEB1 or CPEB2 did not change HIF-1 α mRNA levels (Fig. 4D).

To determine whether CPEBs affect HIF-1 α expression levels via the HIF-1 α 3'-UTR, we performed chimeric reporter gene assays (Fig. 5A). To this end, we transfected cells with constructs harbouring the 5'-UTR (pGLmHIFI.2), the 5'-UTR and 3'-UTR (pGLmHIFI.2 3'UTR) or the 3'-UTR of HIF-1 α (pGLmHIF 3'UTR) in frame with the firefly luciferase coding region. Co-expression of CPEB1 or 2 with the HIF-1 α 5'-UTR (pGLmHIFI.2) did not decrease luciferase activity, whereas the presence of the 3'-UTR alone (pGLmHIF 3'UTR) or in combination with the 5'-UTR (pGLmHIFI.2 3'UTR) significantly diminished firefly luciferase activity under the influence of CPEB1 and CPEB2 (Fig. 5B and C). As a control for the reliability of the assay, we transfected the 3'-UTR of the known CPEB target mRNA cyclin B1 fused to the firefly luciferase coding region. For cyclin B1 a CPEB1-mediated repression was shown previously [36]. In line with the fact that cyclin B1 mRNA is a target for CPEB1, co-expression of CPEB1 attenuated luciferase activity (Fig. 5E).

CPEBs contain two RNA recognition motifs and one cysteine-histidine (C/H) region reminiscent of a zinc finger. For CPEB1 the C/H region has been shown to be critical for RNA binding [35]. Deletion of aa 514-544 coding for the C/H region abolished the CPEB1-induced repression of the luciferase activity after co-transfection with pGLmHIF 3'UTR (Fig. 5D).

HIF-1 α mRNA is poly-adenylated after injection in *Xenopus* oocytes

To determine whether the HIF-1 α mRNA poly A tail is elongated by cytoplasmic polyadenylation after CPEB stimulation, we performed a commonly used assay to test for cytoplasmic polyadenylation [37]. To this end, stage VI *Xenopus* oocytes were injected with radiolabeled rat HIF-1 α cRNA 3'-UTR, containing the CPE and NC-CPEs (Fig. 6A). Oocytes were subsequently incubated with progesterone until a white spot of the animal pole was observed as an indication of germinal vesicle breakdown and thus oocyte maturation and CPEB activation. RNA from the matured oocytes was then extracted and analyzed by gel electrophoresis and autoradiography. As demonstrated in Fig. 6B, the poly(A) tail length of the 3'-UTR was increased after injection in oocytes. This increase in length was due to polyadenylation

since treatment of the isolated RNA with oligo dT and RNase H shortened the fragment. In addition, deletion of the CPE and NC-CPEs in the HIF-1 α 3'-UTR abrogated the polyadenylation.

CPEB mediates insulin-stimulated HIF-1 α expression

CPEB is posttranslationally phosphorylated and thereby activated after stimulation of *Xenopus* oocytes with progesterone. In mammalian cells, the phosphorylation of CPEB can be triggered by treatment with insulin [33, 38]. CPEB activation determines the switch from CPEB-mediated repression to CPEB-induced polyadenylation resulting in translational activation of target mRNAs. Thus we determined the influence of insulin on CPEB-mediated effects on the HIF-1 α 3'-UTR in SK-N-MC cells (Fig. 7A). To this end, we treated the neuroblastoma SK-N-MC cells with insulin after transfection with the chimeric 3'-UTR reporter gene plasmid pGLmHIF3'UTR. Stimulation of the cells with insulin increased the reporter gene activity to a minor extent in normoxia. However, exposing cells to hypoxic conditions and simultaneous treatment with insulin resulted in a significant increase in reporter gene activity. Regarding the reporter gene assay, a similar effect was not detectable after transfecting pGLmHIF1.2, which contains the HIF-1 α 5'-UTR in frame with the luciferase coding region, excluding a non-specific insulin effect (Fig. 7A). In addition, the insulin-stimulated increase in luciferase activity could be abrogated by deleting the CPE and the NC-CPE (Fig. 7B). The insulin-dependent effect on the HIF-1 α 3'-UTR was in accordance with increased endogenous HIF-1 α protein levels after treating SK-N-MC cells with insulin in hypoxia (Fig. 7C). To test the effects of CPEBs on the hypoxic stabilization of HIF-1 α as well as the insulin-dependent increase of HIF-1 α in hypoxia, we transiently transfected the SK-N-MC cells with siRNAs targeting CPEB1 or CPEB2. Since there are no suitable antibodies available for detecting endogenous CPEB1 or CPEB2 protein expression in human cells, we determined the efficacy of the siRNA effects at the mRNA level. As a result of the transient transfection a significant downregulation of CPEB1 and CPEB2 was observed, which was not accompanied by a change in the HIF-1 α mRNA levels or the hypoxia-induced mRNA expression of the HIF target gene CAIX (Fig. 8). In line with the results obtained with the 3'-UTR reporter gene assays, downregulation of CPEB1 or CPEB2 did also not affect

hypoxic stabilization of the HIF-1 α protein but prevented the insulin-dependent increase of HIF-1 α protein levels in hypoxia (Fig. 9A). Mirroring this effect, CPEB1 and CPEB2-downregulation inhibited an insulin-stimulated increase of the CAIX mRNA expression in hypoxia (Fig. 9B). This was not accompanied by changes in the expression of the HIF-1 α mRNA levels, excluding for examples changes in HIF-1 α mRNA expression or stability.

DISCUSSION

The hexanucleotide sequence AAUAAA is required for nuclear polyadenylation of mRNAs. The same sequence is necessary for cytoplasmic polyadenylation, which is facilitated by CPEB1 and 2 (for review [39]). CPEBs are the key factors for regulated mRNA translation via defined changes in the poly(A) tail length. They bind CPEs in the 3'-UTR of specific target mRNAs. After stimulation, CPEB promotes polyadenylation. The newly elongated tail then is bound by the poly(A)-binding protein, which stimulates general translation by augmenting the assembly of the eIF4F initiation complex and thus recruitment of the 40S ribosomal subunit to the 5'-end of the mRNA [40]. This complex formation is inhibited under dormant conditions by the association of the translation inhibiting protein Maskin [41]. Results obtained with CPEB1 knock-out mice demonstrate that CPEB1 plays a role in germ cell development, neuronal synaptic plasticity as well as in cellular senescence [42-45].

The hypoxic response of HIF-1 α is mainly regulated by hydroxylation-dependent protein stability. However, after stimulation of cells with hormones or growth factors or under strong hypoxic/anoxic conditions, an additional translational regulation of HIF-1 α expression has been described [16, 46-48]. Whereas general cap-dependent translation is inhibited during hypoxia/anoxia, the HIF-1 α mRNA is refractory to translational repression. Consistently, while most mRNAs shift to less actively translated monosomes, HIF-1 α mRNA remains in actively translated polysomes under hypoxic/anoxic conditions [11].

In the present study, we demonstrate that the HIF-1 α 3'-UTR contains a functional CPE element and NC-CPE elements. The CPE element is able to bind CPEB1 and CPEB2, resulting in HIF-1 α 3'-UTR

cytoplasmic polyadenylation. In terms of the functional consequences *in vivo*, a simple overexpression of CEPB1 or CPEB2 decreased HIF-1 α protein levels, in line with the assumption that CPEBs need to be activated for polyadenylation activity. The CPEB-mediated repression under resting conditions is in accordance with the specific arrangements of the CPE and the NC-CPE in the HIF-1 α 3'-UTR. Activation of CPEBs has been described after treatment with insulin or progesterone [33, 38]. Different phosphorylation pathways including the PI3 kinase, GSK-3, the aurora A kinase or the cdc2 kinase are associated with CPEB activation [32, 38]. Accordingly, stimulation of SK-N-MC cells with insulin resulted in a CPEB-dependent increase in the chimeric reporter gene activity mediated by the HIF-1 α 3'-UTR. This result resembles the described effects of progesterone and insulin on the β -casein regulation. Both, progesterone and insulin, stimulate synergistically CPEB-dependent polyadenylation, resulting in an increased rate of β -casein mRNA translation [33].

Clear evidence has emerged during the last years that HIF-1 α is affected by many stimuli including insulin under normoxic conditions. We have found that insulin affects HIF-1 α expression through a 3'-UTR-mediated mechanism most prominently under hypoxia. These data indicate that the combination of hypoxia and stimulation of insulin results in the integration of various signals affecting CPEB activation. One candidate pathway beside the above mentioned phosphorylation-dependent CPEB activation could be the mTOR pathway, which is affected by hypoxia and insulin. In addition mTOR has been described to be involved in CPEB activation [49]. Further studies are needed to analyze this hypothesis.

The presented data contribute to the knowledge about the complex regulation of the HIF-system. Whereas hypoxia-regulated protein stability of HIF-1 α probably is the most general mechanism of the HIF system, additional regulatory pathways may account for the precise control of the HIF system after stimulation. In this regard the specific organ expression pattern of CPEB1 and CPEB2 should be noted, which indicates that the function of CPEB may be tissue-specific. Most interestingly, high expression levels of CEPB1 and CEPB2 were found in brain and testis. The high expression level of CPEB2 in testis

is in accordance with a previous report [26]. The spatial pattern of the HIF-1 protein expression *in vivo* in response to oxygen tension as a single specific physiological stimulus in a healthy organism has been examined in detail by Stroka et al [50]. Most interestingly, although in all organs HIF-1 α expression responded to a decrease in the inspiratory oxygen concentration, basal HIF-1 α levels differed greatly between various organs with high levels in brain and testis. These data indicate that organ-specific and mitogen-inducible factors like CPEB may contribute to the oxygen-dependent HIF-1 α expression.

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FIGURE LEGENDS

Fig. 1. Organ expression of CPEB1 and CPEB2. Total RNA was extracted from tissue samples of various organs from mice exposed to inspiratory 20.9% O₂ (**A** and **B**) or 0.1% carbon monoxide (**C** and **D**) for four hours. Subsequently, CPEB1 and CPEB2 mRNA expression was quantified by RT-qPCR and normalized to S12 mRNA levels.

Fig. 2. CPEB1 and 2 mRNA levels are not induced by hypoxia. SK-N-MC and H4 cells were exposed to 21% O₂ or 1% O₂ for 24 hours. Subsequently total RNA was extracted and CPEB1, CPEB2 and CAIX mRNA expression was quantified by RT-qPCR and normalized to L28 mRNA. Shown are mean values \pm SEM of the ratio of the respective RNA/L28 obtained from six independent samples.

Fig. 3. CPEB1 and CPEB2 bind to the HIF-1 α 3'-UTR. (**A**) Schematic drawing of the human, mouse and rat HIF-1 α 3'-UTR. The CPE, the non-consensus CPE (NC-CPE) and the hexanucleotide polyadenylation signal AAUAAA (H) in the 3'-UTR are marked. Numbers represent the distance in nt between the different elements (**B**) Binding of MBP-CPEB1 and MBP-CPEB2 to the mouse HIF-1 α 3'-UTR was tested using fragment 950-989 nt (post stop codon), which harbours the CPE, in a filter binding assay as described in Materials and Methods. Based on the concentration-dependent binding the K_d was calculated. Shown are mean values \pm SEM of three independent experiments. (**C**) Binding of MBP-CPEB2 to mouse HIF-1 α 3'-UTR fragment 777-950 nt post stop codon (which does not contain the CPE or the NC-CPE) or 950-1098 nt post stop codon (which does contain the CPE and NC-CPE) was performed as described in B in the presence of increasing amounts of polyA, polyC or polyU.

Fig. 4. CPEB1 and CPEB2 affect HIF-1 α protein levels and HIF activity. (**A**) HeLa cells were transfected with GFP, GFP-CPEB1 or GFP-CPEB2. 24 hours later, the cells were exposed to 21% O₂ or 1% O₂ for 4 hours. Subsequently, cells were fixed and HIF-1 α protein levels were determined by immunofluorescence. At least 100 transfected cells (which appear with bright green fluorescence) per

slide were analysed and judged based on their HIF-1 α immunofluorescence signal compared to the non-transfected cells (which do not show green fluorescence signals). The percentage of cells with a diminished HIF-1 α signal was calculated. Shown are mean values \pm SEM of three independent experiments **(B)** Representative immunofluorescence pictures for the experiment as described in A. **(C)** HeLa cells were transfected with increasing amounts of the V5-CPEB2 expression plasmid. Subsequently cells were incubated for 24 hours at hypoxic conditions (1% O₂), lysed and analysed by immunoblot analysis. **(D)** HeLa cells were transfected and treated as described in A. 24 hours after transfection, cells were lysed, total cellular RNA isolated and analyzed for the expression of CPEB1, CPEB2, HIF-1 α or L28 mRNA. Shown are mean values \pm SEM of the ratio of the respective RNA/L28 from six independent samples. **(D)** HeLa cells were transfected with different expression plasmids together with the HIF reporter gene plasmid pH3SVL and the control reporter gene plasmid pRLSV40. Subsequently, cells were exposed to 21% O₂ or 1% O₂ for 24 hours. Cells were lysed and firefly/renilla luciferase activities were determined. Shown are the induction factors of cells exposed to hypoxia compared to the normoxic control cells. **(E)** HeLa cells were transfected with pcDNA3.1, pcDNA3.1 V5CPEB1 or pcDNA3.1 V5CPEB2. Subsequently cells were exposed to 21% O₂ or 1% O₂ for 24 hours. Subsequently total cellular RNA was extracted and CAIX mRNA expression was quantified by RT-qPCR and normalized to L28 mRNA. Shown are mean values \pm SEM from six independent samples.

Fig. 5. CPEB1 and 2 change the translational efficiency of HIF-1 α via its 3'-UTR. **(A)** Schematic drawings of the chimeric reporter gene plasmids used in B, C, D and E. **(B)** HeLa cells were transfected using a chimeric reporter gene plasmid containing the HIF-1 α 3'-UTR, HIF-1 α 5'-UTR or both in fusion with the firefly coding region together with the empty expression vector pcDNA3.1V5 or pcDNA3.1V5CPEB1. As a control for the transfection efficiency, the renilla luciferase encoding plasmid pRLSV40 was co-transfected. 24 hours after transfection, the cells were lysed and analyzed for firefly and renilla luciferase activities. **(C)** HeLa cells were transfected and treated as described in B using a chimeric reporter gene plasmid containing the HIF-1 α 3'-UTR, HIF-1 α 5'-UTR or both in fusion with the firefly

coding region together with the empty expression vector pcDNA3.1V5 or pcDNA3.1V5CPEB2. **(D)** HeLa cells were transfected and treated as described in B using a chimeric reporter gene plasmid containing the HIF-1 α 3'-UTR in fusion with the firefly coding region together with pcDNA3.1CPEB1 or the zinc finger deletion variant pcDNA3.1V5CPEB1 Δ aa 514-544. **(E)** HeLa cells were transfected and treated as described in B using a chimeric reporter gene plasmid containing the Cyclin B1 3'-UTR in fusion with the firefly coding region together with pcDNA3.1CPEB1. In B, C, D and E results were normalized to the values of pcDNA3.1-transfected control cells, which were arbitrarily defined as 1. Shown are mean values \pm SEM of three independent experiments.

Fig. 6. HIF-1 α mRNA is polyadenylated after injection into *Xenopus* oocytes. **(A)** Schematic overview of the injection of radioactively labeled *in vitro* HIF-1 α 3'-UTR in *Xenopus* oocytes with subsequent RNA isolation and RNase H digestion. **(B)** Radioactively labeled *in vitro* transcribed rat HIF-1 α 3'-UTR cRNAs (IVT) containing the wild type 3'-UTR or a mutated 3'-UTR, in which the CPE and the two NC-CPEs were deleted, were injected into *Xenopus* oocytes. After oocyte maturation, which was determined by germinal vesicle break down, total RNA was extracted. One part of the extracted RNA was digested by RNase H in the presence of oligo dT to remove the polyA tail. IVT, non-digested and RNase H-digested RNA was separated on a denaturing 4% polyacrylamide/6M urea gel. Radioactive bands were visualized using a phosphoimager.

Fig. 7. Insulin stimulates CPEB-dependent translational efficiency of HIF-1 α . **(A)** SK-N-MC cells were transiently transfected with a chimeric reporter gene plasmid containing the mouse 3'-UTR (pGLmHIF3'UTR) or 5'-UTR (pGLmHIF1.2) of HIF-1 α in fusion with the firefly coding region. As a control for transfection efficiency, the renilla luciferase coding plasmid pRLSV40 was co-transfected. Subsequently, cells were cultured in normoxic (21% O₂) or hypoxic (1% O₂) conditions for 24 hours without fetal calf serum. During the last 6 hours of incubation, 10 nM or 100 nM insulin was added to the indicated samples. Cells were lysed and analyzed for firefly and renilla luciferase activities. Shown are

mean values \pm SEM (n=6). **(B)** SK-N-MC cells were transiently transfected with a chimeric reporter gene plasmid containing the mouse 3'-UTR of HIF-1 α in fusion with the firefly coding region or the indicated deletion/mutation variants. During the last 4 hours of incubation 10 nM insulin was added to the indicated samples. Cells were lysed and analyzed for firefly and renilla luciferase activities. Shown are mean values \pm SEM of one representative experiment (n=6). In A and B, results were normalized to the values of the transfected control cells incubated at 21% O₂ without insulin treatment. **(C)** SK-N-MC cells were cultured in normoxic (21% O₂) or hypoxic (1% O₂) conditions for 24 hours without fetal calf serum. During the last 6 hours of incubation, 10 nM insulin was added to the indicated samples. Cells were lysed and analyzed for expression of HIF-1 α and β -actin by immunoblotting.

Fig. 8. CPEB1 and 2 knockdown do not affect HIF-1 α RNA and CAIX mRNA levels after hypoxic incubation. SK-N-MC cells were transiently transfected with siRNAs targeting CPEB1, CPEB2 or a non-targeting control siRNA. After transfection cells were cultured in normoxic (21% O₂) or hypoxic (1% O₂) conditions for 24 hours. Subsequently cells were lysed, total cellular RNA was extracted and CPEB1, CPEB2, CAIX, HIF-1 α and L28 mRNA levels were analyzed by RT-qPCR.

Fig. 9 CPEB1 and 2 mediate the stimulatory effect of insulin on HIF-1 α protein levels in hypoxia. **(A)** SK-N-MC cells were transiently transfected with siRNAs targeting CPEB1, CPEB2 or a non-targeting control siRNA. After transfection cells were cultured in normoxic (21% O₂) hypoxic (1% O₂) conditions for 24 hours without fetal calf serum. During the last 6 hours of incubation, 10 nM insulin was added to the indicated samples. Cells were lysed and analyzed for expression of HIF-1 α and β -actin by immunoblotting or **(B)** total RNA was extracted and the CAIX, HIF-1 α and L28 mRNA levels were analyzed by RT-qPCR.

Figure 1

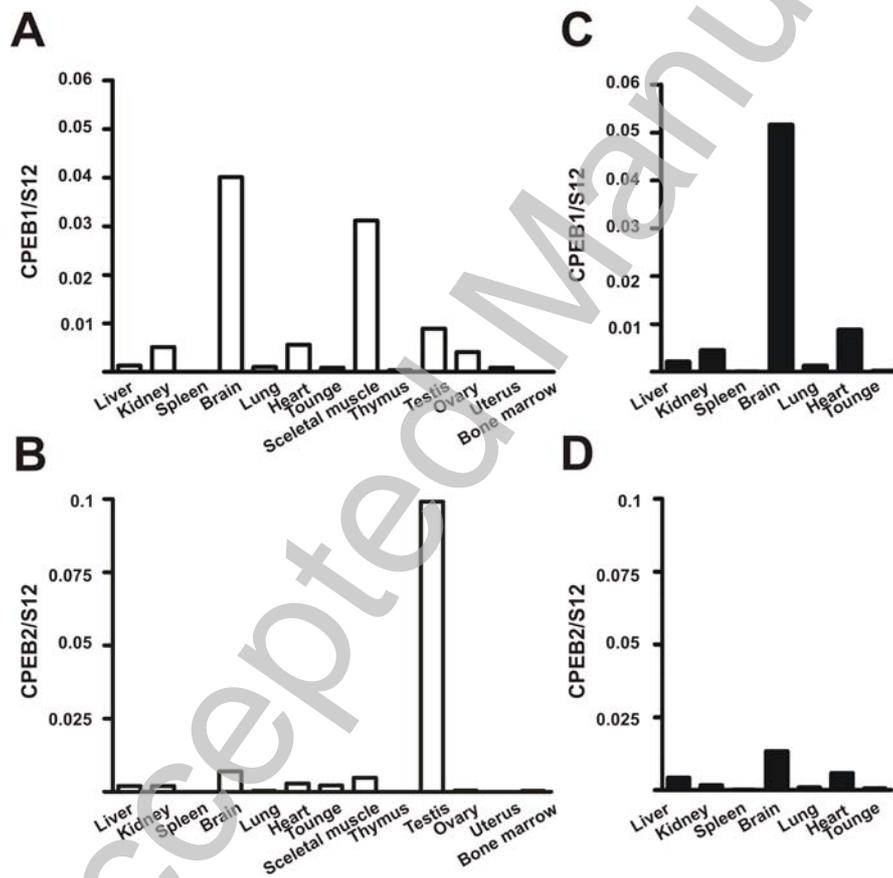


Figure 2

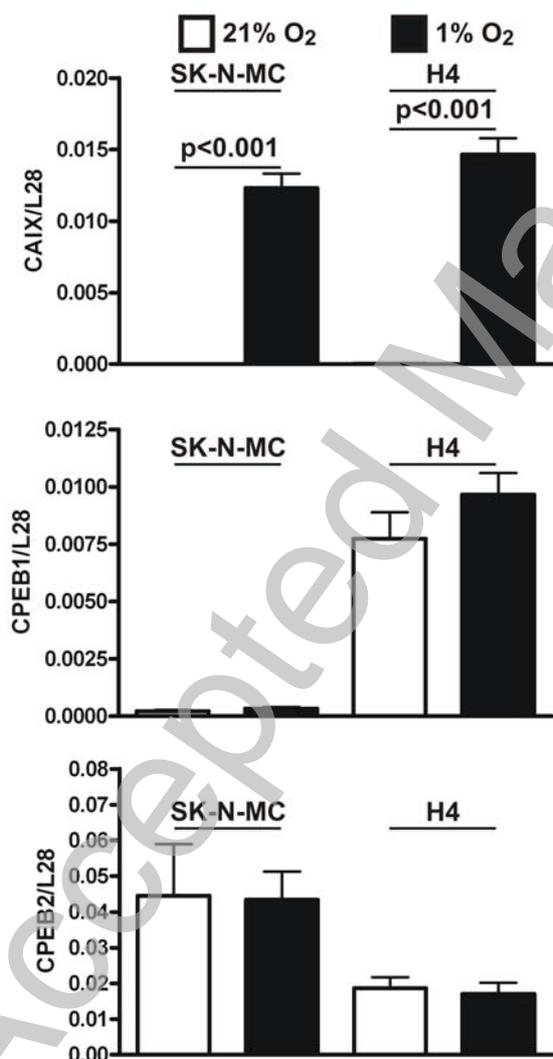
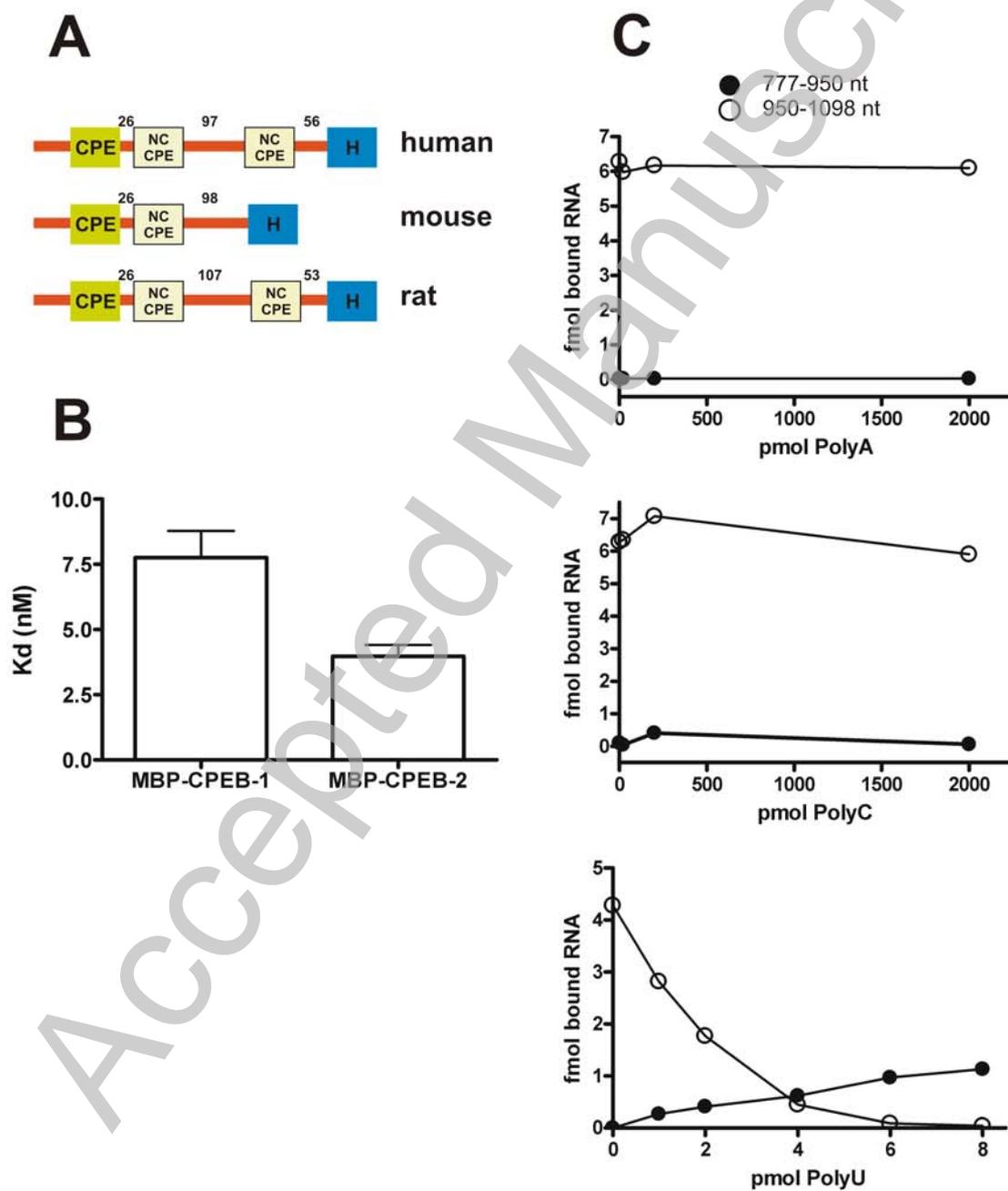
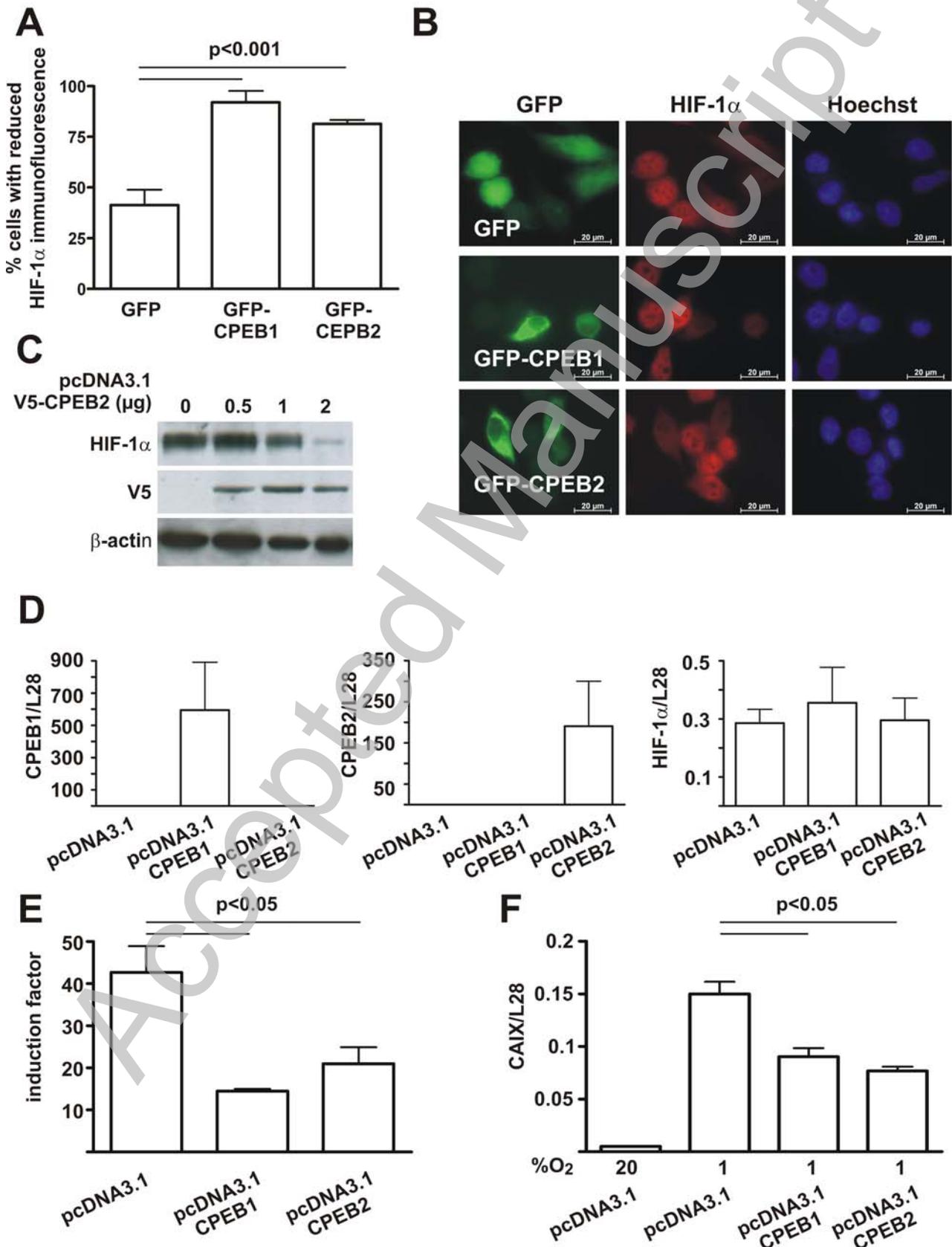


Figure 3



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Figure 4



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Figure 5

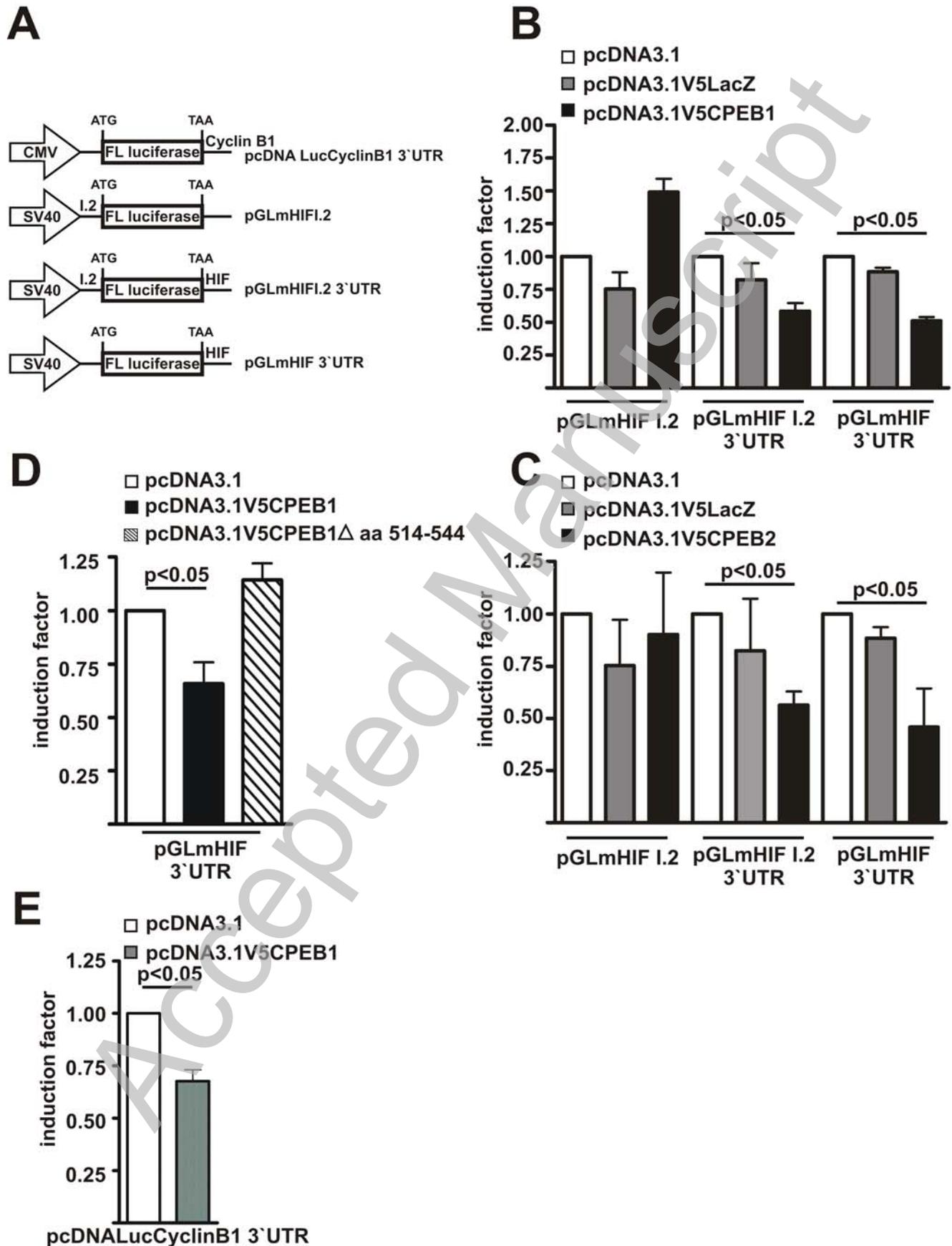


Figure 6

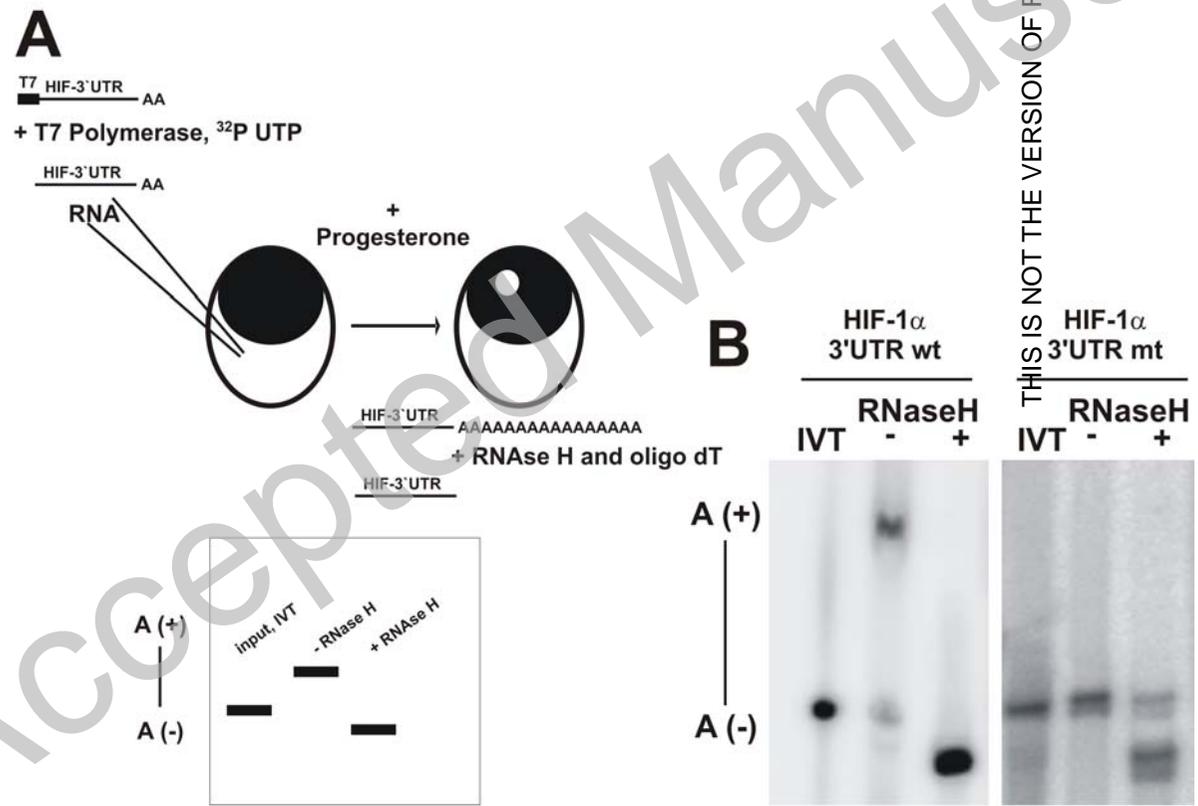


Figure 7

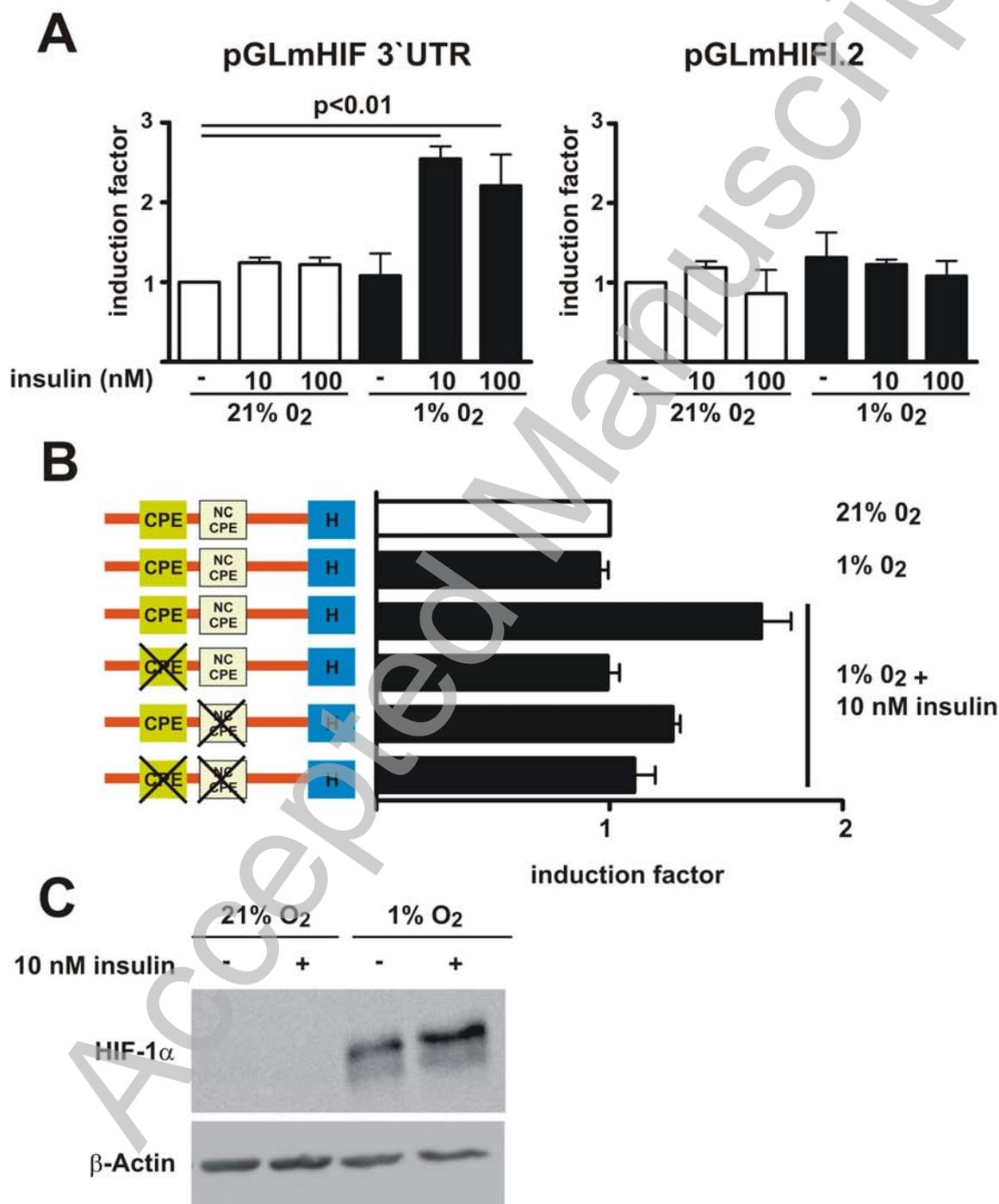
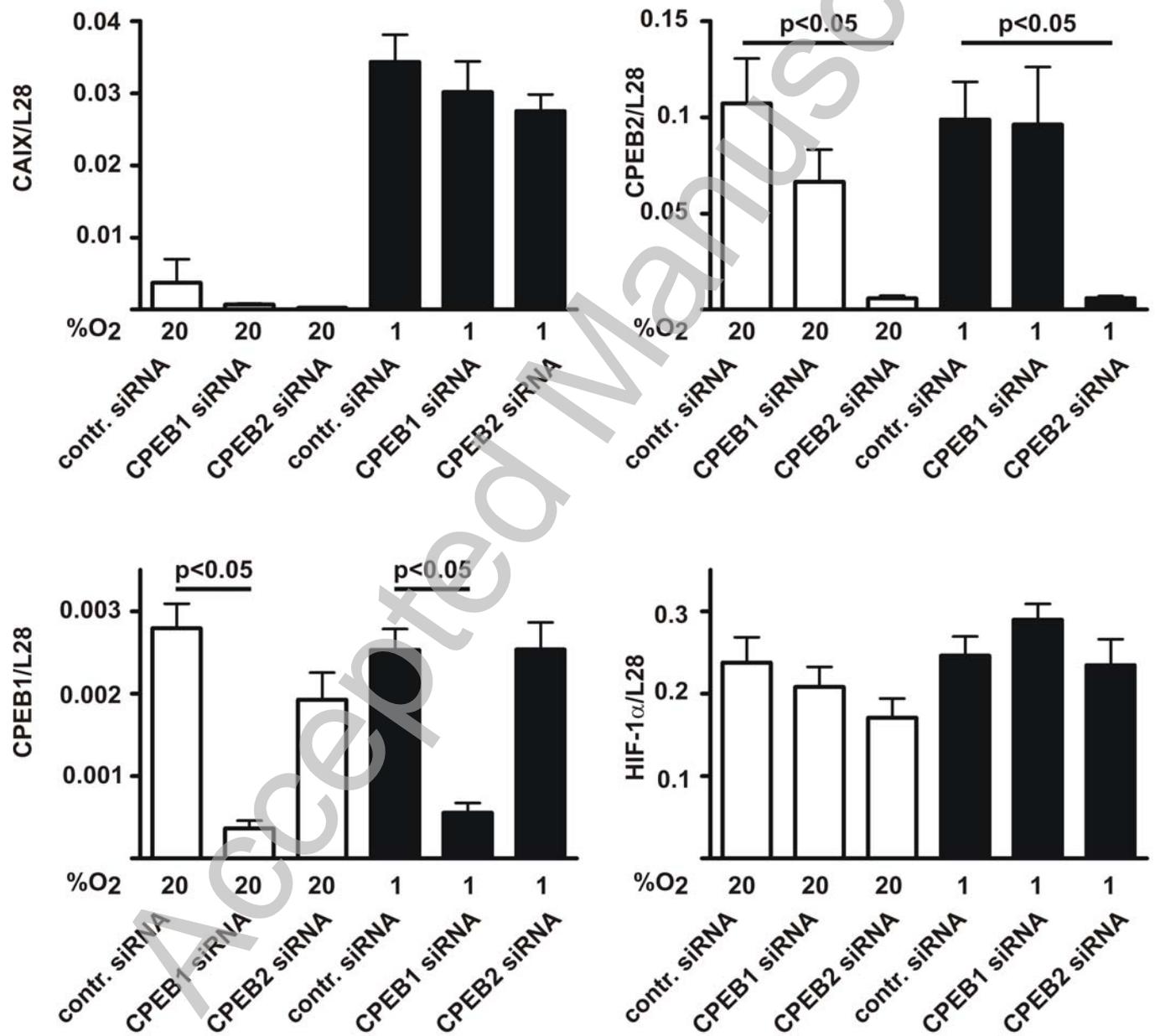
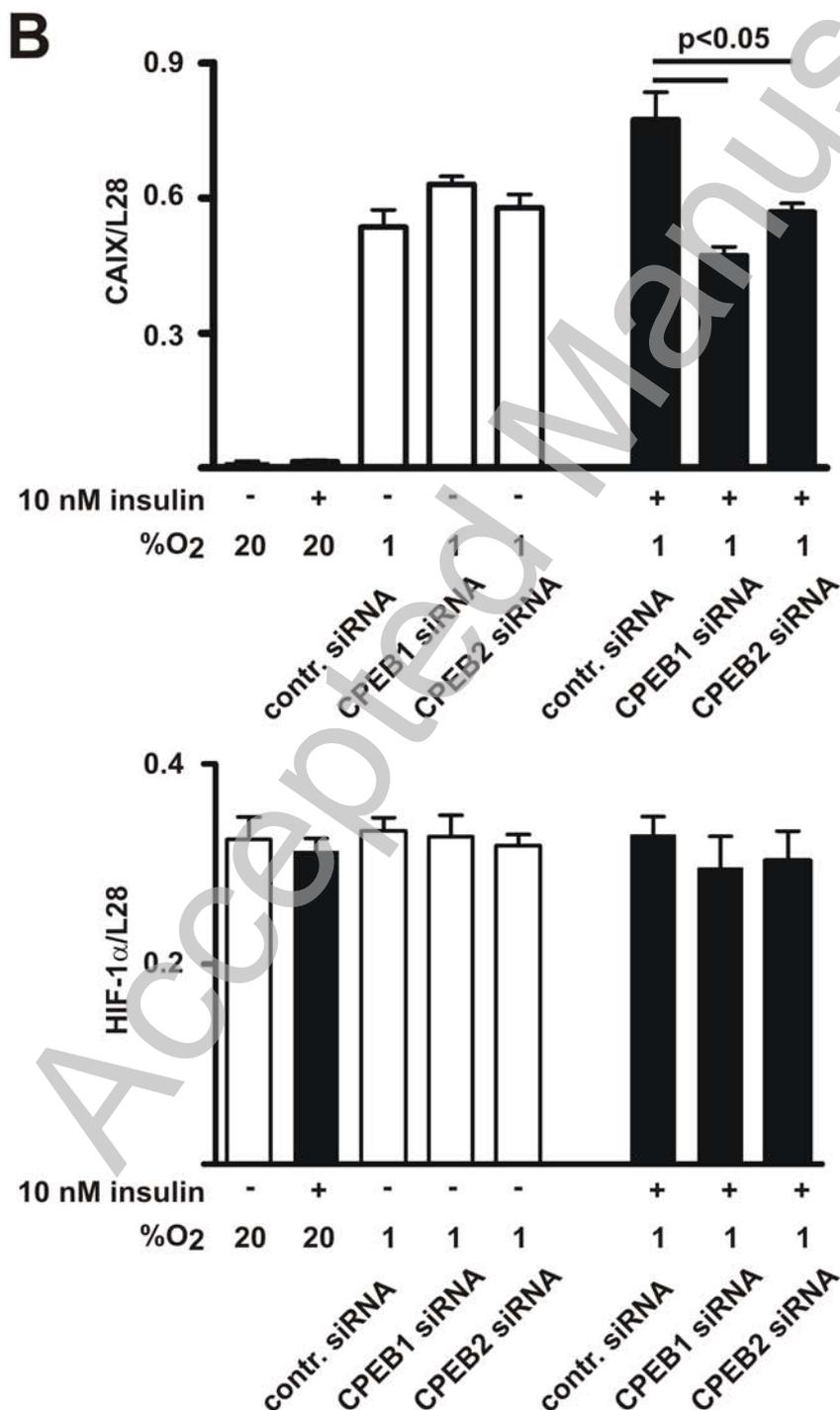
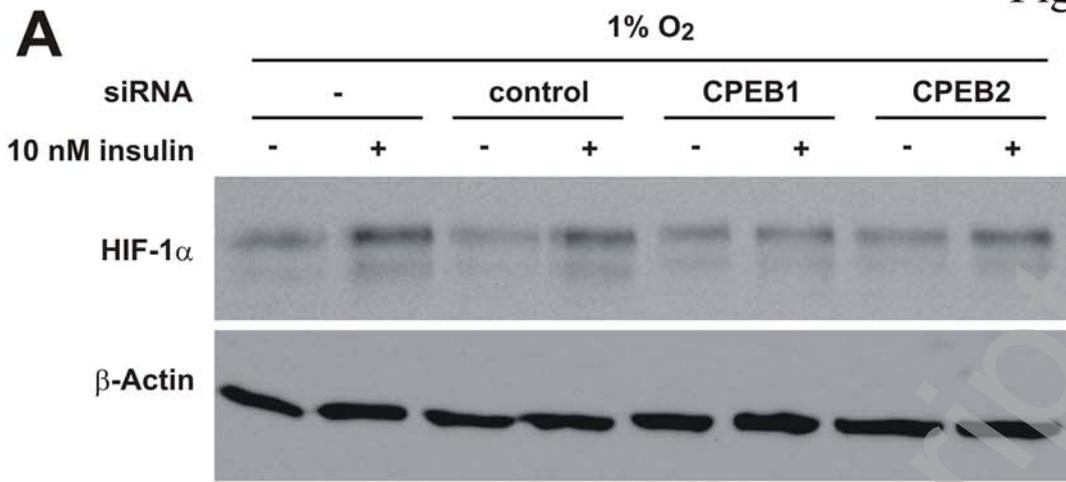


Figure 8



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Figure 9



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