

Role of CBP in regulating HIF-1-mediated activation of transcription

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Summary

The hypoxia-inducible factor-1 (HIF-1) is a key regulator of oxygen homeostasis in the cell. We have previously shown that HIF-1 α and the transcriptional coactivator CBP colocalize in accumulation foci within the nucleus of hypoxic cells. In our further exploration of the hypoxia-dependent regulation of HIF-1 α function by transcriptional coactivators we observed that coexpression of SRC-1 (another important coactivator of the hypoxia response) and HIF-1 α did not change the individual characteristic nuclear distribution patterns. Colocalization of both these proteins proved to be mediated by CBP. Biochemical assays showed that depletion of CBP from cell extracts abrogated interaction between SRC-1 and HIF-1 α . Thus, in contrast to the current model for the assembly of complexes between nuclear hormone receptors and coactivators, the present data suggest that it is CBP that

recruits SRC-1 to HIF-1 α in hypoxic cells. We also observed that CBP, HIF-1 α /Arnt and HIF-1 α /CBP accumulation foci partially overlap with the hyperphosphorylated form of RNA polymerase II, and that CBP had a stabilizing effect on the formation of the complex between HIF-1 α and its DNA-binding partner, Arnt. In conclusion, CBP plays an important role as a mediator of HIF-1 α /Arnt/CBP/SRC-1 complex formation, coordinating the temporally and hierarchically regulated intranuclear traffic of HIF-1 α and associated cofactors in signal transduction in hypoxic cells.

Key words: Hypoxia-inducible factor-1, CREB-binding protein, Steroid receptor coactivator-1, Intranuclear distribution, Cyan/yellow fluorescent protein

Introduction

Oxygen homeostasis is tightly regulated in multicellular organisms by the transcription factor hypoxia-inducible factor-1 (HIF-1). In response to decreased oxygen levels HIF-1 activates transcription of target genes encoding proteins critical for several developmental and physiological processes including glycolysis and angiogenesis (Semenza, 2002). HIF-1 is a heterodimeric complex composed of the transcription factors HIF-1 α and Arnt. In contrast to Arnt, oxygen levels regulate both the expression and the activity of HIF-1 α . At normoxia (21% O₂) HIF-1 α is rapidly ubiquitinated and degraded by the 26S proteasome (Huang et al., 1998; Kallio et al., 1997; Kallio et al., 1999; Salceda and Caro, 1997) whereas at hypoxia (1% O₂) the protein is stabilized. Degradation of HIF-1 α is regulated by hydroxylation of specific proline residues that are recognized by the von Hippel-Lindau tumor suppressor protein which is part of an E3 ubiquitin ligase complex (Cockman et al., 2000; Ivan et al., 2001; Jaakkola et al., 2001; Kamura et al., 2000; Masson et al., 2001; Ohh et al., 2000; Pereira et al., 2003; Tanimoto et al., 2000; Yu et al., 2001).

At low oxygen levels HIF-1 α translocates to the nucleus (Kallio et al., 1998) where the functionally active HIF-1 α /Arnt complex activates transcription of target genes after binding to cognate hypoxia-responsive elements (HRE). HIF-1-mediated activation of transcription requires the recruitment of coactivators such as CBP/adenovirus E1A-binding protein

p300 (p300) and factors belonging to the steroid receptor coactivator (SRC)/p160 family of proteins (Arany et al., 1996; Carrero et al., 2000; Ebert and Bunn, 1998; Ema et al., 1999; Gu et al., 2001; Kung et al., 2000; Ruas et al., 2002). Hydroxylation of an asparagine residue at normoxia by the factor inhibiting HIF-1 α (FIH-1) regulates the transactivation activity of HIF-1 α by abrogating the interaction between the C-terminal transactivation domain (C-TAD) of HIF-1 α and the CH1 domain of the CREB-binding protein (CBP) (Hewitson et al., 2002; Lando et al., 2002a; Lando et al., 2002b; Mahon et al., 2001). CBP/p300 regulate chromatin structure through histone acetylation and interaction with other histone acetyltransferases including P/CAF (Yang et al., 1996) and are known to acetylate other proteins involved in the regulation of transcription (Sterner and Berger, 2000). The p160/SRC proteins, constituting a family of coactivators, have been identified mainly as nuclear hormone receptor-interacting proteins (Xu and Li, 2003). SRCs have been proposed to function as nuclear hormone receptor coactivators by recruiting histone acetyltransferases such as CBP/p300 and P/CAF (Li et al., 2000; Spencer et al., 1997) and histone methyltransferases such as coactivator-associated arginine methyltransferase-1 (CARM1) and protein arginine N-methyltransferase-1 (PRMT1) (Chen et al., 1999; Koh et al., 2001).

Previous reports have shown that the function of both the N-terminal (N-TAD) and C-terminal (C-TAD) transactivation domains of HIF-1 α can be enhanced by CBP and SRC-1 in

reporter gene assays (Carrero et al., 2000; Ema et al., 1999; Kallio et al., 1998; Ruas et al., 2002) and that the C-TAD interacts directly with the CH1 domain of CBP (Bhattacharya et al., 1999; Dames et al., 2002; Freedman et al., 2002; Gu et al., 2001; Kung et al., 2000; Ruas et al., 2002). We have previously shown that colocalization of CBP and HIF-1 α in nuclear accumulation foci is dependent on the integrity of both transactivation domains of HIF-1 α , correlating with HIF-1-mediated activation of transcription (Ruas et al., 2002). In the present study we have investigated the dynamics and architecture of the assembly of the HIF-1/CBP/SRC-1 complex in vivo. Interestingly, our results showed that the interaction between CBP and SRC-1 is necessary for colocalization of HIF-1 α with SRC-1. The data suggest an indirect mode of interaction between SRC-1 and HIF-1 α that is mediated by CBP. This study presents the first evidence for a coordination of the intranuclear trafficking of several proteins involved in transduction of the hypoxic signal (i.e. HIF-1 α , Arnt, CBP and SRC-1) assessing their relative contributions to establish a transcriptionally active form of HIF-1 α .

Materials and Methods

Plasmids and fusion proteins

Amino acid mutations were inserted into pRc/RSV-CBP-HA in order to generate pRc/RSV-CBP/H3P-HA using the QuickChange site-directed mutagenesis kit (Stratagene) following the protocol provided by the manufacturer. To exclude the possibility of random mutations introduced by the PCR reaction, a *SgrAI-NotI* fragment of CBP encoding amino acids 1867-2441 carrying the F2101P and K2103P mutations was cloned into wild-type pRc/RSV-CBP-HA previously digested with the same enzymes. The inserted fragment was completely sequenced using the Dyanamic sequencing kit (Amersham Pharmacia) according to the manufacturer's recommendations. pYFP-CBP/H3P was generated by inserting a *BamHI* fragment of pRc/RSV-CBP/H3P-HA into pEYFP-C1 (Clontech) digested with the same restriction enzyme. pCFP-SRC-1 and pYFP-SRC-1 were generated by insertion of a *SmaI-XbaI* fragment of pSG5/hSRC-1 into pECFP-C1 or pEYFP-C1 digested with the same enzymes. pCFP-mArnt and pYFP-mArnt were a kind gift from I. Pongratz (Karolinska Institutet, Stockholm, Sweden). pT81/HRE-luc and the plasmid containing the GAL4-driven luciferase reporter gene have been previously described (Carrero et al., 2000). pRc/RSV-mCBP-HA (expressing full-length mouse CBP) was obtained from R. H. Goodman (Vollum Institute, Portland, OR) and pSG5/hSRC-1 (encoding human full-length SRC-1) was a kind gift from B. W. O'Malley (Baylor College of Medicine, Houston, TX). Expression plasmids for Flag-GAL4 DNA-binding domain, Flag-GAL4-C-TAD, YFP-CBP, Flag- and CFP-C-terminal fusions of mHIF-1 α and mHIF-1 α (532 Δ 583)(L808A/L809A) have been previously described (Ruas et al., 2002).

Cell culture and transient transfection experiments

A 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) (10% fetal calf serum) and F-12 medium (5% fetal calf serum) containing 50 IU/ml penicillin and 50 μ g/ml streptomycin sulfate was used to maintain human embryonic kidney (HEK) 293 cells. All media and growth factors were purchased from Invitrogen. For reporter gene assays cells were seeded in six-well plates 24 hours before transfection and medium was changed to OPTI-MEM medium (Invitrogen) before transfection. HEK 293 cells were transfected with LipofectAMINE (Invitrogen) according to the manufacturer's instructions. After transfection for 4 hours, medium was changed to culture medium and cells were allowed to grow for 36 hours at

normoxia (21% O₂) or hypoxia (1% O₂). Cells were then harvested and extracts were prepared and analysed for luciferase activity. Total protein concentration of whole cell extracts was determined by a colorimetric method (Bio-Rad).

Nuclear translocation and colocalization studies

HEK 293 cells were cultured on coverslips in 3-cm diameter dishes for 24 hours and transfected as before with 400 ng of each plasmid using pFlag-CMV-2 as carrier DNA to keep the amount of DNA constant at 1.2 μ g. 36 hours after transfection medium was changed for treatment in the presence or absence of 100 μ M CoCl₂ for 2 hours. Coverslips were then removed from the culture dishes and cells were fixed for 20 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS). The coverslips were then mounted on glass slides and observed using a Axiovert 35 Zeiss microscope (Carl Zeiss Jena GmbH, Jena, Germany) with epifluorescence and illumination from a Gixenon burner, and equipped with selective filter sets for CFP (exciter: D436/20, emitter D480/40) and YFP (exciter: HQ500/20, emitter: HQ535/30) (Chroma Tech). Images were captured by a CCD-CH250 camera (Photometrics, Tuscon, AZ) and signal superimposition obtained with the camera software (QED imaging). Subcellular and intranuclear distribution of CFP- and YFP-fused proteins was determined by counting around 300 cells.

Immunocytochemistry for RNA polymerase II

HEK 293 cells were grown on coverslips, transfected and fixed as described above, and endogenous RNA polymerase II was detected by immunofluorescence. Briefly, cells were washed with PBS, and for blocking cells were incubated in 10% fetal calf serum in PBS for 15 minutes at room temperature. After removing blocking solution and washing three times with PBS, cells were incubated for 1 hour at room temperature with a 1:200 dilution of the CC-3 antibody (Thibodeau et al., 1989) in PBS containing 1% BSA and 0.1% Triton X-100. CC-3 anti-RNA polymerase II antibody was obtained from Michel Vincent (Université Laval, Québec, Canada). When mara3 antibody (Patturajan et al., 1998) was used, lyophilized tissue culture supernatant was reconstituted in 700 μ l 1% BSA, 0.1% Triton X-100 in PBS. Mara3 anti-RNA polymerase II antibody was a kind gift from Bartholomew M. Sefton (The Salk Institute, La Jolla, CA). After three washes with PBS anti-mouse IgG-coupled Texas-Red was added (1:200 in 1% BSA, 0.1% Triton X-100 in PBS) as secondary antibody and incubated for 1 hour at room temperature. Coverslips were washed with PBS and mounted on slides for observation. Samples were scanned with a Zeiss LSM 510 laser scanning confocal device coupled to a Axiovert 100 M microscope using a 63 \times Pan-Apochromat oil objective (Carl Zeiss).

Immunoprecipitation assays

HEK 293 cells were grown in 10-cm diameter plates for 24 hours and transfected with 1 μ g of expression plasmid as described above. Cells were allowed to grow for 36 hours and then treated with 100 μ M CoCl₂ or kept at normoxia for 6 hours. Cells were harvested and lysed in RIPA lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Igepal, 0.1% SDS) containing 100 μ M phenyl-methylsulphonyl fluoride (PMSF), 1 mM dithiothreitol (DTT) and a protease inhibitor mixture (Roche Molecular Biochemicals). Total protein concentration was determined as before. Total proteins (3 mg) from whole cell extracts were used for co-immunoprecipitation experiments performed as follows. 30 μ l protein G Sepharose was incubated for 1 hour at room temperature with 5 μ l of anti-SRC-1 (MA1-840, Affinity Bioreagents) or anti-CBP (sc-7300, Santa Cruz Biotech.) or normal mouse IgG (Santa Cruz Biotech.) antibodies in 50 μ l TBS. After incubation at room temperature for 1-2 hours with rotation, the pellet was washed four times with RIPA lysis buffer and

proteins were eluted by adding loading buffer and boiling. The immunoprecipitated protein complexes were submitted to SDS-PAGE, blotted onto a nitrocellulose filter and probed with specific antibodies as described below.

Immunoblotting and detection

Fifty μ g of the total cell proteins were blotted after separation by SDS-PAGE on a nitrocellulose filter and blocked overnight with 5% non-fat milk in TBS. Antibodies for Flag epitope (F3165; Sigma), CBP (sc-7300; Santa Cruz Biotechnology), SRC-1 (MA1-840; Affinity Bioreagents) and GFP (8362-1; Clontech) were diluted according to the manufacturer's instructions in 1% non-fat milk in TBS and incubated at room temperature for 1 hour. After washes, a 1:1000 dilution of anti-rabbit or anti-mouse IgG horseradish peroxidase conjugate (Amersham Biosciences) in TBS containing 1% non-fat milk, was used as secondary antibody. The filters were then extensively washed with TBS containing 0.05% Tween 20 and complexes were visualized using enhanced chemiluminescence (Amersham Biosciences) according to the manufacturer's recommendations.

Results

Regulation of the interaction of HIF-1 α with CBP and SRC-1 in the hypoxic cell

In the present study we wanted to investigate the architecture and distribution of the HIF-1 α /coactivator complex. As we have shown earlier (Ruas et al., 2002) the nucleoplasmic localization of CFP-HIF-1 α is altered by coexpression of CBP, resulting in recruitment of HIF-1 α to CBP-containing accumulation foci. In vivo interaction of HIF-1 α and CBP is dependent on the integrity of HIF-1 α transactivation domains since a HIF-1 α mutant deficient in transactivation [HIF-1 α (532 Δ 583)(L808A/L809A)] does not colocalize with CBP (Ruas et al., 2002). Here we further demonstrate by protein-protein interaction assays that HIF-1 α (532 Δ 583)(L808A/L809A), which does not transactivate or colocalize with CBP (Ruas et al., 2002) has lost its ability to interact with CBP. As shown in Fig. 1A, Flag-HIF-1 α was efficiently co-precipitated with endogenous CBP from whole cell extracts prepared from HEK 293 cells either kept at normoxia (lane 1) or treated with CoCl₂ (Lane 2). Iron antagonists such as CoCl₂, desferrioxamine or 2,2'-dipyridyl are known to act as hypoxia mimicking agents since Fe(II) is a critical cofactor for the hydroxylation reactions that regulate HIF-1 α activity (Bruick and McKnight, 2001; Epstein et al., 2001). Consistent with earlier observations (Ruas et al., 2002), the binding of HIF-1 α to CBP observed at normoxia may be the result of the interaction mediated by the N-TAD when the HIF-1 α protein is overexpressed in HEK 293 cells, indicating an important role of the HIF-1 α N-TAD in forming an interaction interface between the two proteins.

In addition to CBP, SRC-1 has been shown to be involved in HIF-1 α -mediated activation of transcription (Carrero et al., 2000; Ruas et al., 2002) but no direct interaction between these proteins has been reported. We therefore tested whether co-immunoprecipitation of Flag-HIF-1 α by SRC-1 was CBP-dependent, using an

anti-SRC-1 antibody targeting the endogenous protein in HEK 293 cells. As shown in Fig. 1B, HIF-1 α was able to specifically interact with endogenous SRC-1 in HEK 293 whole cell extracts (lanes 1 and 2). However, when cell extracts were depleted of CBP by immunoprecipitation using an anti-CBP antibody no interaction was observed between HIF-1 α and SRC-1 (Fig. 1B, lanes 3 and 4), indicating that CBP is required for the formation of HIF-1 α complexes containing SRC-1.

Colocalization of CFP-HIF-1 α and YFP-SRC-1 in accumulation foci is dependent on CBP

Based on the results obtained in the co-immunoprecipitation experiments, we next examined the distribution of the different proteins tagged with CFP or YFP in HEK 293 cells. As shown in Fig. 2A, and in agreement with previous reports (Ruas et al., 2002), the subcellular localization of YFP-CBP and CFP-HIF-1 α in cells treated with CoCl₂ is exclusively nuclear but both proteins have distinct intranuclear distribution patterns. YFP-CBP shows a distinctly punctate intranuclear pattern whereas CFP-HIF-1 α has a diffuse nuclear distribution. At normoxia

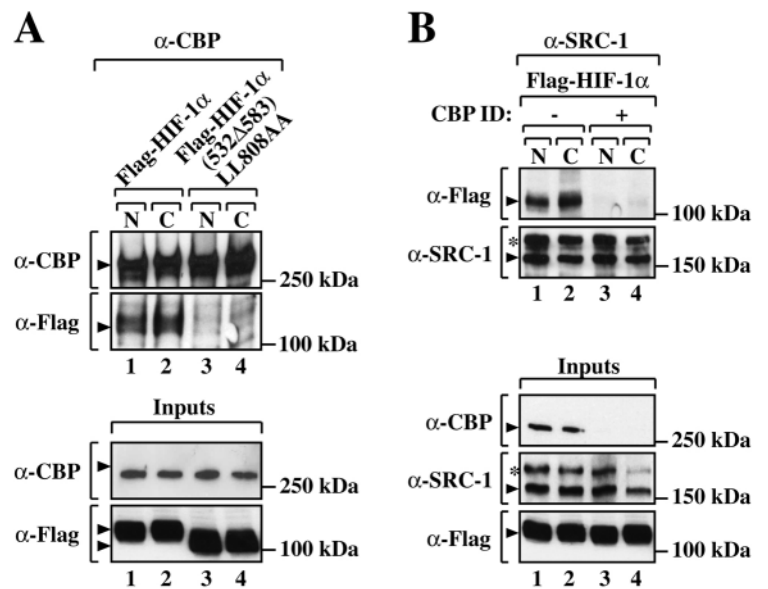
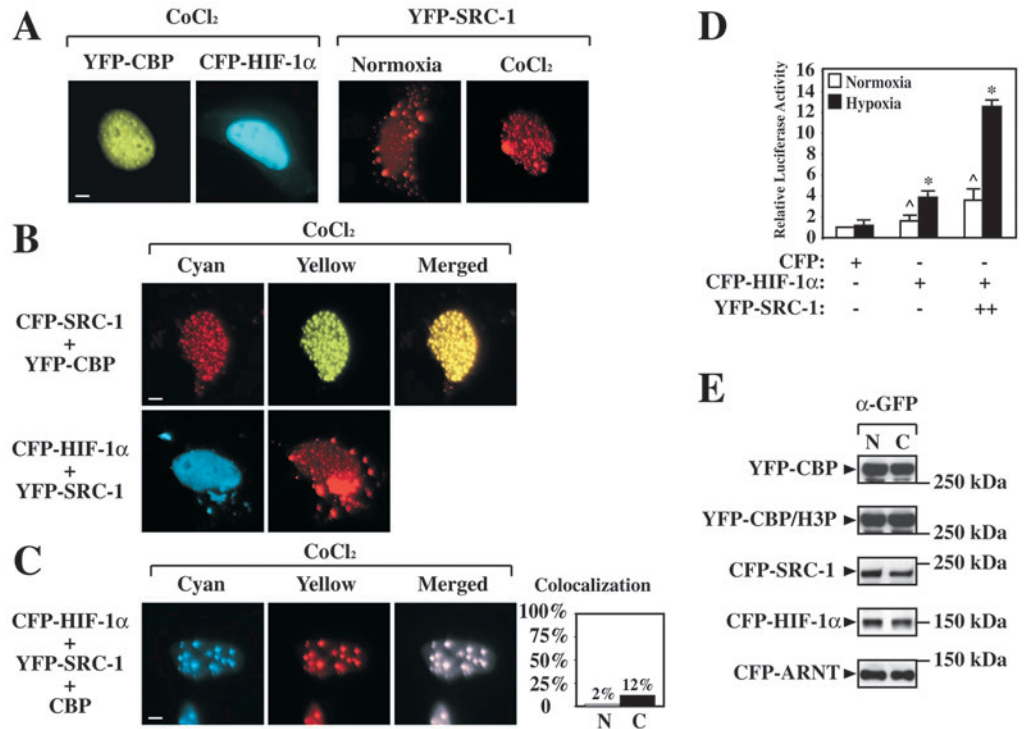


Fig. 1. CBP mediates the interaction of SRC-1 with HIF-1 α . (A) Interaction of HIF-1 α with endogenous CBP is dependent on the integrity of both HIF-1 α transactivation domains. Cells were transfected with 1 μ g pFlag-mHIF-1 α (Flag-HIF-1 α) or pFlag-mHIF1 α (532 Δ 583)(L808A/L809A) [Flag-HIF-1 α (532 Δ 583)LL808AA] and kept at normoxia (N) or treated with 100 μ M CoCl₂ (C) for 3 hours. 3 mg protein from HEK 293 whole cell extracts was incubated with anti-CBP antibody (α -CBP), and precipitated complexes were separated by SDS-PAGE followed by immunoblotting using anti-CBP and anti-Flag (α -Flag) antibodies. (B) CBP immunodepletion abolishes interaction between HIF-1 α and SRC-1. HEK 293 cells were transfected with 1 μ g of pFlag-mHIF-1 α and kept at normoxia (N) or treated with 100 μ M CoCl₂ for 3 hours (C). CBP immunodepletion (CBP ID) was performed by incubating whole cell extracts with either IgG (-) or anti-CBP antibodies (+) for 2 hours at room temperature. Following immunodepletion whole cell extracts were recovered and incubated with anti-SRC-1 antibody (α -SRC-1) for an additional 2 hours. Precipitated complexes were separated by SDS-PAGE and analyzed by immunoblotting using anti-Flag, anti-CBP and anti-SRC-1 antibodies. *Non-specific band. Input gels were loaded with 50 μ g protein of each whole cell extract.

Fig. 2. CBP mediates in vivo interaction between HIF-1 α and SRC-1. (A) Subcellular and intranuclear distribution of YFP-CBP, CFP-HIF-1 α and YFP-SRC-1. HEK 293 cells were transfected with 400 ng of pYFP-CBP (YFP-CBP), pCFP-mHIF-1 α (CFP-HIF-1 α) or pYFP-SRC-1 (YFP-SRC-1) and observed using fluorescence microscopy. The YFP-SRC-1-fluorescent signal is pseudocolored red. Bars, 3 μ m. (B) SRC-1 colocalizes with CBP but not with HIF-1 α in accumulation foci. Cells were transfected with 400 ng of expression plasmids for CFP-SRC-1 or YFP-SRC-1 and YFP-CBP or CFP-HIF-1 α , respectively. CFP- and YFP-SRC-1-fluorescent signals are pseudocolored red. Bars, 3 μ m. (C) Redistribution of CFP-HIF-1 α by YFP-SRC-1 is dependent on CBP. HEK 293 cells were transfected with 400 ng of pCFP-mHIF-1 α (CFP-HIF-1 α), pYFP-SRC-1 (YFP-SRC-1) and pRc/RSV-CBP-HA (CBP). The bar chart shows the percentage of cells in which colocalization foci were observed at normoxia (N) or upon treatment with CoCl₂ (C). Bars, 3 μ m. (D) YFP-SRC-1 enhances the hypoxia-dependent transactivation function of CFP-HIF-1 α . HEK 293 cells were transfected with 500 ng HRE-responsive reporter plasmid, 50 ng of the CFP or CFP-HIF-1 α expression plasmids and 400 ng of YFP-SRC-1 expression plasmid. Total amount of DNA was kept at 1 μ g using pFlag-CMV-2. After transfection, cells were kept at normoxia (21% O₂) or hypoxia (1% O₂) for 36 hours, harvested and luciferase activity was measured. Data are presented as fold induction over the luciferase activity obtained following transfection of cells using pECFP-C1 and incubation of the cells under normoxic conditions. Relative luciferase values obtained with expression of CFP-HIF-1 α at normoxia (\wedge) or hypoxia (*) in the presence or absence of expressed YFP-SRC-1 are significantly different ($P < 0.05$). Values represent the mean \pm s.e.m. of three independent experiments performed in duplicate. (E) Expression levels of the different CFP- and YFP-fusion proteins. HEK 293 cells were transfected with 400 ng of each expression plasmid (as indicated). 36 hours after transfection medium was changed for treatment in the presence or absence of 100 μ M CoCl₂ for 2 hours. 50 μ g whole cell extracts were submitted to SDS-PAGE followed by immunoblotting using anti-GFP (α -GFP) antibodies.



SRC-1 was distributed throughout the cell with a characteristic cytoplasmic distribution pattern restricted to discrete foci (Fig. 2A). Interestingly, under hypoxic conditions YFP-SRC-1 became predominantly nuclear indicating that hypoxia may also increase nuclear retention of the protein (Fig. 2A). No differences were observed between the subcellular distributions of YFP- and CFP-SRC-1 (data not shown).

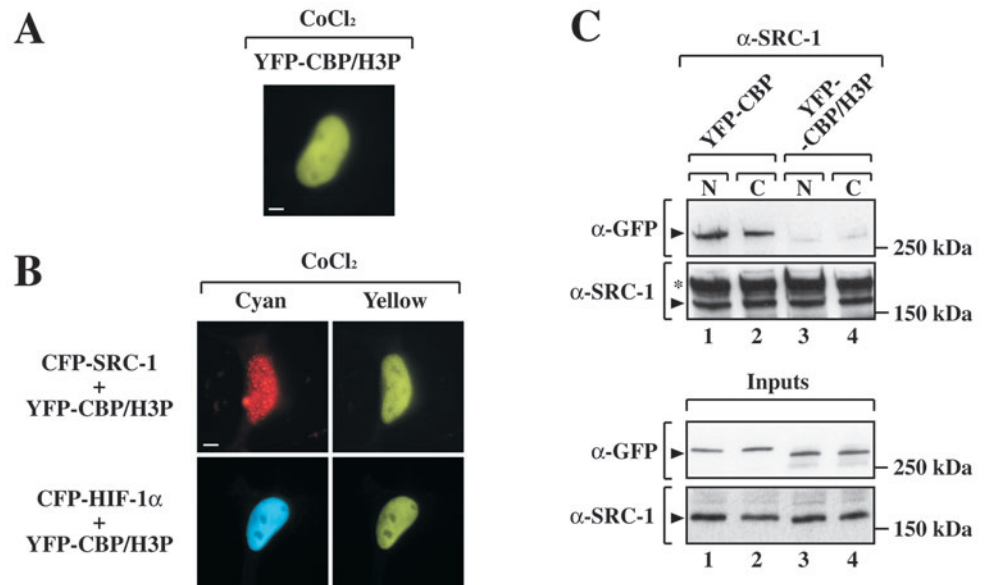
As expected (Yao et al., 1996), coexpression of CFP-SRC-1 and YFP-CBP induced the redistribution of both proteins from their characteristic patterns to intranuclear accumulation foci where both fluorescence signals could be observed, indicating colocalization of CFP-SRC-1 and YFP-CBP (Fig. 2B). Surprisingly, coexpression of CFP-HIF-1 α and YFP-SRC-1 did not lead to the formation of accumulation foci or colocalization of both proteins under any of the tested conditions (Fig. 2B). In summary, both CFP-HIF-1 α and CFP-SRC-1 can independently colocalize with YFP-CBP in discrete accumulation foci, whereas no colocalization is observed upon coexpression of CFP-HIF-1 α and YFP-SRC-1 in HEK 293 cells. These results suggest that SRC-1 and HIF-1 α do not directly interact in vivo.

We next investigated whether CBP could be a limiting factor in the organization of the complexes that leads to the formation

of the accumulation foci where HIF-1 α , SRC-1 and CBP colocalize. As shown in Fig. 2C, colocalization of CFP-HIF-1 α and YFP-SRC-1 in discrete accumulation foci could be observed in the presence of non-fluorescent tagged CBP (CBP-HA). Colocalization of CFP-HIF-1 α , CBP-HA and YFP-SRC-1 proved to be hypoxia-inducible since the number of cells where these complexes were observed could be increased from 2% at normoxia to 12% in the presence of CoCl₂ (as assessed by the total number of cells simultaneously expressing CFP and YFP chimeras that colocalized in nuclear accumulation foci) (Fig. 2C). This observation indicates that in the presence of expressed CBP, CFP-HIF-1 α and YFP-SRC-1 are able to colocalize in accumulation foci, consistent with a mechanism where CBP recruits SRC-1 to HIF-1 α -containing foci.

In order to establish if the tagging of SRC-1 affected its ability to enhance transactivation mediated by HIF-1 α we performed HRE-driven luciferase reporter gene assays in which CFP-HIF-1 α and YFP-SRC-1 were coexpressed (Fig. 2D). Fig. 2E shows the equal expression levels of the chimeric proteins in HEK 293 cells at normoxic or hypoxic conditions, as analyzed by immunoblot assays using anti-GFP antibodies. As observed earlier (Kallio et al., 1998) the CFP entity stabilizes HIF-1 α at normoxia. These results demonstrate that YFP-SRC-

Fig. 3. F2101P/K2103P mutations in full-length CBP disrupt protein-protein interaction and colocalization with SRC-1. (A) Subcellular localization of YFP-CBP(F2101P/K2103P) (YFP-CBP/H3P). HEK 293 cells were transfected with 400 ng of pYFP-CBP/H3P and treated for 2 hours with 100 μ M CoCl₂. Bar, 3 μ m. (B) YFP-CBP/H3P shows diffuse intranuclear distribution and does not colocalize with SRC-1. HEK 293 cells were transfected with 400 ng of expression plasmids encoding CFP-SRC-1, CFP-HIF-1 α and/or YFP-CBP/H3P as described in Fig. 1A. Bar, 3 μ m. (C) YFP-CBP/H3P fails to coprecipitate with endogenous SRC-1 in HEK 293 cells. Cells were transfected with 1 μ g of either pYFP-CBP or pYFP-CBP/H3P and kept at normoxia (N) or treated with 100 μ M CoCl₂ (C) for 3 hours. Whole cell extracts corresponding to a total protein concentration of 3 mg were incubated with anti-SRC-1 antibody (α -SRC-1) for 2 hours at room temperature. Immunoprecipitated protein complexes were separated as before and immunoblotted using anti-GFP (α -GFP) and α -SRC-1 antibodies. *Non-specific band. 50 μ g of whole cell extract protein was loaded on input gels.



I was able to potentiate the transactivation function of HIF-1 α in a hypoxia-dependent manner. Similar results were obtained with CFP-SRC-1 (data not shown). Taken together these results showed that the presence of the YFP or CFP tag has no significant effect on the activity of the chimeric protein.

A full-length CBP point mutant that does not interact with SRC-1 is not able to enhance the HIF-1 transactivation function

To further examine if the interaction between CBP and SRC-1 was necessary for colocalization of HIF-1 α with SRC-1 we generated CBP(F2101P/K2103P) (hereafter referred to as CBP/H3P), by double point mutation of full-length mouse CBP. Both these mutations have been shown to abolish interaction between the fragments of the SRC-1 interaction domain of CBP and the activation domain 1 of SRC-1, as assessed by *in vitro* protein-protein interaction assays (Sheppard et al., 2001). Here we analyzed the effect of the double mutation on the function of full-length CBP in hypoxic cells. We also monitored the intranuclear distribution of YFP-CBP/H3P in HEK 293 cells. The YFP-CBP/H3P chimera showed a subcellular distribution comparable to that of the wild-type protein (i.e. exclusively nuclear). However, in contrast to wild-type CBP, YFP-CBP/H3P showed a diffuse intranuclear distribution pattern (Fig. 3A). This observation suggests that this mutant form of CBP may have lost its ability to interact with structural proteins involved in the formation of the accumulation foci. CFP-SRC-1 did not colocalize with YFP-CBP/H3P (Fig. 3B) and coexpression of both proteins in HEK 293 cells did not affect the punctate distribution pattern of CFP-SRC-1. Since CFP-HIF-1 α is only distributed in foci when coexpressed with a protein able to form accumulation foci, coexpression of CFP-HIF-1 α and YFP-CBP/H3P did not result in formation of accumulation foci as observed with wild-type YFP-CBP (Fig. 3B). Furthermore, CBP/H3P was unable

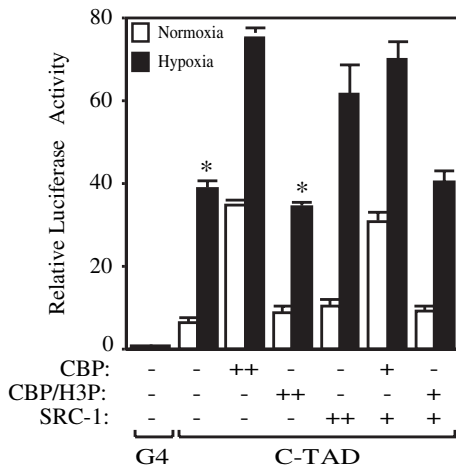
to mediate colocalization of CFP-HIF-1 α with YFP-SRC-1 with no accumulation foci observed (data not shown). In order to confirm that the introduction of mutations F2101P and K2103P in CBP abolished the interaction between the full-length SRC-1 and CBP proteins we expressed YFP-CBP/H3P in HEK 293 cells and investigated whether the CBP mutant was able to interact with endogenous SRC-1 in immunoprecipitation experiments. As shown in Fig. 3C, YFP-CBP was efficiently immunoprecipitated by endogenous SRC-1 (lanes 1 and 2) while no interaction was detected between the mutant YFP-CBP/H3P and SRC-1 (lanes 3 and 4).

The ability of CBP/H3P to enhance the transactivation function of HIF-1 α was analyzed in transient transfection experiments using HEK 293 cells. As expected (Carrero et al., 2000; Ruas et al., 2002) both CBP and SRC-1 were able to enhance, independently of one another and in a synergistic way, transcriptional activation of a GAL4-responsive luciferase reporter gene driven by a GAL4 fusion protein containing the C-terminal transactivation domain of HIF-1 α , GAL4-C-TAD (Fig. 4A). However, no effect was observed when CBP/H3P was used either in the presence or absence of SRC-1 (Fig. 4A). Accordingly, CBP/H3P was unable to enhance HIF-1 α -mediated transactivation of an HRE-driven luciferase reporter gene, and no effect was observed upon coexpression of SRC-1 (Fig. 4B). These results suggest that interaction between CBP and HIF-1 α was required for the recruitment of SRC-1 to the HIF-1 complex.

CFP-Arnt is redistributed by YFP-CBP into discrete accumulation foci but not by YFP-SRC-1

In order to investigate a possible role of Arnt in intranuclear trafficking of HIF-1 α and in the formation of accumulation foci with CBP and SRC-1, mArnt was fused to either CFP or YFP and transiently expressed in HEK 293 cells. Both CFP-Arnt and YFP-Arnt showed exclusive nuclear localization

A



B

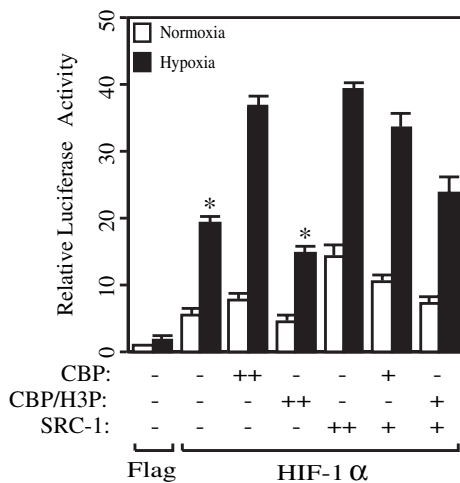


Fig. 4. SRC-1 interacts with HIF-1 α via CBP. (A) Enhancement of HIF-1 α C-TAD-mediated transcription by SRC-1 is dependent on interaction with CBP. Cells were transfected with 500 ng GAL4-driven reporter gene, 10 ng GAL4 (G4) or GAL4-C-TAD expression plasmid (C-TAD) and 200 ng (+) or 400 ng of expression plasmids (++) encoding CBP, CBP/H3P or SRC-1 and carrier DNA, pFLAG-CMV-2 to keep a constant total DNA concentration of 1 μ g. No significant difference was observed in the luciferase values obtained with expression of the C-TAD (*) in the presence or absence of coexpressed CBP/H3P (*) ($P < 0.05$). (B) CBP/H3P does not potentiate HIF-1 α -mediated transactivation. Cells were transfected with 500 ng HRE-driven reporter gene, 50 ng Flag (Flag) or mHIF-1 α (HIF-1 α) expression plasmids and 400 ng (+) or 800 ng (++) of expression plasmids encoding CBP, CBP/H3P, or SRC-1. Total DNA concentration was kept at 1.35 μ g using pFlag-CMV-2. Data are presented as fold induction over the luciferase activity obtained following transfection of cells using GAL4 expression plasmid (A) or pFlag-CMV-2 (B) and incubation of the cells under normoxic conditions. No significant difference was observed in the luciferase values obtained with expression of HIF-1 α (*) in the presence or absence of coexpressed CBP/H3P (*) ($P < 0.05$). Values represent the mean \pm s.e.m. of three independent experiments performed in duplicate.

under normoxic and hypoxic conditions (Fig. 5A) and could be detected in large accumulation foci (3-17 per nucleus). Coexpression of CFP-Arnt and YFP-CBP caused redistribution of both proteins with a clear change of the distribution pattern to smaller foci (40-100 per nucleus) where colocalization could be observed (Fig. 5B). Again, treatment with CoCl₂ had no effect on the pattern of colocalization between CFP-Arnt and YFP-CBP. However, when CFP-Arnt and YFP-SRC-1 were coexpressed only YFP-SRC-1 was redistributed from its punctate pattern into large accumulation foci characteristic of CFP-Arnt (Fig. 5B). Cotransfection of CFP-Arnt and YFP-CBP/H3P resulted in a diffuse intranuclear distribution of both proteins (Fig. 5B) indicating that YFP-CBP/H3P was able to interact with CFP-Arnt disrupting the characteristic organization of CFP-Arnt in a few discrete accumulation foci. Taken together these results show that Arnt is able to interact with CBP and SRC-1 in vivo, forming complexes with distinct intranuclear localization patterns.

Colocalization of CFP-HIF-1 α and YFP-Arnt in accumulation foci is enhanced by CBP and hypoxia but not by SRC-1

As shown in Fig. 2E, transfection in HEK 293 cells of equal amounts of pCFP-HIF-1 α and pYFP-Arnt resulted in different expression levels of CFP-HIF-1 α and YFP-Arnt. Under these conditions, no colocalization was observed between CFP-HIF-1 α and YFP-Arnt (Fig. 6A). We next used these conditions to evaluate the role of CBP on the colocalization of CFP-HIF-1 α and YFP-Arnt. Interestingly, coexpression of non-fluorescent-tagged CBP (CBP-HA) resulted in the redistribution of both proteins into characteristic accumulation foci (normoxia: 17%; in the presence of CoCl₂: 36% of cells) suggesting that CBP can have a stabilizing effect on the formation of the HIF-1 α /Arnt complex (Fig. 6A). In agreement with this hypothesis, expression of CBP/H3P that has no ability to form foci, failed to induce colocalization of CFP-HIF-1 α and YFP-Arnt (data not shown). As expected, cotransfection of SRC-1 had no effect on the intranuclear redistribution of either CFP-HIF-1 α or YFP-Arnt (Fig. 6A). In order to examine if CFP-HIF-1 α and YFP-Arnt were able to colocalize independently of expressed CBP, we cotransfected pYFP-Arnt with increasing amounts of pCFP-HIF-1 α , obtaining the protein levels shown in Fig. 3C. Under these conditions a discrete hypoxia-inducible colocalization pattern consisting of accumulation foci could be observed, albeit in fewer cells (normoxia: 5%; in the presence of CoCl₂: 23% of cells) when compared to the results obtained in the presence of expressed CBP (Fig. 6B). Intranuclear distribution of the CFP-HIF-1 α /YFP-Arnt complex in the absence of CBP corresponds to a distinct colocalization pattern that could be observed with any of the HIF-1 α mutants [HIF-1 α (L808A/L809A), HIF-1 α (532 Δ 583), HIF-1 α (772 Δ 822) and HIF-1 α (532 Δ 583)(L808A/L809A)] containing one or both of the transactivation domains inactivated by mutation (data not shown). The fact that similar results were obtained with a HIF-1 α mutant that did not colocalize with CBP and is not able to activate transcription [e.g. CFP-HIF-1 α (531 Δ 584)(L808A/L809A) normoxia: 3%, in the presence of CoCl₂: 20% of cells] indicates that interaction between HIF-1 α and Arnt is independent of the integrity of the transactivation domains of HIF-1 α . This notion is in perfect agreement with previous reports showing that HIF-

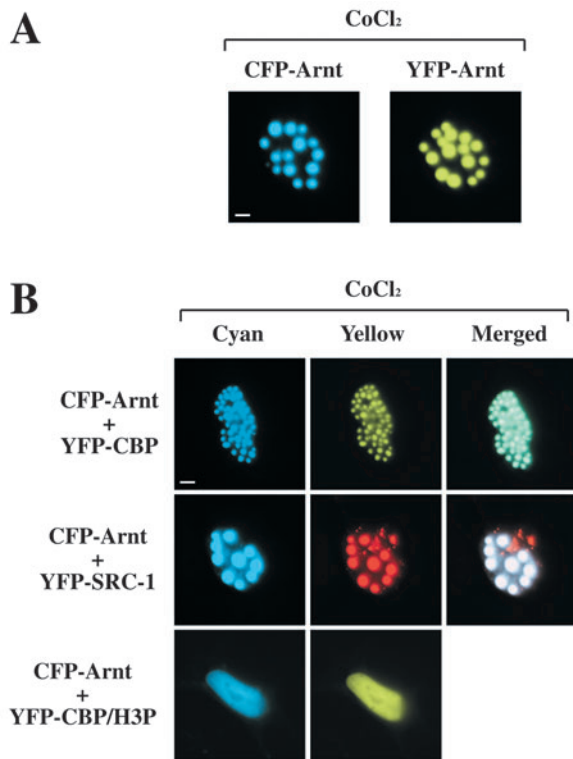


Fig. 5. In vivo analysis of colocalization of Arnt, CBP and SRC-1 using CFP and YFP chimeric proteins. (A) CFP- and YFP-Arnt localize in discrete nuclear accumulation foci. Cells were transfected with CFP- and YFP-Arnt expression plasmids and analyzed as described in the legend of Fig. 1A. (B) CFP-Arnt colocalizes with YFP-CBP and YFP-SRC-1 but not with YFP-CBP/H3P. Cells were transfected with 400 ng expression plasmids encoding CFP-Arnt and YFP-CBP or YFP-SRC-1 or YFP-CBP/H3P and analyzed as described in Fig. 1A. Bars, 3 μ m.

1 α /Arnt heterodimer formation is dependent on the bHLH-PAS domains of the two proteins (Jiang et al., 1996). Our results show that in vivo, CFP-HIF-1 α and YFP-Arnt can colocalize in discrete accumulation foci in a CBP-independent manner although in a less efficient way, suggesting that further stabilization of the heterodimer can result from interaction of the transactivation domains of Arnt with CBP. Fig. 6D shows that the CFP and YFP tag had no significant effect on the ability of the HIF-1 α /Arnt dimer to activate transcription of a HRE-driven luciferase reporter gene in a hypoxia-inducible manner.

The hyperphosphorylated form of RNA polymerase II localizes to some HIF-1 α -containing accumulation foci

In order to further analyze the nature and function of the hypoxia-induced discrete accumulation foci we performed immunocytochemistry using CC-3 and mara3 antibodies (Patturajan et al., 1998; Thibodeau et al., 1989) that recognize the hyperphosphorylated COOH-terminal domain of the largest subunit of RNA polymerase II (RNA polymerase IIo) (Albert et al., 1999; Vincent et al., 1996; von Mikecz et al., 2000). RNA polymerase IIo is involved in RNA elongation and

is known to colocalize with the majority of sites of active mRNA transcription, as assessed by incorporation of BrUTP (Zeng et al., 1997). Subnuclear localization of RNA polymerase IIo and YFP-tagged proteins was analyzed by laser scanning confocal microscopy. As shown in Fig. 7A, and in agreement with previous reports (von Mikecz et al., 2000; Zeng et al., 1997), endogenous RNA polymerase IIo was distributed in the nucleus in discrete accumulation foci. No significant alteration was observed when comparing the distribution pattern of RNA polymerase IIo at normoxia (data not shown) or following treatment of the cells with CoCl₂ (Fig. 7A). In cells expressing YFP-CBP several intranuclear foci of colocalization with RNA polymerase IIo could be observed (Fig. 7Bc). This observation is in agreement with previous reports showing that CBP colocalizes partially with RNA polymerase IIo (von Mikecz et al., 2000). We observed that YFP-SRC-1 colocalized with RNA polymerase IIo in just a few foci in the nucleus (Fig. 7Cc). Interestingly, in HEK 293 cells expressing YFP-Arnt no colocalization was observed between RNA polymerase IIo (Fig. 7Da) and YFP-Arnt (Fig. 7Db). In contrast, coexpression of CFP-HIF-1 α and YFP-Arnt (under the conditions described in the legend of Fig. 6B) resulted in several foci of colocalization with RNA polymerase IIo (Fig. 7Ec). Similar results were obtained with the mara3 anti-RNA polymerase IIo antibody (data not shown). Finally, in cells expressing CFP-HIF-1 α and YFP-CBP several intranuclear accumulation foci could be observed that colocalized with the hyperphosphorylated form of RNA polymerase II (Fig. 7Fc), suggesting that some of the observed foci may correspond to sites of active mRNA elongation.

Discussion

In this study we provide novel insights into the mechanism of activation of HIF-1 α to a transcriptionally active form in hypoxic cells. Our data strongly indicate that CBP is a limiting factor in the formation of the HIF-1 α /coactivator complex and that SRC-1 does not interact directly with HIF-1 α but is recruited to the complex by CBP in a hypoxia-dependent manner. This mechanism contrasts with the ligand-dependent recruitment of SRC-1 and CBP to the ligand-binding domain of nuclear hormone receptors, where SRC-1 has been demonstrated to function as a bridging protein between nuclear hormone receptors and CBP (Li et al., 2003; Liu et al., 2001; Xu and Li, 2003). Furthermore our results indicate that CBP stabilizes the formation of the HIF-1 α /Arnt heterodimer.

Since SRCs, CBP and P/CAF all possess functional histone acetyltransferase (HAT) activities (Bannister and Kouzarides, 1996; Chen et al., 1997; Spencer et al., 1997; Yang et al., 1996), the formation of the nuclear hormone receptor/coactivator complex has been proposed to be essential for chromatin remodeling. However, the HAT activity of SRCs has been described as very weak and, in line with these observations, only the HAT domains of CBP and P/CAF have been demonstrated to be essential for nuclear hormone receptor/coactivator complex-mediated transcription (Korzus et al., 1998; Kraus et al., 1999; Puri et al., 1997). We therefore speculate that SRC-1 may be present in the HIF-1 α complex in order to recruit other cofactors such as protein arginine methyltransferases (Chen et al., 1999; Koh et al., 2001; Qi et al., 2002).

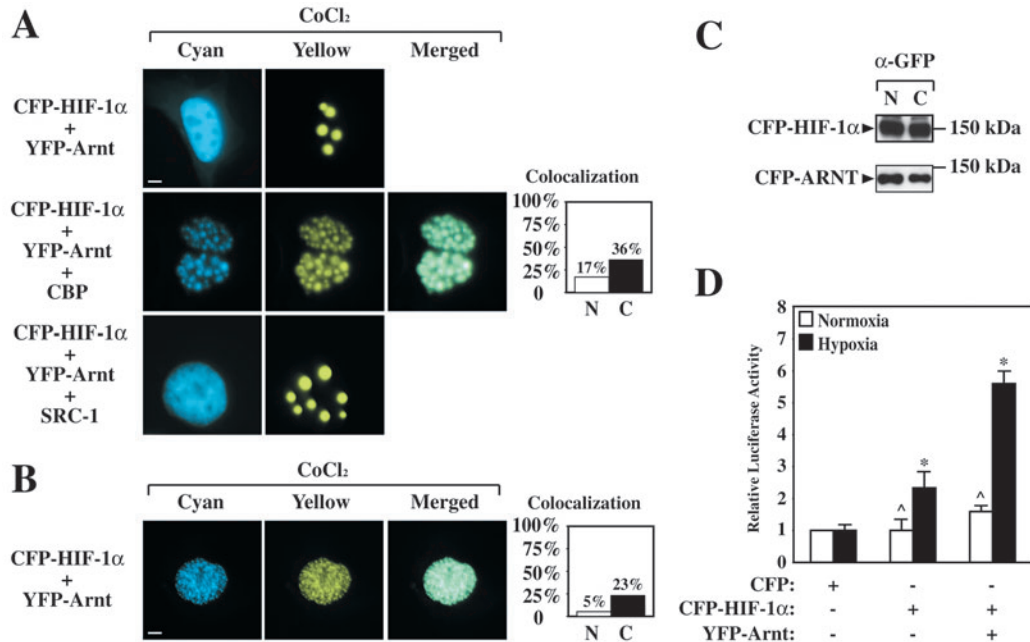


Fig. 6. CBP enhances colocalization between CFP-HIF-1 α and YFP-Arnt. (A) Cells were transfected with 400 ng of pCFP-mHIF-1 α (CFP-HIF-1 α), pYFP-Arnt (YFP-Arnt) and pRc/RSV-CBP-HA (CBP) or pSG5/hSRC-1 (SRC-1) as indicated and analyzed as described in the legend of Fig. 1A. The bar chart shows the percentage of cells in which colocalization foci were observed at normoxia (N) or upon treatment with CoCl₂ (C). Bars, 3 μ m. (B) Colocalization between CFP-HIF-1 α and YFP-Arnt. HEK 293 cells were transfected with 300 ng pYFP-Arnt (YFP-Arnt) and 700 ng pCFP-mHIF-1 α (CFP-HIF-1 α). The bar chart shows the percentage of cells in which colocalization foci were observed at normoxia (N) or upon treatment with CoCl₂ (C). Bars, 3 μ m. (C) Protein expression levels of CFP-HIF-1 α and YFP-Arnt under the conditions described in B. 36 hours after transfection, medium was changed and cells were given a further 2 hours at normoxia or in the presence of 100 μ M CoCl₂. 50 μ g whole cell extracts were submitted to SDS-PAGE followed by immunoblotting using anti-GFP (α -GFP) antibodies. (D) The CFP-HIF-1 α /YFP-Arnt heterodimer efficiently activates transcription of an HRE-driven reporter gene in a hypoxia-inducible way. HEK 293 cells were transfected with 500 ng HRE-driven reporter gene, 50 ng of the indicated expression plasmid and carrier DNA, pFlag-CMV-2, to keep the total DNA concentration at 1 μ g. After transfection cells were kept at normoxia (21% O₂) or hypoxia (1% O₂) for 36 hours, harvested and luciferase activity was measured. Data are presented as fold induction over the luciferase activity obtained with transfection of the pECFP-C1 and incubated at normoxia. Relative luciferase values obtained with expression of CFP-HIF-1 α at normoxia (\wedge) or hypoxia (\ast) in the presence or absence of expressed YFP-Arnt are significantly different ($P < 0.05$). Values represent the mean \pm s.e.m. of three independent experiments performed in duplicate.

The DNA-binding partner of HIF-1 α , Arnt, has been shown to interact with coactivators of the p160/SRC family, namely SRC-1 and SRC-2 (Beischlag et al., 2002). The same study has shown Arnt to be recruited by SRC-1 to nuclear foci (Beischlag et al., 2002). Here, we show that Arnt interacts in vivo with SRC-1 in HEK 293 cells by recruiting the coactivator to its accumulation foci. However, Arnt was not able to colocalize with HIF-1 α in the presence of SRC-1, which may indicate that the interaction of Arnt and SRC-1 does not play an important role in the formation of the transcriptionally active HIF-1 α /Arnt coactivator complex. Interaction between Arnt and SRC-1 has been reported to be mediated by helix 2 of the bHLH domain of Arnt (Beischlag et al., 2002) that is also required for heterodimerization with HIF-1 α . The formation of the HIF-1 α /Arnt dimer under hypoxic conditions may preclude the binding of SRC-1 to the same Arnt domain and therefore be incompatible with the formation of a HIF-1 α /Arnt/SRC-1 ternary complex. We also present evidence that Arnt can interact in vivo with CBP and that this interaction is important for the formation of the HIF-1 α /Arnt complex. Taken together these data indicate that CBP may interact simultaneously with the transactivation domains of HIF-1 α and Arnt, thus increasing the stability of this complex.

In the present study, we observed that certain proteins localize in the nucleus of HEK 293 cells in accumulation foci with different patterns of distribution. Although the nature and function of the different foci observed are not known, we regard these as the result of specific in vivo protein-protein interactions since (i) formation of the various foci could be disrupted by expression of mutant proteins that do not interact in co-immunoprecipitation assays, and (ii) generation of HIF-1 α -containing foci was dependent on the expression of a HIF-1 α -interacting protein. Accumulation foci in the nucleus have been extensively reported for nuclear hormone receptors after addition of ligand (Fejes-Toth et al., 1998; Georget et al., 1997; Htun et al., 1996; Htun et al., 1999; Prifer et al., 2000; Racz and Barsony, 1999; Stenoien et al., 2000). Moreover, coactivators or components of chromatin remodeling complexes have been shown to be recruited to these ligand-dependent foci (Matsuda et al., 2002; Rivera et al., 2003; Stenoien et al., 2000; Stenoien et al., 2001). In order to further characterize accumulation foci containing proteins involved in the HIF-1 α -mediated transactivation response in hypoxic cells we investigated if RNA polymerase IIo, the hyperphosphorylated form of RNA polymerase II, was present in some of the foci. Transcription elongation is mediated by

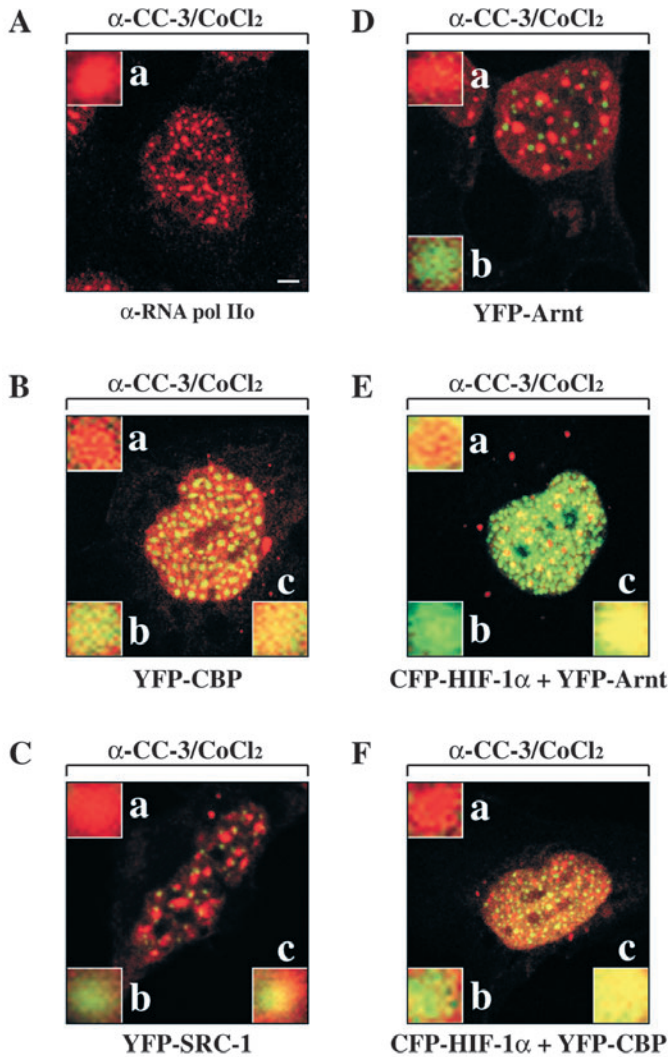


Fig. 7. Intranuclear distribution of RNA polymerase IIo and analysis of colocalization with HIF-1 α , Arnt, CBP and SRC-1. Insets show enlarged examples of the different observed foci in each image. (A) RNA polymerase IIo shows an intranuclear distribution in discrete accumulation foci. Immunocytochemistry for RNA polymerase IIo (RNA pol IIo) was performed using anti-RNA polymerase IIo antibody (CC-3) as described in Materials and Methods. (a) Enlargement of a nuclear accumulation focus of endogenous RNA polymerase IIo. (B) CBP colocalizes with RNA polymerase IIo in a large number of nuclear accumulation foci. Cells were transfected with pYFP-CBP as described in Fig. 1A, and anti-RNA polymerase IIo immunocytochemistry was performed using the CC-3 antibody. An RNA polymerase IIo accumulation focus (a), a YFP-CBP accumulation focus (b) and a focus where RNA polymerase IIo colocalized with YFP-CBP (c) are shown. (C) Colocalization of SRC-1 and RNA polymerase IIo in a few discrete foci. Cells were transfected with pYFP-SRC-1 and immunocytochemistry was performed as indicated above. Enlargement of an RNA polymerase IIo accumulation focus (a), a YFP-SRC-1 accumulation focus (b) and a focus of colocalization between RNA polymerase IIo and YFP-SRC-1 are shown. (D) The intranuclear distribution of Arnt in discrete foci does not overlap with RNA polymerase IIo. Cells were transfected with pYFP-Arnt. Enlarged images of an RNA polymerase IIo accumulation focus (a) and a YFP-Arnt accumulation focus (b) are shown. (E) The intranuclear distribution pattern of HIF-1 α and Arnt partially overlaps with that of RNA polymerase IIo. HEK 293 cells were transfected with pCFP-HIF-1 α (CFP-HIF-1 α) and pYFP-Arnt (YFP-Arnt) as indicated in the legend of Fig. 6B. An RNA polymerase IIo accumulation focus (a), a CFP-HIF-1 α /YFP-Arnt accumulation focus (b) and a focus of colocalization between RNA polymerase IIo and CFP-HIF-1 α /YFP-Arnt (c) are shown. (F) Distribution of CFP-HIF-1 α and YFP-CBP into some discrete foci of colocalization with RNA polymerase IIo. Enlargements of an RNA polymerase IIo accumulation focus (a), a CFP-HIF-1 α /YFP-CBP accumulation focus (b) and a focus of colocalization between RNA polymerase IIo and CFP-HIF-1 α /YFP-CBP (c) are shown. All figures are single confocal sections obtained from HEK 293 cells treated with 100 μ M CoCl₂ for 2 hours. Bar, 3 μ m for all images.

RNA polymerase IIo that has been shown to colocalize but not completely overlap with sites of active transcription (Zeng et al., 1997). In our study we observed that some foci are not related to the distribution of RNA polymerase IIo (e.g. Arnt foci), whereas others where CBP, HIF-1 α /CBP and HIF-1 α /Arnt are present contain a significant fraction of nuclear domains that colocalized with the polymerase.

The nucleus is a highly complex organelle containing specific intranuclear domains that ensure efficiency of nuclear processes including transcription, RNA processing and DNA synthesis (Stein et al., 1999). Several recent reports suggest that efficient signal transduction depends on the spatial and temporal colocalization of the different partner proteins (Teruel and Meyer, 2000). These events seem to be dependent on the nuclear architecture, and several intranuclear accumulation foci have been characterized that contain different complexes (e.g. PML, Cajal bodies, speckles) (Dirks et al., 1999; Ogg and Lamond, 2002; Zhong et al., 2000). The dynamic nature of the different transcription factor/coactivator complexes described in this study suggests the existence of a highly regulated constant traffic between different intranuclear foci containing one or more HIF-1 α partner factors in order to form a transcriptionally competent HIF-1 α complex. In conclusion,

our results show that the HIF-1 α -mediated signal transduction pathway involves intranuclear redistribution of several partner proteins such as Arnt, CBP and SRC-1 and that this process is strictly temporally and hierarchically regulated by hypoxia.

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References

- Albert, A., Lavoie, S. and Vincent, M. (1999). A hyperphosphorylated form of RNA polymerase II is the major interphase antigen of the phosphoprotein antibody MPM-2 and interacts with the peptidyl-prolyl isomerase Pin1. *J. Cell Sci.* **112**, 2493-2500.
- Arany, Z., Huang, L. E., Eckner, R., Bhattacharya, S., Jiang, C., Goldberg, M. A., Bunn, H. F. and Livingston, D. M. (1996). An essential role for p300/CBP in the cellular response to hypoxia. *Proc. Natl. Acad. Sci. USA* **93**, 12969-12973.

- Bannister, A. J. and Kouzarides, T.** (1996). The CBP co-activator is a histone acetyltransferase. *Nature* **384**, 641-643.
- Beischlag, T. V., Wang, S., Rose, D. W., Torchia, J., Reisz-Porszasz, S., Muhammad, K., Nelson, W. E., Probst, M. R., Rosenfeld, M. G. and Hankinson, O.** (2002). Recruitment of the NCoA/SRC-1/p160 family of transcriptional coactivators by the aryl hydrocarbon receptor/aryl hydrocarbon receptor nuclear translocator complex. *Mol. Cell. Biol.* **22**, 4319-4333.
- Bhattacharya, S., Michels, C. L., Leung, M. K., Arany, Z. P., Kung, A. L. and Livingston, D. M.** (1999). Functional role of p35srj, a novel p300/CBP binding protein, during transactivation by HIF-1. *Genes Dev.* **13**, 64-75.
- Bruick, R. K. and McKnight, S. L.** (2001). A conserved family of prolyl-4-hydroxylases that modify HIF. *Science* **294**, 1337-1340.
- Carrero, P., Okamoto, K., Coumilleau, P., O'Brien, S., Tanaka, H. and Poellinger, L.** (2000). Redox-regulated recruitment of the transcriptional coactivators CREB-binding protein and SRC-1 to hypoxia-inducible factor 1alpha. *Mol. Cell. Biol.* **20**, 402-415.
- Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S. M., Schurter, B. T., Aswad, D. W. and Stallcup, M. R.** (1999). Regulation of transcription by a protein methyltransferase. *Science* **284**, 2174-2177.
- Chen, H., Lin, R. J., Schiltz, R. L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y. and Evans, R. M.** (1997). Nuclear receptor coactivator ACTR is a novel histone acetyltransferase and forms a multimeric activation complex with P/CAF and CBP/p300. *Cell* **90**, 569-580.
- Cockman, M. E., Masson, N., Mole, D. R., Jaakkola, P., Chang, G. W., Clifford, S. C., Maher, E. R., Pugh, C. W., Ratcliffe, P. J. and Maxwell, P. H.** (2000). Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. *J. Biol. Chem.* **275**, 25733-25741.
- Dames, S. A., Martinez-Yamout, M., de Guzman, R. N., Dyson, H. J. and Wright, P. E.** (2002). Structural basis for Hif-1 alpha/CBP recognition in the cellular hypoxic response. *Proc. Natl. Acad. Sci. USA* **99**, 5271-5276.
- Dirks, R. W., Hattlinger, C. M., Molenaar, C. and Snaar, S. P.** (1999). Synthesis, processing, and transport of RNA within the three-dimensional context of the cell nucleus. *Crit. Rev. Eukaryot. Gene Expr.* **9**, 191-201.
- Ebert, B. L. and Bunn, H. F.** (1998). Regulation of transcription by hypoxia requires a multiprotein complex that includes hypoxia-inducible factor 1, an adjacent transcription factor, and p300/CREB binding protein. *Mol. Cell. Biol.* **18**, 4089-4096.
- Ema, M., Hirota, K., Mimura, J., Abe, H., Yodoi, J., Sogawa, K., Poellinger, L. and Fujii-Kuriyama, Y.** (1999). Molecular mechanisms of transcription activation by HLF and HIF1alpha in response to hypoxia: their stabilization and redox signal-induced interaction with CBP/p300. *EMBO J.* **18**, 1905-1914.
- Epstein, A. C., Gleadle, J. M., McNeill, L. A., Hewitson, K. S., O'Rourke, J., Mole, D. R., Mukherji, M., Metzzen, E., Wilson, M. I., Dhanda, A. et al.** (2001). C. elegans EGL-9 and mammalian homologs define a family of dioxygenases that regulate HIF by prolyl hydroxylation. *Cell* **107**, 43-54.
- Fejes-Toth, G., Pearce, D. and Naray-Fejes-Toth, A.** (1998). Subcellular localization of mineralocorticoid receptors in living cells: effects of receptor agonists and antagonists. *Proc. Natl. Acad. Sci. USA* **95**, 2973-2978.
- Freedman, S. J., Sun, Z. Y., Poy, F., Kung, A. L., Livingston, D. M., Wagner, G. and Eck, M. J.** (2002). Structural basis for recruitment of CBP/p300 by hypoxia-inducible factor-1 alpha. *Proc. Natl. Acad. Sci. USA* **99**, 5367-5372.
- Georget, V., Lobaccaro, J. M., Terouanne, B., Mangeat, P., Nicolas, J. C. and Sultan, C.** (1997). Trafficking of the androgen receptor in living cells with fused green fluorescent protein-androgen receptor. *Mol. Cell. Endocrinol.* **129**, 17-26.
- Gu, J., Milligan, J. and Huang, L. E.** (2001). Molecular mechanism of hypoxia-inducible factor 1alpha-p300 interaction. A leucine-rich interface regulated by a single cysteine. *J. Biol. Chem.* **276**, 3550-3554.
- Hewitson, K. S., McNeill, L. A., Riordan, M. V., Tian, Y. M., Bullock, A. N., Welford, R. W., Elkins, J. M., Oldham, N. J., Bhattacharya, S., Gleadle, J. M. et al.** (2002). Hypoxia-inducible factor (HIF) asparagine hydroxylase is identical to factor inhibiting HIF (FIH) and is related to the cupin structural family. *J. Biol. Chem.* **277**, 26351-26355.
- Htun, H., Barsony, J., Renyi, I., Gould, D. L. and Hager, G. L.** (1996). Visualization of glucocorticoid receptor translocation and intranuclear organization in living cells with a green fluorescent protein chimera. *Proc. Natl. Acad. Sci. USA* **93**, 4845-4850.
- Htun, H., Holth, L. T., Walker, D., Davie, J. R. and Hager, G. L.** (1999). Direct visualization of the human estrogen receptor alpha reveals a role for ligand in the nuclear distribution of the receptor. *Mol. Biol. Cell* **10**, 471-486.
- Huang, L. E., Gu, J., Schau, M. and Bunn, H. F.** (1998). Regulation of hypoxia-inducible factor 1alpha is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc. Natl. Acad. Sci. USA* **95**, 7987-7992.
- Ivan, M., Kondo, K., Yang, H., Kim, W., Valiando, J., Ohh, M., Salic, A., Asara, J. M., Lane, W. S. and Kaelin, W. G., Jr** (2001). HIF1alpha targeted for VHL-mediated destruction by proline hydroxylation: implications for O2 sensing. *Science* **292**, 464-468.
- Jaakkola, P., Mole, D. R., Tian, Y. M., Wilson, M. I., Gielbert, J., Gaskell, S. J., Kriegsheim, A., Hebestreit, H. F., Mukherji, M., Schofield, C. J. et al.** (2001). Targeting of HIF-alpha to the von Hippel-Lindau ubiquitylation complex by O2-regulated prolyl hydroxylation. *Science* **292**, 468-472.
- Jiang, B. H., Rue, E., Wang, G. L., Roe, R. and Semenza, G. L.** (1996). Dimerization, DNA binding, and transactivation properties of hypoxia-inducible factor 1. *J. Biol. Chem.* **271**, 17771-17778.
- Kallio, P. J., Pongratz, I., Gradin, K., McGuire, J. and Poellinger, L.** (1997). Activation of hypoxia-inducible factor 1alpha: posttranscriptional regulation and conformational change by recruitment of the Arnt transcription factor. *Proc. Natl. Acad. Sci. USA* **94**, 5667-5672.
- Kallio, P. J., Okamoto, K., O'Brien, S., Carrero, P., Makino, Y., Tanaka, H. and Poellinger, L.** (1998). Signal transduction in hypoxic cells: inducible nuclear translocation and recruitment of the CBP/p300 coactivator by the hypoxia-inducible factor-1alpha. *EMBO J.* **17**, 6573-6586.
- Kallio, P. J., Wilson, W. J., O'Brien, S., Makino, Y. and Poellinger, L.** (1999). Regulation of the hypoxia-inducible transcription factor 1alpha by the ubiquitin-proteasome pathway. *J. Biol. Chem.* **274**, 6519-6525.
- Kamura, T., Sato, S., Iwai, K., Czyzyk-Krzeska, M., Conaway, R. C. and Conaway, J. W.** (2000). Activation of HIF1alpha ubiquitination by a constitutively von Hippel-Lindau (VHL) tumor suppressor complex. *Proc. Natl. Acad. Sci. USA* **97**, 10430-10435.
- Koh, S. S., Chen, D., Lee, Y. H. and Stallcup, M. R.** (2001). Synergistic enhancement of nuclear receptor function by p160 coactivators and two coactivators with protein methyltransferase activities. *J. Biol. Chem.* **276**, 1089-1098.
- Korzus, E., Torchia, J., Rose, D. W., Xu, L., Kurokawa, R., McInerney, E. M., Mullen, T. M., Glass, C. K. and Rosenfeld, M. G.** (1998). Transcription factor-specific requirements for coactivators and their acetyltransferase functions. *Science* **279**, 703-707.
- Kraus, W. L., Manning, E. T. and Kadonaga, J. T.** (1999). Biochemical analysis of distinct activation functions in p300 that enhance transcription initiation with chromatin templates. *Mol. Cell. Biol.* **19**, 8123-8135.
- Kung, A. L., Wang, S., Klcio, J. M., Kaelin, W. G. and Livingston, D. M.** (2000). Suppression of tumor growth through disruption of hypoxia-inducible transcription. *Nat. Med.* **6**, 1335-1340.
- Lando, D., Peet, D. J., Gorman, J. J., Whelan, D. A., Whitelaw, M. L. and Bruick, R. K.** (2002a). FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor. *Genes Dev.* **16**, 1466-1471.
- Lando, D., Peet, D. J., Whelan, D. A., Gorman, J. J. and Whitelaw, M. L.** (2002b). Asparagine hydroxylation of the HIF transactivation domain a hypoxic switch. *Science* **295**, 858-861.
- Li, J., O'Malley, B. W. and Wong, J.** (2000). p300 requires its histone acetyltransferase activity and SRC-1 interaction domain to facilitate thyroid hormone receptor activation in chromatin. *Mol. Cell. Biol.* **20**, 2031-2042.
- Li, X., Wong, J., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W.** (2003). Progesterone and glucocorticoid receptors recruit distinct coactivator complexes and promote distinct patterns of local chromatin modification. *Mol. Cell. Biol.* **23**, 3763-3773.
- Liu, Z., Wong, J., Tsai, S. Y., Tsai, M. J. and O'Malley, B. W.** (2001). Sequential recruitment of steroid receptor coactivator-1 (SRC-1) and p300 enhances progesterone receptor-dependent initiation and reinitiation of transcription from chromatin. *Proc. Natl. Acad. Sci. USA* **98**, 12426-12431.
- Mahon, P. C., Hirota, K. and Semenza, G. L.** (2001). FIH-1: a novel protein that interacts with HIF-1alpha and VHL to mediate repression of HIF-1 transcriptional activity. *Genes Dev.* **15**, 2675-2686.
- Masson, N., Willam, C., Maxwell, P. H., Pugh, C. W. and Ratcliffe, P. J.** (2001). Independent function of two destruction domains in hypoxia-inducible factor-alpha chains activated by prolyl hydroxylation. *EMBO J.* **20**, 5197-5206.
- Matsuda, K., Ochiai, I., Nishi, M. and Kawata, M.** (2002). Colocalization

- and ligand-dependent discrete distribution of the estrogen receptor (ER) α and ER β . *Mol. Endocrinol.* **16**, 2215-2230.
- Ogg, S. C. and Lamond, A. I. (2002). Cajal bodies and coilin – moving towards function. *J. Cell Biol.* **159**, 17-21.
- Ohh, M., Park, C. W., Ivan, M., Hoffman, M. A., Kim, T. Y., Huang, L. E., Pavletich, N., Chau, V. and Kaelin, W. G. (2000). Ubiquitination of hypoxia-inducible factor requires direct binding to the beta-domain of the von Hippel-Lindau protein. *Nat. Cell Biol.* **2**, 423-427.
- Patturajan, M., Schulte, R. J., Sefton, B. M., Berezney, R., Vincent, M., Bensaude, O., Warren, S. L. and Corden, J. L. (1998). Growth-related changes in phosphorylation of yeast RNA polymerase II. *J. Biol. Chem.* **273**, 4689-4694.
- Pereira, T., Zheng, X., Ruas, J. L., Tanimoto, K. and Poellinger, L. (2003). Identification of residues critical for regulation of protein stability and the transactivation function of the hypoxia-inducible factor-1 α by the von Hippel-Lindau tumor suppressor gene product. *J. Biol. Chem.* **278**, 6816-6823.
- Prufer, K., Racz, A., Lin, G. C. and Barsony, J. (2000). Dimerization with retinoid X receptors promotes nuclear localization and subnuclear targeting of vitamin D receptors. *J. Biol. Chem.* **275**, 41114-41123.
- Puri, P. L., Avantaggiati, M. L., Balsano, C., Sang, N., Graessmann, A., Giordano, A. and Levrero, M. (1997). p300 is required for MyoD-dependent cell cycle arrest and muscle-specific gene transcription. *EMBO J.* **16**, 369-383.
- Qi, C., Chang, J., Zhu, Y., Yeldandi, A. V., Rao, S. M. and Zhu, Y. J. (2002). Identification of protein arginine methyltransferase 2 as a coactivator for estrogen receptor α . *J. Biol. Chem.* **277**, 28624-28630.
- Racz, A. and Barsony, J. (1999). Hormone-dependent translocation of vitamin D receptors is linked to transactivation. *J. Biol. Chem.* **274**, 19352-19360.
- Rivera, O. J., Song, C. S., Centonze, V. E., Lechleiter, J. D., Chatterjee, B. and Roy, A. K. (2003). Role of the promyelocytic leukemia body in the dynamic interaction between the androgen receptor and steroid receptor coactivator-1 in living cells. *Mol. Endocrinol.* **17**, 128-140.
- Ruas, J. L., Poellinger, L. and Pereira, T. (2002). Functional analysis of hypoxia-inducible factor-1 α -mediated transactivation. Identification of amino acid residues critical for transcriptional activation and/or interaction with CREB-binding protein. *J. Biol. Chem.* **277**, 38723-38730.
- Salceda, S. and Caro, J. (1997). Hypoxia-inducible factor 1 α (HIF-1 α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J. Biol. Chem.* **272**, 22642-22647.
- Semenza, G. (2002). Signal transduction to hypoxia-inducible factor 1. *Biochem. Pharmacol.* **64**, 993-998.
- Sheppard, H. M., Harries, J. C., Hussain, S., Bevan, C. and Heery, D. M. (2001). Analysis of the steroid receptor coactivator 1 (SRC1)-CREB binding protein interaction interface and its importance for the function of SRC1. *Mol. Cell. Biol.* **21**, 39-50.
- Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S. Y., Tsai, M. J. et al. (1997). Steroid receptor coactivator-1 is a histone acetyltransferase. *Nature* **389**, 194-198.
- Stein, G. S., van Wijnen, A. J., Stein, J. L., Lian, J. B., McNeil, S. and Pockwinse, S. M. (1999). Transcriptional control within the three-dimensional context of nuclear architecture: requirements for boundaries and direction. *J. Cell Biochem.* **33**, 24-31.
- Stenoien, D. L., Mancini, M. G., Patel, K., Allegretto, E. A., Smith, C. L. and Mancini, M. A. (2000). Subnuclear trafficking of estrogen receptor- α and steroid receptor coactivator-1. *Mol. Endocrinol.* **14**, 518-534.
- Stenoien, D. L., Nye, A. C., Mancini, M. G., Patel, K., Dutertre, M., O'Malley, B. W., Smith, C. L., Belmont, A. S. and Mancini, M. A. (2001). Ligand-mediated assembly and real-time cellular dynamics of estrogen receptor α -coactivator complexes in living cells. *Mol. Cell. Biol.* **21**, 4404-4412.
- Sterner, D. E. and Berger, S. L. (2000). Acetylation of histones and transcription-related factors. *Microbiol. Mol. Biol. Rev.* **64**, 435-459.
- Tanimoto, K., Makino, Y., Pereira, T. and Poellinger, L. (2000). Mechanism of regulation of the hypoxia-inducible factor-1 α by the von Hippel-Lindau tumor suppressor protein. *EMBO J.* **19**, 4298-4309.
- Teruel, M. N. and Meyer, T. (2000). Translocation and reversible localization of signaling proteins: a dynamic future for signal transduction. *Cell* **103**, 181-184.
- Thibodeau, A., Duchaine, J., Simard, J. L. and Vincent, M. (1989). Localization of molecules with restricted patterns of expression in morphogenesis: an immunohistochemical approach. *Histochem. J.* **21**, 348-356.
- Vincent, M., Lauriault, P., Dubois, M. F., Lavoie, S., Bensaude, O. and Chabot, B. (1996). The nuclear matrix protein p255 is a highly phosphorylated form of RNA polymerase II largest subunit which associates with spliceosomes. *Nucleic Acids Res.* **24**, 4649-4652.
- von Mikecz, A., Zhang, S., Montminy, M., Tan, E. M. and Hemmerich, P. (2000). CREB-binding protein (CBP)/p300 and RNA polymerase II colocalize in transcriptionally active domains in the nucleus. *J. Cell Biol.* **150**, 265-273.
- Xu, J. and Li, Q. (2003). Review of the in vivo functions of the p160 steroid receptor coactivator family. *Mol. Endocrinol.* **17**, 1681-1692.
- Yang, X. J., Ogryzko, V. V., Nishikawa, J., Howard, B. H. and Nakatani, Y. (1996). A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A. *Nature* **382**, 319-324.
- Yao, T. P., Ku, G., Zhou, N., Scully, R. and Livingston, D. M. (1996). The nuclear hormone receptor coactivator SRC-1 is a specific target of p300. *Proc. Natl. Acad. Sci. USA* **93**, 10626-10631.
- Yu, F., White, S. B., Zhao, Q. and Lee, F. S. (2001). HIF-1 α binding to VHL is regulated by stimulus-sensitive proline hydroxylation. *Proc. Natl. Acad. Sci. USA* **98**, 9630-9635.
- Zeng, C., Kim, E., Warren, S. L. and Bergeret, S. M. (1997). Dynamic relocation of transcription and splicing factors dependent upon transcriptional activity. *EMBO J.* **16**, 1401-1412.
- Zhong, S., Salomoni, P. and Pandolfi, P. P. (2000). The transcriptional role of PML and the nuclear body. *Nat. Cell Biol.* **2**, E85-E90.