

The Acetylase/Deacetylase Couple CREB-binding Protein/Sirtuin 1 Controls Hypoxia-inducible Factor 2 Signaling*

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Background: HIF-2 α is acetylated during hypoxia and is deacetylated by Sirt1, but the acetyltransferase is unknown.

Results: CBP selectively acetylates HIF-2 α and augments Sirt1/HIF-2 signaling during hypoxia. Coactivation correlates with stable CBP·HIF-2 α complex formation, which requires intact enzyme/substrate determinants.

Conclusion: Efficient HIF-2 signaling during hypoxia requires CBP as well as Sirt1.

Significance: Acetylation and deacetylation are molecular handles that act in concert to modulate HIF-2 signaling.

Hypoxia-inducible factors (HIFs) are oxygen-sensitive transcription factors. HIF-1 α plays a prominent role in hypoxic gene induction. HIF-2 α target genes are more restricted but include erythropoietin (*Epo*), one of the most highly hypoxia-inducible genes in mammals. We previously reported that HIF-2 α is acetylated during hypoxia but is rapidly deacetylated by the stress-responsive deacetylase Sirtuin 1. We now demonstrate that the lysine acetyltransferases cAMP-response element-binding protein-binding protein (CBP) and p300 are required for efficient *Epo* induction during hypoxia. However, despite close structural similarity, the roles of CBP and p300 differ in HIF signaling. CBP acetylates HIF-2 α , is a major coactivator for HIF-2-mediated *Epo* induction, and is required for Sirt1 augmentation of HIF-2 signaling during hypoxia in Hep3B cells. In comparison, p300 is a major contributor for HIF-1 signaling as indicated by induction of *Pgk1*. Whereas CBP can bind with HIF-2 α independent of the HIF-2 α C-terminal activation domain via enzyme/substrate interactions, p300 only complexes with HIF-2 α through the C-terminal activation domain. Maximal CBP/HIF-2 signaling requires intact CBP acetyltransferase activity in both Hep3B cells as well as in mice.

Signal transduction processes initiated during hypoxia include *de novo* transcriptional events. Hypoxia-inducible factor (HIF)³ family members are heterodimeric transcription factors belonging to the basic helix-loop-helix-Per/ARNT/Sim domain superfamily that contain one of three unique oxygen-

sensitive HIF- α proteins and a shared oxygen-insensitive β protein (1). HIF-1 and HIF-2 regulate unique and shared target genes whose products control adaptive cellular and physiological processes. For example, erythropoiesis is regulated at distinct developmental stages by HIF-1 and HIF-2 through control of erythropoietin (*Epo*), one of the most highly induced hypoxia-responsive target genes in mammals (2, 3).

HIF-2 α is closely related in structure to HIF-1 α (4). The N terminus of HIF-1 α and HIF-2 α contains the basic helix-loop-helix as well as Per/ARNT/Sim domain regions involved in DNA binding and dimerization, respectively, whereas the C terminus contains an N-terminal activation domain (NTAD) with an embedded oxygen-dependent degradation domain, a C-terminal activation domain (CTAD), and a highly divergent sequence in between known as the unique region. HIF- α protein levels increase during hypoxia due to impaired modifications of two proline residues (5, 6) located in the NTAD/oxygen-dependent degradation domain (7), which are otherwise hydroxylated under normoxic conditions by oxygen-dependent prolyl hydroxylases (6, 8, 9) and thereby target the HIF- α proteins for von Hippel-Lindau-mediated proteasomal degradation (10–14). The CTAD recruits p300 or CBP if an asparagine residue in the CTAD is not modified by a second oxygen-dependent hydroxylase, the asparaginyl hydroxylase factor inhibiting HIF-1 (FIH-1) (15, 16).

Changes in HIF- α protein stability are a prominent, but not exclusive, mechanism for HIF regulation (1). We recently reported that the stress-responsive deacetylase Sirt1 selectively augments HIF-2 signaling by deacetylating HIF-2 α at specific lysine residues (3). We therefore postulate that a unique lysine acetyltransferase acetylates HIF-2 α during hypoxia. Here, we evaluate the role of the p300/CBP and PCAF/GCN5 acetyltransferases in HIF-dependent regulation of *Epo* gene expression. We conclude that although CBP and p300 both regulate *Epo* gene expression during hypoxia, CBP and p300 exhibit unique roles in HIF-dependent transactivation with CBP, and not p300, acetylating HIF-2 α during hypoxia and thereby acting in conjunction with Sirt1 to augment HIF-2 signaling.

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³ The abbreviations used are: HIF, hypoxia-inducible factor; ARNT, aryl hydrocarbon receptor nuclear translocator; CBP, cAMP-response element-binding protein-binding protein; CTAD, C-terminal activation domain; HAT, histone acetyltransferase; NTAD, N-terminal activation domain; Sirt1, Sirtuin 1; ANOVA, analysis of variance; NAM, nicotinamide.

EXPERIMENTAL PROCEDURES

Reporter and Expression Plasmids—Full-length human wild-type (WT) p300 cDNA (R. Evans, Salk Institute) and full-length mouse WT CBP cDNA (T.-P. Yao, Duke University) were used to generate siRNA-resistant WT or HAT (K1399Y) mutant p300 and WT or HAT (K1540A and F1541A) mutant CBP by mutagenesis. WT and deacetylase inactive Sirt1 with an amino VSV-G epitope tag were previously described (3). Oxygen-independent (PP and PPN) HIF- α proteins contain substitutions for the two NTAD proline residues (PP) modified by the oxygen-dependent proline hydroxylases without (PP) or with (PPN) a substitution for the CTAD arginine residue modified by the oxygen-dependent asparagine hydroxylase; K3, HIF-2 α containing three lysine residues acetylated during hypoxia (K385, K685, and K741) and deacetylated by Sirt1; R3, HIF-2 α with three arginine substitutions (K385R, K685R, and K741R) for the lysine residues that are acetylated during hypoxia were constructed as described previously (3). The HIF- α Δ CTAD constructs were truncated at the junction between the unique region and the CTAD; all HIF- α constructs contain a C-terminal hemagglutinin A (HA) epitope tag. HIF- α constructs used in coimmunoprecipitations and pulldowns also have an S protein (SP) tag at the N terminus. Sirt1 and HIF expression vectors were based on pIRES-hrGFP-2a (pIRES), which also served as the control (empty) expression vector, except for SP-tagged HIF constructs, which were generated in pSPN (3).

siRNA Knockdown—We used the following siRNA from Thermo Fisher Scientific, Lafayette, CO, in conjunction with DharmaFECT1 (catalog no. T-2001-03): nontargeting control (catalog no. D-001810-10-20), p300 (catalog no. L-003486-00-0005), CBP (catalog no. L-003477-00-0005), PCAF (catalog no. L-005055-00-0005), GCN5 (catalog no. L-009722-00-0005), HIF-1 α (catalog no. L-004018-00-0005), EPAS1/HIF-2 α (catalog no. L-004814-00-0005), or SIRT1 (catalog no. L-003540-00-0005). After siRNA transfection of Hep3B cells, we incubated cells for 48 h (mRNA analysis) or 72 h (protein analysis).

For knockdown/rescue experiments, we performed sequential transfection of siRNA followed by transfection of plasmid DNA using Lipofectamine LTX with PLUS reagent (catalog no. 15338-100, Invitrogen) or by infection with lentivirus that coexpresses a cDNA followed by shRNA against the gene of interest. The rescue expression cassettes harbor a cDNA with silent mutations that confer resistance to siRNA or shRNA directed against the endogenous protein of interest. Cells were incubated an additional 48 h following plasmid transfection prior to harvest.

Immunoblotting and Cell Fractionation—We used nuclear extract-PER[®], nuclear and cytoplasmic extraction reagents (catalog no. 78833, Pierce), and Cytobuster protein extraction reagent (catalog no. 71009, Novagen, Gibbstown, NJ), with 1 $\times$ protease inhibitor mixture (catalog no. P8340, Sigma) and 1 mM PMSF (phenylmethylsulfonyl fluoride) (catalog no. P7626, Sigma). We used 40 μ g of Hep3B whole cell extracts, 20 μ g of Hep3B nuclear extracts, or 10 μ g of mouse liver nuclear extracts for immunoblotting with the following antibodies: p300 (1:500 dilution; catalog no. sc-584, Santa Cruz Biotechnol-

ogy, Santa Cruz, CA); CBP (1:500 dilution; catalog no. 4772, Cell Signaling Technology, MA); PCAF (1:1,000 dilution; catalog no. 3378, Cell Signaling Technology); GCN5 (1:1,000 dilution; catalog no. 3305, Cell Signaling Technology); TATA-binding protein (1:1,000 dilution; catalog no. sc-204, Santa Cruz Biotechnology); tubulin (1:10,000 dilution; catalog no. T9026, Sigma); c-Myc (1:5,000 dilution; catalog no. 2272, Cell Signaling Technology); acetyl-lysine (1:1,000 dilution; catalog no. 9814, Cell Signaling Technology); HIF-1 α (1:1,000 dilution; catalog no. 610958, BD Biosciences); HIF-2 α (1:1,000 dilution; catalog no. NB100-122, Novus Biologicals, Littleton, CO); Sirt1 (1:1,000 dilution; catalog no. 07-131, Millipore/Upstate, MA); or HA (1:5,000 dilution; catalog no. H9658, Sigma).

Exogenous HIF-2 α Acetylation—Hep3B cells were transfected using Lipofectamine 2000 with expression vectors encoding SP:WT or PPN HIF-2 α :HA containing three intact lysine residues in the HIF-2 C terminus that are acetylated (K3) or arginine substitutions (R3). To assess acetylation during hypoxia, cells were transfected, treated with 5 μ M sirtinol (catalog no. 510 8474, Chembridge Corporation, San Diego) plus 10 mM nicotinamide (catalog no. N0636, Sigma) 18 h after transfection, and then incubated for an additional 6 h until harvest. Ectopic HIF-2 α :HA was purified by SP pulldown using SP-agarose and then was first immunoblotted using antibodies recognizing acetylated lysine and second using antibodies recognizing HA (3).

Endogenous HIF-2 α Acetylation—Hep3B cells were cultured in complete medium supplemented with 5 μ M sirtinol plus 10 mM nicotinamide (NAM) and maintained for 6 h under either normoxia or hypoxia. Cells were fractionated, and the nuclear protein was extracted using a nuclear extract kit (catalog no. 78833, ThermoScientific, Rockford, IL) supplemented with 1 $\times$ protease inhibitor mixture (catalog no. P8340, Sigma), 1 mM PMSF (phenylmethylsulfonyl fluoride) (catalog no. P7626, Sigma), 10 mM nicotinamide, and 5 μ M sirtinol. Endogenous HIF-2 α was incubated with a monoclonal human HIF-2 α antibody (catalog no. NB100-132, Novus Biologicals) for 1 h and then immunoprecipitated after incubation for 0.5 h at 4 $^{\circ}$ C with protein A/G-agarose beads (catalog no. sc2003, Santa Cruz Biotechnology). Aliquots were immunoblotted for HIF-2 α or acetyl lysine as described (3).

Cell Culture and Transfections—We maintained Hep3B cells and performed hypoxia exposure (1% O₂) as described (3). For ectopic HIF-2 α studies, Hep3B cells were transfected and harvested 48 h later for total RNA. For sirtinol treatment, sirtinol (5 μ M) or vehicle control (0.1% DMSO) was added to the media for the last 6 h prior to harvest.

In Vitro Deacetylation Experiments—To prepare acetylated HIF-2 α substrate, HEK293 cells were transfected with an expression plasmid encoding SP:WT HIF-2 α :HA or SP:PPN HIF-2 α :HA with control or CBP expression plasmid using Lipofectamine 2000. Media were replaced with complete media 6 h after transfection, and cells were allowed to recover for an additional 12 h. Next, transfected cells were treated with sirtinol (5 μ M) plus NAM (10 mM) and exposed another 6 h to either normoxia for cells expressing the CBP expression plasmid or to hypoxia for cells expressing the control expression plasmid. Ectopic HIF-2 α was purified using SP-agarose chromatogra-

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phy. For Sirt1 protein, wild-type or deacetylase mutant VSV-G: SIRT1 expression plasmid was transfected in HEK293 cells and purified 24 h later using anti-VSV-G antibody agarose (catalog no. A1970, Sigma). Before the deacetylation reaction, the SP-agarose bound HIF-2 α :HA and the VSV-G-bead bound SIRT1 were each spun down, equilibrated with deacetylation reaction buffer (50 mM Tris, pH 9.0, 150 mM NaCl, 5 mM MgCl₂, 5 mM DTT, and 10 mM NAD), and resuspended in deacetylase reaction buffer. The *in vitro* deacetylation reaction proceeded for 0 or 30 min at 30 °C. The reaction was separated using SDS-PAGE and immunoblotted using antibody recognizing acetylated-lysine or HA epitope.

Lentivirus Transient Transduction of Hep3B Cells—Lentiviruses were generated by cotransfection with packaging plasmids pSPAX2 and pMD2G. The day before transduction, Hep3B cells were trypsinized, and 2×10^5 cells per well were plated in 1 ml of complete culture medium in a 12-well plate. Cells were incubated at 37 °C overnight. On the day of transduction, complete media with Polybrene (catalog no. 107689, Sigma) was prepared at a final concentration of 5 μ g/ml. Media were removed from the wells and replaced with 0.5 ml of the Polybrene/media mixture. Lentiviral particles were added to the Hep3B cells (multiplicity of infection = 20), and the plate was gently swirled to mix and then incubated overnight. After 12 h, the culture medium was replaced with 1 ml of complete medium (without Polybrene). For RNA and protein preparations, cells were harvested an additional 48 h following transduction, with hypoxia exposure during the final 8 h when indicated.

Quantitative RT-PCR Analyses—The expression of endogenous *Epo*, *Pgk1*, and cyclophilin B from three independent experiments for Hep3B experiments, or from individual mouse liver samples, was determined by reverse transcription of total RNA followed by real time quantitative PCR assays performed in triplicate, as described previously (3), using human or mouse *Epo*, *Pgk1*, or cyclophilin primers. We used the following human quantitative PCR primer pairs: *EPO* (forward) 5'-GAGGC-CGAGAATATCACGACGGG-3' and (reverse) 5'-TGCCCGA-CCTCCATCCTCTTCCAG-3'; *PGK1* (forward) 5'-TTAAAGG-GAAGCGGGTCGTTA-3' and (reverse) 5'-TCCATTGTCCAA-GCAGAATTTGA-3'; cyclophilin (forward) 5'-ATGTGGTTT-CGGCAAAGTTCTA-3' and (reverse) 5'-GGCTTGTCCTCGGCTGTCT-3'. We used the following mouse quantitative PCR primer pairs: *Epo* (forward) 5'-GAGGCAGAAAATGTCACG-ATG-3' and (reverse) 5'-CTTCCACCTCCATTCTTTTCC-3'; *Pgk1* (forward) 5'-CTCCGCTTTCATGTAGAGGAAG-3' and (forward) 5'-GACATCTCCTAGTTTGGACAGTG-3'; cyclophilin (forward) 5'-ATGTGGTTTTCGGCAAAGTTCTA-3' and (reverse) 5'-GGCTTGTCCTCGGCTGTCT-3'.

Mouse Adenoviral Experiments—All experiments were approved by the institutional IACUC. We injected 7–9-week-old (24–28 g) CD1 female mice (Charles River Laboratories; Wilmington, MA) intravenously with the indicated amount of virus brought to a final volume of 250 μ l with normal saline. For CBP knockdown/rescue, we injected 3×10^{11} particles of PPN HIF-2 α :HA or control (empty) adenovirus that also express control or mouse CBP shRNA (PPN HIF-2 α :HA/shRNA adenovirus), plus 3×10^{11} particles of either control adenovirus or

adenovirus expressing Myc-tagged shRNA-resistant (resist) wild-type (WT) or acetyltransferase activity mutant (HAT) mouse CBP. The PPN HIF-2 α :HA/shRNA adenovirus expresses PPN HIF-2 α :HA followed by an internal ribosome entry site with a downstream DsRed cassette containing multiple shRNA at the 3' end of the DsRed cDNA (17). The resist CBP cDNA maintains amino acid identity but is designed to disrupt binding of the shRNA that targets endogenous mouse CBP. After adenoviral injections, mice were housed for 1 week and fed *ad libitum* during this period.

Statistical Analyses—All findings were rounded to two significant digits. A value of $p < 0.10$ or lower was considered statistically significant. SPSS V18 (IBM Inc., Chicago) was used to analyze the data using Dunnett or Bonferroni correction for multiple comparisons to provide coverage for type I statistical error. Post hoc analyses were performed based on specific data. Where all comparison groups were the same (*i.e.* no control), the Bonferroni correction was used. When one group was a control and all others groups compared only with control, the Dunnett correction was used to minimize the effect of multiple comparisons on the p value.

RESULTS

We first asked if endogenous p300/CBP or PCAF/GCN5 participates in acetylation of endogenous HIF-2 α during hypoxia in Hep3B cells using siRNA knockdown to decrease each candidate acetyltransferase. Knockdown of p300/CBP or PCAF/GCN5 family members singly or in combination revealed that only CBP participated in acetylation of endogenous HIF-2 α during hypoxia in Hep3B cells (Fig. 1, A–C).

To define the role of the candidate acetyltransferases in hypoxia signaling, we next determined how siRNA knockdown of candidate acetyltransferases affects *Epo* and *Pgk1* induction after hypoxia exposure (Fig. 1D). Knockdown of p300 as well as CBP, but not of PCAF or GCN5, resulted in significant impairment in hypoxia-induced *Epo* gene expression. For the HIF-1 selective target gene *Pgk1*, knockdown of p300, but not CBP, affected *Pgk1* induction in response to hypoxia whereas PCAF or GCN5 knockdown had no effect. Efficient knockdown was confirmed by immunoblotting (Fig. 1E).

CBP, but not p300, participates in acetylation of HIF-2 α during hypoxia in Hep3B cells. However, CBP and p300 both contribute to hypoxia-mediated induction of *Epo*. We asked if CBP and p300 act selectively through specific HIF factors to induce target genes during hypoxia. We first defined the contribution of HIF-1 α or HIF-2 α to regulation of *Epo* or *Pgk1* (Fig. 2A) gene expression during hypoxia using siRNA to efficiently knock down HIF-1 α or HIF-2 α (Fig. 2B). As reported previously, *Epo* was predominantly regulated by HIF-2 α , whereas *Pgk1* was exclusively regulated by HIF-1 α .

We next combined knockdown of HIF-1 α or HIF-2 α with knockdown of CBP or p300 to determine the relative role of CBP and p300 in HIF-dependent induction of *Epo* and *Pgk1* gene expression (Fig. 2C). Hypoxia-induced *Epo* gene expression was not significantly reduced by HIF-1 α knockdown, either alone or in conjunction with p300, but it was significantly diminished with combined HIF-1 α and CBP knockdown. HIF-2 α knockdown substantially blunted *Epo* gene induction

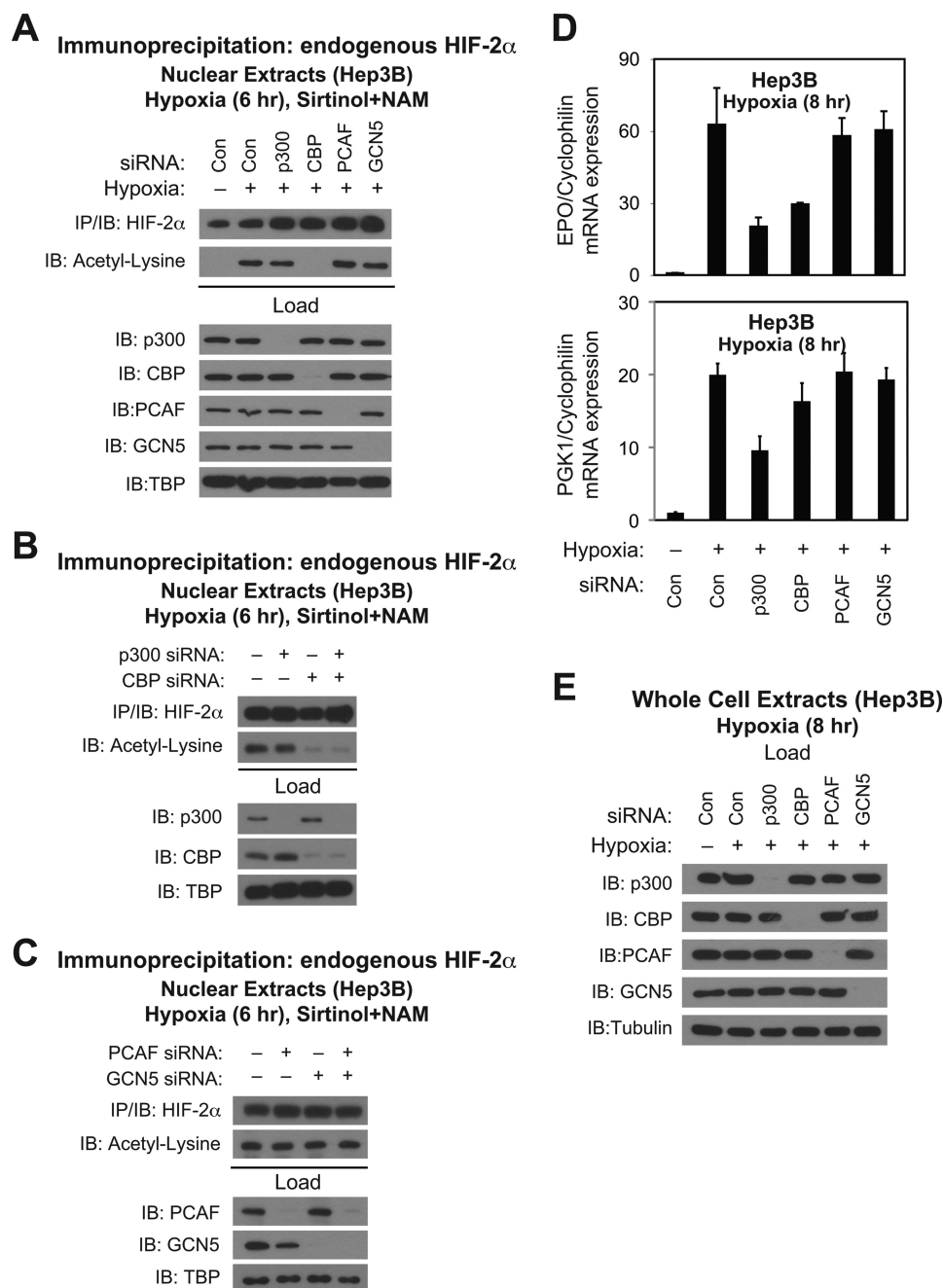


FIGURE 1. Distinct roles for acetyltransferases in hypoxia signaling. A, acetylation of immunoprecipitated (IP) endogenous HIF-2 α from Hep3B nuclear extracts detected by immunoblotting (IB) after control (Con), p300, CBP, PCAF, or GCN5 siRNA knockdown. Acetylation of endogenous HIF-2 α after isolated or combined p300 or CBP knockdown (+) (B) or isolated or combined PCAF or GCN5 knockdown (+) (C). The siRNA levels in B and C were kept constant by use of control siRNA (-). D, *Epo* and *Pgk1* gene expression after hypoxia exposure (8 h) following control (Con), p300, CBP, PCAF, or GCN5 knockdown (mean $\pm$ S.E.; all comparisons of hypoxia samples are by one-way ANOVA with control siRNA alone/hypoxia as reference; *Epo*, $p < 0.03$ for p300 siRNA, $p < 0.07$ for CBP siRNA; *Pgk1*, $p < 0.01$ for p300 siRNA). E, immunoblot (IB) of p300, CBP, PCAF, GCN5, and tubulin in whole cell extracts prepared in parallel to D.

and the addition of p300 knockdown further reduced *Epo* gene expression, whereas the addition of CBP knockdown had no additional effect. For *Pgk1*, HIF-1 α knockdown severely reduced expression to basal levels, whereas HIF-2 α knockdown had no effect; knockdown of p300 alone significantly reduced *Pgk1* induction, but knockdown of CBP, either alone or combined with HIF-2 α knockdown, had no measurable effect upon *Pgk1* induction despite efficient knockdown of CBP (Fig. 2D).

We asked if the acetyltransferase activity of p300 or CBP was required for induction of *Epo* and *Pgk1* gene expression during

hypoxia (Fig. 3A). Knockdown of p300 blunted *Epo* induction, and this reduction could be rescued with an siRNA-resistant cDNA encoding either wild-type (WT) p300 or a mutant lacking acetyltransferase activity (HAT). In comparison, CBP knockdown also resulted in a blunting of *Epo* induction during hypoxia, and this inhibition could be rescued with a cDNA encoding WT CBP but not by a cDNA encoding a CBP HAT mutant. Rescue of the HIF-1 target gene *Pgk1* was effective with either WT or HAT p300, whereas CBP knockdown had no effect, consistent with p300/HIF-1-selective regulation. Knock-

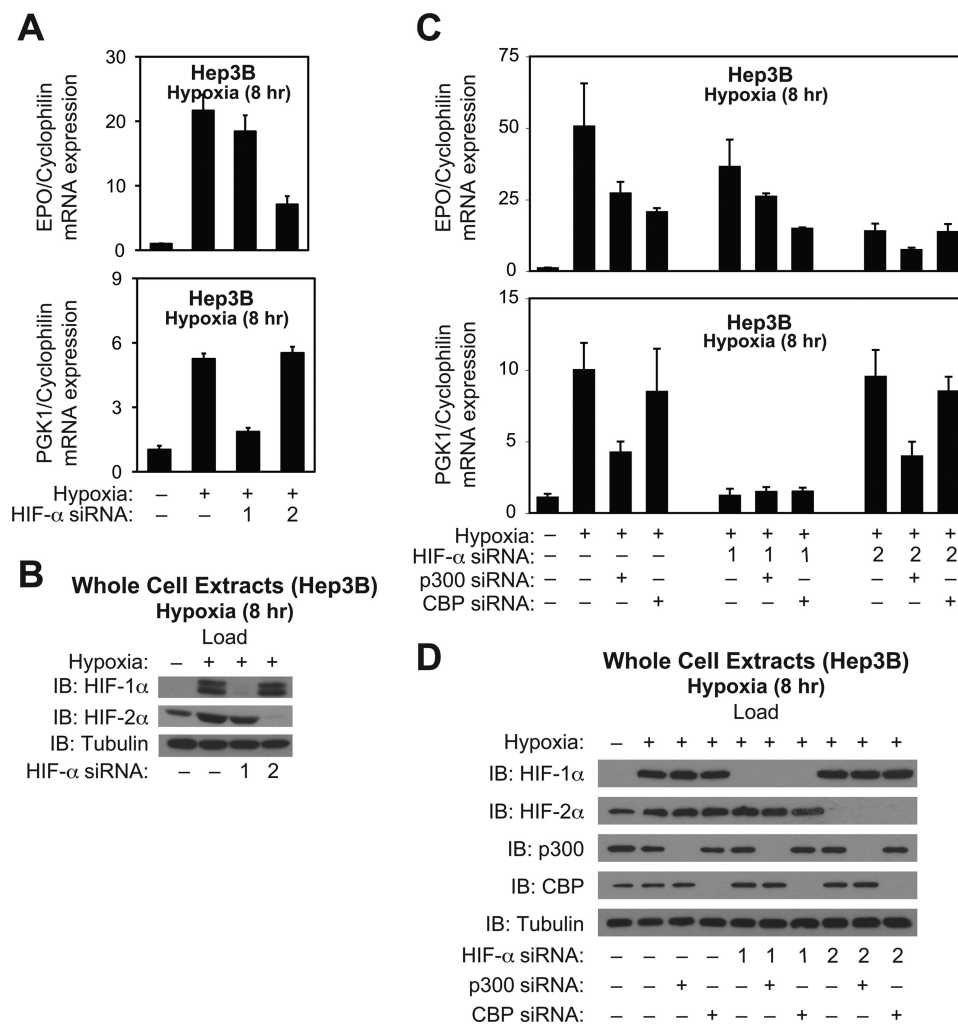


FIGURE 2. CBP and p300 differentially regulate HIF signaling. *A*, *Epo* and *Pgk1* gene expression during hypoxia (8 h) after control (–), HIF-1α (numeral 1), or HIF-2α (numeral 2) knockdown (mean ± S.E.; all comparisons of hypoxia samples are by one-way ANOVA with control siRNA alone/hypoxia as reference; *Epo*, $p < 0.007$ for control versus HIF-2α siRNA; *Pgk1*, $p < 0.0001$ for control versus HIF-1α siRNA). *B*, immunoblot (IB) of HIF-1α, HIF-2α, and tubulin in whole cell extracts prepared in parallel to *A*. *C*, *Epo* and *Pgk1* gene expression during hypoxia (8 h) after control (–) or p300 versus CBP (+) knockdown combined with control (–), HIF-1α (bar 1), or HIF-2α (bar 2) knockdown (mean ± S.E.; all comparisons of hypoxia samples are by one-way ANOVA with control siRNA alone/hypoxia as reference; *Epo*, $p < 0.10$ for p300 siRNA and $p < 0.05$ for CBP siRNA with no HIF siRNA; $p < 0.07$ for p300 siRNA with HIF-1α siRNA, $p < 0.03$ for CBP siRNA with HIF-1α siRNA; $p < 0.04$ for all HIF-2α siRNA samples; *Pgk1*, $p < 0.08$ for control versus p300 siRNA with no HIF siRNA; $p < 0.02$ for all HIF-1α siRNA samples; $p < 0.02$ for p300 siRNA with HIF-2α siRNA). *D*, immunoblot of HIF-1α, HIF-2α, p300, CBP, and tubulin in whole cell extracts prepared in parallel to *C*.

down of p300 as well as CBP was efficient, whereas ectopic p300 or CBP expression levels were only slightly greater than endogenous protein levels (Fig. 3B).

Acetylation of HIF-2α requires CBP, whereas deacetylation of HIF-2α requires Sirt1 during hypoxia. To confirm that Sirt1 inactivation results in acetylation of HIF-2α due to CBP, we examined isolated as well as combined Sirt1 and CBP knockdown (Fig. 4A). CBP knockdown performed in isolation or combined with Sirt1 knockdown eliminated acetylation. HIF-2α was acetylated following hypoxia exposure or CBP overexpression, and the acetylated HIF-2α was efficiently deacetylated by WT, but not by deacetylase, Sirt1 in an *in vitro* reaction (Fig. 4B).

To define the relationship of CBP and p300 to Sirt1/HIF-2 signaling as measured by *Epo* and *Pgk1* induction during hypoxia, we performed knockdown studies of CBP or p300 alone or in combination with Sirt1 knockdown (Fig. 4C). As we reported previously (3), Sirt1 knockdown alone blunted *Epo*

induction following hypoxia exposure; *Epo* expression was further reduced after Sirt1 knockdown in combination with p300, but not CBP, knockdown. For the HIF-1 selective target gene *Pgk1*, only p300 knockdown affected induction and was not further affected when combined with Sirt1 knockdown. Knockdown of p300, CBP, or Sirt1 was efficient in all cases (Fig. 4D).

We next sought to define the role of HIF-2α acetylation in HIF-2 signaling. We first asked if the lysine residues in the HIF-2α C terminus required for acetylation of ectopic HIF-2α during hypoxia are also required for acetylation of ectopic HIF-2α by overexpressed CBP (Fig. 4E). Similar to what we previously reported with endogenous HIF-2α (3), ectopic wild-type (WT) HIF-2α that contains intact lysine residues (K3) was not appreciably acetylated during normoxia, but acetylation was evident following hypoxia exposure. Furthermore, ectopic CBP also acetylated WT K3 HIF-2α. In comparison, a mutant form of WT HIF-2α containing arginine substitutions for the

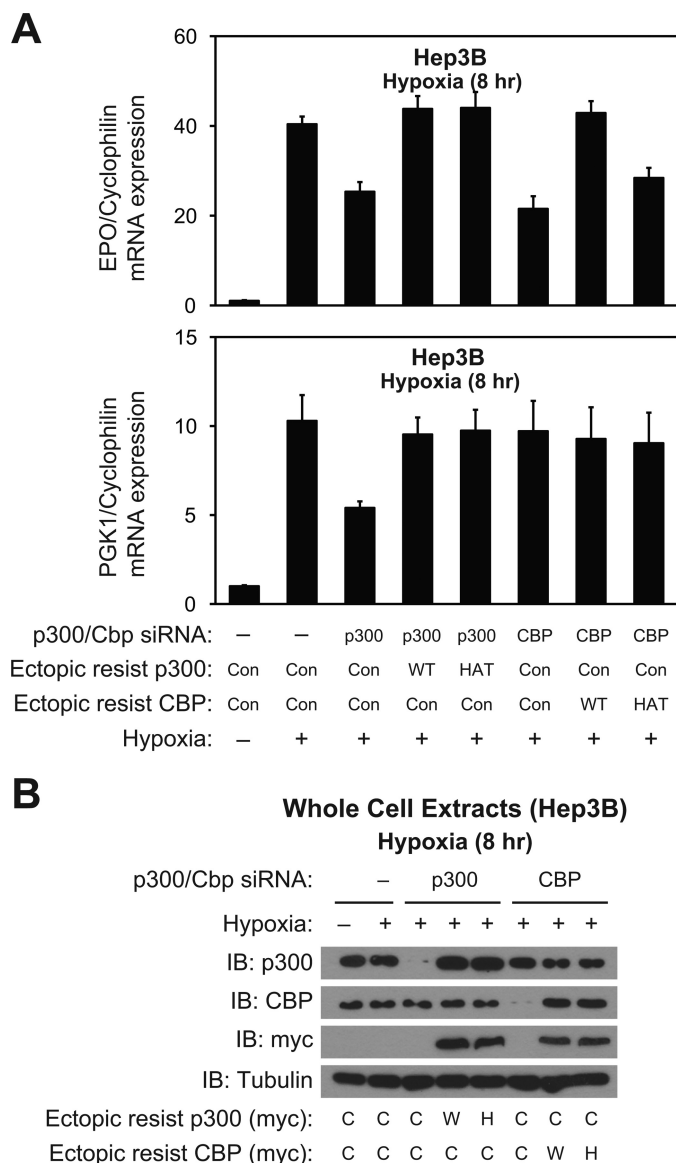


FIGURE 3. CBP/HIF-2 signaling requires acetyltransferase activity. *A*, *Epo* and *Pgk1* gene expression during hypoxia (8 h) after control (–), p300, or CBP siRNA knockdown, followed by rescue with an empty expression vector (Con) or an expression vector harboring a siRNA-resistant (*resist*) cDNA encoding Myc-tagged wild-type (WT) or histone acetyltransferase domain (HAT) point mutant p300 or CBP (mean $\pm$ S.E.; all comparisons of hypoxia samples are by one-way ANOVA with control siRNA/all control expression vectors/hypoxia as reference; *Epo*, $p < 0.002$ for p300 siRNA alone, $p < 0.0001$ for CBP siRNA alone, $p < 0.0001$ for CBP siRNA plus ectopic HAT CBP; *Pgk1*, $p < 0.03$ for p300 siRNA alone). *B*, immunoblot (IB) of ectopic p300 and CBP (Myc), p300, CBP, and tubulin present in whole cell extracts prepared in parallel to *A*. Cells were transfected with expression plasmids encoding control (C), wild-type (W), or histone acetyltransferase domain (H) point mutant p300 and CBP (myc), and subjected to knockdown with control (–), p300, or CBP siRNA and exposed to normoxia or hypoxia as indicated.

acetylated lysines (R3) was not acetylated during hypoxia exposure or by overexpressed CBP.

We next asked if the acetylated HIF-2 α lysine residues are required for Sirt1 augmentation of *Epo* gene expression (Fig. 4F). Knockdown of HIF-2 α severely impaired *Epo* expression following hypoxia exposure. Cells rescued with an siRNA-resistant cDNA encoding WT K3 HIF-2 α efficiently induced *Epo* gene expression during hypoxia, but this induction by WT R3 HIF-2 α was substantially reduced despite comparable levels

of WT K3 HIF-2 α and WT R3 HIF-2 α expression (Fig. 4G). Furthermore, reduced Sirt1 or CBP levels impaired HIF-2 signaling if the HIF-2 α acetylated lysines remained intact, as only WT K3, but not R3, HIF-2 α was affected by Sirt1 or CBP knockdown.

We next sought to define the role of HIF-2 α acetylation in hypoxia-independent HIF-2 signaling (Fig. 5A). Similar to what we previously reported (3), ectopic oxygen-insensitive (PPN) HIF-2 α with intact lysine residues (K3) was acetylated during normoxia, although acetylation substantially increased after hypoxia exposure. Furthermore, ectopic CBP also acetylated PPN K3 HIF-2 α . In comparison, PPN HIF-2 α containing arginine substitutions for the acetylated lysines (R3) was not acetylated during normoxia, after hypoxia exposure, or by overexpressed CBP. Similar to results obtained with WT K3 HIF-2 α , PPN K3 HIF-2 α acetylated following hypoxia exposure or CBP overexpression was efficiently deacetylated *in vitro* by wild-type, but not by deacetylase inactive, Sirt1 (Fig. 5B).

To examine the role of p300 and CBP in *Epo* and *Pgk1* induction conferred by isolated HIF signaling, we next performed knockdown studies using siRNA against p300 or CBP in the presence of ectopic oxygen-insensitive mutant HIF proteins (PPN HIF) (Fig. 5C). Exogenous PPN HIF-1 α modestly, and PPN HIF-2 α greatly, increased *Epo* mRNA levels in Hep3B cells. Induction of *Epo* mRNA levels by exogenous PPN HIF-1 α was reduced solely with p300 knockdown, whereas induction mediated by PPN HIF-2 α was significantly reduced only by CBP knockdown. Consistent with its identity as a HIF-1 target gene, *Pgk1* was only induced by ectopic PPN HIF-1 α and not by PPN HIF-2 α ; knockdown of p300, but not CBP, blunted induction of *Pgk1* by PPN HIF-1 α . Knockdown of CBP as well as p300 was efficient, and expression levels of ectopic PPN HIF-1 α and PPN HIF-2 α were similar (Fig. 5D).

Whereas p300 plays a minor, if any, role in coactivation of HIF-2 signaling, CBP plays a major role in this process. Furthermore, acetylation of HIF-2 α is mediated solely by CBP and not by p300. Using coimmunoprecipitation assays in HEK293 cells, we asked if these observations were due to differences in interactions of CBP and p300 with ectopic HIF- α proteins (Fig. 6A). Oxygen-insensitive (PPN) HIF-1 α bound CBP or p300 during normoxia. Consistent with p300 binding being completely dependent upon the CTAD, oxygen-insensitive HIF-1 α lacking the CTAD region (PPACTAD) did not bind either CBP or p300. In comparison, oxygen-insensitive (PPN) HIF-2 α bound p300 or CBP. However, mutant HIF-2 α that lacked the CTAD region (PPACTAD) retained the ability to complex with CBP but not with p300.

We next asked if the hydroxylated residues were required for CBP complex formation with HIF-2 α (Fig. 6B). Mutant HIF-2 α that was completely oxygen-independent (PPN) bound ectopic p300 or CBP. However, HIF-2 α with substitution mutations for the hydroxylated proline residues only (PP), but not of the hydroxylated asparagine residue in the CTAD, as well as wild-type (WT) HIF-2 α complexed with ectopic CBP but not with ectopic p300.

The above results indicate a CTAD-dependent mechanism for p300-HIF-2 α complex formation and a CTAD-dependent as well as a CTAD-independent mechanism for CBP-HIF-2 α

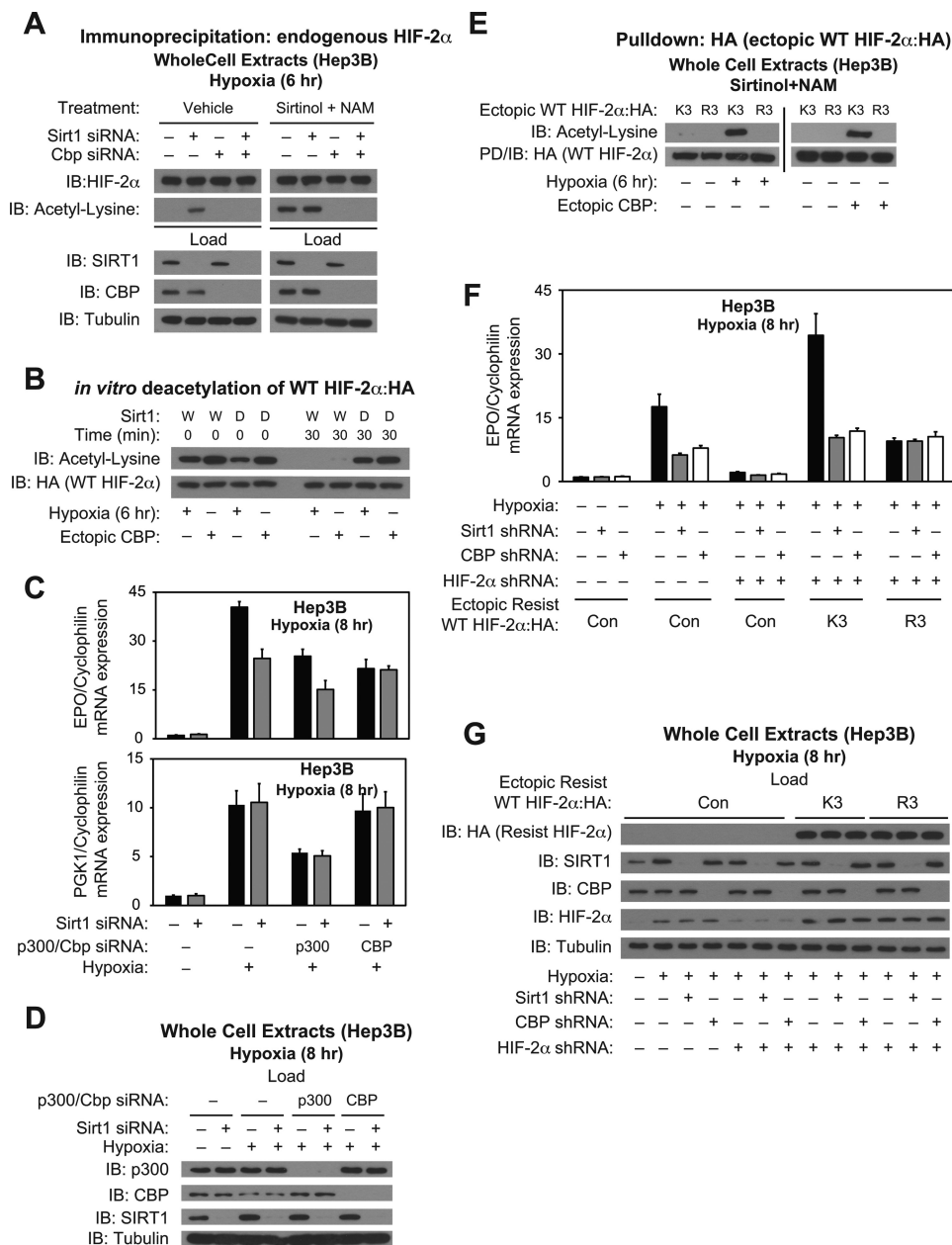


FIGURE 4. CBP/Sirt1 action regulates HIF-2 signaling. A, acetylation of immunoprecipitated (IP) endogenous HIF-2 α during hypoxia as detected by immunoblotting (IB) after control (–) and isolated or combined Sirt1 or CBP (+) siRNA-mediated knockdown in conjunction with vehicle or sirtinol plus NAM treatment. B, purified ectopic wild-type (WT) HIF-2 α , acetylated after hypoxia exposure or CBP overexpression, assessed for acetylation and loading following incubation with immunopurified wild-type (WT) or deacetylase inactive (D) Sirt1. W, wild type; D, deacetylase inactive. C, *Epo* and *Pgk1* gene expression during hypoxia (8 h) after knockdown with control (–), p300, or CBP siRNA, without or with Sirt1 siRNA (+) (mean $\pm$ S.E.; all comparisons of hypoxia samples are by one-way ANOVA; *Epo*, $p < 0.009$ for control siRNA versus Sirt1 siRNA, $p < 0.02$ for p300 siRNA versus Sirt1 plus p300 siRNA, $p < 0.005$ for control siRNA versus p300 siRNA, $p < 0.005$ for control siRNA versus CBP siRNA, $p < 0.04$ for Sirt1 siRNA versus Sirt1 plus p300 siRNA; *Pgk1*, $p < 0.04$ for control siRNA versus p300 siRNA, $p < 0.04$ for Sirt1 siRNA versus Sirt1 plus p300 siRNA). D, immunoblot of endogenous Sirt1, p300, CBP, and tubulin in whole cell extracts (WCE) prepared in parallel to C. E, control expression vector (Con) or an shRNA-resistant cDNA encoding ectopic wild-type (WT) HIF-2 α :HA with intact lysine residues (K3) or with arginine substitutions (R3), all followed by an shRNA targeting endogenous HIF-2 α mRNA, were expressed via lentivirus. Acetylation and loading of ectopic HIF-2 α :HA were examined, following HA-pulldown (PD), by immunoblotting (IB) for acetyl-lysine or HA, respectively, after hypoxia exposure or in the setting of ectopic CBP overexpression. F, *Epo* gene expression following transduction with a lentivirus expressing control (–) or HIF-2 α (+) shRNA and an shRNA-resistant HIF-2 α group as reference; *Epo*, $p < 0.008$ for Sirt1 or CBP shRNA plus control (no HIF-2 α) shRNA with control (Con) expression (no ectopic HIF-2 α), $p < 0.002$ for Sirt1 or CBP shRNA plus HIF-2 α shRNA with ectopic WT K3 HIF-2 α expression). G, immunoblot of ectopic HIF-2 α (HA), total (ectopic plus endogenous) HIF-2 α , Sirt1, CBP, and tubulin in whole cell extracts prepared in parallel.

complex formation. We reasoned that the acetylated lysine residues in the HIF-2 α C terminus provide a stabilizing interaction for CTAD-independent CBP-HIF-2 α complex formation. To test this hypothesis, we generated CTAD deletions of the

mutant PPN HIF-2 α (PPACTAD) containing either intact lysine residues (K3) or arginine substitutions (R3) for use in coimmunoprecipitation assays (Fig. 6C). CBP bound PPACTAD K3 HIF-2 α but not PPACTAD R3 HIF-2 α . As

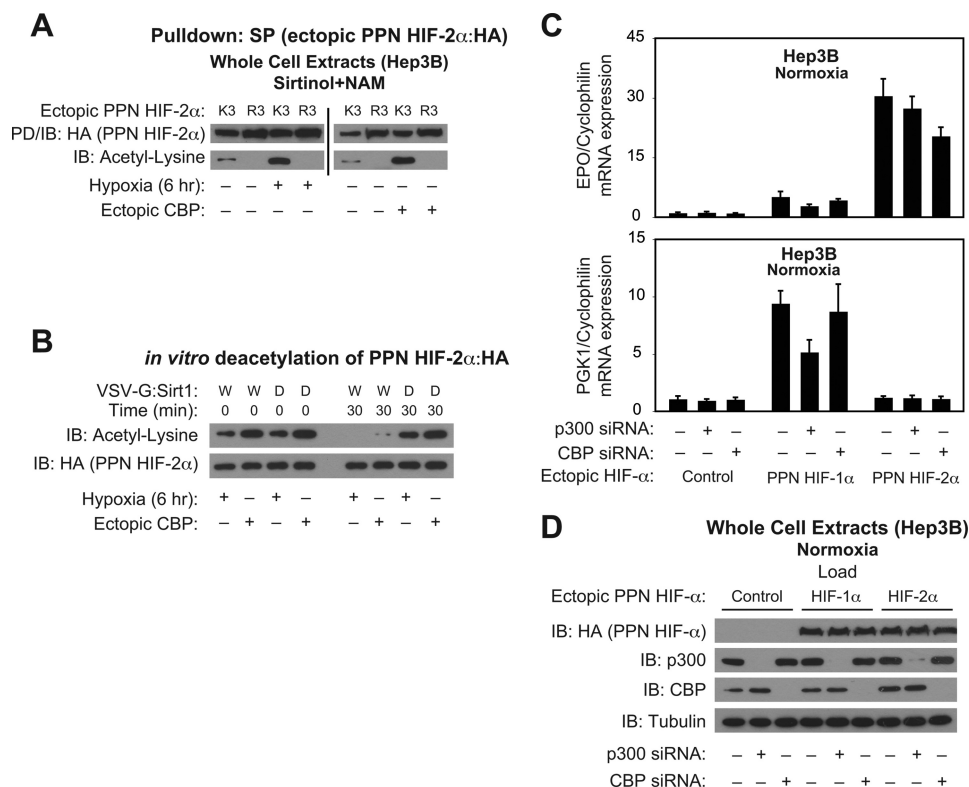


FIGURE 5. CBP contributes to hypoxia-independent HIF-2 signaling. *A*, acetylation of ectopic HA-tagged, oxygen-insensitive (PPN) HIF-2 α containing lysine residues acetylated during hypoxia (K3) or arginine substitutions (R3) after hypoxia exposure or CBP overexpression. Acetylation and loading were examined, following HA-agarose pulldown (PD), by immunoblotting (IB) for acetyl-lysine and HA, respectively. *B*, purified ectopic PPN HIF-2 α , acetylated after hypoxia exposure or CBP overexpression, assessed for acetylation and loading following incubation with immunopurified wild-type (WT) or deacetylase inactive (D) Sirt1. *C*, *Epo* and *Pgk1* gene expression during normoxia after control (–), CBP, or p300 (+) knockdown in conjunction with ectopic PPN HIF-1 α or PPN HIF-2 α expression (mean $\pm$ S.E.; all comparisons by one-way ANOVA with all control siRNA/control expression vector/hypoxia within each PPN group as reference; *Epo*, $p < 0.09$ for p300 siRNA with ectopic PPN HIF-1 α expression, $p < 0.05$ for CBP siRNA with ectopic PPN HIF-2 α expression; *Pgk1*, $p < 0.09$ for p300 siRNA with ectopic PPN HIF-1 α expression). *D*, immunoblot of PPN HIF-1 α and PPN HIF-2 α (HA), p300, CBP, and tubulin present in whole cell extracts prepared in parallel to *C*.

expected, p300 could not bind either PPACTAD K3 HIF-2 α or PPACTAD R3 HIF-2 α .

If enzyme/substrate interactions stabilize CTAD-independent CBP·HIF-2 α complex formation, then elimination of CBP acetyltransferase activity should affect CBP·HIF-2 α complex formation. Consistent with this hypothesis, WT CBP bound to PPN K3 HIF-2 α , PPN R3 HIF-2 α , and PPACTAD K3 HIF-2 α . HAT CBP, in comparison, only bound to PPN K3 or R3 HIF-2 α , presumably through the CTAD (Fig. 6D). Thus, prevention of acetylation, either by mutations in the enzyme (CBP) or substrate (HIF-2 α lysine residues), affects CTAD-independent CBP·HIF-2 α complex formation.

We next asked if prevention of deacetylation affects CBP·HIF-2 α complex formation (Fig. 6E). CBP complexed with PPN K3 HIF-2 α , PPN R3 HIF-2 α , or PPACTAD K3 HIF-2 α in vehicle-treated cells, which have minimally detectable acetylated HIF-2 α levels. In comparison, CBP only complexed with PPN K3 HIF-2 α or PPN R3 HIF-2 α in cells treated with sirtinol plus NAM to inhibit Sirt1 deacetylase activity, which results in marked acetylated HIF-2 α levels.

To assess if HIF-2 α , Sirt1, and CBP interact in a single complex, we first immunoprecipitated ectopic CBP, and second we assessed for coimmunoprecipitation of ectopic Sirt1 and ectopic HIF-2 α (Fig. 6F). Sirt1 and CBP formed a macromolecular complex in the presence of HIF-2 α , but not if HIF-2 α was

absent, suggesting that HIF-2 α is a scaffold for Sirt1/CBP assembly. Reversal of the immunoprecipitation order (Sirt1 first and CBP second) resulted in a similar outcome.

To assess the *in vivo* role of CBP in HIF-2 signaling, we used adenoviral gene delivery of PPN HIF-2 α to induce hepatic *Epo* (Fig. 7A) as we have done previously (3). Knockdown of endogenous CBP by shRNA blunted *Epo* induction by ectopic HIF-2 α , which was alleviated by coexpression of an shRNA-resistant cDNA encoding WT CBP but not HAT CBP. The effects upon *Epo* gene expression were reflected at the physiological level (Fig. 7B). Knockdown of endogenous CBP was efficient, and ectopic CBP expression levels in liver was only modestly elevated compared with endogenous CBP levels (Fig. 7C).

DISCUSSION

Transactivation by transcription factors requires recruitment of coactivators, including HAT, recently renamed lysine acetyltransferases because of their ability to acetylate nonhistone substrates. Of the mammalian acetyltransferases, the most widely studied is p300 (also termed EP300, KAT3B), which has been implicated in multiple signal transduction pathways. CBP (also termed Crebbp, KAT3A) is closely related to p300. CBP and p300 have unique biological roles (18), yet there are limited examples demonstrating selective cellular actions of,

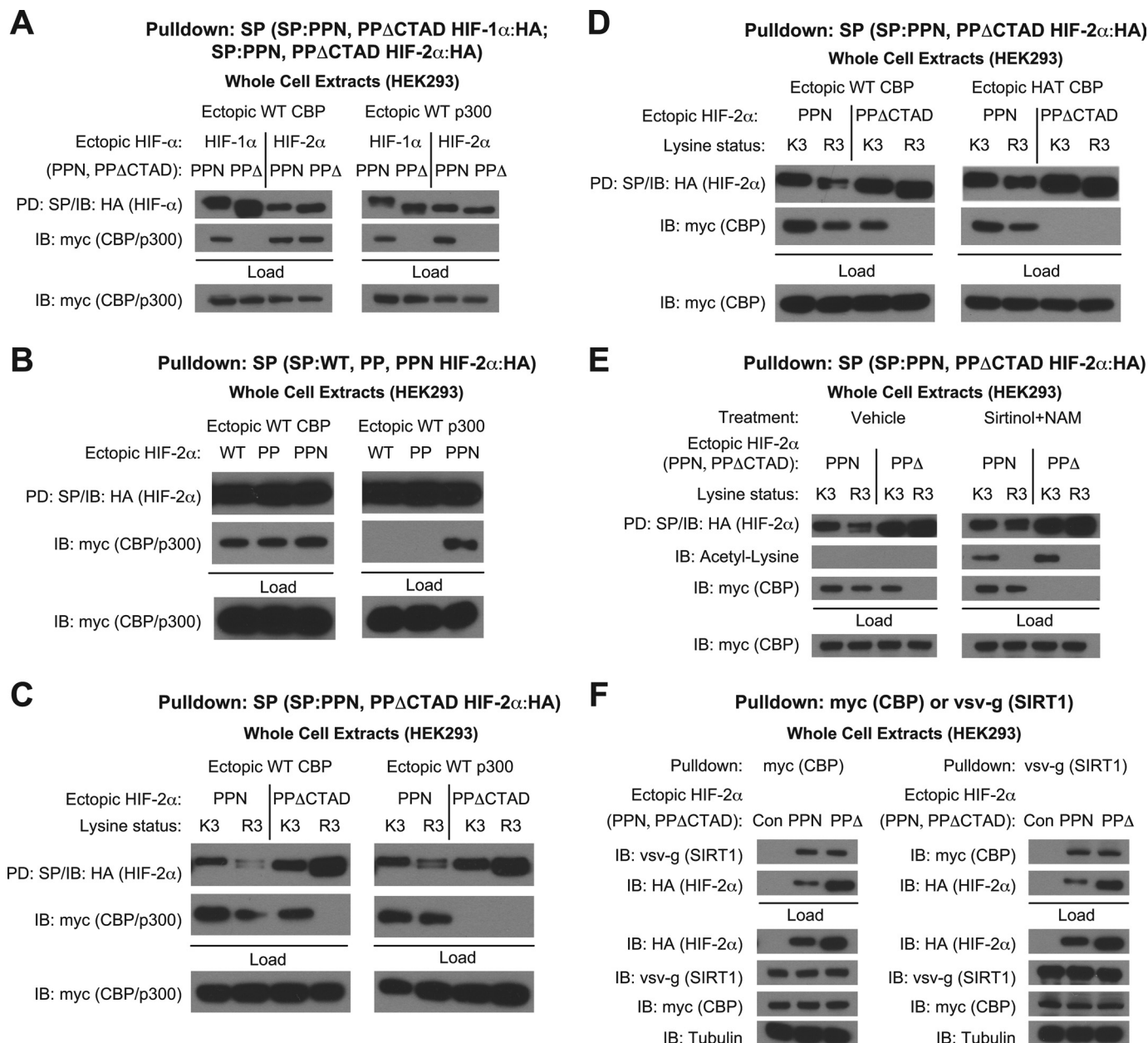


FIGURE 6. CBP and p300 differ in HIF-2 α binding requirements. *A*, interaction assays during normoxia of ectopic wild-type (WT) CBP or p300 with ectopic HIF-1 α or HIF-2 α containing substitution mutations for the hydroxylated prolines alone (PP) or with a substitution mutation for the hydroxylated asparagine (PPN). Following SP-agarose pulldown (PD), immunoblotting (IB) was performed for ectopic HIF-2 α (HA) or CBP or p300 (myc). *B*, interaction assays of ectopic CBP or p300 with ectopic full-length WT, PP, or PPN HIF-2 α . *C*, interaction assays of ectopic CBP or p300 with PPN HIF-2 α or a deletion mutant lacking the CTAD (PP Δ), each containing either intact lysine residues acetylated during hypoxia (K3) or arginine substitutions for these residues (R3). *D*, interaction assays of ectopic wild-type (WT) CBP or HAT mutant CBP along with K3 or R3 PPN HIF-2 α , or with K3 or R3 PP Δ HIF-2 α . *E*, interaction assays of ectopic CBP with K3 or R3 PPN HIF-2 α , or with K3 or R3 PP Δ HIF-2 α following pretreatment with vehicle or sirtinol plus NAM. To confirm Sirt1 inhibition, immunoblotting was performed for acetyl-lysine. *F*, macromolecular complex formation with cells expressing ectopic Sirt1 and CBP and either control (Con), PPN HIF-2 α , or PP Δ HIF-2 α . Interactions were assessed after initial immunoprecipitation of either ectopic CBP (myc) or ectopic Sirt1 (VSV-G), followed by immunoblotting for Myc, VSV-G, and HA (HIF-2 α).

and even fewer examples of selective acetylation by, CBP and p300 (19).

HIF-1 (2, 20) and HIF-2 (3, 21–24) contribute to regulation of *Epo* gene expression to a differing extent in cells and in mice. Candidate acetyltransferases that modulate HIF signaling include p300/CBP (25) and PCAF/GCN5 (26) family members. Most studies have focused on HIF-1 α interactions with p300 (25, 27) or CBP (27–29), used isolated domains rather than full-length HIF protein (30–32), or used synthetic reporters

instead of measuring endogenous target gene expression (31). A recent report examined the role of CBP and p300 in HIF-dependent *Epo* regulation (33), but primarily used desferrioxamine for HIF activation, which differs in its mechanism of activation compared with hypoxia (34). Thus, the role of endogenous p300, CBP, or other acetyltransferases in transactivation of HIF signaling during hypoxia was unclear.

Our experiments focused on two hypoxia-induced genes, *Pgk1* and *Epo*, and revealed unique aspects of CBP and p300 in

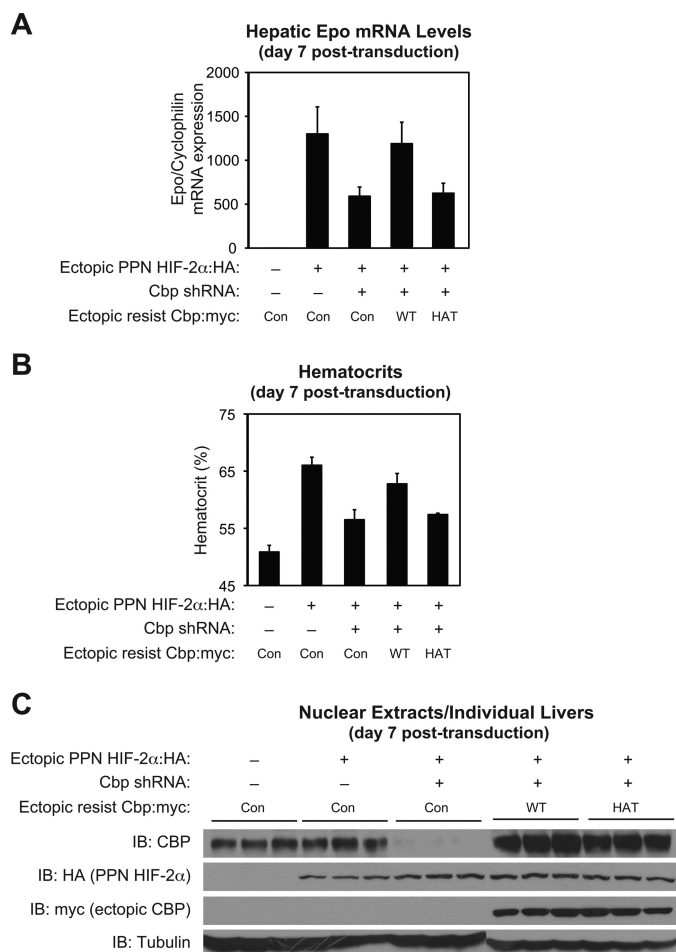


FIGURE 7. CBP regulates HIF-2 signaling in mice. *A*, *Epo* gene expression in mouse livers 1 week after transduction with a first adenovirus expressing control or ectopic oxygen-insensitive (PPN) HIF-2α:HA followed by control (–) or CBP (+) shRNA and with a second adenovirus expressing control (Con) or shRNA-resistant (resist) cDNA encoding ectopic Myc-tagged wild-type (WT) or HAT domain CBP (mean ± S.D.; *n* = 4 per group; all comparisons by one-way ANOVA with PPN HIF-2α/control shRNA/control expression vector as reference; *p* < 0.04 for CBP shRNA (+) with control expression vector; *p* < 0.05 for CBP shRNA with ectopic HAT CBP). *B*, hematocrits of same mice (*p* < 0.0001 for CBP shRNA (+) with control expression vector, *p* < 0.0001 for CBP shRNA with ectopic HAT CBP). *C*, immunoblot (IB) of ectopic CBP (myc) or PPN HIF-2α (HA), endogenous CBP, or tubulin in individual livers.

HIF signaling. During hypoxia, HIF-2 acts primarily in conjunction with CBP, which acetylates HIF-2α, to regulate *Epo* induction. In comparison, p300, which does not acetylate HIF-2α, plays a minor, if any, role in HIF-2-mediated *Epo* induction. However, p300 acts as a major coactivator for factors other than HIF-2 that also regulate *Epo* induction, the combined effect of which is comparable with CBP/HIF-2 signaling, but the stimulatory action of p300 does not require its acetyltransferase activity.

HIF-1 is the only HIF that induces *Pgk1* during hypoxia, and it requires p300, but not CBP, in Hep3B cells. Other coactivators may be involved in HIF-1 signaling as p300 knockdown severely blunted, but did not eliminate, hypoxia induction of *Pgk1*. Src members, which serve as adaptor proteins between p300/CBP or PCAF and transcription factors and also reportedly have weak acetyltransferase activity, contribute to HIF-1 signaling, possibly through interactions with CBP (27, 28).

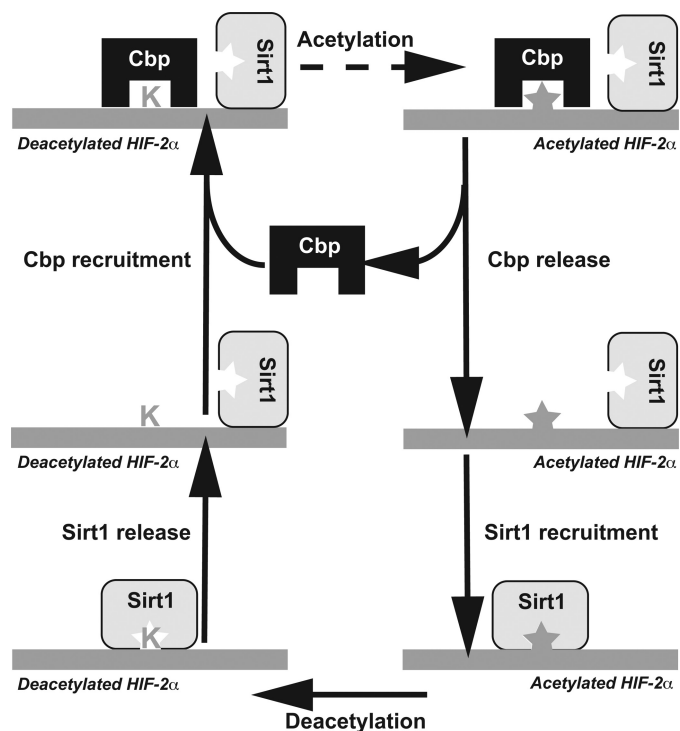


FIGURE 8. Sirt1/CBP regulates CTAD-independent HIF-2 signaling. When the asparagine residue in the HIF-2α CTAD remains hydroxylated by active FIH-1 (not indicated), CBP, and p300 cannot interact with HIF-2α through the CTAD. However, deacetylated HIF-2α is nevertheless capable of recruiting CBP independent of the CTAD through a process of complex formation that requires intact enzyme (CBP acetyltransferase domain) and substrate (HIF-2α lysine residues) determinants. Acetylation of HIF-2α by CBP (dotted line) is likely the rate-limiting and slow step in the acetylation/deacetylation process. After acetylation, CBP cannot remain bound to, and therefore dissociates from, acetylated HIF-2α. Acetylated HIF-2α is then deacetylated by Sirt1, which associates with HIF-2α irrespective of its acetylation status (3). Deacetylated HIF-2α can now engage CBP to reinitiate the entire cyclical process.

However, Src members also bind to the HIF-1/2β protein ARNT and prevent formation of HIF-1α/ARNT dimers (35), the presumed target of CBP (29). Thus, it is difficult to predict what role, if any, Src would play in CBP acetylation, binding, or activation of HIF-2α during hypoxia.

In contrast to a previous study (36), we have no evidence to date that ectopic or endogenous HIF-1α is acetylated to a substantial degree or is deacetylated by Sirt1 during acute hypoxia in HEK293, HT1080, or Hep3B cells (3, 37). Moreover, we did not find that PCAF or Sirt1 have a direct role in HIF-1 signaling as assessed by the effect of knockdown on *Pgk1* gene expression induced by hypoxia or by ectopic oxygen-insensitive HIF-1α. However, Sirt1 and HIF-1 signaling are intricately linked in other respects as Sirt1 itself is induced during acute hypoxia in an HIF-1 and HIF-2 codependent manner (37).

Although CBP, rather than p300, evidently plays a selective role in Sirt1/HIF-2 induction of *Epo* gene expression, and p300, but not CBP, clearly is important for HIF-1-mediated induction of *Pgk1* in Hep3B cells during hypoxia, generalizations of p300 and CBP selectivity in HIF signaling should not be made for several reasons. First, the architecture of specific HIF-responsive regulatory regions may also dictate CBP or p300 responsiveness (38). Second, p300 and CBP association with regulatory regions does not necessarily imply function as many genes,

including hypoxia-induced genes (39), recruit, but are not regulated by, p300 and CBP (38). Third, cell-specific factors may also dictate p300 or CBP function in HIF signaling. Nevertheless, interaction of CBP with and its subsequent acetylation of HIF-2 α suggest CBP is a key coactivator of HIF-2 signaling.

Acetylation of HIF-2 α , which is conferred by CBP, is not simply an activating modification. Rather, the coupled activities of CBP and Sirt1 are required for maximal HIF-2 signaling. Because prevention of either HIF-2 α acetylation or deacetylation inhibits stable CTAD-independent CBP-HIF-2 α complex formation and HIF-2 signaling, we postulate that cyclical acetylation and deacetylation facilitates CTAD-independent recruitment and retention of CBP onto HIF-2 α (Fig. 8), particularly at times when FIH-1, but not PHDs, remains active (40, 41).

Among the p300/CBP and PCAF/GCN5 acetyltransferases, only CBP acetylates HIF-2 α during hypoxia. Future studies will be needed to define the precise molecular determinants for substrate recognition, acetylation, and transactivation of HIF-2 α by CBP, as well as to determine precisely how Sirt1 deacetylation acts in conjunction with CBP acetylation to augment HIF-2 signaling. Identifying other scenarios besides hypoxia in which CBP/Sirt1 augments HIF-2 signaling may also broaden the role of this signal transduction pathway in mammalian physiology and stress response.

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