Hypoxic Repression of Endothelial Nitric-oxide Synthase Transcription Is Coupled with Eviction of Promoter Histones*

Received for publication, September 22, 2009, and in revised form, October 26, 2009 Published, JBC Papers in Press, October 30, 2009, DOI 10.1074/jbc.M109067868

Jason E. Fish†§1, Matthew S. Yan‡2, Charles C. Matouk§, Rosanne St. Bernard*, J. J. David Ho§, Anna Gavryushova§, Deepak Srivastava†, and Philip A. Marsden*§3

From the †Department of Medical Biophysics, University of Toronto, Toronto, Ontario M5S 1A8, Canada, the ‡Renal Division and Department of Medicine, St. Michael’s Hospital and University of Toronto, Toronto, Ontario M5B 1W8, Canada, and the §Gladstone Institute of Cardiovascular Disease and University of California, San Francisco, California, 94158

Hypoxia elicits endothelial dysfunction, in part, through reduced expression of endothelial nitric-oxide synthase (eNOS). Here we present evidence that hypoxia causes a rapid decrease in the transcription of the eNOS/NOS3 gene, accompanied by decreased acetylation and lysine 4 (histone H3) methylation of eNOS proximal promoter histones. Surprisingly, we demonstrate that histones are rapidly evicted from the eNOS proximal promoter during hypoxia. We also demonstrate endothelium-specific H2A.Z incorporation at the eNOS promoter and find that H2A.Z is also evicted by hypoxic stimulation. After longer durations of hypoxia, histones are reincorporated at the eNOS promoter, but these histones lack substantial histone acetylation. Additionally, we identify a key role for the chromatin remodeler, BRG1, in re-establishing eNOS expression following reoxygenation of hypoxic cells. We posit that post-translational histone modifications are required to maintain constitutive eNOS transcriptional activity and that histone eviction rapidly resets histone marks and is a proximal event in the hypoxic repression of eNOS. Although nucleosome eviction has been reported in models of transcriptional activation, the observation that eviction can also accompany transcriptional repression in hypoxic mammalian cells argues that eviction may be broadly relevant to both positive and negative changes in transcription.

Hypoxia has long been associated with alterations to blood vessel function. For example, hypoxia elicits dramatic changes in the expression of genes in the vascular endothelium (1). These gene expression changes result in alterations to endothelial phenotype and can ultimately result in endothelial activation and dysfunction. It is known, for example, that the vasoconstrictor, endothelin-1 (2), and the mitogen, platelet-derived growth factor β (3), are induced in endothelial cells exposed to hypoxic conditions. In contrast, hypoxia potently decreases the expression of eNOS in endothelial cells by both transcriptional and post-transcriptional mechanisms (4). In the pulmonary circulation, hypoxia induces vasoconstriction that is thought to match ventilation to perfusion (reviewed in Ref. 5). This vasoconstriction can be partly attributed to decreased nitric oxide (NO)-elicited effects on vascular tone (reviewed in Ref. 6). For example, loss of eNOS-derived NO contributes to the phenotype of pulmonary hypertension (7–9), whereas reintroduction of the NOS3 gene in NOS3−/− mice can reverse pulmonary vascular defects (10).

Although the transcription of several genes, including eNOS, is decreased upon exposure to hypoxic conditions, it is not clear what mechanisms underlie these changes. Models of transcriptional repression by hypoxia include the induction of transcriptional repressors (11–15) and direct inhibition elicited by HIF binding to proximal promoter elements (16–18). We found that the chromatin structure at the eNOS proximal promoter and 5′-coding region plays a prominent role in regulating the constitutive transcriptional activity of the NOS3 gene (19). A specific histone code exists at these regions in endothelial cells, consisting of acetylation of the N terminus of histones H3 and H4, particularly at lysine 9 of histone H3 and lysine 12 of histone H4, and di- and trimethylation of lysine 4 of histone H3 (H3K4Me). We considered whether alterations to the histone code of the NOS3 gene may play a causative role in the down-regulation of eNOS transcription that occurs following exposure of endothelial cells to pathological stimuli, such as hypoxia. Indeed, several studies have pointed to a role for modulation to chromatin structure in hypoxic gene repression. For example, silencing of MLH1 by hypoxia is reversible upon treatment with histone deacetylase (HDAC) inhibitors (20), and the expression of p53 and von Hippel Lindau mRNA is repressed by HDAC induction in hypoxic endothelial cells (21). Additionally, the CAMP-mediated transcriptional induction of surfactant protein A is also compromised under hypoxic conditions. Reduced histone acetylation and increased lysine 9 methylation of histone H3 (H3K9Me) at the promoter of this gene have been

* The recipient of a CIHR Frederick Banting and Charles Best Canada Graduate Scholarship.
† Recipient of an Natural Sciences and Engineering Research Council Canada Graduate Scholarship.
‡ The recipient of a CIHR Frederick Banting and Charles Best Canada Graduate Scholarship.
§ Recipient of a Career Investigator Award from the Heart and Stroke Foundation of Canada. To whom correspondence should be addressed: Rm. 7358, Medical Sciences Bldg., University of Toronto, 1 King’s College Circle, Toronto, Ontario M5S 1A8, Canada. Tel.: 416-978-2441; Fax: 416-978-8765; E-mail: p.marsden@utoronto.ca.

The on-line version of this article (available at http://www.jbc.org) contains supplemental Figs. S1–S3.

‡§1 Recipient of an Natural Sciences and Engineering Research Council Canada Graduate Scholarship.
§2 Recipient of a scholarship.
* This work was supported by Grant CIHR MOP 79475 from the Canadian Institutes of Health Research (CIHR).

† This article was selected as a Paper of the Week.

The abbreviations used are: eNOS, endothelial nitric-oxide synthase; NO, nitric oxide; pol II, RNA polymerase II; HIF, hypoxia-inducible factor; HRE, HIF-responsive element; HDAC, histone deacetylase; HAT, histone acetyltransferase; VEGF, vascular endothelial growth factor; CREB, cAMP-response element-binding protein; BHQ, butylhydroquinone; ORF, open reading frame; HUVEC, human umbilical vein endothelial cell; VSMC, vascular smooth muscle cell; ChIP, chromatin immunoprecipitation; IP DNA, immunoprecipitated DNA; siRNA, small interfering RNA; RT-PCR, reverse transcription-PCR; CHART-PCR, chromatin accessibility and real-time PCR.

810 JOURNAL OF BIOLOGICAL CHEMISTRY
suggested to be important in this hypoxic repression (22). Similar hypoxia-dependent global and gene-specific increases in H3K9Me (23) and decreases in global levels of histone H4 acetylation (24) have also been described by others. Taken together, these findings suggest that hypoxia negatively regulates gene expression, in part, through chromatin-based pathways.

To determine whether modulation of chromatin structure is involved in eNOS repression during hypoxia, we exposed endothelial cells to hypoxic conditions and assessed the changes in post-translational histone modifications at the eNOS promoter. We found that hypoxia decreased histone acetylation and H3K4Me at the eNOS promoter, even after very short durations of hypoxic exposure. We considered whether the histones were being deacetylated in a general fashion by HDAC activity or whether the histones might actually be completely removed upon hypoxic stimulation. Here we present evidence that argues against enzymatic deacetylation of eNOS promoter histones during short term hypoxia. By assessing the total levels of histone H3 and H4 at the eNOS proximal promoter, we found that histones were rapidly evicted during short term hypoxia and were reincorporated following long term hypoxia. We also found that the H2A variant, H2A.Z, was basally present at the eNOS promoter and was also transiently evicted from the eNOS proximal promoter during hypoxia. The eviction of histones was highly localized; the eNOS proximal promoter lost histone proteins, whereas regions upstream of the eNOS promoter were not affected. The histones (H3 and H4) that were reincorporated at the eNOS promoter following long term hypoxia were functionally distinct from eNOS promoter histones present during normoxic conditions because they were not highly acetylated. Additionally, using siRNA to target nucleosome remodeling proteins, we identified an unexpected role for Brahma-related gene 1 (BRG1) in the re-establishment of eNOS expression following reoxygcnation of hypoxic endothelial cells. We hypothesize that nucleosome eviction is a rapid method to remove post-translational histone modifications that are required for eNOS transcriptional activity. This is the first demonstration, to our knowledge, of a role for histone eviction in mammalian gene repression and provides a new paradigm for hypoxic gene regulation.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Hypoxia Model**—Human umbilical vein endothelial cells (HUVEC) were cultured and characterized as described previously (25), and experiments were performed in early passage cells (passages 3–4). Hypoxia experiments were performed using a ThermoForma (Marietta, OH) normobaric anoxia chamber that maintains less than 1% oxygen. This was established by the use of ultra-high purity gases (5% CO2, 10% O2, and 85% N2, Praxair, Mississauga, Ontario, Canada).

**Antibodies Used**—Antibodies used for chromatin immunoprecipitation and Western blot experiments were purchased from Millipore (Lake Placid, NY) (anti-acetyl-histone H3 (Lys-9 and Lys-14) (catalog number 06-599), anti-acetyl-histone H3 (Lys-9) (06-942), anti-acetyl-histone H3 (Lys-14) (06-911), anti-acetyl-histone H4 (Lys-5, Lys-8, Lys-12, and Lys-16) (06-866), anti-acetyl-histone H4 (Lys-5) (06-759), anti-acetyl-histone H4 (Lys-8) (06-760), anti-acetyl-histone H4 (Lys-12) (06-761), anti-acetyl-histone H4 (Lys-16, chromatin immunoprecipitation (ChIP) grade) (07-329), anti-dimethyl-histone H3 (Lys-4) (07-030), anti-histone H4 (unmodified/pan) (05-858); from Santa Cruz Biotechnology (Santa Cruz, CA) (anti-pol II (N-20; antibody number sc-899), anti-BRG1 (H-88; sc-10768), anti-BRM (N-19; sc-6450), anti-Lamin A/C (346: sc-7293)); and from Abcam (Cambridge, MA) (anti-histone H3 (ab1791), anti-dimethyl-histone H3 (Lys-9) (ab7312)). For BRM Western blots, an anti-BRM antibody was used from Abcam (ab15597). Normal rabbit IgG (sc-2027) was purchased from Santa Cruz Biotechnology. H2A.Z and NC2 antibodies were kind gifts from Benoit Guillemette (Universite de Sherbrooke, Quebec, Canada) (26), and T. Oelgeschlager (The Rockefeller University, New York, NY) (27), respectively.

**RNA Interference**—40 nM siRNA was transfected into 90% confluent HUVEC grown on 60-mm gelatin-coated tissue culture plates using 33 μl of Oligofectamine transfection reagent (Invitrogen) in a total volume of 2000 μl. The BRG1, BRM, and control siRNAs were from Santa Cruz Biotechnology (sc-29831, sc-29827, and sc-37007, respectively). A second independent set of Stealth control and BRG1 siRNAs from Invitrogen were also used (12935-300 and 1299003, respectively). Following 3 h of transfection at 37 °C in Opti-MEM medium (Invitrogen), M199 medium (Invitrogen) containing fetal bovine serum (HyClone), heparin, and endothelial cell growth supplementation (Biomedical Technologies) was added. Cells were cultured for 18 h and were then transferred to the hypoxia chamber and grown for an additional 24 h. In some experiments, hypoxic cells were returned to a normoxic environment for an additional 24 h.

**Real-time RT-PCR**—2 μg of DNase I-treated (5 units, 30 min, 37 °C; Fermentas, Burlington, Ontario, Canada) total cellular RNA were treated with heparinase (1 unit, 1 h, room temperature) and then used in first-strand cDNA synthesis using random primers and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer’s recommendations. cDNA was diluted to a final volume of 120 μl. All quantitative reverse transcriptase-PCR analyses were performed in triplicate on 2 μl of cDNA using the ABI PRISM 7900 HT sequence detection system (Applied Biosystems, Foster City, CA). Quantification of eNOS, and cyclophilin A was performed using TaqMan® technology. Human eNOS mRNA was quantified using primers spanning the junction between exon 11 and 12: 5′-GCG ATC ACC AGG AAG AAG ACC-3′, 5′-TCA CTC GTC TCG CCA TCA C-3′, and probe 5′-FAM TM CCA ACG CCG TGA AGA TCT CCG C TAMRA TM-3′. Human cyclophilin A/CPYA mRNA (exon 1/2) was quantified using primers 5′-GAC GCC GAG CCC TTT G-3′ and 5′-TCT GCT TTT GGC ACC TTG T-3′ and probe 5′-FAM CGC GTC TCT TTT GAG CTT TGG GCA BHQ-3′. Levels of 18S, VEGFA, BRM, and BRG1 were quantified using SYBR Green chemistry. 18S was quantified using the following primer set: 5′-AGG AAT TGA CCG AAG GGC AC-3′ and 5′-GGA CAT CTA AGG AGG CCA TCA CA-3′. VEGFA mRNA levels were quantified using primers 5′-GCA GAC CAA AGA AAG ATA GAC CAA G-3′ and 5′-CGC CTC GCC TTT GCA CAT-3′ (28). The forward and reverse primers are located in exon 4 and 8, respectively, and detect all alterna-
Hypoxic Repression of eNOS Transcription

Real-time RT-PCR Analysis of eNOS Pre-mRNA—Analysis of unspliced eNOS pre-mRNA was determined by reverse transcribing (Superscript II, Invitrogen) 2 μg of DNase I (5 units; Fermentas) and heparinase-treated (1 unit (Sigma), 1 h at room temperature) total cellular RNA and diluting to a final volume of 120 μL. Real-time RT-PCR was performed on 2 μL of first-strand cDNA and performed in triplicate on multiple biological replicates. To exclude the presence of contaminating genomic DNA, a similar analysis was performed in the absence of reverse transcriptase. This analysis revealed that <0.5% of the pre-mRNA copies could be attributed to genomic DNA contamination. Copies of pre-mRNA were quantified by comparison with a standard curve of known concentrations of genomic DNA, where 1 ng of genomic DNA was taken to reflect 300 copies of a single copy gene in the haploid genome. Real-time amplicons were located in exon 1/intron 1, intron 1/exon 2, intron 4/exon 5, exon 14/intron 14, and intron 25/exon 26 of the NOS3 gene (Table 1). The intron 25/exon 26 primer pair also amplifies the cis-antisense transcribed transcripts. BRM was quantified using primers 5′-GGG TCC CAG TCC TAC TAC ACC GT-3′ and 5′-TTT CCA AGC CCC ATTT TCA TCG-3′. BRG1 was quantified using primers 5′-CCT TCC TAC TCG TGC TTC TGG TCC-3′ and 5′-GCC AGA AGG TGG AGA GTG CTG G-3′.

Western Blotting—Histones were harvested from hypoxia-treated HUVEC as described previously (19). Total cellular protein was isolated in Laemmli buffer. The following antibodies were used: anti-acetyl histone H3 (Upstate Biotechnology: 06-599), anti-acetyl lysine (Upstate Biotechnology: 06-933) (to detect histone H4 acetylation), anti-histone H3 (Abcam: ab1791), anti-BRG1 (Santa Cruz Biotechnology: sc-10768), anti-BRM (Abcam: ab15597), and anti-Lamin A/C (Santa Cruz Biotechnology: sc-7293). Detection was performed using an horseradish peroxidase-conjugated secondary antibody with SuperSignal West Pico chemiluminescent substrate (Pierce) and the Fluor-S Max multimager (model 170-772), Bio-Rad.

ChiP—ChiP was performed essentially as described previously (19, 30). Briefly, ~1 × 10^6 cells were used per ChiP. Formyl-aldehyde was added directly to the medium to a final concentration of 1% and incubated at 37 °C for 10 min. Sonication was performed on ice using a Sonics and Materials, Inc. Vibra-Cell sonicator with a 3-mm tip set at 30% maximum power using 10-s pulses with a 10-s interval between sonications to completely solubilize chromatin and achieve documented chromatin fragmentation ranging in size between 200 and 400 bp. Chromatin was precleared using 80 μl of salmon-sperm DNA/protein-A/agarose for 2 h. Immunoprecipitation was performed overnight using 2–5 μg of antibody or a background control (no antibody or 5 μg of normal rabbit IgG). An 18-μl (1800 μl total volume) aliquot of chromatin was removed prior to immunoprecipitation to serve as an input control. Immune complexes were collected for 2 h with 60 μl of salmon-sperm DNA/protein-A/agarose. After extensive washing, formaldehyde cross-links were reversed, DNA was purified by proteinase K digestion and phenol/chloroform extraction, and following ethanol precipitation, samples were resuspended in 40 μl of water. Real-time PCR was performed in triplicate on 2 μl of bound chromatin, 2 μl of a no antibody control or control IgG immunoprecipitation, and 2 μl of a 10-fold dilution of input chromatin in a 10-μl reaction. The number of copies of target sequence was determined by comparison with a standard curve using plasmids containing varied human genomic NOS3 fragments (31), a bacterial artificial chromosome clone containing the NOS3 locus, or human genomic DNA, with comparable results. Immunoprecipitated DNA (IP DNA) was determined by subtracting the number of target DNA molecules in the control immunoprecipitation from the number of copies in the immunoprecipitated samples using a specific antibody and dividing by the number in the diluted input sample. In these studies, findings were comparable whether a no antibody control or control IgG was used to control for nonspecific immunoprecipitation. Real-time PCR primer sequences can be found in Table 1.

Luminometric Methylation Assay—Analysis of global levels of DNA methylation in control and hypoxic HUVEC was determined essentially as described (32). This technique takes advantage of an isoschizomer pair that cleaves 5′-CCCGG sites but differs in its methylation sensitivity. HpaII cleaves only unmethylated sequences, whereas MspI cleaves both unmethylated and methylated sites. Genomic DNA (500 ng) derived

---

**Table 1**

| Primer name and location | Primer sequence |
|--------------------------|-----------------|
| eNOS HRE (−5351 to −2500) | 5′-CCT CAG CTC GCT GGT GTG CAT CAG-3′ |
| eNOS promoter (−891 to −797) | 5′-TGA AGG CCC TCT CAG AGA TTC CAG-3′ |
| eNOS promoter (−694 to −564) | 5′-GCT TCC TGC TGG TTC TGG CTC CAG-3′ |
| eNOS promoter (−488 to −398) | 5′-GCC AGA ACC AGC ACT CAG TCA GGA-3′ |
| eNOS promoter (−306 to −210) | 5′-TAC CTT ACT CAG CTC CCA TCG CAG-3′ |
| eNOS promoter (−237 to −112) | 5′-CCA ATG ATC ACC ATG AGT CAA-3′ |
| eNOS promoter (−166 to −66) | 5′-GTG GAG CTC AGG CTT TAG AGC-3′ |
| eNOS exon 1/intron 1 | 5′-TTT CTT TAC GAA GAG GAG GGT-3′ |
| eNOS intron 1/exon 2 | 5′-GCC TCG CAG ACT CAG CTA CAA-3′ |
| eNOS exon 4/exon 5 | 5′-GCC AGA ACC ACC TCT TCC TCA GGA-3′ |
| eNOS exon 14/intron 14 | 5′-AGC TCC GCT GAT GAT GTC CTT-3′ |
| eNOS intron 25/exon 26 | 5′-GCC GGG GGG TTC CTC CAG TCT-3′ |
| Cyclophilin A promoter | 5′-TCA GTC GCA GCA GAG GAG GGT-3′ |
| VEGFA HRE (−934 to −785) | 5′-TCA ACC CCA CCA CCA CCA GAT-3′ |
| E-Cadherin exon 10 | 5′-TGC CAA CAG CAG CAG CAG CAG-3′ |

script, sONE/NOS3AS, which overlaps with NOS3 in a tail-to-tail fashion (29).
from hypoxia-treated HUVEC was digested with HpaII + EcoRI and MspI + EcoRI (New England Biolabs, Ipswich, MA) in two separate 20-μl reactions for 4 h at 37 °C. Digests were subsequently inactivated for 20 min at 65 °C. EcoRI (cleaves 5′-GAAATTCC sequence) was used for normalization. Pyrosequencing using a PSQ96™HS instrument (EpigenDx Inc.) was used to assess each restriction site. The HpaIIMspI ratio was calculated as (HpaII/EcoRI)/(MspI/EcoRI).

**Sodium Bisulfite Genomic Sequencing**—Analysis of DNA methylation was performed as described in Ref. 30. 5 μg of genomic DNA from HUVEC that were exposed to hypoxia for 24 h were bisulfite-treated. The eNOS proximal promoter was PCR-amplified from bisulfite-treated DNA using nested PCR. The outer primer set was 5′-GTG TTA TAT TAT AGA AGG ATT TTT ATG-3′ and 5′-ACC CAA CAA CCA ACC ACC ACC CCA ACC CAAC-3′ (−346 to +95), and the inner primer set was 5′-TGTTT TTG TAT GTT GTA GTT TTA G-3′ and 5′-ACT ACC TAC TCC AAG AAA ACC CTA ACC-3′ (−286 to +3). The PCR product was gel-purified and cloned into pcDNA III vector, and 10–15 individual clones were sequenced.

**Chromatin Accessibility and Real-time PCR (CHART-PCR)**—CHART-PCR was essentially performed as described (33) with minor modifications. Briefly, two 100-mm plates of confluent normoxic, hypoxia-treated HUVEC or normoxic vascular smooth muscle cells were used for CHART-PCR analysis. Cell pellets were lysed for 10 min on ice in 1 ml of lysis buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40, 150 μM spermine, 500 μM spermidine) to release nuclei. Pelleted nuclei were washed with 1 ml of wash buffer (10 mM Tris-HCl, pH 7.4, 15 mM NaCl, 60 mM KCl, 150 μM spermine, 150 μM spermidine) at 4 °C for 10 min, with rotation. Nuclei were resuspended in 700 μl of digestion buffer (wash buffer plus 1 mM CaCl₂). Following optimization of approaches, 100 μl of nuclei were either digested with 7.5 units of micrococcal nuclease (USB Corp., Cleveland, OH) for 5 min or left undigested. The reaction was stopped by adding 100 μl of stop buffer (20 mM EDTA, 2 mM EGTA, 1% SDS) followed by incubation on ice for 10 min. Protein was removed by the addition of 75 μg of proteinase K and digestion overnight at 50 °C followed by phenol-chloroform extraction and ethanol precipitation of DNA. DNA from digested and undigested samples was resuspended in 30 μl of water. Samples were adjusted to 10 ng/μl for real-time PCR analysis. Real-time PCR was performed in triplicate on 2 μl of DNA, and transcript copy number was determined by comparison with a standard curve consisting of serial dilutions of a bacterial artificial chromosome clone containing the NOS3 genomic locus or serial dilutions of genomic DNA (each of which gave similar results). The percentage of accessibility was determined by comparing the number of copies in the digested and undigested DNA samples. See Table 1 for a list of primers used for CHART-PCR analysis.

**Statistics**—Unless otherwise stated, all experiments were performed a minimum of three times, and data represent the mean ± S.E. Statistical analyses were performed using a Student’s t test or analysis of variance and Newman–Keuls post hoc test, as appropriate. A p value less than 0.05 was considered to be statistically significant.

**RESULTS**

**Hypoxia Down-regulates eNOS Transcription**—Prior studies by us and others have investigated hypoxia-mediated decreases in eNOS expression using in vitro approaches (4, 34, 35). We have also previously determined the effect of hypoxic exposure on the transcriptional activity of the chromatin-integrated eNOS promoter in vivo by exposing eNOS insertional promoter/reporter transgenic mice to hypoxia (8%, 48 h) and assessing reporter expression in the endothelial cells of the renal papilla (36). In this transgene, 5.2 kb of the murine eNOS promoter directs expression of the reporter β-galactosidase. Hypoxia dramatically decreased eNOS promoter activity. This was not due to a general decrease in β-galactosidase translation as similar treatment of nNOS promoter/reporter transgenic mice led to an increase in β-galactosidase activity (36). Thus, hypoxia elicits a dramatic decrease in eNOS transcription in a living animal.

To further study the mechanisms of eNOS transcriptional repression in hypoxic endothelial cells, we exposed HUVEC to hypoxic conditions (<1% O₂) for various durations of time. We have previously demonstrated that eNOS mRNA levels decrease following longer durations (6–24 h) of hypoxic exposure (34). Because hypoxia is known to regulate eNOS mRNA levels through both transcriptional and post-transcriptional mechanisms, RNA polymerase II (pol II) ChIP assays of the native eNOS promoter in HUVEC were used to assess the effect of hypoxia on eNOS transcription. We assessed the eNOS proximal promoter (−166 to −26) and a downstream region in the NOS3 gene (exon 14/intron 14). At the proximal promoter of eNOS, hypoxia resulted in a significant decrease in the amount of pol II after only 1 h of hypoxia. Levels of pol II continued to decrease throughout hypoxic treatment, with a 50% reduction in pol II recruitment observed by 24 h (Fig. 1A).

Although we have previously demonstrated that long term hypoxia inhibits eNOS transcription using nuclear run-off analyses (4), these findings reveal that transcriptional repression is extremely rapid, occurring after only 1 h of exposure to hypoxic conditions, and that transcriptional repression continues during long term hypoxia. We previously demonstrated that pol II was highly enriched at the eNOS proximal promoter when compared with the coding region under basal conditions. This suggested that pol II was selectively loaded at the promoter, forming a preinitiation complex (19). Analysis of pol II density in the coding region of NOS3 (exon 14/intron 14) demonstrated decreased basal loading of pol II when compared with the proximal promoter, as we described previously (19). Similar to the proximal promoter, pol II levels in the coding region also decreased rapidly upon exposure of endothelial cells to hypoxia, with a more than 70% decrease in the density of pol II at exon 14/intron 14 evident by 24 h (Fig. 1A). Because others have suggested that a HIF-responsive element (HRE) is involved in eNOS hypoxic regulation of transiently transfected eNOS promoter/reporters (37), we also assessed pol II recruitment to this element in basal and hypoxia-stimulated HUVEC. Only background levels of pol II were present at the putative HRE, and hypoxia did not result in altered pol II recruitment to this region (data not shown).
As an independent method of measuring the transcriptional activity of the NOS3 gene during hypoxia, we measured the levels of eNOS pre-mRNA by real-time RT-PCR (Fig. 1, B and C). Measurement of unspliced pre-mRNA is a surrogate of transcriptional activity due to the very short half-life of such nascent RNA species, which are very rapidly processed in the nucleus co-transcriptionally (38). Quantitative pre-mRNA measurement has been used by others to monitor transcriptional activity (39). Quantification by real-time RT-PCR revealed that the abundance of pre-mRNA generated across the NOS3 locus represented $4.4 \pm 1.2\%$ of the levels of fully processed eNOS mRNA (data not shown). Levels of eNOS pre-mRNA were assessed across the NOS3 locus during hypoxic exposure (Fig. 1C). The mature processed eNOS mRNA is a very stable mRNA under basal conditions in normal vascular endothelium (40). In agreement with prior studies (34), we found that processed eNOS mRNA levels do not begin to decrease until 6–24 h of hypoxic exposure (Fig. 1C). In contrast, levels of eNOS pre-mRNA, measured across the NOS3 locus, are rapidly reduced, even after only 1–2 h of hypoxic exposure (Fig. 1C). This is consistent with a rapid inhibition of eNOS transcription. In contrast to the down-regulation of pre-mRNA levels across the majority of the NOS3 locus, pre-mRNA levels detected at the 3′ end of the NOS3 gene

![Figure 1](http://example.com/figure1.png)

**FIGURE 1.** Hypoxia represses eNOS transcription. A, ChIP using anti-RNA pol II antibodies was performed in HUVEC exposed to various durations of hypoxia (<1% O₂), and real-time PCR was used to determine the density of pol II at the eNOS proximal promoter and exon 14. IP DNA was calculated for each time point (see “Experimental Procedures” for details), and changes in IP DNA relative to normoxia were determined. IP DNA values for exon 14 of eNOS are expressed relative to the values for the proximal promoter. Error bars indicate S.E. B, schematic of the NOS3 locus including locations of primers used to detect eNOS pre-mRNAs. Note that primer set preE (intron 25/exon 26) also amplifies the overlapping sONE gene. C, expression of eNOS mRNA and pre-mRNA were analyzed during hypoxia. Levels were normalized to those in normoxic HUVEC. * indicates a statistically significant difference between normoxic and hypoxic HUVEC.
Hypoxia Decreases Active Chromatin Marks at the eNOS Proximal Promoter—We have previously identified a functional role for histone post-translational modifications in eNOS transcriptional activity, with high levels of histone acetylation and H3K4Me on the histones at the eNOS proximal promoter (19). To determine whether changes in histone modifications were associated with the decreased transcriptional activity of eNOS during hypoxia, we used anti-acetyl histone H3 (H3Ac), anti-acetyl histone H4 (H4Ac), and anti-dimethyl lysine 4 of H3 (H3K4Me2) antibodies in ChIP assays and analyzed the eNOS proximal promoter (−166/−26) (Fig. 2A, solid lines). Hypoxia resulted in dynamic changes in these modifications at the eNOS proximal promoter, with levels of H3Ac decreasing ∼30% and levels of H4Ac decreasing ∼50% within the first 1–4 h of hypoxia treatment. H4Ac levels were stably decreased during longer durations of hypoxia. Although H3Ac levels tended to increase during long term hypoxia (6–24 h) treatment, H3Ac failed to reach baseline values (20–25% lower acetylation when compared with normoxia). Assessment of H3K4Me2 revealed a similar loss of this modification when compared with H3Ac levels. These findings demonstrate that post-translational modifications are dynamically altered at eNOS proximal promoter histones during hypoxic exposure.

To determine whether changes in post-translational histone modifications were confined to the eNOS proximal promoter or whether they extend into other regions of the NOS3 transcriptional unit, these modifications were also assessed in exon 14/intron 14 (Fig. 2A, dashed line). Basal levels of histone acetylation and H3K4Me are lower at exon 14 than at the proximal promoter (19). In contrast to the proximal promoter, histone acetylation and methylation did not significantly decrease with short durations of hypoxic treatment (1–4 h) at exon 14. However, following 24 h of hypoxia, levels of histone H3 and H4 acetylation were

FIGURE 2. Hypoxia results in decreased histone acetylation and lysine 4 methylation of histone H3 at the eNOS proximal promoter. A, ChIP analysis was performed on hypoxic HUVEC chromatin using anti-acetylated histone H3 (H3Ac), anti-acetylated histone H4 (H4Ac), and anti-dimethyl lysine 4 of H3 (H3K4Me2) antibodies at the eNOS promoter (solid line) and exon 14 (dashed line) of eNOS. IP DNA values for eNOS exon 14 are relative to the IP DNA values determined for the eNOS proximal promoter. Error bars indicate S.E. Where error bars are not visible, they are below the resolution of the figure. * indicates a statistically significant difference between normoxic and hypoxic HUVEC. B, ChIP analysis of the VEGF HRE was performed in hypoxic endothelial cells using anti-RNA pol II, anti-H3Ac, and anti-H4Ac antibodies. C, global changes in histone acetylation (H3Ac and H4Ac) were measured by performing Western blots on acid-extracted histones from hypoxic HUVEC. Total histone H3 was used as a loading control. A representative Western blot of three independent experiments is shown. Densitometric analysis is indicated above.
Hypoxic Repression of eNOS Transcription

reduced by ~70 and 40%, respectively, at exon 14 (Fig. 2A). Additionally, H3K4Me decreased modestly at this genomic region following long term hypoxia.

As an additional control, histone acetylation and RNA pol II recruitment were assessed at the HIF binding site of the VEGF-A promoter (VEGFA HRE) (Fig. 2B). Although it has previously been demonstrated that acetylation of histone H3 increases at the VEGFA HRE in response to hypoxia (41), the kinetics of histone acetylation are poorly understood. In contrast to the decrease in acetylation at the eNOS proximal promoter during short durations of hypoxia, acetylation increased at the VEGFA HRE after 2 h of hypoxia (Fig. 2B). Levels of histone acetylation then slowly declined following 4 h of hypoxia, with levels returning to near base-line levels by 24 h. This change in histone acetylation correlated well with recruitment of pol II to the VEGFA HRE (Fig. 2B).

To determine whether hypoxia elicited global changes in histone acetylation patterns, Western blots on acid-extracted histones were performed to assess the acetylation of histone H3 and H4 in hypoxic HUVEC (Fig. 2C). Total histone H3 levels were used as a loading control. Histone acetylation was not significantly altered over the first few hours of exposure to hypoxia. However, levels of H3Ac and H4Ac decreased markedly during longer durations of hypoxia. Therefore, when taken together with the VEGFA HRE ChIP data, it is apparent that the rapid changes in histone acetylation observed at the eNOS proximal promoter are a specific, rather than a global phenomenon.

Hypoxia Does Not Alter Repressive Chromatin Marks at the eNOS Promoter—We demonstrate above that active chromatin marks, including histone acetylation and H3K4Me, are lost from the eNOS promoter during hypoxia. We additionally sought to determine whether a corresponding gain of repressive chromatin marks, such as DNA methylation and H3K9Me, occurred. H3K9Me is a well characterized repressive histone modification, and we have previously demonstrated modest levels of this modification at the eNOS promoter in non-expressing cell types (19). This is in contrast to the markedly elevated levels of this repressive mark at the human inducible NOS gene in endothelial cells and vascular smooth muscle cells (42). It is now appreciated that cellular H3K9Me content is dynamically regulated in mammalian tissues and that hypoxia can modulate genome-wide and gene-specific levels of H3K9Me (23, 43). We failed to detect an increase in H3K9me at the eNOS promoter in hypoxic endothelial cells (supplemental Fig. S1A) despite positive H3K9Me signal at the inducible NOS promoter in these cells (data not shown). We have also previously demonstrated that DNA methylation represses eNOS transcription in non-endothelial cell types (30). Of interest, others have argued that hypoxia can elicit site-specific DNA hypermethylation in endothelial cells (44). We therefore assessed whether the eNOS promoter was methylated in endothelial cells during chronic hypoxia. Similar to H3K9Me analyses, DNA methylation did not increase during long term hypoxia. (supplemental Fig. S1B) The eNOS promoter remained completely unmethylated following long term hypoxia (24 h). Additionally, analysis of global levels of DNA methylation revealed a lack of regulation of bulk DNA methylation level by hypoxia (supplemental Fig. S1C). These negative findings suggest that loss of active chromatin marks, specifically histone H3 and H4 acetylation and H3K4Me, are the primary epigenetic alterations that occur at the eNOS promoter during hypoxia.

Reoxygenation Restores Histone Acetylation at the eNOS Promoter and Transcription of the eNOS Gene—To determine whether hypoxia-mediated repression of eNOS expression was reversible, we returned hypoxic HUVEC (24 h) to normoxic conditions and measured eNOS expression following reoxygenation for 24 h (supplemental Fig. S2). Reoxygenation of hypoxic cells resulted in a return of eNOS RNA levels to that of untreated control cells. This rapid restoration of eNOS expression is in contrast to other studies demonstrating that hypoxic repression can continue even after cells are returned to a normoxic environment for 48 h (45). To determine whether the histone acetylation code at the NOS3 locus is reset following reoxygenation and whether hypoxia-mediated transcriptional repression is relieved, we also performed ChIP assays on reoxygenated cells (supplemental Fig. S2). Levels of H4Ac, which decrease nearly 50% following 6 or 24 h of hypoxia, returned to normoxic levels following reoxygenation, suggesting that histone acetylation is reset at the eNOS promoter. In addition, pol II levels at the eNOS promoter drop by ~50% following hypoxia but return to baseline levels following reoxygenation. These findings suggest that the hypoxia-mediated transcriptional repression of eNOS is reversible.

Hypoxia Decreases Acetylation at All Assessed Lysine Residues—We previously suggested that a specific histone code exists in endothelial cells at the eNOS proximal promoter (19). The histone acetylation code consisted of high levels of lysine 9 acetylation of histone H3 (H3K9Ac) and lysine 12 acetylation of histone H4 (H4K12Ac). Because others have suggested that hypoxia can lead to the induction of HDAC activity (21), we determined whether lysine residues of histones at the eNOS promoter were selectively deacetylated during hypoxia by performing ChIP assays with antibodies that recognize acetylation of specific lysine residues of histone H3 and H4 tails (Fig. 3). Surprisingly, rather than altering specific acetylation marks at the eNOS promoter, such as selective changes in either H3K9Ac or H4K12Ac, we observed that all of the acetylation marks were decreased during hypoxia. All of the lysine residues that we assessed on histone H3 and H4 were deacetylated by ~50% throughout the hypoxic treatment.

Hypoxia Results in Loss of Histone Proteins from the eNOS Promoter—A decrease in ChIP signal for acetylated lysine residues of histone proteins could be attributed to a loss of histone proteins rather than to enzymatic removal of the acetylation mark. We sought to determine whether decreased levels of histone H3 or H4 could account for the decrease in histone acetylation levels at the eNOS proximal promoter during hypoxia. To this end, we performed ChIP assays using antibodies that detect total levels of histone H3 and H4, independent of post-translational histone modifications. Indeed, histone H3 and H4 occupancy was observed to decrease at the eNOS proximal promoter (~166/−26) during hypoxia (Fig. 4A). Interestingly, although histone H3 and H4 ChIP signals were decreased at the proximal promoter during short durations of hypoxia (1–4 h), levels began to increase after 6 h and returned to baseline values
following 24 h of treatment with hypoxia. This suggests that histones are rapidly evicted and then return to the proximal promoter after longer durations of hypoxia. To determine whether this loss of histones was localized to the proximal promoter or was more widespread, we analyzed two regions upstream of the proximal promoter as well. This analysis revealed that the loss of histone proteins was largely confined to the proximal promoter as regions upstream in the eNOS promoter did not demonstrate significant histone loss. Additionally, histone density was unaltered in the open reading frame (ORF) of E-cadherin/CDH1, a gene that is not expressed in HUVEC (data not shown).

We demonstrated in Fig. 3 that histone acetylation rapidly decreased following only 1 h of hypoxia and remained at decreased levels throughout the duration of hypoxia. Taking histone density into account, by normalizing changes in lysine acetylation to total levels of histone proteins, revealed that the acetylation of the remaining eNOS proximal promoter histones decreased at a steady rate during hypoxia, with only modest changes (~25%) occurring during the first 2 h of hypoxia (Fig. 4B). Following long term hypoxia (6–24 h), histone acetylation decreased ~50%. This suggests that histone eviction during short term hypoxia has a major effect on the total acetylation level of the eNOS proximal promoter. These data also suggest that the histones that are reincorporated at the eNOS proximal promoter following long term hypoxia are not highly acetylated. Because acetylation of histones is reset following reoxygenation, this suggests that either increased HDAC activity and/or decreased HAT activity in hypoxic endothelial cells maintains the eNOS promoter in an inactive state by preventing the acetylation of reincorporated histone proteins. In contrast to histone acetylation, H3K4Me decreased modestly during hypoxia when normalized to total histone H3 levels, suggesting that H3K4Me levels at the eNOS promoter are more stable during hypoxia (Fig. 4B).

Although these studies reveal an important role for histone eviction in repressing transcription, they also emphasize the importance of controls in ChIP analyses. Measuring total levels of histone H3 and H4 at a genomic locus is key to interpreting changes or differences in post-translational modifications of histones.

**The Histone Variant H2A.Z Is Preferentially Incorporated into the eNOS Promoter in an Endothelium-specific Fashion, and Hypoxia Results in a Loss of H2A.Z from the eNOS Proximal**
Promoter—In addition to histone post-translational modifications, histone variants can also play a role in regulating transcription. The H2A variant, H2A.Z, has been suggested to play a role in regulating chromatin architecture and transcriptional processes (46). Although the function of H2A.Z is still not fully understood, it is known that H2A.Z is non-randomly incorpo-
rated into chromatin. In genome-wide studies in yeast, H2A.Z has been found to be concentrated at the promoters of both active and inactive genes (47). However, H2A.Z was localized almost exclusively to the promoters and enhancers of highly expressed genes in human cells (48, 49). To determine whether H2A.Z was incorporated into the NOS3 gene locus, we used ChIP to assess the presence of H2A.Z at several regions across the eNOS promoter, at exon 1/intron 1 and exon 14/intron 14 of the NOS3 gene, at the promoter of the housekeeping gene, cyclophilin A (CYP A), and in the ORF of a non-expressed gene, E-cadherin (CDH1), in both eNOS-expressing cells (HUVEC) and eNOS non-expressing (VSMC) cell types (Fig. 5A). H2A.Z was preferentially incorporated at the eNOS promoter in endothelial cells, with the highest levels present at the −166/−26 region. We failed to detect significant basal levels of H2A.Z at the eNOS promoter in VSMC. H2A.Z incorporation at the CYP A promoter, which is transcriptionally active in both cell types, and which we have previously shown to be similarly acetylated between cell types (19), was similar between HUVEC and VSMC. Similar to the paucity of H2A.Z in ORFs that have been noted in global H2A.Z localization studies in human and yeast cells, H2A.Z levels were low in the coding region of eNOS and CDH1. These data demonstrate that H2A.Z is incorporated at the promoter of eNOS in an endothelium-specific fashion and represents the first example of cell-specific incorporation of H2A.Z at the promoter of a gene that exhibits cell-specific transcription.

Because H2A.Z-containing chromatin has been argued to be more prone to histone loss (49, 50), we measured the levels of H2A.Z at the eNOS promoter during hypoxia (Fig. 5B). Similar to the rapid loss of histones H3 and H4 at the eNOS proximal promoter (−166/−26), H2A.Z levels were also diminished at the proximal promoter with short term hypoxia. Levels increased back to base-line values following 24 h of hypoxia. Surprisingly, levels of H2A.Z were not significantly decreased at genomic regions upstream in the eNOS promoter (−488/−398) and (−891/−797)) (Fig. 5B), despite higher basal levels of this variant at −488/−398 in endothelial cells. This argues against H2A.Z levels being directly responsible for histone eviction at the eNOS promoter and suggests that H2A.Z is lost together with histones H3 and H4 at the proximal promoter (−166/−26). That H2A.Z is only incorporated at the eNOS promoter in endothelial cells, where the NOS3 gene is highly expressed, argues for a role for H2A.Z in regulating eNOS expression. Future studies will be needed to directly test this hypothesis.

**FIGURE 5.** H2A.Z is highly enriched in endothelial cells at the eNOS promoter and is evicted from the proximal promoter during hypoxia. A, ChIP was used to assess H2A.Z incorporation across the eNOS/NOS3 promoter and coding region (A–E). The housekeeping gene, CYP A, which is equivalently expressed in HUVEC and VSMC, and E-cadherin (CDH1), which is not expressed in either cell type, were used as positive and negative controls, respectively. * denotes a statistically significant difference between HUVEC and VSMC (p < 0.05). Error bars indicate S.E. B, ChIP was used to assess the levels of H2A.Z at the eNOS proximal promoter and two upstream regions of the eNOS promoter during hypoxia. * denotes a statistically significant difference between normoxia and hypoxia (p < 0.05).
regions. The high levels of chromatin accessibility observed at the proximal promoter (−200 to +1) did not continue into the coding region of the NOS3 gene as chromatin accessibility was ~25% at exon 1/intron 1, intron 1/exon 2, and exon 14/intron 14. For comparison, we performed CHART-PCR on VSMCs, which do not express eNOS, and demonstrated a marked difference in chromatin accessibility between cell types, nucleosome accessibility at the promoter of the ubiquitously expressed housekeeping gene, CYPα, and at the ORF of the non-expressed E-cadherin gene (CDH1), were performed. B, histone H3 and H4 density was measured in HUVEC and VSMC across the eNOS promoter and at the ORF of CDH1 by ChIP analysis. C, nucleosome accessibility of the eNOS proximal promoter (−166/−26) was measured during hypoxia. As a control, accessibility at the VEGF HRE was also monitored during hypoxia treatment. Hypoxia resulted in a gradual decrease in chromatin accessibility at the eNOS proximal promoter. The data represent the mean ± S.E. (error bars) of three independent experiments. * denotes a statistically significant difference between normoxia and hypoxia.

FIGURE 6. eNOS proximal promoter chromatin is highly accessible to nuclease digestion and hypoxia results in decreased nucleosome accessibility. Nucleosome accessibility was measured using the CHART-PCR analysis, which measures the ability of micrococcal nuclease to cleave chromatin. The percentage of accessibility was determined by measuring the loss of PCR product in micrococcal nuclease-digested vs undigested chromatin. The amount of genomic DNA was assessed with quantitative real-time PCR. A, nucleosome accessibility was measured across the eNOS (NOS3) promoter and coding region in eNOS expressing endothelial cells (HUVEC) and in VSMC, which do not express eNOS. Distance from the eNOS transcriptional start site corresponds to the midpoint of the PCR amplicon used. As a control for micrococcal nuclease digestion between cell types, nucleosome accessibility at the promoter of the ubiquitously expressed housekeeping gene, CYPα, and at the ORF of the non-expressed E-cadherin gene (CDH1), were performed. B, histone H3 and H4 density was measured in HUVEC and VSMC across the eNOS promoter and at the ORF of CDH1 by ChIP analysis. C, nucleosome accessibility of the eNOS proximal promoter (−166/−26) was measured during hypoxia. As a control, accessibility at the VEGF HRE was also monitored during hypoxia treatment. Hypoxia resulted in a gradual decrease in chromatin accessibility at the eNOS proximal promoter. The data represent the mean ± S.E. (error bars) of three independent experiments. * denotes a statistically significant difference between normoxia and hypoxia.

To determine whether differential nucleosome density between HUVEC and VSMC might explain the large differences in nucleosome accessibility observed between these two cell types, we assessed the total level of histone H3 and H4 across the eNOS promoter using ChIP assays. Basal levels of
histone H3 and H4 were dramatically reduced in HUVEC when compared with VSMC across the eNOS promoter but were similar in the coding region of CDH1, a gene that is not expressed in either cell type (Fig. 6B). We previously demonstrated that levels of acetylated histone H3 and H4 at the eNOS proximal promoter (−166/−26) were elevated ~3–4-fold in HUVEC when compared with VSMC (19). Taking total levels of histone H3 and H4 into account reveals an even more striking difference in histone acetylation between eNOS expressing and non-expressing cell types (~10–15-fold difference). Nucleosome accessibility, measured by CHART-PCR, may be partly attributed to differences in nucleosome density between cell types. However, this cannot be the only determining factor because histone density is similar between the proximal promoter (−166/−26) and a region upstream (−488/−398), yet nucleosome accessibility at these two regions is 82 and 13%, respectively.

**Hypoxia Reduces Nucleosome Accessibility at the eNOS Proximal Promoter**—We have found that hypoxic treatment of endothelial cells results in decreased histone acetylation at the eNOS promoter mediated, in part, by an initial loss of histone proteins followed by reincorporation of unacetylated histones. We next sought to determine whether changes in chromatin accessibility accompany these changes in chromatin modifications. Nucleosome accessibility at the eNOS proximal promoter (−166/−26) did not change significantly during short term hypoxia (1–4 h) (Fig. 6C). This was a surprising finding given that we have provided clear evidence that histone H3 and H4 are evicted from this same genomic region. Consistent with the hypoaecytlation of reincorporated nucleosomes and the lack of transcriptional activity following chronic hypoxia, nucleosome accessibility was significantly decreased following 24 h of hypoxia. For comparison, we assessed accessibility at the VEGFA HRE. This region was highly accessible in normoxic endothelial cells (data not shown), and accessibility tended to increase during prolonged hypoxia, although this change did not reach statistical significance (Fig. 6C).

**Nucleosome Remodeling Proteins Are Recruited to the eNOS Promoter during Hypoxia and Are Functionally Important for the Re-expression of eNOS following Reoxygenation**—Several studies have demonstrated repressive roles for nucleosome remodeling complexes (51–54). For example, the SWI/SNF chromatin remodeling complex has been shown to negatively regulate the expression of cyclin A (52) and c-Fos (55). Brahma (BRM) and BRG1 are ATPase subunits of the SWI/SNF complex. They are present in SWI/SNF complexes in a mutually exclusive manner. Considering that histones are evicted from the eNOS promoter during hypoxia-mediated eNOS repression, we sought to define whether nucleosome remodeling proteins were involved in this process. To this end, we used antibodies to BRM and BRG1 in ChIP assays in hypoxic HUVEC (Fig. 7A). These proteins were not highly abundant at the eNOS promoter under basal conditions. However, these two proteins were recruited to the eNOS promoter during hypoxia with distinct kinetics. Although BRG1 recruitment peaked at 2 h of hypoxia, BRM was recruited later at 4 h of hypoxia (Fig. 7A). BRG1 recruitment appeared to be more prominent during hypoxia than BRM. Assessment of global levels of BRG1 revealed that the increase in BRG1 at the eNOS promoter was not the result of altered global BRG1 protein levels during hypoxia (supplemental Fig. S3A). Of note, the recruitment of BRM and BRG1 occurred after the onset of histone loss during hypoxia and continued during the time when histones were depleted from the eNOS promoter. BRG1 and BRM recruitment was subsequently lost at 6 h of hypoxia, at a time point when H3/H4 proteins were returning to the eNOS promoter.

To determine whether BRM and/or BRG1 played a functional role in regulating eNOS expression during hypoxia, we used RNA interference to knock down BRG1 and/or BRM prior to exposing endothelial cells to hypoxia and measured changes in eNOS mRNA following hypoxia. Levels of BRG1 and BRM protein were significantly decreased following a 48-h knockdown with specific siRNAs when compared with nonspecific, non-targeting control siRNAs (Fig. 7B). Despite substantial knockdown of BRG1 and BRM prior to exposure of endothelial cells to hypoxia, no significant effects on eNOS RNA levels were noted (Fig. 7C). Levels of eNOS RNA continued to fall following 24 h of hypoxia despite the knockdown of these SWI/SNF proteins. This finding was confirmed using an independent set of BRG1 siRNAs (supplemental Fig. S3, B and C). These findings suggest that other factors must be involved in hypoxia-mediated eNOS repression.

Because BRG1 and BRM are recruited to the eNOS promoter following histone eviction and remain associated until histone density is re-established at 6 h of hypoxia, we reasoned that BRG1 and/or BRM might play a role in re-establishing nucleosome density following long term hypoxia. We therefore tested whether BRG1 and/or BRM were required for the re-expression of eNOS mRNA following reoxygenation of hypoxic cells (Fig. 8A). Indeed, we found that BRG1 siRNA had a potent effect on eNOS re-expression following reoxygenation, whereas BRM siRNA did not have an effect. Following BRG1 knockdown with RNA interference, eNOS expression was not induced upon reoxygenation. As a control, we assessed VEGF expression following reoxygenation and found no effect of BRG1 or BRM siRNA (data not shown). Assessment of histone density revealed that BRG1 knockdown did not affect histone density at the eNOS promoter following chronic hypoxia (Fig. 8B); however, lack of BRG1 did lead to a loss of histone H4 at the eNOS promoter following reoxygenation when compared with control siRNA treated cells (Fig. 8B). A more detailed analysis of the kinetics of eNOS induction during reoxygenation revealed that eNOS mRNA levels return to normoxic levels after only 2 h (Fig. 8C). This coincided with the time point at which BRG1 was recruited to the eNOS promoter during reoxygenation (Fig. 8D). This suggests that BRG1 may play a key functional role in the re-establishment of eNOS transcription during reoxygenation.

**DISCUSSION**

Decreased eNOS-derived NO contributes to several diseases of the vasculature, including atherosclerosis (56) and pulmonary hypertension (9, 57). Although the eNOS promoter is active in a variety of cell types when it is located in episomes (30), it is highly restricted in expression to endothelial cells when the promoter is integrated into chromatin (58). We and
others have shown that this cell specificity is regulated by DNA methylation (30, 59) and post-translational histone modifications (19, 59). Considering the importance of chromatin structure to the cell-specific expression of eNOS (reviewed in Refs. 60 and 61), it was anticipated that expression of the NOS3 gene in disease states might involve changes to chromatin structure. Here we have analyzed changes in eNOS transcription and chromatin modifications in hypoxic endothelial cells. We demonstrate that eNOS transcription is rapidly inhibited by hypoxia and that repression coincides with changes in histone acetylation and H3K4Me at the eNOS proximal promoter. Surprisingly, histone proteins (H3 and H4) appear to be evicted from the proximal promoter of the NOS3 gene during acute hypoxia (1–4 h), resulting in the resetting of histone marks, including lysine acetylation and methylation. In support of this model, we demonstrate endothelium-specific incorporation of the histone variant H2A.Z at the eNOS proximal promoter and find that levels of H2A.Z also decrease rapidly with hypoxia. Following chronic hypoxic exposure (6–24 h), histones H3, H4, and H2A.Z return to the locus. However, histones H3 and H4 have reduced levels of acetylation. Histone acetylation can be re-established to normoxic levels following reoxygenation, and this is coincident with recruitment of pol II, suggesting that reduced levels of histone acetylation may functionally repress eNOS transcriptional activity during hypoxia. This suggests that histone acetylation may play a role in the recruitment of factors to the eNOS promoter that regulate eNOS transcription. These findings provide a new model for gene repression, namely histone eviction.

Although the mechanisms involved in the transcriptional up-regulation of several hypoxia-inducible genes have been dissected in detail (62–64), hypoxia-mediated repression is less well understood. For example, a role for negative cofactor 2 (NC2) has been implicated in the hypoxia-mediated repression of several genes (11). Levels of NC2/α and β-protein are potently increased during hypoxia, and binding of this protein to promoter regions can displace components of the RNA polymerase II complex, such as TFIIB (11). We considered whether NC2 might also facilitate decreased binding of pol II to the eNOS promoter during hypoxia but failed to detect an increase in NC2 binding using ChIP analysis (data not shown). Several studies have also demonstrated direct transcriptional repression by HIF binding to promoter elements (16–18). Although HIF can interact with co-activators, including the acetyltransferase p300, to mediate increases in hypoxia-mediated transcription (65), HIF can also interact with transcriptional repres-
sors including factor inhibiting HIF-1 (FIH) (66), von Hippel Lindau (VHL)-associated Kruppel-associated box-A domain-containing protein (VHLaK) (67), and inhibitor of growth family member 4 (ING4) (68). Additionally, HIF can associate with HDAC activity (66). We considered whether HIF might bind to the proximal eNOS promoter to repress transcription, but a consensus HIF site was not present in this region. An HRE has been identified at position -5375/-5366 upstream of the eNOS transcriptional start site, and HIF2α has been implicated in the regulation of eNOS transcriptional activity from transiently transfected eNOS promoter/reporter constructs (37). However, we have found that eNOS promoter/reporter mice containing genomic regions that lack this HIF site still demonstrated reduced eNOS promoter activity in hypoxic mice (36). We also found only background levels of RNA pol II at the eNOS HRE, and hypoxia did not result in alterations to pol II binding (data not shown). These findings argue that this HIF site is unlikely to play a direct role in the repression of chromatin-based eNOS transcription during hypoxia.

The importance of histone post-translational modifications to hypoxic gene regulation is beginning to be appreciated. For example, the VEGFα HRE is hyperacetylated following the hypoxic induction of this gene (41). We have recapitulated these prior findings here and have further defined the kinetics of hypoxia-induced chromatin changes at the VEGFα HRE. Other studies have suggested a potential role for HDACs in repressing genes under hypoxic conditions (20, 21), but these studies did not measure the effects of hypoxia on promoter acetylation or on the transcription of the regulated genes. A role for histone acetylation has also been implicated in the cAMP induction of surfactant protein A (SPA) in normoxic cells (22). Hypoxia inhibits this hyperacetylation through the concerted up-regulation of HDACs and the down-regulation of the HAT, CREB-binding protein. Hypoxia also increased H3K9Me at the SPA promoter, preventing its induction (22). Increases in promoter-associated and global levels of H3K9Me2 and H3K9Me3 in hypoxic cells have also been reported by others (23). In addition to these prior studies, our current findings have suggested a new molecular mechanism, histone eviction, in eliciting changes to post-translational histone modifications in the setting of hypoxia. These findings are significant because they provide a new model for how hypoxia may elicit decreases in gene transcription.

Dynamic histone removal has been observed at the enhancer (69), promoter, and/or coding regions (70, 71) of highly active/inducible genes. Additionally, evidence indicates that histones can be dynamically evicted and replaced genome-wide, even at transcriptionally inactive loci (72). The specific loss of histones at the promoter of a repressed gene has not been observed, at least to our knowledge. We have shown that the total level of histone H3 and H4 at the eNOS promoter decreased during short durationsofhypoxia but then returned to baseline levels following 24 h of hypoxia treatment. However, the histones that return to the eNOS proximal promoter are distinct from the histones present during normoxia because they are not as highly acetylated. During replication-coupled histone deposition, histones are deposited in an acetylated form, with histone H3 being acetylated at lysines 9 and 14 and histone H4 being acetylated at lysines 5 and 12 (73). Because histone eviction and deposition occur quickly (<24 h) and because hypoxia is known to inhibit DNA replication (74), replication-independent histone deposition is likely to occur. Histone H3.3, which is deposited in a replication-independent manner, is highly acetylated before incorporation into chromatin (75). Because eNOS pro-
Hypoxic Repression of eNOS Transcription

Hypoxia has reduced levels of acetylation following chronic hypoxia, increased HDAC activity and/or reduced HAT activity may be implicated in maintaining a hypoacetylated state of these replacement histones. Levels of histone acetylation at the eNOS promoter return to baseline values following reoxygenation, suggesting that HAT/HDAC activity is reset following reoxygenation. Importantly, transcription of the NOS3 gene resumes, implying that decreased histone acetylation at the eNOS promoter may be functionally relevant to decreased eNOS transcription in the setting of hypoxia.

Our findings suggest that there are two distinct kinetic stages involved in the reduction in acetylation of eNOS promoter histones during hypoxia. First, rapid eviction of histones occurs, followed by reincorporation of histones with reduced acetylation levels. Alternatively, deacetylation of acetylated replacement histones may occur through HDAC activity or decreased HAT activity. When levels of lysine acetylation are compared with the total levels of histone H3 and H4, it is evident that the acetylation status of the remaining histones does not significantly change during the first 1–2 h of hypoxia (Fig. 6B). However, during longer durations of hypoxia (6–24 h), histones lose acetylation. A prior study demonstrated a transient hyperacetylation of histones prior to histone eviction (71). When normalized to total histone levels, we found that levels of lysine 9 and lysine 14 acetylation of histone H3 did increase modestly after 1 h of hypoxia (Fig. 6B). Because histones are already evicted by 1 h, we currently cannot exclude a role for hyperacetylation prior to histone eviction due to the fast kinetics of histone eviction.

We hypothesize that post-translational modifications to the histones themselves may contribute to the recruitment of components of the preinitiation complex. Although chromatin accessibility can be a determinant of the binding of the transcriptional apparatus, the nucleosomes themselves also play an important role in directing the binding of the components of the pol II complex. For example, BRD4, a component of the pol II holoenzyme, binds to hyperacetylated chromatin and recruits P-TEFb to facilitate the transition to transcriptional elongation (76), and BDF1 binds to hyperacetylated histone H4 and recruits TFIID to TATA-less promoters in lower species (77). Note, eNOS is a TATA-less promoter, suggesting that histone acetylation may be required for recruitment of the basal transcriptional machinery. H3K4Me also acts as a docking site for a variety of plant homeodomain (PHD)-containing proteins (78, 79). Global analysis of the binding of c-Myc to promoters reveals a strict prerequisite for the presence of specific post-translational histone marks (80). Therefore, the removal of post-translational histone marks from the eNOS proximal promoter during hypoxia may impede the formation of functional transcriptional complexes. Recacylation (and presumably remethylation of lysine 4) of eNOS promoter histones following reoxygenation would be expected to re-establish the marks responsible for recruitment of the transcriptional apparatus.

Similar to the loss of histone H3 and H4 at the eNOS proximal promoter during hypoxia, H2A.Z is also lost transiently with similar kinetics, implying that whole nucleosomes are evicted during this process. Because the region of histone loss does not correlate with the highest levels of H2A.Z incorporation, this argues against an important role for H2A.Z levels in mediating histone removal. Recent genome-wide localization studies in human cells have identified peaks of H2A.Z enrichment at the promoters and enhancers of highly active genes (48, 49). We found that this variant was preferentially incorporated at the eNOS promoter in endothelial cells, but not in non-endothelial cells, identifying cell-specific H2A.Z incorporation for the first time. H2A.Z incorporation is facilitated by the Swr1 complex (47), and efficient H2A.Z deposition requires histone acetylation of H3 and H4 at yeast promoters. Bdf1 and Bdf2, which both contain bromo domains, mediate this acetylation-dependent deposition (47). It is of interest that the most important acetylation sites for H2A.Z deposition were found to be lysine 9 of histone H3 (H3K9Ac) and lysines 5 and 12 of histone H4 (H4K5Ac/H4K12Ac) (47). In light of our dissection of the eNOS acetylation histone code (19), it is interesting that the cell-specific modifications that we observed were at H3K9Ac and H4K12Ac. Perhaps acetylation of these lysine residues plays a functional role in the cell-specific deposition of H2A.Z at the eNOS promoter.

The mechanism by which eNOS proximal promoter histones are evicted during short term hypoxia is currently not known. We have demonstrated dynamic recruitment of the ATPase subunits of the SWI/SNF complex, namely BRM and BRG1, to the eNOS promoter during short durations of hypoxia. However, BRM and BRG1 engage with the eNOS promoter following, rather than concordant with, histone eviction. Because BRM and BRG1 are found in mutually exclusive SWI/SNF complexes, it is likely that this represents an ordered recruitment of two separate complexes: one that contains BRG1 at 2 h of hypoxia and one that contains BRM at 4 h of hypoxia. We also demonstrate that Brg1 is recruited transiently to the eNOS promoter during reoxygenation, demonstrating that BRG1 recruitment to the eNOS locus is highly dynamic. How these chromatin-remodeling complexes are recruited to the eNOS promoter is currently not known, but these complexes are known to interact with various transcription factors (53, 54, 81). SWI/SNF complexes have also been demonstrated to bind to naked DNA (82), suggesting that they may be recruited to nucleosome-depleted region of the eNOS promoter during short term hypoxia. This is consistent with the finding that recruitment of BRG1 and BRM occurs after histone eviction has occurred. Interestingly, we demonstrated by CHART-PCR analysis of the eNOS proximal promoter (−166/−26) that chromatin accessibility does not change during short durations of hypoxia (1–4 h), although whole nucleosomes are removed from this region. It should be emphasized that the proximal promoter (−200 to the start site of transcription) is especially accessible to nucleases at baseline. Perhaps the removal of histones from this region is not able to further increase the already elevated accessibility at this region. Additionally, due to the large size of the SWI/SNF complex (83) and the kinetics of their recruitment at 2 and 4 h of hypoxia, it is possible that the binding of this complex prevents gross changes in chromatin accessibility. Following 24 h of exposure to hypoxia, chromatin accessibility significantly decreases, suggesting that reduced levels of acetylation of eNOS promoter histones may modulate chromatin structure...
Hypoxic Repression of eNOS Transcription

at the eNOS proximal promoter, especially after long term exposure to hypoxia.

Although SWI/SNF complexes have been demonstrated to alter chromatin structure by numerous mechanisms, including nucleosome sliding and movement of nucleosomes in trans (reviewed in Ref. 83), we provide evidence that BRG1 and BRM are not involved in the repression of eNOS transcription. We used siRNA to knock down levels of BRG1 and/or BRM prior to exposing endothelial cells to hypoxic conditions and found that levels of eNOS mRNA were decreased similarly in control, BRG1, or BRM knockdown cells. We demonstrated, however, that BRG1 is functionally important for the re-establishment of eNOS expression following reoxygenation of hypoxic cells. eNOS expression is rapidly induced following reoxygenation (2 h), and this induction occurs at a time point in which BRG1 is recruited to the eNOS promoter. Knockdown of BRG1 results in altered histone density during reoxygenation, suggesting that BRG1 perhaps plays a functional role in re-establishing eNOS chromatin to a transcriptionally active state during reoxygenation.

Although well established histone eviction models exist for the transcriptional induction of several yeast genes, the mechanisms responsible for eviction of histones remain elusive. Importantly, the model that we propose for the regulation of eNOS in yeast systems because our findings reveal for the first time that histone eviction can also be involved in the repression of transcription. The findings presented here have important implications for the regulation of vascular gene expression in disease settings. We have recently shown that eNOS mRNA can be regulated post-transcriptionally in the setting of hypoxia by the up-regulation of a cis-antisense gene, sONE/NOS3AS (34). In addition to post-transcriptional regulation of eNOS, we posit that histone biochemistry plays an important role in the process of eNOS transcriptional repression during decreased cellular oxygen levels and that both rapid histone eviction and longer term alterations in histone post-translational modifications are functionally relevant.

Acknowledgments—We thank Dr. Michael Ohh (University of Toronto, Canada) for the use of the hypoxia incubator and Benoit Guillemette (Universite de Sherbrooke, Canada) and T. Oelgeschla¨ger (The Rockefeller University, New York, NY) for contributing antibodies.

REFERENCES

1. Chi, J. T., Wang, Z., Nuyten, D. S., Rodriguez, E. H., Schaner, M. E., Salim, A., Wang, Y., Kristensen, G. B., Helland, A., Berresen-Dale, A. L., Giaccia, A., Longaker, M. T., Hasteie, T., Yang, G. P., van de Vijver, M. J., and Brown, P. O. (2006) PLoS Med. 3, e47
2. Kourembanas, S., Marsden, P. A., McQuillan, L. P., and Faller, D. V. (1991) J. Clin. Invest. 88, 1054–1057
3. Kourembanas, S., Hannan, R. L., and Faller, D. V. (1990) J. Clin. Invest. 86, 670–674
4. McQuillan, L. P., Leung, G. K., Marsden, P. A., Kostyk, S. K., and Kourembanas, S. (1994) Am. J. Physiol. 267, H1921–1927
5. Moudgil, R., Michelakis, E. D., and Archer, S. L. (2005) J. Appl. Physiol. 98, 390–403
6. Humbert, M., Morrell, N. W., Archer, S. L., Stenmark, K. R., MacLean, M. R., Lang, I. M., Christman, B. W., Wein, E. K., Eickelberg, O., Voelkel, N. F., and Rabinovitch, M. (2004) J. Am. Coll. Cardiol. 43, 135–145
7. Fagan, K. A., Fouty, B. W., Tyler, R. C., Morris, K. G., Jr., Hepler, L. K., Sato, K., LeCras, T. D., Abman, S. H., Weinberger, H. D., Huang, P. L., McMurtrey, I. F., and Rodman, D. M. (1999) J. Clin. Invest. 103, 291–299
8. Ozaki, M., Kawashima, S., Yamashita, T., Ohashi, Y., Rikitake, Y., Inoue, N., Hirata, K. I., Hayashi, Y., Itoh, H., and Yokoyama, M. (2001) Hypertension 37, 322–327
9. Giaid, A., and Saleh, D. (1995) N Engl. J. Med. 333, 214–221
10. Champion, H. C., Bivalacqua, T. J., Greenberg, S. S., Giles, T. D., Hyman, A. L., and Kadowitz, P. J. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 13248–13253
11. Denko, N., Wernke-Dollries, K., Johnson, A. B., Hammond, E., Chiang, C. M., and Barton, M. C. (2003) J. Biol. Chem. 278, 5744–5749
12. Ivanov, S. V., Salnikov, K., Ivanova, A. V., Bai, L., and Lerman, M. I. (2007) Oncogene 26, 802–812
13. Kitamura, T., Takahashi, K., Ogawa, K., Udono-Fujimori, R., Takeda, K., Furuyama, K., Nakayama, M., Sun, J., Fujita, H., Hida, W., Hattori, T., Shirato, K., Igarashi, K., and Shibahara, S. (2003) J. Biol. Chem. 278, 9125–9133
14. Nakamura, H., Tanimoto, K., Hiyama, K., Yonokawa, M., Kawamoto, T., Kato, Y., Yoshioka, K., Poellinger, L., Hiyama, E., and Nishiyama, M. (2008) Oncogene 27, 4200–4209
15. Choi, S. M., Cho, H. J., Cho, H., Kim, K. H., Kim, J. B., and Park, H. (2008) Nucleic Acids Res. 36, 6372–6385
16. Mazure, N. M., Chauvet, C., Bois-Joyeux, B., Bernard, M. A., Nacer-Cherif, H., and Danan, J. L. (2002) Cancer Res. 62, 1158–1165
17. Chen, K. F., Lai, Y. Y., Sun, H. S., and Tsai, S. J. (2005) J. Biol. Chem. 280, 3265–3273
18. Eltzschig, H. K., Abdulla, P., Hoffman, E., Hamilton, K. E., Daniels, S., Schonfeld, C., Löffler, M., Reyes, G., Duszenko, M., Karhausen, J., Robinson, A., Westerman, K. A., Coe, I. R., and Colgan, S. P. (2005) J. Exp. Med. 202, 1493–1505
19. Fish, J. E., Matoouk, C. C., Rachlis, A., Lin, S., Tai, S. C., D’Abreo, C., and Marsden, P. A. (2005) J. Biol. Chem. 280, 24824–24838
20. Mihaylova, Y. T., Bindra, R. S., Yuan, J., Campisi, D., Narayanlan, L., Jensen, R., Giordano, F., Johnson, R. S., Rockwell, S., and Glazer, P. M. (2003) Mol. Cell. Biol. 23, 3265–3273
21. Kim, M. S., Kwon, H. J., Lee, Y. M., Baek, J. H., Jang, J. E., Lee, S. W., Moon, E. J., Kim, H. S., Lee, S. K., Chung, H. Y., Kim, C. W., and Kim, K. W. (2001) Nat. Med. 7, 437–443
22. Islam, K. N., and Mendelsson, C. R. (2006) Mol. Cell. Biol. 26, 2901–2912
23. Chen, H., Yan, Y., Davidson, T. L., Shinkai, Y., and Costa, M. (2006) Cancer Res. 66, 9009–9016
24. Li, Q., and Costa, M. (2009) Biochimie 91, 1307–1310
25. Marsden, P. A., Schappert, K. T., Chen, H. S., Flowers, M., Sundell, C. L., Wilcox, J. N., Lamas, S., and Michel, T. (1992) FEBS Lett. 307, 287–293
26. Golub, M., Baille, B. R., Gévré, N., Adam, M., Blanchette, M., Robert, F., and Gaudreau, L. (2005) PLoS Biol. 3, e384
27. Nery, J. A., and Rabinovitch, M. (1992) J. Biol. Chem. 267, 322–327
28. Robb, G. B., Casson, A. R., Tai, S. C., Fish, J. E., Singh, S., Yamada, T., Scherer, S. W., Nakabayashi, K., and Marsden, P. A. (2004) J. Biol. Chem. 279, 37982–37996
29. Chan, Y., Fish, J. E., D’Abreo, C., Lin, S., Robb, G. B., Teichert, A. M., Karantzas-Zigos, F., Keightley, A., Steer, B. M., and Marsden, P. A. (2004) J. Biol. Chem. 279, 35087–35100
30. Robb, G. B., Casson, A. R., Tai, S. C., Fish, J. E., Singh, S., Yamada, T., Scherer, S. W., Nakabayashi, K., and Marsden, P. A. (1999) J. Biol. Chem. 274, 3076–3093
31. Karimi, M., Johansson, S., Stach, D., Corcoran, M., Grandér, D., Schalling, M., Bakalkin, G., Lyko, F., Larsson, C., and Ekström, T. J. (2006) Exp. Cell Res. 312, 1899–1995
32. Rados, S., Kroo, E., and Shannon, M. F. (2001) J. Immunol. 167, 4494–4503
Hypoxic Repression of eNOS Transcription

34. Fish, J. E., Matouk, C. C., Yeboah, E., Bevan, S. C., Khan, M., Patil, K., Ohh, M., and Marsden, P. A. (2007) J. Biol. Chem. 282, 15652–15666
35. Phelan, M. W., and Faller, D. V. (1996) J. Cell Physiol. 167, 469–476
36. Ward, M. E., Torspolders, M., Scott, I. A., Teoh, H., Govindaraju, V., Quan, A., Wener, A. D., Wang, G., Bevan, S. C., Newton, D. C., and Marsden, P. A. (2005) J. Clin. Invest. 115, 3128–3139
37. Coulet, F., Nadaud, S., Agrapart, M., and Soubrier, F. (2003) J. Biol. Chem. 278, 46230–46240
38. Bentley, D. (1999) Curr. Opin. Cell Biol. 11, 347–351
39. Batsche, E., Yaniv, M., and Muchardt, C. (2006) Nat. Struct. Mol. Biol. 13, 22–29
40. Tai, S. C., Robbins, G. B., and Marsden, P. A. (2004) Arterioscler. Thromb. Vasc. Biol. 24, 405–412
41. Jung, J., Lee, H. G., Chu, I. H., Chung, D. H., Yoon, S. H., Yang, Y. M., Lee, J. W., Choi, S., Park, J. W., Ye, S. K., and Chung, M. H. (2005) FASEB J. 19, 1296–1298
42. Chan, G. C., Fish, J. E., Mawji, I. A., Leung, D. D., Rachlis, A. C., and Marsden, P. A. (2005) J. Immunol. 175, 3846–3861
43. Wellmann, S., Bazyk, A., Schaffer, M., Zelmer, A., Seeger, K., Faigle, M., Eltzschig, H. K., and Bührer, C. (2008) Biochem. Biophys. Res. Commun. 372, 892–897
44. Hu, C. J., Chen, S. D., Yang, D. I., Lin, T. N., Chen, C. M., Huang, T. H., and Hsu, C. Y. (2006) J. Cereb. Blood Flow Metab. 26, 1519–1526
45. Bindra, R. S., Schaffer, P. J., Meng, A., Woo, J., Madeira, K., Roth, M. E., Lizardi, P., Hedley, D. W., Bristow, R. G., and Glazer, P. M. (2004) Mol. Cell. Biol. 24, 8504–8518
46. Li, B., Pattenden, S. G., Lee, D., Gutiérrez, J., Chen, J., Seidel, C., Gerton, J., and Workman, J. L. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 18385–18390
47. Raisner, R. M., Hartley, R. G., Meneghini, M. D., Bunn, H. F., and Livingston, D. M. (1996) J. Biol. Chem. 271, 2764–2770
48. Raisner, R. M., Hartley, P. D., Schaffer, M., Lizardi, P., Chen, J., Seidel, C., Gerton, J., and Workman, J. L. (1999) Proc. Natl. Acad. Sci. U.S.A. 96, 1979–1984