Hypoxia induces a novel signature of chromatin modifications and global repression of transcription

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Abstract

Tumor cells respond to the harsh hypoxic microenvironment, in part, by transcriptional regulation of specific target genes. We found that hypoxia-mediated activation of selected genes occurs amidst widespread repression of transcription that is neither cell type-specific nor HIF-1-dependent. Despite overall repression, hypoxia induces a pool of histone modifications typically associated with transcriptional activation or repression. Chromatin immunoprecipitation analyses showed that this global mixture of hypoxia-modified histones is sorted in a gene-specific manner to correlate with transcriptional response to hypoxia. Exceptions to this were unexpected increases in H3K4me3 levels, typically associated with transcriptional activation, and decreased H3K27me3 levels, generally a marker of transcriptional silencing, at core promoters of both hypoxia-activated and -repressed genes. These data suggest that a novel signature of chromatin modifications is induced under hypoxic stress, which may play a role in gene regulatory switches active in proliferating tumor cells undergoing cycles of hypoxia and reoxygenation.

1. Introduction

Tissue hypoxia, or low oxygen tension, occurs as a result of inadequate blood flow to tissues or reduced oxygen transport capacity. Within normal cells, hypoxic stress generally leads to cell cycle arrest, apoptosis, and/or necrosis [1]. However, cells within tumors, which often have transient and chronic areas of hypoxia or anoxia, can survive and proliferate in this adverse environment by inducing genes that increase angiogenesis, glycolysis and/or oppose cell death. These hypoxia-induced changes in gene expression result in a more clinically aggressive phenotype with increased resistance to typical cancer treatments [2].

Stress-induced changes in gene expression are accompanied by or a result of changes in chromatin structure; however, the relationship between hypoxia-regulated alterations of chromatin and downstream effects on transcription is addressed in relatively few studies [3]. It is known that hypoxia-inducible factor 1 (HIF-1) drives much of hypoxic gene activation and interacts with histone acetyltransferases p300, CBP and SRC-1 [4]. Although the interaction between HIF-1 and CBP/p300 is critical for the activation of a large percentage of

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hypoxia-inducible genes [5], there are few determinations of histone acetylation levels at the promoters of hypoxia-responsive genes [6]. Additionally, the variety of histone modifications investigated globally and at the promoters of hypoxia-responsive genes is even more limited.

In this study, we found that hypoxia causes widespread repression of total RNA and mRNA synthesis, independently of HIF-1, and globally induces a mixture of histone modifications, typically associated with either transcriptional activation or repression. In general, our chromatin immunoprecipitation (ChIP) analyses of core promoters of hypoxia-repressed and -activated genes showed that gene-specific profiles of repressed or activated chromatin structure correlate with hypoxia-regulated, decreased or increased gene expression. However, at each hypoxic-responsive promoters tested, whether activated or repressed, we found increased H3K4me3 and decreased H3K27me3 induced by hypoxia. These specific exceptions to the generalities of the "histone code" [7] reveal a hypoxia-induced signature of histone modifications that may be indicative of the often transient state of tumor hypoxia.

2. Materials and Methods

2.1. Cells and treatments

Hepa 1–6 (ATCC) were cultured as in [8]. HIF-1α. null MEFs and HIF-1β deficient (Hepa-1C4) cell lines were obtained as in [9]. To achieve <0.01% oxygen, cells were treated as in [10]. Other oxygen concentrations were generated in a Ruskinn Invivo2 400 Hypoxia Workstation. 150μM CoCl (in H_2O, Sigma, CAS# 7791-13-1) or 10μg/mL actinomycin D (Sigma, CAS# 50-76-0)was added to fresh media.

2.2. Cell extracts and immunoblot analysis

Preparation of histone-enriched fractions is described in supplemental information. Proteins were separated on a 15% SDS-PAGE gel, transferred onto a PVDF membrane, and probed with antibodies indicated in supplemental information.

2.3. Pulse-labeling and RNA synthesis analysis

Cells were labeled with 2–4μCi/mL of tritiated uridine (5-^{3}H uridine, Amersham) for 1 hour. RNA was isolated with TRIzol (Invitrogen) (Fig. 1A) or TCA precipitated and collected on GF/C filters (Milipore) (Fig. 1B and C). Poly-adenylated RNA was purified via an Oligotex mRNA miniprep kit (Qiagen).

2.4. RT-PCR

Quantitative PCR was performed and analyzed on an ABI 7500 Fast instrument, from cDNA prepared as described [8]. Primers and PCR conditions are listed in supplemental information.

2.5. Chromatin immunoprecipitation

ChIPs were performed as in [8] with antibodies listed in supplemental information. Fragmentation of chromatin to less than 300bp was verified by electrophoresis and ethidium bromide staining. DNA was analyzed by quantitative PCR; Taqman probes are listed in supplemental information.

3. RESULTS AND DISCUSSION

3.1. Hypoxia induces global repression of transcription independently of HIF1

Gene-specific response to hypoxia is often mediated by the HIF transcription factor and includes both repression and activation of expression [2]. Here, we assessed general transcription levels to define a global, cellular response to hypoxic stress. We pulse-labeled

Hepa 1–6 cells subjected to normoxia or a time-course of hypoxia with tritiated uridine in order to measure ongoing total RNA and mRNA synthesis. After 24h or 48h at 0.2% oxygen, total RNA synthesis was repressed 42% and 65%, respectively (Fig. 1A), and mRNA synthesis was repressed 22% and 51%, respectively (Fig. 1B). Repression of RNA synthesis is dependent upon the severity and duration of hypoxic exposure, as 75% of transcription is repressed in cells exposed to 24h of <0.01% oxygen (Fig 1B, left). Hypoxia-induced, widespread transcriptional repression proved independent of cell type-specificity or tumor origin, as repression of total RNA synthesis occurs in normal primary fibroblasts (Fig. 1B, right). We assessed potential, indirect effects of cellular growth and density by plating cells to achieve cultures of equal density under normoxic and hypoxic incubation conditions (Fig. 1A) or by establishing equal numbers of cells at t=0 and growth to sub-confluent levels (Fig. 1B and C). In all cases, effects of hypoxia in mediating repression of global transcription were apparent over time.

Treatment with $CoCl_2$ mimics hypoxia by inducing HIF-1α protein stability; however, it did not induce substantial transcriptional repression (Fig. 1B), suggesting that HIF-1α is not required for this effect. To test this hypothesis, we measured RNA synthesis in mouse embryonic fibroblast (MEF) cells derived from HIF-1α-null mouse embryos (Fig. 1C left) and in hepatoma cells mutant for HIF-1β (Fig. 1C right). Analyses over a time course of severe hypoxia (<0.01% oxygen) or $CoCl_2$ treatment show that neither loss of functional HIF-1α nor HIF-1β inhibited hypoxia-induced repression of total RNA synthesis. A HIF-1-independent mechanism of widespread transcription repression may facilitate energy conservation during hypoxia, an ATP-limiting stress [11], and allow hypoxic tumor cells to channel their energy toward activation of genes that promote survival.

3.2. Hypoxia-induced global histone modifications

To determine a potential mechanism for the observed, widespread repression of transcription, we conducted an analysis of histone modifications present in extracts of normoxic (21% oxygen) or hypoxic (0.2% oxygen) hepatoma-derived Hepa 1–6 cells (Fig. 2 and Supplemental Fig. 1). During hypoxia, there was 32% loss of H3K9ac, an event generally associated with transcriptional repression; however, we observed an increase in H3K14ac (1.9-fold) and induced histone arginine methylation (H4R3me2, 2.5-fold), which can facilitate acetylation of histones normally associated with activation of transcription [12]. Methylation of lysine residues of histones is associated with either repression or activation of transcription, depending on the specific residue modified by methylation and the extent of methylation. We observed an increase in H3K4me2 (2-fold), H3K4me3 (3.6-fold), and H3K79me2 (1.4-fold) in response to hypoxia, all of which generally correlate with transcriptional activation [13]. Analysis with an antibody that recognizes multiple acetylated lysines on histone H4 showed little quantifiable change between normoxic and hypoxic conditions.

Additional complexity emerged when we investigated histone modifications associated with transcriptional repression. H3K9/27me2 and H3K9me2 levels, which are concentrated at repressed euchromatin and heterochromatin [14], increased 1.6-fold and 1.8-fold, respectively, after hypoxic exposure. Other histone modifications correlated with repression of transcription, H3K27me3 (2-fold) and monomethylated H3K4 (H3K4me1, 2.2-fold), were increased during hypoxia. Taken together, we found that the cellular pool of bulk histones, readily extracted from nuclei and chromatin, is altered in response to hypoxia and comprises a mixture of histone modifications typically associated with transcriptional activation or repression.

3.3. H3 occupancy inversely correlates with gene activity

To assess a direct relationship between hypoxia-induced alterations of chromatin and regulation of transcription at specific genes, we analyzed chromatin structure, at the level of

nucleosomal occupancy and histone modifications, at the core promoters of hypoxia-repressed and -activated genes. We analyzed both *albumin (ALB)*, a constitutively expressed hepatic gene, and *alpha-fetoprotein (AFP)* gene, a marker of hepatocellular carcinoma, as models of hypoxia-repressed genes [10]. To investigate hypoxia-activated genes, we used *early growth response* 1 (*EGR1*), which has both oncogenic and tumor-suppressor properties, and *vascular endothelial growth factor (VEGF)*, an angiogenesis-promoting gene, as models of hypoxia-activated genes [15,16]. We found that endogenous *ALB* and *AFP* are repressed and *EGR1* and *VEGF* are induced in Hepa 1–6 cells during 24 hours of the indicated hypoxic treatments (Fig. 3A).

Whether active core promoters of mammalian genes lie within nucleosome-free regions and/ or density of nucleosomes changes with transcriptional activity has been somewhat controversial [17]. To determine if hypoxia influences nucleosomal occupancy at the core promoters of these genes, we performed ChIP analyses with an antibody that recognizes the C-terminal domain of histone H3 (total histone H3). We observed an increase in total histone H3 occupancy at core promoters of repressed *AFP* and *ALB*, and a decrease at hypoxia-activated *VEGF* and *EGR1* (Fig. 3B). Thus, we saw an inverse relationship between nucleosome occupancy and transcriptional activation that shifts proportionally as a regulatory response to hypoxia. These findings suggest a model of hypoxia-responsive remodeling of chromatin structure with direct links to downstream regulation of transcription.

3.4. Hypoxia-induced, gene-specific histone modifications

We performed ChIP analyses with antibodies that recognize H3K9ac and H3K4me3, markers of transcriptional activation, and H3K9/27me2 and H3K27me3, associated with transcriptional repression, at hypoxia-repressed *AFP* and *ALB*, hypoxia-activated *EGR1* and *VEGF*, and the silent, hypoxia-nonresponsive Brn3b gene. These data were quantified as %-input precipitated by each modified histone antibody, histone H3 antibody, and nonspecific IgG, at each region (Supplemental Fig. 2). Hypoxia induced little change in overall H3K9ac levels at the *AFP* and *ALB* core promoters, relative to normoxia (Suppl. Figs. 2 and 3). However, per bound nucleosome, hypoxia induced a 1.8-fold decrease in H3K9ac and a 3.4-fold increase in H3K9/27me2 at *AFP* (Fig. 4A) and similar trends at repressed *ALB* (Fig. 4B), indicating active changes in these modifications that correlate with transcriptional repression. Interestingly, Brn3-b, which does not alter in its expression in response to hypoxia, showed little alteration in nucleosomal occupancy or in histone modifications with the exception of a 3.5-fold increase in repression marks H3K9/27me2 (Supplemental Fig. 2E). In contrast, at hypoxia-activated *EGR1* and *VEGF*, induction of transcription was marked by increased H3K9ac levels per bound histone H3 and a decrease in total, quantified H3K9/27me2 at both *VEGF* and *EGR1* promoters during hypoxia (Fig. 4C and D; Supplemental Figs. 2 and 3). When H3K9/27me2 was normalized per bound histone H3, *EGR1* exhibited a 41% loss at its core promoter (Fig. 4B), and *VEGF* was relatively unchanged (Fig. 4C) during hypoxia.

Unexpectedly, we saw a marked increase in H3K4me3 levels at all core promoters, whether activated or repressed (Fig. 4A–D), but little was detected at the hypoxia-inert *Brn3b* gene (Suppl. Fig. 2E). The relative change in H3K4me3 induced by hypoxia varied in a gene-specific manner (*AFP>VEGF>EGR1>ALB*), rather than by regulatory response of activation versus repression, likely reflecting gene-specific paradigms of chromatin regulation interwoven with global, hypoxic response. A measured decrease in bound H3K27me3 at all four hypoxia-responsive promoters was equally unanticipated, as H3K27me3 is normally associated with gene repression (Fig. 4A–D). The combination of histone modifications induced by hypoxia, increased H3K4me3 and decreased H3K27me3, is especially surprising at hypoxia-repressed *AFP* and *ALB* promoters.

It is speculated that nucleosomes bearing H3K4me3 reside at genes poised for activation by specific stimuli [18,19]. Genes that are inducible, though minimally transcribed, in resting T-cells contain relatively high levels of H3K4me3 [19]. Notably, bivalent domains consisting of stretches of H3K27me3 and pockets of H3K4me3 are found within mouse embryonic stem cells at silenced, developmentally regulated genes, which are poised for regulatory response during differentiation [18]. In hypoxic cells, which quickly respond to changes in oxygen tension by altering gene expression levels [1], repressed chromatin that is detectably enriched in H3K4me3 and low in H3K27me3 may be rapid activated once hypoxic conditions are reversed.

Our findings of hypoxia-induced, widespread repression of transcription suggest that limited energy resources are directed, independently of HIF-1 activation, toward survival-promoting gene responses under hypoxic stress. Interestingly, the recently discovered H3K27 demethylases can associate as protein complexes with enzymes that methylate H3K4, offering a two-pronged response to stimuli [20]. Hypoxia may additionally mediate rapid control of these histone and protein modifiers by directly altering the reaction components of their enzymatic processes. Both amine oxidases and JmjC-domain proteins demethylate by oxidation or hydroxylation, processes directly affected under hypoxia [21]. One potential mechanism driving increased H3K4me3 may be hypoxia-induced inactivation of H3K4me3-specfic histone demethylases, which utilize molecular oxygen during enzymatic removal of methyl groups [22–24]. The observed modifications of histones provide a hypoxia-regulated platform for binding and interactions of additional chromatin modifiers, remodelers or regulators of transcription, further amplifying gene-specific response to hypoxic stress.

A bivalent, chromatin signature of increased H3K4me3 and decreased H3K27me3 under hypoxia may poise hypoxia-responsive chromatin and facilitate downstream flexibility in response to induced or inhibited trans-acting regulators of gene expression. Identification of "hypoxia-signature" alterations in chromatin structure may further guide use or development of therapeutic, "epigenetic" agents that target specific histone modifications and shift hypoxia-regulated gene expression patterns. Further studies are needed to understand how chromatin responds to the demands of fluctuating signals, such as cycles of hypoxia and reoxygenation, and its role in mechanisms that mediate rapid changes in gene regulation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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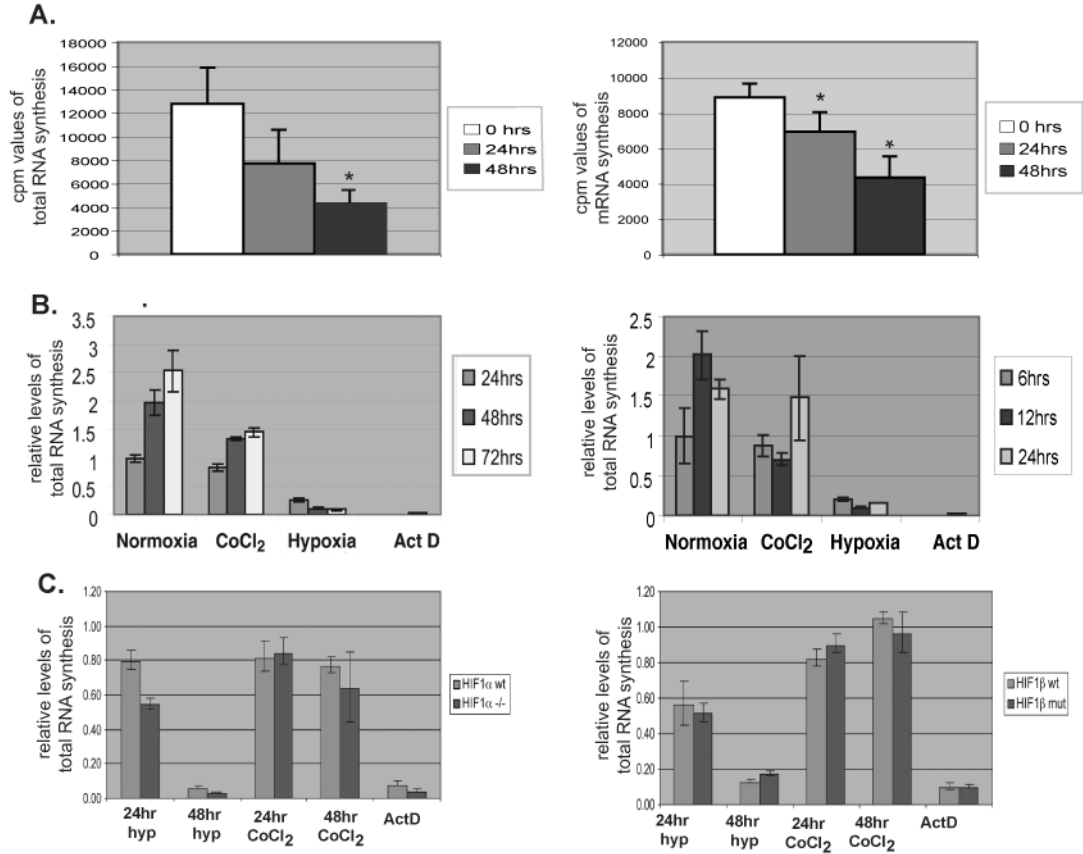
Fig. 1. Hypoxia induces global repression of total RNA and mRNA synthesis
A) Total RNA (left) and mRNA (right) labeled with tritiated uridine (cpm) during 1 hr synthesis in Hepa 1–6 cells subjected to normoxia or a time-course of 0.2% oxygen. Values, normalized to cell number, represent an average of 5 replicates. Error bars denote SD. *P<0.005. B) Relative total RNA synthesis in three replicates, Hepa 1–6 cells (left) or normal human fibroblasts (right) subjected to indicated time of normoxia, $CoCl_2$ treatment, <0.01% oxygen (hypoxia), or actinomycin D. C) Relative total RNA synthesis in immortalized HIF-1α-null MEFs (left) or HIF-1β mutant hepatoma cells (right) exposed for indicated time as in (B). Values normalized to normoxic controls.
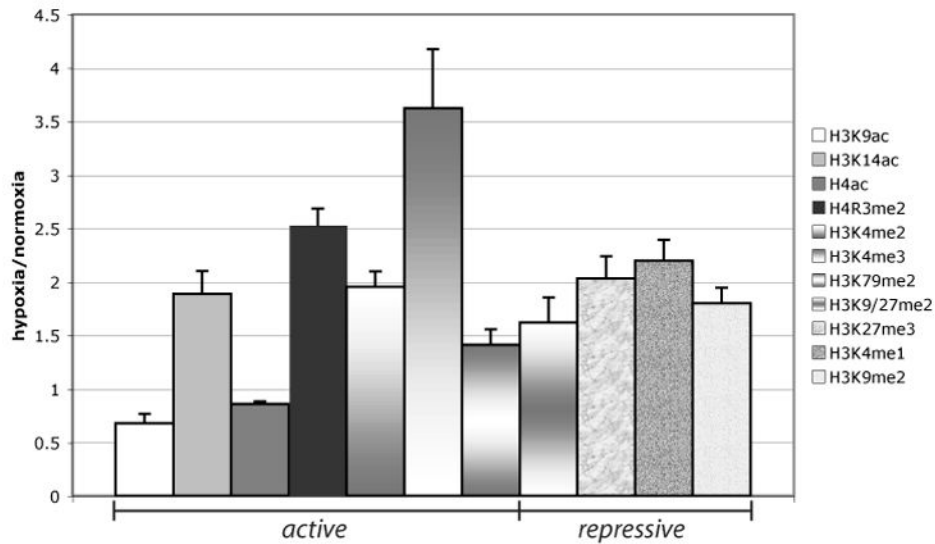
Fig. 2. Hypoxia induces altered post-translational modifications of total histone populations
Modifications of histones, associated with transcriptional activation and repression, were determined by immunoblotting of protein extracts of Hepa 1–6 cells grown under normoxia or 0.2% oxygen for 48 hrs (representative blots, Supplemental Fig. 1). Quantified values, generated by NIH image, were normalized to H3-C-terminus, and fold change (H/N) is graphed. The average was generated from quantified values, obtained by NIH image, from three independent immunoblots of three independently obtained normoxic and hypoxic extracts. Error bars denote the SEM.
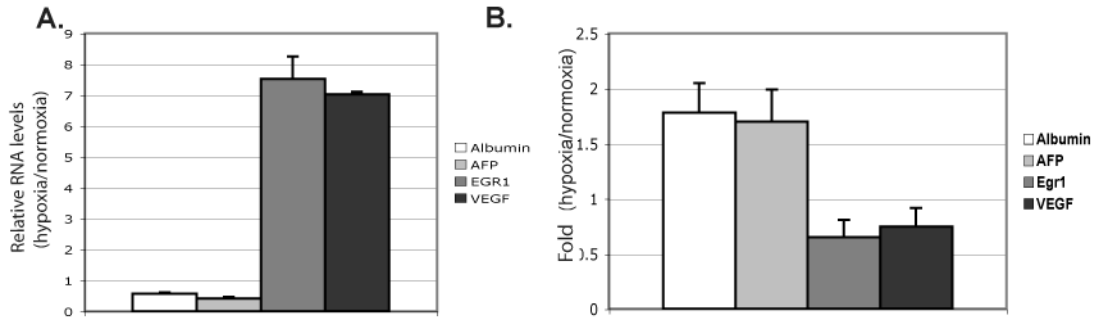
Fig. 3. Nucleosome-occupancy at specific core promoters inversely correlates with transcription activation in response to hypoxia
A) Relative levels of *Albumin*, *AFP*, *EGR1*, *VEGF*, and *Albumin* expression (hypoxia/ normoxia), generated by quantitative RT-PCR, in Hepa 1–6 cells exposed for 48h to normoxia or 0.2% oxygen; each sample normalized to *18S*. An average of at least four experiments is shown, and error bars denote SD. B) ChIP of histone H3 in Hepa 1–6 cells after 48h at normoxia or 0.2% oxygen, followed by quantitative PCR of core promoter regions of *Albumin*, *AFP*, *EGR1*, and *VEGF*. The %-input bound by histone H3, minus background (IgG), in hypoxia relative to normoxia shown. An average of at least three independent experiments shown, each PCR performed in duplicate, with error bars representing SD.
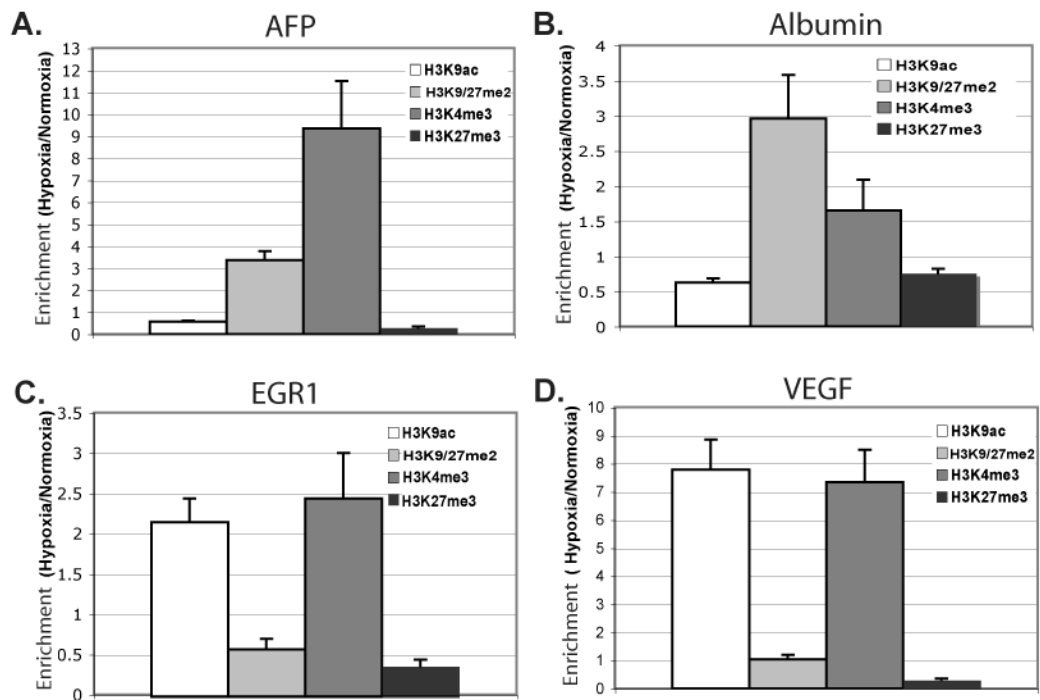
Fig. 4. Specific histone modifications induced at hypoxia-responsive genes
ChIP of indicated modified histones in Hepa 1–6 cells after 48h at normoxia or 0.2% oxygen, followed by quantitative PCR for core promoter regions of *AFP* (A), *Albumin* (B), *EGR1* (C), and *VEGF* (D). The %-input bound by each modified histone H3, minus background (IgG), was normalized by total bound histone H3; fold-change hypoxia versus normoxia is graphed. Averages represent of two to three independent experiments, each experiment quantified in duplicate, with error bars representing SEM.