Stable Chromatin Binding Prevents FoxA Acetylation,
Preserving FoxA Chromatin Remodeling*

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Sarah Kohler and Lisa Ann Cirillo

From the Department of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, Milwaukee, Wisconsin 53226

FoxA1–3 (formerly HNF3α, -β, and -γ), members of the FoxA subfamily of forkhead transcription factors, function as initial chromatin-binding and chromatin-remodeling factors in a variety of tissues, including liver and pancreas. Despite essential roles in development and metabolism, regulation of FoxA factors is not well understood. This study examines a potential role for acetylation in the regulation of FoxA chromatin binding and remodeling. Using in silico analysis, we have identified 11 putative p300 acetylation sites within FoxA1, five of which are located within wings 1 and 2 of its winged-helix DNA-binding domain. These polypeptide structures stabilize FoxA DNA and chromatin binding, and we have demonstrated that acetylation attenuates FoxA binding to DNA and diminishes its ability to remodel chromatin. FoxA acetylation is inhibited by chromatin binding. We propose a model whereby stable chromatin binding protects the FoxA DNA-binding domain from acetylation to preserve chromatin binding and remodeling by FoxA factors in the absence of extracellular cues.

The FoxA subfamily of winged-helix/forkhead transcription factors, FoxA1–3 (previously HNF3α, -β, and -γ), binds to and activates numerous genes in the liver and pancreas required for differentiation, metabolism, and tissue integrity (1–5). In the embryo, FoxA1 and FoxA2 cooperate to specify the liver (3), and based upon their ability to stably bind and remodel target sites within compacted chromatin in vivo and in vitro (6–10), FoxA1 and FoxA2 are believed to function as “pioneer factors” for liver specification, directing hepatic competence through chromatin opening of liver genes. In the adult, all three FoxA proteins control glucose metabolism through the regulation of multiple target genes in the liver and pancreas (reviewed in Ref. 5). FoxA homologs are also critical for gut development and metabolic regulation in Drosophila and Caenorhabditis elegans (11–14), demonstrating the general importance of this factor across metazoan evolution. Despite this, little is known about how the binding or transactivation activities of FoxA factors are themselves regulated in the cell in response to extracellular events. This work investigates a possible role for acetylation in the regulation of FoxA chromatin binding and remodeling.

During early development, FoxA functions as a pioneer transcription factor, recognizing and binding to its sites within compacted chromatin (8, 10), and is able to open chromatin in the absence of ATP-dependent remodeling complexes (10, 15). This process has been extensively studied using the enhancer of the serum albumin gene, a developmentally regulated, liver-expressed FoxA target. The albumin enhancer and gene, which is expressed exclusively in the liver, are first activated when the liver is first formed in the early embryo (6, 16). In the liver, the albumin enhancer is organized into an array of three positioned nucleosome particles and bound transcription factors (17). The nucleosome particle designated N1 contains three FoxA-binding sites, eG, eH, and NS. The ability of FoxA to bind and remodel chromatin is due in large part to the structural similarity of its winged-helix DNA-binding domain (DBD)2 to that of the linker histone (18–20). FoxA has a higher affinity for its sites than the linker histone, which allows FoxA to displace the linker histone to bind to its albumin enhancer sites within transcriptionally silent chromatin in embryonic liver (8, 10). Once bound, FoxA remodels albumin enhancer chromatin into a localized open domain, thereby promoting and stabilizing the binding of additional transcription factors to the albumin enhancer (6, 9, 10).

In comparison with the majority of transcription factors, which interact with chromatin in a highly dynamic fashion (21, 22), FoxA binding to both in vitro assembled and cellular chromatin is extremely stable (9, 23). Stable chromatin binding is believed to enable FoxA factors to potentiate gene activation by maintaining chromatin at its target genes in an opened and remodeled state (8). Recent studies have demonstrated that stable nucleosome binding resides within the FoxA “winged-helix” DNA-binding domain shared with the linker histone H1 (23). This domain, which is highly conserved among FoxA and all other forkhead proteins (24), consists of three α-helices flanked by two loops or “wings” of polypeptide (wings 1 and 2) (20). The FoxA wing 1 and 2 polypeptides, which make multiple contacts with the DNA backbone, stabilize binding of the recognition helix to the DNA (18, 25). Not surprisingly, a recent study has demonstrated that wings 1 and 2 are necessary for stable FoxA binding to nucleosomal DNA; mutations in these domains destabilize FoxA nucleosome binding and enable more dynamic interactions with cellular chromatin (25).

In adult liver and pancreas, FoxA proteins continue to play important roles in metabolism, protecting the organism from hypoglycemia through their regulation of multiple target genes involved in gluconeogenesis, lipolysis, and glucose uptake (26–

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1 To whom correspondence should be addressed: Dept. of Cell Biology, Neurobiology, and Anatomy, Medical College of Wisconsin, 8701 Watertown Plank Rd., Milwaukee, WI 53226. Fax: 414-456-6517; E-mail: lcirillo@mcw.edu.

2 The abbreviations used are: DBD, DNA-binding domain; HAT, histone acetyltransferase; EMSA, electrophoretic mobility shift assay.
Surprisingly, despite their important regulatory roles in development and metabolism, regulation of FoxA proteins themselves in response to extracellular cues is poorly understood. Considering the importance of chromatin binding and remodeling to the function of FoxA as a transcriptional activator, it would make sense that regulation of FoxA proteins would be directed at its chromatin-binding and/or chromatin-remodeling functions. In agreement with this idea, binding of the small heterodimer partner to FoxA, in response to excess bile acids, has been shown to inhibit FoxA DNA binding (29) at genes with roles in maintenance of bile acid homeostasis. Similarly, interaction with the developmental co-regulator TLE (transducin-like enhancer of split) was recently demonstrated to inhibit FoxA1-mediated chromatin remodeling required for developmental activation of the albumin enhancer (30). In addition to indirect regulation via protein-protein interactions, FoxA2 has been shown to directly respond to changes in insulin via phosphorylation through the Akt2/protein kinase B pathway at a non-conserved threonine (Thr^546) adjacent to the DNA-binding domain (31, 32). This phosphorylation event attenuates binding of FoxA2 to chromatin at insulin target genes (33) and triggers its export from the nucleus (31), thereby inhibiting FoxA2 transcriptional activity in response to insulin. Phosphorylation of FoxA2 is the only documented post-translational modification of FoxA; additional post-translational modifications have not been examined.

In addition to phosphorylation, acetylation has recently emerged as a critical regulator of transcription factor activity (34). Acetylation is catalyzed by numerous acetyltransferases present in the cell; many of these function as transcriptional co-regulatory proteins for the transcription factors they acetylate (34). Acetylation has been demonstrated to positively and negatively influence DNA and chromatin binding by transcription factors (34). When these transcription factors are expressed in a developmental or cell type-dependent fashion, this can, in turn, influence cell fate decisions and/or dictate complex physiological responses. In the case of the hematopoietic pioneer factor GATA1, acetylation of its DNA-binding domain, which stimulates its DNA-binding capabilities (35), is required for GATA1 to activate target genes necessary for establishment of the hematopoietic program in cellular chromatin (36). In contrast, acetylation of the architectural factor required for GATA1 to activate target genes necessary for chromatin binding and/or chromatin remodeling required for developmental activation of the albumin enhancer (30).

As a first step toward defining a potential role for acetylation in regulation of FoxA function, we examined the FoxA protein sequence for consensus sites for p300 acetylation. We found numerous putative p300 acetylation sites throughout the FoxA1 protein, including two clusters of lysine residues residing within wings 1 and 2 of the winged-helix DNA-binding domain conserved among FoxA1–3 of multiple species. Because, as noted above, wings 1 and 2 are essential for stabilizing FoxA binding to DNA and chromatin (23, 25), this suggested that acetylation might regulate DNA and chromatin binding and/or chromatin remodeling by FoxA. Here, we demonstrate that FoxA1 is acetylated both in vitro and in vivo and that acetylation of lysine residues residing within wings 1 and 2 of its DNA-binding domain curtails FoxA binding to its sites within free DNA and curtails nucleosome binding and remodeling by this factor. However, following stable binding to its sites in nucleosomal DNA, FoxA1 is no longer capable of being acetylated. We propose a model whereby stable chromatin binding protects the FoxA DNA-binding domain from acetylation to preserve chromatin binding and remodeling by FoxA factors in the absence of extracellular cues.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction and Mutagenesis**—The bacterial expression plasmid encoding histidine-tagged full-length FoxA1 (pET28b-6xhis-FoxA) contains the mouse FoxA1 cDNA subcloned into the pET28b plasmid (Novagen, Gibbstown, NJ) as described (8). Mutations were introduced into this plasmid to convert lysine residues to either glutamine (acetylation mimics) or arginine (acetylation controls) in wing 1 of the DNA-binding domain (lysines 237 and 240; W1-KQ and W1-KR), wing 2 (lysines 264, 267, and 270; W2-KQ and W2-KR), or all five sites (W1W2-KQ and W1W2-KR) using the PCR-based strategy described in the QuikChange mutagenesis kit (Stratagene, La Jolla CA).Mutations were verified by sequencing.

**Protein Preparation and Purification**—Purified recombinant FoxA1 proteins were expressed in Escherichia coli and purified through a combination of anion-exchange and nickel-agarose chromatography as described previously (10). Purified recombinant full-length p300 and p300 histone acetyltransferase (HAT) domain were obtained from Active Motif (Carlsbad, CA). In vitro translated FoxA1 proteins were prepared using the Tn^T T7 transcription/translation Kit from Promega (Madison, WI) according to manufacturer’s instructions.

**In Vitro Acetylation**—Purified recombinant FoxA1 (2 μg) was incubated for 3 h at 22 °C in HAT buffer (50 mM Tris-HCl, pH 8.0, 10% glycerol, 0.1 mM EDTA, and 1 mM dithiothreitol) with and without 0.8 pm [3H]acetyl-CoA (MP Biomedicals, Solon OH) and 0.5 mM unlabeled acetyl-CoA with and without 0.1 μg of p300 HAT domain or 0.5 μg of full-length p300 as indicated. To verify acetylation of FoxA1, an aliquot of the in vitro acetylated protein was electrophoresed on a 10% SDS-polyacrylamide gel, stained with Coomassie Brilliant Blue (Bio-Rad), subjected to fluorographic enhancement by incubation in ENHANCE (PerkinElmer Life Science) for 30 min, and visualized by exposure to Kodak XAR film. The remainder, to be used for electrophoretic mobility shift assay (EMSA) and DNase...
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footprinting analysis, was stored at −80 °C. For these experiments, mock-acetylated protein (FoxA1 incubated with p300 in the absence of acetyl-CoA) was used to compare binding of non-acetylated and acetylated FoxA1 and the control for possible deleterious effects of p300 on FoxA1 binding. Mock-acetylated FoxA1 is labeled non-acetylated FoxA1 in all figures and graphs for simplicity.

EMSA—Double-stranded DNA oligonucleotides corresponding to the eG FoxA-binding site in the mouse serum albumin enhancer (42) were purchased from IDT (Coralville, IA), annealed, and end-labeled with [γ-32P]ATP using T4 polynucleotide kinase to form the DNA probe. The sequence of the top strand is 5′-GCT CCA GGG AAT GTT TGT TCT TAA ATA CCA TC-3′. The indicated amounts of purified recombinant in vitro or mock-acetylated FoxA1 protein (wild-type or mutant) or in vitro translated wild-type, acetylation mimic, and acetylation mutant FoxA1 proteins were incubated with end-labeled eG site probe (7.8 ng for purified proteins and 24 ng for in vitro translated proteins) for 30 min at room temperature in binding buffer (10 mM Tris, pH 7.5, 1% Ficoll, 0.33 mM MgCl2, 0.001 M dithiothreitol, and 130 ng/μl bovine serum albumin). Acetylated and mock-acetylated FoxA1 were diluted in 20 mM Hepes, pH 6.5, 80 mM NaCl, 1 mM MgCl2, 20% glycerol, and 250 μg/ml bovine serum albumin. Binding carried out with in vitro translated proteins included 100 ng of poly(dI/dC) to prevent nonspecific binding. After the incubation, binding reactions were electrophoresed through a 10% nondenaturing polyacrylamide gel in 0.5× Tris borate/EDTA. The gel was dried, and reaction products were detected by exposure to a phosphorimaging screen.

Nucleosome Template Preparation and DNase Footprinting Assays—Procedures for mononucleosome core assembly from purified liver core histone proteins and PCR-generated DNA with the mouse albumin enhancer sequence from positions 472–638 and DNase footprinting have been described (8). To examine the effect of acetylation on FoxA1 binding, binding reactions were carried out in a 20-μl volume containing 5 ng of free DNA probe or 10 ng of nucleosomes (final concentration of 4 nM DNA or chromatin) and the indicated amounts of purified recombinant in vitro or mock-acetylated FoxA1 under final buffer conditions of 10 mM Tris, pH 7.5, 1% Ficoll, 1 mM MgCl2, 35 mM KCl, 5 mM dithiothreitol, 300 ng/μl bovine serum albumin, and 4.8% glycerol at 21–25 °C for 1 h. Following DNase I digestion, reactions were stopped by addition of 30 mM EDTA, 0.35 M NaCl, 0.1% SDS, and 50 μg/μl tRNA, and DNA fragments were extracted with phenol/chloroform (1:1) and then chloroform alone, followed by ethanol precipitation. The DNA fragments were separated on 6% polyacrylamide and 7 M urea sequencing gels in 1× Tris borate/EDTA buffer and detected by exposure to a phosphorimaging screen. To examine acetylation after stable binding, purified recombinant unmodified FoxA1 was incubated with nucleosomes overnight, followed by further incubation with p300 HAT in the presence and absence of acetyl-CoA as indicated. The bound nucleosome templates were digested and evaluated as described above.

Cell Culture, Immunoprecipitation, and Western Blot Analysis—HepG2 cells were cultured in Eagle’s minimal essential medium supplemented with 10% fetal bovine serum. HepG2 cells were grown until ~80% confluent and then treated with 1 nM trichostatin A and 5 mM nicotinamide for 24 h. Following incubation with the deacetylase inhibitors, the cells were harvested in radioimmune precipitation assay buffer (1.25% (w/v) Triton X-100, 1.25% sodium deoxycholate, 0.0125 M sodium phosphate, pH 7.2, 2 mM EDTA, 50 mM sodium fluoride, and protease inhibitor cocktail (Sigma)) containing 10 mM sodium butyrate and 10 mM nicotinamide, and the lysates were subjected to immunoprecipitation.

For immunoprecipitation, cell lysates (~0.5 μg of protein) were incubated at 4 °C overnight in one-third volume of immunoprecipitation buffer (50 mM Tris, pH 7.5, 15 mM EGTA, 100 mM NaCl, and 0.1% (w/v) Triton X-100) with 5 mM nicotinamide, 10 mM sodium butyrate, and 4 μg of antibody (anti-FoxA (sc-6553, Santa Cruz Biotechnology), anti-acetyllysine (ICP380, Immunchem), or rabbit IgG (Santa Cruz Biotechnology)). Protein A/G-agarose beads (Millipore) were added and incubated for an additional 2 h. The agarose was pelleted and washed three times with immunoprecipitation buffer. NuPAGE SDS sample buffer (Invitrogen) was added directly to the beads, which were incubated for 10 min at 95 °C to prepare for SDS-PAGE and Western blotting.

For Western blot analysis, samples were run on a 10% SDS-acrylamide gel and transferred to polyvinylidene difluoride membrane (Bio-Rad). The membranes were blocked in 5% milk in 1× Tris-buffered saline/Tween and incubated with anti-FoxA or anti-acetyllysine antibody in blocking solution overnight at 4 °C. The membrane was washed with 1× Tris-buffered saline/Tween and incubated for 1 h at room temperature with a biotinylated secondary antibody in blocking solution. The membrane was washed with 1× phosphate-buffered saline, incubated for 1 h in ABC (Pierce) mixed in 1× phosphate-buffered saline, and then washed a final time before exposure to ECL Western blot detection reagent (GE Healthcare) and Kodak XAR film.

RESULTS

FoxA1 Is Acetylated by p300 In Vitro—p300 acetylates proteins at lysines that are part of either a KXXX or KXXXK motif or lysines that are located −3 or +4 from a positive charge (lysine, arginine, or histidine) (43). Our in silico analysis of the FoxA1 protein sequence revealed 11 putative p300 acetylation sites, including five in the DNA-binding domain, as illustrated in Fig. 1A. To determine whether FoxA1 is acetylated by p300, we performed an in vitro acetylation assay in which we incubated purified recombinant FoxA1 with full-length protein p300 or the catalytic (HAT) domain of p300 in the presence or absence of [3H]acetyl-CoA. As demonstrated by the autoradiograph in Fig. 1B, FoxA1 could be acetylated at one or more sites in vitro by both the p300 HAT domain and full-length p300 (lanes 1 and 2). FoxA1 was not acetylated in the absence of acetyl-CoA (lanes 7 and 8). A small amount of acetylated FoxA1 was observed when incubated in the presence of [3H]acetyl-CoA alone (lane 6); this acetylation is most likely due to non-enzymatic acetylation of cysteines as described previously by Dormeyer et al. (44). The much higher levels of acetylation in the presence of acetyltransferase enzyme relative to [3H]acetyl-CoA alone suggest that FoxA1 is acetylated by p300 in vitro.
As mentioned above, 5 of the 11 putative acetylation sites were located in the FoxA1 DNA-binding domain. To determine whether the FoxA1 DNA-binding domain is acetylated, we subjected the FoxA1 DNA-binding domain to the in vitro acetylation assay. As demonstrated in Fig. 1B (lanes 3 and 4), the DNA-binding domain was acetylated by both the p300 HAT domain and full-length p300, indicating that at least one of the five sites located in the DNA-binding domain of FoxA1 is capable of being acetylated. Additionally, purified recombinant FoxA1 in which all five lysine residues located within the DNA-binding domain were mutated to glutamine (FoxA1-DBD-KQ) exhibited a >50% reduction in acetylation levels compared with the wild-type protein when subjected to the in vitro acetylation (Fig. 1C, compare lanes 1 and 2). We conclude that the FoxA1 DNA-binding domain is acetylated by p300 in vitro.

Endogenous FoxA1 Is Acetylated in Hepatocyte Cell Lines—To determine whether endogenous FoxA1 is acetylated in hepatocytes, we treated HepG2 cells with deacetylase inhibitors for 24 h and then harvested the cells for immunoprecipitation with antibodies directed against either FoxA or acetylated lysine. HepG2 cells were treated for 24 h with deacetylase inhibitors (trichostatin A and nicotinamide), and the cells were harvested for immunoprecipitation. Upper band: anti-FoxA antibody; lower band: anti-acetyllysine antibody. IB, immunoblot; IN, input whole cell lysate.
Proteins can be acetylated in HepG2 cells. Similar results were seen in the H4IIE liver cell line (data not shown). We conclude that FoxA1 can be acetylated in hepatocytes in vivo.

**Acetylation Attenuates FoxA1 Binding to DNA**—Because the DNA-binding domain of FoxA1 is acetylated (Fig. 1B), and the putative acetylation sites are located within wings 1 and 2 of the FoxA1 winged-helix DNA-binding domain, which stabilize binding of the FoxA DNA-binding domain to DNA (25), this suggested that acetylation might affect the ability of FoxA1 to bind to its sites in DNA. To examine the effect of acetylation on FoxA1 DNA binding, we compared binding of mock-acetylated FoxA1 and FoxA1 acetylated with p300 HAT to a 32P-labeled DNA probe corresponding to the eG FoxA-binding site from the mouse serum albumin enhancer in an EMSA (Fig. 2A). To control for deleterious effects of p300 HAT on FoxA1 binding, FoxA1 protein designated “non-acetylated” was incubated with p300 HAT in the absence of acetyl-CoA (Fig. 1B, lane 7). A similar notation is used throughout this work. As shown in Fig. 2B, binding of acetylated FoxA1 protein to the eG binding site probe (lanes 6–8) was markedly reduced compared with non-acetylated FoxA1 (lanes 2–4). Acetylation attenuated FoxA1 binding to such an extent that it took a 2-fold greater concentration of acetylated FoxA1 than the maximal amount of mock-acetylated FoxA1 used in the gel shift assay to obtain discernible binding to the eG probe (lane 11). p300 alone did not bind to the eG probe (lane 9). Similar results were obtained with FoxA1 protein acetylated with full-length p300 (data not shown). Because acetylation of FoxA1 with full-length p300 and the p300 HAT domain similarly curtailed FoxA1 DNA binding, the remaining binding experiments were performed using FoxA1 acetylated by the p300 HAT domain.

Diminished DNA binding by in vitro acetylated FoxA1 compared with its non-acetylated counterpart was also evident in DNase footprinting assays (Fig. 2C) conducted on DNA corresponding to the N1 region of the albumin enhancer (Fig. 2A). Non-acetylated FoxA1 bound to its eG and eH sites as described previously (17) and indicated by the region of protected bands in Fig. 2C (lanes 2–4, bars to the left). Acetylation of FoxA1 attenuates this binding at both the eG and eH sites.
Acetylation Curtails Nucleosome Binding and Remodeling by FoxA1—As an initial chromatin-binding pioneer transcription factor for liver development, FoxA1 stably binds to its sites in the DNA-binding domain on FoxA1 binding, we studied DNA binding by mutant FoxA1 proteins containing substitution mutations mimicking acetylation of lysine residues located within wings 1 and 2 of the FoxA1 DNA-binding domain (Fig. 1A). Three different sets of acetylation mimics and their counterpart substitution controls were generated: W1-KQ and W1-KR, in which the two lysine residues residing within wing 1 of the FoxA1 DNA-binding domain were substituted with either glutamine (which mimics acetylation by substituting a neutrally charged amino acid for the basic lysine residue) or arginine (which functions as a control for amino acid substitution by replacing the basic lysine residue with an amino acid of a similar charge); W2-KQ and W2-KR, containing substitutions for the three lysine residues residing within wing 2; and W1W2-KQ and W1W2-KR, containing substitutions for all five lysines. DNA constructs encoding the substituted proteins, together with wild-type FoxA1, were translated in vitro (Fig. 3A) and subjected to EMSA with the \(^{32}\)P-labeled eG probe. As demonstrated in Fig. 3B, binding of mutant FoxA1 proteins containing substitution mutations mimicking acetylation of lysine residues located within either wing 1 (W1KQ; lanes 4 and 5) or wing 2 (W2KQ; lanes 8 and 9) bound less efficiently to the DNA probe than either the wild-type protein (lanes 2 and 3) or corresponding KR controls (lanes 6 and 7 and lanes 10 and 11, respectively). Similar to in vitro acetylated FoxA1, FoxA1 acetylation mimics in which all five wing 1 and 2 lysine residues were substituted with glutamine failed to bind the eG probe (lanes 12 and 13), suggesting that in vitro acetylation targets lysine residues located within both wing motifs. In agreement with this assessment, in vitro acetylation of the corresponding FoxA1 mutant by p300 was significantly reduced compared with the wild-type protein (Fig. 1C, lane 2). Furthermore, acetylation of the W1W2-KR mutant, in which the five putative acetylation sites in the FoxA1 DNA-binding domain were mutated to prevent their acetylation, had no effect on DNA binding by this protein (Fig. 3C, compare lanes 6–8 with lanes 2–4). This supports the notion that acetylation of lysine residues within the DNA-binding domain is directly responsible for attenuated FoxA1 binding, as opposed to acetylation of putative sites located outside this domain.

![Figure 2](image2.png)

**Figure 2. Acetylation of FoxA1 attenuates binding to the albumin enhancer.** A, diagram of three nucleosome particles that comprise the albumin enhancer, N1, N2, and N3. The N1 region has been enlarged to illustrate the three FoxA1-binding sites (NS, eG, and eH). B, EMSA using a \(^{32}\)P-labeled probe corresponding to the eG binding site on the albumin enhancer bound without protein (lanes 1, 5, and 10) or with decreasing concentrations (0.0025, 0.00125, and 0.000625 pmol) of non-acetylated (lanes 2–4) and acetylated (lanes 6–8) FoxA1 or p300 alone (lane 9). An additional lane with an increased amount of acetylated FoxA1 (0.005 pmol) was also included (lane 11). The proteins were incubated with 24 ng of the probe for 30 min at room temperature and resolved on a polyacrylamide gel. C, non-acetylated and acetylated FoxA1 were bound to 5 ng of a \(^{32}\)P-labeled probe corresponding to the N1 region of the albumin enhancer at varying concentrations of FoxA (0.0625, 0.125, and 0.25 pmol) for 1 h and then digested with DNase I for 1 min. The positions of the FoxA-binding sites are indicated (eG and eH). Arrowheads designate hypersensitive sites. D, quantitation of six independent DNase I footprinting gels. *, p < 0.05.

![Figure 3](image3.png)

**Figure 3. Wing 1/wing 2 FoxA1 acetylation mimics exhibit attenuated binding to DNA.** A, autoradiograph of \(^{32}\)P-methionine-labeled, in vitro translated wild-type FoxA1 (WT), wing 1 acetylation mimic (W1-KQ) or mutant (W1-KR), wing 2 acetylation mimic (W2-KQ) or mutant (W2-KR), and DNA-binding domain mimic (DBD-KQ) or mutant (DBD-KR). B, EMSA using a \(^{32}\)P-labeled probe corresponding to the eG binding site on the albumin enhancer bound without protein (lanes 1 and 5) or with decreasing concentrations (0.0025, 0.00125, and 0.000625 pmol) of non-acetylated (lanes 2–4) and acetylated (lanes 6–8) FoxA1. The proteins were incubated with 24 ng of the probe for 30 min at room temperature and resolved on a polyacrylamide gel.

(Fig. 2C, compare lanes 5–7 with lanes 2–4). Quantification of data from six independent footprinting experiments revealed that acetylated FoxA1 bound ~20% less well than non-acetylated FoxA1 to both of these sites (Fig. 2D). Additionally, a hypersensitive DNase I cleavage site created within the eH footprint by the non-acetylated and acetylated FoxA1 proteins was considerably less pronounced in the case of acetylated FoxA (band in Fig. 2C, compare lanes 5–7 with lanes 2–4, indicated by the arrowhead to the right). A comparative quantitation of the hypersensitive signal generated by non-acetylated versus acetylated FoxA1 is shown in Fig. 2D (lower panel). We conclude that acetylation of FoxA1 curtails its binding to DNA.

Finally, to confirm the consequences of in vitro acetylation of the wing motifs within the FoxA1 winged-helix DNA-binding domain on FoxA1 binding, we studied DNA binding by mutant FoxA1 proteins containing substitution mutations mimicking acetylation of lysine residues located within wings 1 and 2 of the FoxA1 DNA-binding domain (Fig. 1A). Three different sets of acetylation mimics and their counterpart substitution controls were generated: W1-KQ and W1-KR, in which the two lysine residues residing within wing 1 of the FoxA1 DNA-binding domain were substituted with either glutamine (which mimics acetylation by substituting a neutrally charged amino acid for the basic lysine residue) or arginine (which functions as a control for amino acid substitution by replacing the basic lysine residue with an amino acid of a similar charge); W2-KQ and W2-KR, containing substitutions for the three lysine residues residing within wing 2; and W1W2-KQ and W1W2-KR, containing substitutions for all five lysines. DNA constructs encoding the substituted proteins, together with wild-type FoxA1, were translated in vitro (Fig. 3A) and subjected to EMSA with the \(^{32}\)P-labeled eG probe. As demonstrated in Fig. 3B, binding of mutant FoxA1 proteins containing substitution mutations mimicking acetylation of lysine residues located within either wing 1 (W1KQ; lanes 4 and 5) or wing 2 (W2KQ; lanes 8 and 9) bound less efficiently to the DNA probe than either the wild-type protein (lanes 2 and 3) or corresponding KR controls (lanes 6 and 7 and lanes 10 and 11, respectively). Similar to in vitro acetylated FoxA1, FoxA1 acetylation mimics in which all five wing 1 and 2 lysine residues were substituted with glutamine failed to bind the eG probe (lanes 12 and 13), suggesting that in vitro acetylation targets lysine residues located within both wing motifs. In agreement with this assessment, in vitro acetylation of the corresponding FoxA1 mutant by p300 was significantly reduced compared with the wild-type protein (Fig. 1C, lane 2). Furthermore, acetylation of the W1W2-KR mutant, in which the five putative acetylation sites in the FoxA1 DNA-binding domain were mutated to prevent their acetylation, had no effect on DNA binding by this protein (Fig. 3C, compare lanes 6–8 with lanes 2–4). This supports the notion that acetylation of lysine residues within the DNA-binding domain is directly responsible for attenuated FoxA1 binding, as opposed to acetylation of putative sites located outside this domain.
compacted chromatin and remodels the underlying nucleosome structure (8, 10, 17, 45). To determine whether acetylation affects FoxA1 binding to nucleosomes, we performed DNase footprinting analysis of FoxA1 binding to nucleosome particles corresponding to the N1 region of the albumin enhancer (Fig. 4). Binding of non-acetylated FoxA1 to the N1 nucleosome particles generated three footprints, one each at its eG and eH binding sites, which straddle the nucleosome dyad axis, and one located at a nucleosome-specific binding site, NS, located at the nucleosome edge. Each footprint is characterized by multiple protected DNA bands (located within the three FoxA-binding sites in Fig. 4A); the eH and NS footprints also exhibit a hypersensitive site at the center of the protected region (arrowheads to the right). As illustrated by the reduction in protections and hypersensitive sites at each footprint depicted in Fig. 4A and quantitated in Fig. 4B, binding of in vitro acetylated FoxA1 protein to all three sites (lanes 5–7) was markedly reduced compared with non-acetylated FoxA1 (lanes 2–4). FoxA binding to N1 nucleosome particles also generated a ladder of hypersensitive sites outside its footprints, indicative of nucleosome remodeling/positioning (Fig. 4A, dots to the right). Not surprisingly, the intensity of these hypersensitive sites was significantly diminished when comparing nucleosomes bound by acetylated FoxA1 with those bound by non-acetylated FoxA1. We conclude that acetylation negatively impacts nucleosome binding and remodeling by FoxA1. Curtailed binding by in vitro acetylated FoxA1 to nucleosome arrays assembled from the albumin enhancer (10) and to previously characterized FoxA-binding sites on nucleosomes assembled from the IGFBP-1 (insulin-like growth factor-binding protein-1) promoter (46) (data not shown) provides further evidence that acetylation of FoxA1 attenuates its ability to bind to and remodel chromatin.

**Stable Nucleosome Binding Inhibits FoxA1 Acetylation—**

FoxA1 has been demonstrated to stably bind to its sites in chromatin in vitro and, more recently, in vivo (9, 23). Because the putative sites of lysine acetylation within wings 1 and 2 of the FoxA1 DNA-binding domain make intimate contacts with the DNA backbone necessary for stable chromatin binding (23), it was possible that stable nucleosome binding might interfere with the ability of p300 to acetylate the FoxA1 DNA-binding domain through steric hindrance. Therefore, we were interested in whether FoxA1 could be acetylated once bound to nucleosomal DNA and, if so, what affect this would have on its nucleosome-binding and nucleosome-remodeling capabilities. To determine this, we bound FoxA1 to nucleosome particles overnight to establish stable nucleosome binding (9) and then subjected the prebound FoxA1 to in vitro acetylation by addition of p300 HAT and [3H]acetyl-CoA. As demonstrated by the fluorograph in Fig. 5A, FoxA1 was not acetylated to any appreciable level after it was stably bound to its sites on a nucleosome (lane 1). In contrast, neither overnight binding of FoxA1 to free DNA (lane 3), to which it does not exhibit stable binding (9), nor incubation of FoxA1 in the presence of nucleosomes for short time periods (lane 5), which is not amenable to stable nucleosome binding (data not shown), inhibited in vitro acetylation of FoxA1. In agreement with these results, DNase footprinting of the corresponding reaction products clearly showed no appreciable decrease in nucleosome binding or remodeling by stably bound FoxA1 subjected to in vitro acetylation (lanes 5–7) compared with non-acetylated FoxA1 (lanes 2–4). We conclude that, once stably bound to nucleosomal DNA, FoxA1 no longer serves as a suitable substrate for acetylation.

**DISCUSSION**

In this study, we have demonstrated that FoxA1 can be acetylated in vitro and in vivo (Fig. 1) and that acetylation attenuates binding of FoxA1 to its regulatory elements assembled within nucleosomal DNA (Fig. 4), curtailing its nucleosome-remodeling capabilities. Acetylation sites for FoxA1 were identified within wings 1 and 2 of its winged-helix DNA-binding domain (Figs. 1A and C) and 3. Wings 1 and 2 have previously been demonstrated to stabilize FoxA DNA and nucleosome binding (23, 25), and acetylation at these sites is likely responsible for the observed decrease in FoxA1 DNA and nucleosome binding following acetylation. This is the first work to demonstrate that
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Our data demonstrate that FoxA is not acetylated once stably bound to nucleosomal DNA (Fig. 5). A recent article by Sekiya et al. (23) identified key amino acid residues in wings 1 and 2 of the FoxA DNA-binding domain that are important for stable binding of FoxA proteins within cellular chromatin. Many of these amino acid residues are in close proximity to the acetylated lysines identified in this study (Fig. 4), suggesting that steric hindrance due to wing 1/wing 2-DNA interactions might inhibit FoxA acetylation. DNA binding by GATA1 has similarly been demonstrated to inhibit acetylation of its DNA-binding domain (47).

FoxA transcription factors play essential roles in development and metabolism (5, 48). How FoxA acetylation might influence these biological processes remains unclear. In light of the fact that chromatin-bound FoxA is not a suitable substrate for acetylation, it seems unlikely that acetylation would be directly responsible for the displacement of FoxA from its target sites in cellular chromatin. In fact, acetylated FoxA protein is not readily detected in vivo. Addition of deacetylase inhibitors was required to observe acetylation of endogenous FoxA in this study, suggesting that levels of acetylated FoxA in the cell are normally quite low. It is more probable that FoxA acetylation cooperates with FoxA protein partners such as the small heterodimer partner and/or other post-translational modifications such as phosphorylation in response to specific physiological conditions to permanently disable FoxA binding following its initial removal from chromatin at target genes. This scenario is particularly attractive in the case of Akt-mediated phosphorylation of FoxA2 in response to insulin stimulation (31), which has been shown to attenuate FoxA2 binding at insulin-responsive genes (33). Acetylation and phosphorylation have been shown to synergize in their regulation of other proteins. For example, acetylation of FoxO1 increases its sensitivity to Akt-mediated phosphorylation (38), whereas, in the case of p53, phosphorylation stimulates its subsequent acetylation (49). It is possible that acetylation could similarly cooperate with phosphorylation to permanently quench binding of FoxA2 and facilitate its translocation from the nucleus, as demonstrated by Wolfrum et al. (31). FoxA factors exhibit nonspecific chromatin binding (23). This nonspecific binding has been hypothesized to facilitate FoxA target site recognition by allowing FoxA to scan for its binding sites within chromatin. Not surprisingly, the same amino acid residues located in wings 1 and 2 that facilitate stable chromatin binding also specify nonspecific chromatin binding. By inhibiting nonspecific FoxA chromatin binding, acetylation of lysine residues within wings 1 and 2 would be expected to hamper FoxA target site recognition and eliminate cellular chromatin as a “sink” for FoxA binding, promoting FoxA removal from the nucleus.

Our finding that acetylation of wing 1 and 2 lysine residues within the FoxA DNA-binding domain inhibits DNA and nucleosome binding by FoxA would seem to contradict previous studies demonstrating that FoxA proteins synergize with acetyltransferases, either alone or as participants in multiprotein complexes, to activate gene expression (40, 41, 50). How FoxA acetylation might influence these biological processes remains unclear. In light of the fact that chromatin-bound FoxA is not a suitable substrate for acetylation, it seems unlikely that acetylation would be directly responsible for the displacement of FoxA from its target sites in cellular chromatin. In fact, acetylated FoxA protein is not readily detected in vivo. Addition of deacetylase inhibitors was required to observe acetylation of endogenous FoxA in this study, suggesting that levels of acetylated FoxA in the cell are normally quite low. It is more probable that FoxA acetylation cooperates with FoxA protein partners such as the small heterodimer partner and/or other post-translational modifications such as phosphorylation in response to specific physiological conditions to permanently disable FoxA binding following its initial removal from chromatin at target genes. This scenario is particularly attractive in the case of Akt-mediated phosphorylation of FoxA2 in response to insulin stimulation (31), which has been shown to attenuate FoxA2 binding at insulin-responsive genes (33). Acetylation and phosphorylation have been shown to synergize in their regulation of other proteins. For example, acetylation of FoxO1 increases its sensitivity to Akt-mediated phosphorylation (38), whereas, in the case of p53, phosphorylation stimulates its subsequent acetylation (49). It is possible that acetylation could similarly cooperate with phosphorylation to permanently quench binding of FoxA2 and facilitate its translocation from the nucleus, as demonstrated by Wolfrum et al. (31). FoxA factors exhibit nonspecific chromatin binding (23). This nonspecific binding has been hypothesized to facilitate FoxA target site recognition by allowing FoxA to scan for its binding sites within chromatin. Not surprisingly, the same amino acid residues located in wings 1 and 2 that facilitate stable chromatin binding also specify nonspecific chromatin binding. By inhibiting nonspecific FoxA chromatin binding, acetylation of lysine residues within wings 1 and 2 would be expected to hamper FoxA target site recognition and eliminate cellular chromatin as a “sink” for FoxA binding, promoting FoxA removal from the nucleus.

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FoxA factors are capable of being acetylated and only the second post-translational modification associated with FoxA (31). Our data demonstrate that FoxA is not acetylated once stably bound to nucleosomal DNA (Fig. 5).
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directed at histones or other transcription regulatory proteins, not the FoxA DNA-binding domain stably bound to chromatin. The same scenario would also apply in the situation in which HNF6/p300 binding accentuates FoxA2 transcriptional activity (50). Additional putative consensus sites for p300 were identified in our study. Mutation of the wing 1 and 2 acetylation sites does not completely eliminate acetylation of FoxA by p300, suggesting that some of these sites are capable of being acetylated. Acetylation of these sites, one of which is located in the FoxA N-terminal transactivation domain (51), could positively influence transactivation by FoxA. We conclude that the fortuitous location of the acetylation sites of FoxA within a region of its DNA-binding domain controlling both stable and nonspecific binding enables the effect of acetyltransferase recruitment on FoxA activity to be adjusted in response to specific physiological cues.

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