NuA4-dependent Acetylation of Nucleosomal Histones H4 and H2A Directly Stimulates Incorporation of H2A.Z by the SWR1 Complex*

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Structural and functional analyses of nucleosomes containing histone variant H2A.Z have drawn a lot of interest over the past few years. Important work in budding yeast has shown that H2A.Z (Htz1)-containing nucleosomes are specifically located on the promoter regions of genes, creating a specific chromatin structure that is poised for disassembly during transcription activation. The SWR1 complex is responsible for incorporation of Htz1 into nucleosomes through ATP-dependent exchange of canonical H2A-H2B dimers for Htz1-H2B dimers. Interestingly, the yeast SWR1 complex is functionally linked to the NuA4 acetyltransferase complex in vivo. NuA4 and SWR1 are physically associated in higher eukaryotes as they are homologous to the Tip60/p400 complex, which encompasses both histone acetyltransferase (Tip60) and histone exchange (p400/Domino) activities. Here we present work investigating the impact of NuA4-dependent acetylation on SWR1-driven incorporation of H2A.Z into chromatin. Using in vitro histone exchange assays with native chromatin, we demonstrate that prior chromatin acetylation by NuA4 greatly stimulates the exchange of H2A for H2A.Z. Interestingly, we find that acetylation of H2A or H4 N-terminal tails by NuA4 can independently stimulate SWR1 activity. Accordingly, we demonstrate that mutations of H4 or H2A N-terminal lysine residues have similar effects on H2A.Z incorporation in vivo, and cells carrying mutations in both tails are nonviable. Finally, depletion experiments indicate that the bromodomain-containing protein Bdf1 is important for NuA4-dependent stimulation of SWR1. These results provide important mechanistic insight into the functional cross-talk between chromatin acetylation and ATP-dependent exchange of histone H2A variants.

Genetic information within the eukaryotic cell nucleus is organized in a highly conserved structural polymer, chromatin, which supports and controls crucial functions of the genome. Chromatin remodeling and post-translational modifications of histones are critical processes regulating genome expression and maintenance by controlling access to DNA and signaling local regions for specific molecular interactions. Incorporation of different histone variants in specific chromosomal regions is also known to be associated with important biological processes controlling gene expression and genome integrity.

A specific histone variant, H2A.Z, has been the focus of intense research over the past few years. Delineation of its impact on nucleosome structure and stability and on gene expression has turned into a long-heated debate involving apparently contradicting experimental data. Recent findings led to an emerging model that could partly reconcile these contradictions. Genome-wide analyses of H2A.Z localization in eukaryotes indicate that it is preferentially found on gene promoter/regulatory regions within nucleosomes flanking more accessible DNA sequences. Importantly, H2A.Z is found within the promoters of inactive or weakly transcribed genes in yeast euchromatin. Additional data suggest that H2A.Z-containing nucleosomes form a special chromatin structure that presents nucleosomes for disassembly upon gene activation.

Activities responsible for incorporation of H2A.Z in chromatin have been characterized and form a new family of SWI/SNF-related ATP-dependent chromatin remodelers. The yeast SWR1 complex uses ATP hydrolysis to perform histone dimer exchange within a nucleosome, replacing canonical H2A-H2B with H2A.Z-H2B dimers. Protein complexes homologous to SWR1 have been identified in higher eukaryotes (e.g. human SRCAP and p400 complexes) and shown to possess similar histone exchange activity in vitro and in vivo.

Interestingly, a double bromodomain protein has been identified as a component of both SWR1 and p400 complexes. In yeast, the SWR1 subunit Bdf1 associates with acetylated histone H4 in vitro and functionally interacts in vivo with Esal, the enzyme responsible for the bulk of histone H4 and H2A acetylation. Accordingly, there is a strong genetic and functional link between the Esal histone acetyltransferase (HAT) complex, named NuA4, and the SWR1 complex. The two complexes share four subunits, and...
the NuA4 subunit Eaf1 has significant homology to Swr1 outside of the SWI/SNF-related ATPase domain (31). Supporting a role for chromatin acetylation in the mechanism of histone dimer exchange, it was shown that HAT complexes and histone N-terminal domains regulate the efficient incorporation of H2A.Z at specific chromosomal loci in vivo (8, 10, 31, 33, 34). Such cooperation between histone acetylation and ATP-dependent exchange of histone H2A-H2B dimers is further supported by the fact that Drosophila and human NuA4 HAT complexes (also known as TIP60) contain subunits homologous to those found in the yeast Swr1 complex, including p400 (23–25, 31, 35). Furthermore, it was shown that the Drosophila TIP60/p400 complex is able to exchange histone H2Av-H2B dimers on chromatin in vitro and that this reaction was stimulated by prior TIP60-dependent acetylation of histone H2Av (23). Finally, to further functionally tie NuA4 and SWR1 activities, it was shown that H2A.Z N terminus is acetylated by NuA4 in yeast (36–38). This acetylation occurs only after incorporation of H2A.Z in chromatin by SWR1 and is thought to be involved in the specific destabilization of H2A.Z-containing nucleosomes on gene promoters during transcription activation (36–39).

In this study we investigated the mechanisms implicated in the cross-talk between NuA4-dependent acetylation of chromatin and SWR1-dependent incorporation of H2A.Z into nucleosomes through ATP-dependent exchange of histone dimers. We used a highly purified in vitro histone exchange assay with yeast native chromatin, NuA4 and SWR1 complexes, and H2A.Z-H2B dimers. We found that prior acetylation of chromatin by NuA4 greatly stimulates the ability of SWR1 to replace canonical H2A-H2B with H2A.Z-H2B. Interestingly, mutation of all acetylatable lysine residues on histone H4 does not diminish the effect of pretreatment with NuA4 and acetyl-CoA on SWR1 activity. The same results were obtained when H2A lysine residues were mutated. Because stimulation of SWR1 activity is strictly acetyl-CoA-dependent and NuA4 cannot acetylate the SWR1 complex, we conclude that acetylated histone H4 and H2A tails function in an independent and likely redundant manner to promote incorporation of H2A.Z by the SWR1 complex. Accordingly, mutations of H4 and H2A lysine residues affect H2A.Z presence at specific loci in vivo, and simultaneous mutations in both tails are lethal in vivo. Finally, bromodomain protein Bdf1, a component of the SWR1 complex, is critical for the stimulatory effect of NuA4-mediated acetylation. Altogether, these results bring new mechanistic understanding of the evolutionarily conserved intimate relationship between chromatin acetylation by the NuA4/TIP60 complex and exchange of histone H2A variants by SWR1/p400.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains**—Genomic SWR1 was fused to three copies of the FLAG epitope in BY4741 using PCR and p3XFLAG-KanMX6 as template (40). BDF1 was fused to the Tandem affinity-purified (TAP) epitope using pBS1539 as template (41). Eppl-TAP, H4 K5Q/K8Q/K12Q/K16Q, H4 K5R/K12R, and H4 K5Q/K12Q strains and their isogenic wild type strains have been described (27, 42, 43). For the H2A K4R/K7R/K13R, H2A K4Q/K7Q/K13Q, H4 K5R/K8R/K12R-H2A K3R/K7R/K13R, and H4 K5Q/K8Q/K12Q-H2A K4Q/K7Q/K13Q strains, plasmids pJHA2 (wild type H2A-H2B ARS/CEN/His3 vector) (44) and pQQ18 (wild type H3-H4-H2A-H2B ARS/CEN/LEU2 vector) (45) were used in site-directed mutagenesis (Strategene QuikChange) and introduced into FY406 (46) and JHY205 (45), respectively, followed by 5-fluoro-orotic acid treatment to chase the wild type H2A-H2B and H3-H4-H2A-H2B ARS/CEN/URA3 vectors. Wild type control strains carrying the pJHA2 and pQQ18 vectors are used to compare the mutant phenotypes.

**Purification of the SWR1 and NuA4 Complexes**—The Swr1-FLAG complex was purified similarly to what has been described (20) using 2 liters of culture grown to an A_{600} 2.5. Cells were lysed in 10 mM Tris-HCl, pH 8, 270 mM NaCl, 10% glycerol, 0.1% Nonidet P-40 containing the desired protease inhibitors. Cellular extract was first precleared using 200 μl of Sepharose CL-6B. The resulting flow-through was affinity-bound to 200 μl of anti-FLAG M2-agarose beads (Sigma) for 3 h at 4 °C. Beads were washed with 30 ml of lysis buffer containing 500 mM NaCl and then with 2 ml of lysis buffer with 100 mM NaCl. Proteins were eluted twice with 100 μg of competing 3×FLAG peptide diluted in 150 μl of lysis buffer (100 mM NaCl) at 4 °C. TAP-tagged NuA4 complex (Eppl-TAP) and Bdf1 were purified essentially as described (27) from 6 liters of yeast cultures grown to an A_{600} of 2.5. Cell extracts were precleared with Sepharose and fractionated sequentially on 100 μl of immuno-globulin G and calmodulin resins for every liter of culture. The final elution of purified TAP-protein complex was done with 10 mM EGTA-containing buffer. In some cases, NuA4 complex was purified through gel filtration on Superose 6 after elution from the IgG resin. Protein complexes were visualized by SDS-PAGE on a 10% gel and silver staining. For the Bdf1 depletion experiment, SWR1 complex was FLAG-purified from Swr1-FLAG/Bdf1-TAP whole cell extracts, and the purified material was incubated with the same ratio of IgG-Sepharose beads. Flow-through material was used in histone exchange assays. Bound material was also eluted with tobacco etch virus protease, but no exchange activity could be detected.

**Purification of Recombinant Histones**—Recombinant yeast histones were purified from bacteria and assembled as dimers and octamers essentially as described (47). Nucleosome reconstitution on 5 S DNA sequences was also done as described but using DNA previously biotinylated by the Kleobacteriophage polymerase and purified on streptavidin magnetic beads (see below) (48).

**Preparation of Native Chromatin Substrates for Exchange Assays**—Native chromatin from yeast cells was purified as previously described (33). 5 μg of yeast native chromatin was incubated with T4 DNA polymerase for 30 min at 30 °C. The reaction was then supplemented with dGTP, dTTP, and biotinylated dATP and dCTP for another 30 min. The reaction mixture was passed through a Sephadex G50 column equilibrated with binding buffer containing 10 mM HEPES-KOH, pH 7.6, 0.5 mM Mgta, 5 mM MgCl₂, 10% glycerol, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, 70 mM KCl. Buffer exchanged fractions were pooled and incubated for 3 h at room temperature with 60 μl of streptavidin-coupled paramagnetic beads (Invitrogen). Beads were washed 3
times with binding buffer and resuspended in 100 μl of binding buffer.

**Histone Exchange Assays**—The exchange assays were carried out similarly to what has been described previously with minor modifications (22). Briefly, a total of 40 ng of DNA equivalents of di/trinucleosomes immobilized on streptavidin-coupled magnetic beads were preincubated with 15 μl of SWR1 complex in 50 μl of exchange buffer (25 mM HEPES-KOH, pH 7.6, 0.1 mM EDTA, 5 mM MgCl₂, 10% glycerol, 0.02% Nonidet P-40, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 70 mM KCl) for 30 min at 37 °C. The beads were washed twice with 500 μl of exchange buffer and resuspended in a 100-μl reaction volume with 37 ng of recombinant H2A.Z-H2B dimers. The reactions were further incubated for 60 min at 37 °C in the absence or presence of 1 mM ATP. 1 unit of apyrase is also included in the reactions without ATP (except in Fig. 3B). Beads were washed twice with exchange buffer containing 400 mM KCl and twice with exchange buffer containing 70 mM KCl. The bound proteins were eluted using SDS-PAGE loading buffer, fractionated on an 18% SDS-PAGE gel, and probed with anti-Htz1 (yeast H2A.Z, Millipore), hyperacetyl-H4 (penta, Millipore), H2B, H3, and H4 (Abcam) antibodies. For the NuA4 pre-acetylation step, the beads were first incubated with NuA4 and acetyl-CoA (0.15 mM) for 30 min at 37 °C followed by 2 washes with exchange buffer before adding the SWR1 complex.

**Histone Acetyltransferase Assays**—HAT assays were performed in a 15-μl total volume using 0.5 μg of yeast chromatin, recombinant histones, or nucleosomes and 0.125 μCi of [3H]acetyl-CoA (3.5 Ci/mm) in HAT buffer (50 mM Tris/HCl, pH 8, 50 mM KCl, 0.1 mM EDTA, 5% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride) for 30 min at 37 °C. To visualize radio-labeled acetylated histones, the reactions were resolved on 15% SDS-polyacrylamide gels followed by fluorography/autoradiography.

**FIGURE 1.** Specificity of the NuA4 complex on recombinant histones and native yeast chromatin. Purified complexes from Swr1-FLAG (A) and Epl1-TAP (B) strains were loaded on 8% SDS-PAGE and visualized by silver staining. Swc4 and Eaf2 are the same protein present in both complexes along Yaf9, Arp4, and Act1. Asterisks denote nonspecific proteins obtained with the respective FLAG and TAP purification protocols. Note that a strong nonspecific band migrates close to Swc3 after FLAG purification and that Bdf1-expected migration is put in parentheses because of an apparent substoichiometry compared with other SWR1 subunits. Histone acetyltransferase assays with purified NuA4 complex were performed with recombinant yeast histones (C) and native yeast chromatin (D). Chromatin acetylation levels were measured by loading the assays on 15% SDS-PAGE followed by fluorography and autoradiography. The monomeric recombinant H2A.Z protein is different from the others because it contains an HA epitope (lane 7). The asterisk indicates the streptavidin band coming from the magnetic beads linked to the reconstituted recombinant chromatin (lanes 1 and 2, it migrates at the same position as H4).
Chromatin Immunoprecipitation Assays—Chromatin immunoprecipitations were performed as described previously (33). Cells from 200-ml cultures were cross-linked with 1% formaldehyde for 20 min at room temperature. Cross-linked chromatin was sonicated (Diagenode Bioruptor) to yield DNA fragments of an average size of 500 base pairs, and 100 μg was used for immunoprecipitations with antibodies against Htz1 (Upstate/Millipore) and the H3 C terminus (Abcam). Primers used in the PCR reactions were analyzed for linearity range and efficiency with a LightCycler (Roche Applied Science) to accurately evaluate occupancy (% of immunoprecipitation/input). All PCR reactions were at least duplicates, and variation was less than 15%. Immunoprecipitation; chromatin samples were also repeated with similar results. The numbers presented with standard errors are based on at least two independent experiments.

RESULTS

To characterize a putative cooperativity between NuA4 and SWR1 activities, we set up a highly purified in vitro enzymatic assay to test how histone acetylation could affect incorporation of histone variant H2A.Z in chromatin. Using FLAG and TAP affinity chromatography, we obtained highly purified SWR1 and NuA4 complexes (Fig. 1, A and B). The two complexes share four subunits, Eaf2/Swc4, Yaf9, Arp4, and Act1, and Eaf1 has homology with parts of Swr1 (31).

Histones H2A and H2A.Z Are Equivalent Preferred Targets of NuA4 for Acetylation of Chromatin—Our initial plan was to use recombinant yeast histones purified from bacteria to reconstitute nucleosomes for the histone exchange assays, as previously reported in the literature (20, 23). Thus, we purified recombinant yeast core histones H4, H3, H2A, and H2B along with histone variant H2A.Z (named Htz1 in yeast) and assembled them into dimers and octamers. HAT assays using NuA4 and these recombinant histones indicate that histones H2A and H2A.Z are equivalently preferred targets of NuA4 in vitro (Fig. 1C, lanes 3, 4, 7, and 8). Even when those histones are reconstituted in chromatin, NuA4 shows equivalent activity on H2A and H2A.Z (lanes 1 and 2).

NuA4 Shows Different Specificity toward Recombinant and Native Chromatin—It was already shown that, although NuA4 targets only H4 and H2A on chromatin in vitro and in vivo, the use of free histones leads to nonspecific acetylation of histone H3 and a weaker targeting of H2A (27, 49) (Fig. 1C, lanes 5 and 6). On the other hand, when we incubated NuA4 with nucleosomes reconstituted with recombinant yeast or *Xenopus* histones on beads or in solution, we repeatedly saw a marked preference for histone H2A over histone H4 (Fig. 1C, lanes 1 and 2; data not shown). This is in clear contrast with what is normally obtained using native chromatin purified from human cells (27). To further investigate this discrepancy and because histone H2A tail shows some sequence variation between yeast and human, we performed a HAT assay with NuA4 on native chromatin purified from yeast cells (Fig. 1D). It is clear that on yeast native chromatin, NuA4 displays specificity toward histone H4 and H2A tails, with no obvious preference for one versus the other. These results suggest that nucleosomes reconstituted with recombinant histones are not recognized by NuA4 in the same way as are native nucleosomes. Similar results were previously obtained with human NuA4/TIP60 HAT complex using native chromatin versus recombinant nucleosomes from a different source (50).

Thus, we decided to use purified native chromatin for the in vitro histone exchange assay.

Native chromatin is typically purified from yeast cells by micrococcal nuclease (MNase) treatment to obtain different lengths of soluble oligonucleosomes by gel filtration, as seen in
H4 and H2A Acetylation Stimulates SWR1

![Diagram](image)

**FIGURE 3. The SWR1 complex incorporates H2A.Z-H2B dimers into native chromatin in an ATP-dependent manner.** *A*, shown is an experimental scheme of *in vitro* histone swapping assays. Immobilized native chromatin is incubated with the SWR1 complex for 30 min to allow binding. ATP and recombinant H2A.Z-H2B dimers are then added after washes to remove unbound SWR1. *B*, native chromatin immobilized on paramagnetic beads was bound by purified SWR1 complex and incubated in the presence or absence of ATP. After washes, incorporated H2A.Z was analyzed by SDS-PAGE and Western blotting with anti-Htz1 (yeast H2A.Z). Anti-H3 and H2B signals are used as chromatin loading/dimer stoichiometry controls.

Fig. 2A. Usually, oligonucleosome fractions are pooled away from non-histone contaminants and subnucleosome particles (mono- to tetranucleosomes) (Fig. 2, A and B). Because micrococcal nuclease digestion leaves 3'-overhangs, labeling with biotinylated dNTPs required prior exonuclease digestion with T4 DNA polymerase before allowing the polymerase reaction by the addition of the nucleotides (Fig. 2C). After buffer exchange, the biotinylated chromatin was bound to streptavidin magnetic beads followed by washes. As shown in Fig. 2D, a significant amount of native yeast chromatin is efficiently bound to the beads and is stable when stored at 4 °C.

The SWR1 Complex Uses ATP to Incorporate H2A.Z into Native Chromatin in Vitro—When we tested the purified native yeast chromatin in histone exchange assays, we used a protocol adapted from related work done with yeast, *Drosophila*, and human activities (20, 22, 23). The SWR1 complex was first allowed to bind to the chromatin on beads; the unbound SWR1 complexes was then washed away before the addition of ATP and H2A.Z-H2B dimers (Fig. 3A). Incorporation of H2A.Z can then be evaluated by Western analysis of the beads after stringent washes. As shown in Fig. 3B, no H2A.Z was detected on the beads in the absence of SWR1 complex (lane 2). The addition of SWR1 led to incorporation of some H2A.Z into chromatin even in the absence of ATP (lane 3). This has been seen by other groups and is due to ATP presence in the purified SWR1 complex, as treatment with apyrase inhibits this incorporation (Refs. 15 and 22 and data not shown). When exogenous ATP was added after SWR1 was allowed to bind, a much higher level of H2A.Z was incorporated into chromatin (lane 4). Importantly, histone H2B signal did not change on the beads, validating the exchange reaction compared with nonspecific binding of H2A.Z-H2B dimers. In our hands, the use of native chromatin was a big improvement for the histone exchange assay, as the use of recombinant nucleosomes often led to nonspecific binding of H2A.Z-H2B dimers even in the absence of SWR1 (data not shown).

The NuA4 Complex Stimulates the Incorporation of H2A.Z into Chromatin by SWR1 in an Acetyl-CoA-dependent Manner—To analyze the putative cooperativity between NuA4 and SWR1 in the incorporation of H2A.Z into nucleosomes, we performed the histone exchange assay with different steps described in Fig. 4A. First, NuA4 was allowed to acetylate the chromatin substrate on beads. The chromatin was then washed before the addition of SWR1 to avoid potential acetylation events occurring while SWR1 is present. Preincubation of chromatin with NuA4 did not lead to incorporation of H2A.Z even with the addition of SWR1 but in the absence of ATP (Fig. 4B, lane 4). Preincubation of chromatin with NuA4 and acetyl-CoA led to a significant increase of SWR1/ATP-dependent incorporation of H2A.Z in chromatin compared with SWR1 + ATP alone (compare lanes 3 and 5). This effect cannot be due to NuA4-dependent acetylation of H2A.Z in the exogenous dimers as repeated washes remove acetyl-CoA before the dimers were added.

On the other hand, one could argue that stimulation of SWR1 activity by NuA4 is through more efficient binding to nucleosomes by direct physical interaction with NuA4 (possibly due to four shared subunits). If this were the case, the stimulation would not require acetylation per se. Results in Fig. 4C clearly demonstrate that NuA4 stimulates incorporation of H2A.Z by SWR1 through acetylation of the nucleosome substrate. When acetyl-CoA was absent during the incubation step with NuA4, subsequent loading of H2A.Z by SWR1 was decreased (Fig. 4C, compare lanes 4 to 3 and lanes 6 to 5). Importantly, the use of less SWR1 activity led to a stronger stimulation by the NuA4 complex (compare lanes 5 and 6 versus 3 and 4), suggesting that histone acetylation is more important when SWR1 is limiting. In parallel, adding more NuA4 complex did not lead to a further increase of SWR1 activity (Fig. 5, A–C, lanes 6 and 7).

**Mutation of H2A or H4 Acetylation Sites Does Not Hamper NuA4 Ability to Enhance SWR1-dependent Loading of H2A.Z—** Because NuA4 acetylates chromatin on both histone H4 and H2A N-terminal tails *in vivo* and *in vitro*, it was important to determine whether both tails were implicated in stimulating SWR1 activity. Previous work had linked SWR1 subunit Bdf1 to acetylated H4 (10, 29, 30), whereas histone exchange assays with *Drosophila* Tip60/p400 had implicated acetylation of nucleosomal H2Av in the exchange reaction (23). To determine the relative importance of H4 and H2A tails in SWR1 stimulation by NuA4, we purified native chromatin from yeast cells carrying mutations on all lysines targeted by NuA4 on either H4 or H2A tails. After binding the chromatin to beads, we per-
formed histone exchange assays with SWR1 after pretreatment with NuA4 (Fig. 5). As seen in Fig. 5A, preincubation of chromatin with NuA4 led to increased level of H4 and H2A acetylation and stimulation of ATP- and SWR1-dependent incorporation of H2A.Z (lanes 3–6). Adding more NuA4 did not lead to further incorporation of H2A.Z, but chromatin hyperacetylation was apparently less efficient (lane 7). This is not due to NuA4/SWR1-dependent loss of nucleosomes on the beads as the total histone H4 signal remained unchanged (Fig. 5A, bottom panel). Based on our experience with the NuA4 complex, we concluded that the ratio of NuA4 to substrate is too high under these conditions, which leads to inhibition of the acetyltransferase reaction (data not shown). Nevertheless, enhanced H2A.Z incorporation was similar to the conditions with more hyperacetylation, suggesting that a threshold of acetylation is required to stimulate SWR1, but further acetylation of all NuA4-targeted lysines is not.

We then used chromatin containing an H2A tail on which the three lysine residues have been changed to arginines. As seen in Fig. 5B, NuA4-dependent acetylation could still enhance SWR1 activity on this native chromatin (compare lanes 3–6). These results suggested that H2A acetylation was not essential in the mechanistic cross-talk between NuA4-directed acetylation and SWR1-dependent exchange of histone dimers. Thus, we expected that mutation of all four lysine residues of the H4 tail would have an impact. Surprisingly, native chromatin with H4 tail mutations also showed enhancement of SWR1 activity by NuA4 (Fig. 5C, compare lanes 3–6). Because chromatin is the only substrate present in the reaction with NuA4 and acetyl-CoA, and NuA4 only targets H4 and H2A tails in nucleosomes, we conclude that NuA4-dependent acetylation of either H4 or H2A N-terminal tails is sufficient to enhance SWR1 activity and H2A.Z incorporation. Thus, these results indicate that H4 and H2A acetylation events play independent and likely redundant roles in stimulating the SWR1 complex in our in vitro histone exchange assay.

On a separate note, although H2A lysine mutation retains the positive charge of the histone tail (substitution to arginines), H4 mutant replaced the four lysine residues with glutamine, which is thought to mimic acetylation by removing the positive charge. If the stimulation of SWR1 activity by NuA4 was only indirect through destabilization of the nucleosome core (loss of positive charge on histone tails), then the H4 K5Q/K8Q/K12Q/K16Q mutant chromatin should by itself stimulate SWR1 activity when compared to controls with wild-type H4.

**FIGURE 4.** NuA4-mediated acetylation of chromatin stimulates the histone exchange ability of SWR1 complex. A, shown is an experimental scheme of the histone swapping assay with prior acetylation by the NuA4 complex. Immobilized chromatin is preacetylated with NuA4 followed by washes to remove acetyl-CoA and the addition of the SWR1 complex. ATP and H2A.Z-H2B dimers are added last after washes to remove unbound SWR1. B, NuA4 enhances SWR1 histone exchange activity. Immobilized chromatin was preacetylated with NuA4 followed by the addition of SWR1 and H2A.Z-H2B dimers. Beads were loaded on 15% gel and probed with anti-Htz1 (yH2A.Z) antibodies. Anti-H3 and H2B are used as chromatin loading/dimer stoichiometry controls. C, stimulation of SWR1 activity by NuA4 requires acetyl-CoA. Reactions were incubated with NuA4 in the presence or absence of acetyl-CoA followed by washes and SWR1 addition. After incubation with H2A.Z/H2B dimers and ATP, the beads were washed and analyzed as in B, except that an anti-H4 signal was used as chromatin loading control. More SWR1 activity was used in lanes 2–4 compared with 5–6.
with wild type chromatin. This is clearly not the case (compare lanes 3 in Fig. 5, A–C), although there might be a slightly higher background of ATP-independent binding of H2A.Z to this chromatin (lanes 2, 4, and 5). These results suggest that NuA4-directed acetylation per se is required to stimulate SWR1, presumably through specific recognition by the bromodomain protein Bdf1 present in the SWR1 complex (see below).

H2A and H4 Lysine Residues Targeted by NuA4 Are Important for Normal Localization of H2A.Z on Specific Gene Promoters and Near Telomeric Regions in Vivo—To support the conclusions drawn from our in vitro experiments, we performed chromatin immunoprecipitation experiments to analyze H2A.Z localization in cells carrying the H4 or H2A acetylation mutants. Previous reports had shown that mutations of some histone H4 acetylation sites, NuA4 components, and Bdf1 impact the efficient deposition of H2A.Z into specific locations on chromatin in vivo (8, 10, 30, 31, 33, 34). Taking into account any variation of nucleosome occupancy, we analyzed H2A.Z enrichment at two specific promoters that have been reported to be affected by NuA4 and H4 mutations (10). As reported, we could detect a significant decrease of H2A.Z deposition in H4 K5R/K12R mutant at both SGF29 and ABP1 promoters (Fig. 6, A and B). The H4 K5Q/K12Q and K5Q/K8Q/K12Q/K16Q mutants have similar effects on H2A.Z deposition in vivo. Similar if not stronger decreases were also detected at well studied telomere-proximal regions (Fig. 6, C and D; data not shown). These effects are similar to what was reported for the H4 K16-specific HAT Sas2 on the same locations, an important regulator of chromatin boundaries (33, 34).

When the lysine residues on histone H2A tail were instead mutated, a very similar effect was seen on H2A.Z deposition (Fig. 6, A–D). A 40–50% drop of H2A.Z incorporation was detected at both SGF29 and ABP1 promoters and near telomere regions. Again, removing or keeping the positive charge of the lysine residues did not change the effect on H2A.Z deposition. Thus, NuA4-dependent acetylation of both histone H4 and H2A tails in vivo is required for efficient incorporation of H2A.Z at specific gene promoters.

H2A and H4 Lysine Residues Targeted by NuA4 Are Together Essential for Cell Viability—To determine whether independent H4 and H2A acetylation play redundant or cooperative roles in the stimulation of H2A.Z incorporation by SWR1, we produced a yeast strain carrying point mutations in all lysine
FIGURE 6. Histone H4 and H2A acetylation-defective mutants show decreased incorporation of H2A.Z at specific chromosomal loci in vivo, and their combination is lethal for the cell. H2A.Z (Htz1) enrichment at the promoters of SGF29 (−273 to −60 bp from transcription start site) (A), ABP1 (−310 to −29) (B), 10 kb from TelVIR (C), and 12 kb from TelVL (D) was analyzed by chromatin immunoprecipitations. Putative variation in nucleosome occupancy was corrected by analyzing in parallel total histone H3 signals at the same locations. H2A.Z enrichment was measured as a ratio of Htz1 to H3 immunoprecipitation/input (values range from 0.2 to 0.3 Htz1/H3 ratio in wild type cells). Data are presented as a relative change compared with isogenic wild type strain (set to 1). Standard errors are based on two to four independent experiments. E, a yeast strain with all core histone genes deleted and covered by a URA3 vector containing a single copy of H4, H3, H2A, and H2B genes was transformed with a Leu2 vector expressing wild type histones or histones mutated on the lysine residues targeted by the NuA4 complex in vivo (H4 Lys-5/8/12 and H2A Lys-3/7/13) to arginines or glutamines. Exponentially growing cells were then serially diluted (10-fold) and spotted on control plates lacking leucine and uracil or on 5-fluoro-orotic acid (5′ FOA) plates to get rid of the URA3 expressing vector.
residues targeted by NuA4 in vivo, i.e. H4 Lys-5/8/12 and H2A Lys-4/7/13. Unfortunately, chasing wild type copies of the genes indicate that these mutants, as many NuA4 deletion mutants including esa1, cannot support growth (Fig. 6E). These results indicate that loss of both H4 and H2A lysine residues targeted by NuA4 is lethal, suggesting that they may play redundant roles in vivo.

Depletion of Bromodomain Protein Bdf1 from the SWR1 Complex Cripples NuA4-mediated Stimulation—Our chromatin immunoprecipitation data support the conclusions drawn from our in vitro experiments and the literature, i.e. the loss of positive charges on H4 and H2A tails is not sufficient to stimulate SWR1-dependent deposition of H2A.Z, implicating a direct recognition of the H4/H2A-acetylated lysines by SWR1 subunit Bdf1, a double bromodomain-containing protein. These results also support the idea of a sufficient threshold of NuA4-dependent acetylation to stimulate SWR1 as K5Q/K12Q and K5Q/K8Q/K12Q/K16Q mutants have similar effects on H2A.Z deposition in vivo. Importantly, mutation of the NuA4 HAT subunit, Esa1, has been shown to decrease SWR1, H2A.Z, and Bdf1 binding to many loci in vivo (30).

We wanted to determine whether Bdf1 was the key effector that recognizes NuA4-acetylated chromatin in order to stimulate SWR1-mediated histone exchange. Based on our several purifications of the SWR1 complex through different affinity tags and the literature, it seems clear that Bdf1 is loosely associated with the complex or is only present in a small population (Refs. 19, 20, 31, and 32 and data not shown; also see Fig. 7C). Thus, we tried to enrich our fraction of SWR1 complex containing Bdf1 by directly tagging Bdf1. Purification of TAP-tagged Bdf1 led to a fraction containing a good quantity of purified protein along a full set of core histones (Fig. 7A). Subunits of the SWR1 complex can be detected but clearly form a minority of Bdf1-associated proteins. We tested this fraction in histone exchange assays but could not detect any activity, likely because of the core histones present in the fraction (data not shown). We then decided to use a Swr1-FLAG/Bdf1-TAP strain to purify only the Bdf1-containing SWR1 complex. Unfortunately, the yield after FLAG and TAP affinity steps was too low to obtain sufficient material for enzymatic assays (data not shown; fur-
thermore, we were never successful at obtaining active SWR1 through a TAP protocol). Then we decided to use the SWR1 complex fraction that went through the IgG-Sepharose beads without binding (Fig. 7, B–D). Western analysis confirmed that Bdf1 is specifically depleted from this SWR1 fraction (Fig. 7C, compare lanes 1 and 2). As shown in Fig. 7E, this Bdf1-depleted SWR1 complex, although active in histone exchange assay (lanes 2 and 3), was unable to respond to prior acetylation of chromatin by NuA4 (compare lanes 3 and 5). These results indicate that Bdf1 has to be part of the SWR1 complex to allow stimulation of histone exchange by NuA4-dependent chromatin acetylation.

**DISCUSSION**

The results presented in this study clearly augment our understanding of the precise mechanisms implicated in the incorporation of histone variant H2A.Z in eukaryotic chromatin. They support a model previously proposed based on functional genomic data that lacked direct molecular evidence (8, 10, 30). Our *in vitro* histone exchange experiments using native chromatin and purified NuA4/SWR1 complexes clearly established a direct role of histone H4 and H2A acetylation in enhancing SWR1 function and H2A.Z incorporation. Such a mechanistic cooperativity between NuA4 and SWR1 has been suggested for some time—based on genetic interactions in yeast and the evolutionary physical merge of the two activities into a single TIP60/p400 complex (17, 31, 35). Interestingly, although H4 acetylation had been implicated in modulating SWR1 function in yeast (8, 10, 30), H2A acetylation had more links to reported histone exchange or displacement by the TIP60/p400 complex in higher eukaryotes (23, 51). Our data now support an important function of both H4 and H2A acetylation in modulating histone dimer exchange. Furthermore, our *in vitro* and *in vivo* data support independent and likely redundant roles of H4 and H2A acetylation in enhancing SWR1 activity, the presence of either acetylated tail being sufficient.

To support a model of redundancy between H4 and H2A acetylation in enhancing deposition of H2A.Z by SWR1, it would have been useful to test chromatin from cells carrying lysine mutations on both tails. Unfortunately, such a yeast strain is non-viable (Fig. 6E), like strains deleted for the NuA4 catalytic subunit Esa1. Nevertheless, deletion of a nonessential subunit of NuA4, Eaf1, or a temperature-sensitive mutant of Esa1 led to promoter–specific decreases of nucleosomal H2A.Z in *in vivo* (10, 30, 31). Eaf1 functions as the platform for the NuA4 complex assembly, links Esa1 to the four subunits shared with the SWR1 complex, and has homology with the Swr1 protein (31). The double bromodomain protein Bdf1 was expected to be a key to the cross-talk between NuA4 and SWR1. *esal* temperature-sensitive mutant cells show decreased binding of SWR1 and Bdf1 to promoters *in vivo* (30), and Bdf1 deletion affects loading of H2A.Z in *in vivo* (10, 30). Unfortunately, loss of Bdf1 leads to its partial replacement by Bdf2 *in vivo*, and a *bdf1/bdf2* double mutant strain is non-viable (30). Thus, directly testing the role of Bdf1 in our *in vitro* H2A.Z incorporation assay would require site-directed mutagenesis of both bromodomains rather than simply purifying the SWR1 complex from a deletion strain. In an intermediate approach, we depleted our purified SWR1 complexes of Bdf1 through an affinity step and used this fraction to demonstrate that Bdf1 was required for NuA4-dependent stimulation *in vitro* (Fig. 7E).

In addition, a recent report by Wu et al. (26) further characterized the molecular determinants for SWR1 complex assembly and function. Intriguingly, this study demonstrates that Bdf1 association to SWR1 was dependent on the Arp4-Yaf9-Swc4-Act1 tetrameric subcomplex composed of the four subunits shared with the NuA4 complex. It remains to be determined why in this case Bdf1 is not found associated with NuA4 as well. The human TIP60/p400 complex does contain a homolog of Bdf1, the double bromodomain-containing protein Brd8 (24, 25). Interestingly, in mammalian cells there is a second protein in addition to p400 that is homologous to yeast Swr1, named SRCAP. The SRCAP complex can also incorporate H2A.Z into chromatin *in vitro* and *in vivo* and shares several subunits with the TIP60/p400 complex but lacks Brd8 (21, 22, 25). This has led us to propose that the TIP60/p400 complex was involved in acetylation-dependent exchange of histone H2A variants, whereas the SRCAP complex did not target acetylated chromatin (31). Cooperativity between NuA4-dependent acetylation and SWR1-dependent incorporation of H2A.Z variant plays an important role in presetting the chromatin structure over the promoter of highly inducible genes, poised them for activation, e.g. *PHOS* (Fig. 7F) (52, 53).

The use of native chromatin in our histone exchange assay proved to be very useful by increasing the specificity of the reaction and made the assay more physiological, as demonstrated by a lower nonspecific association of H2A.Z and an *in vivo*-like acetylation by NuA4. It will also be very interesting to use native chromatin prepared from cells carrying other mutations on histones or important cofactors. One interesting line of future experiments is to analyze the cross-talk between NuA4 and SWR1 at sites of DNA damage. Both NuA4 and SWR1 have been implicated at DNA double-strand breaks and interact...
with local H2A phosphorylated at serine 129, the yeast functional homolog of mammalian γ-H2AX (54). Additional findings indicate that NuA4-mediated acetylation near double-strand breaks enhances recruitment of SWR1 and INO80 complexes (54), which in turn regulate phospho-H2A exchange from local chromatin and cell cycle checkpoint (55, 56). It will be very important to investigate, using our in vitro assay, whether phospho-H2A in native chromatin is preferentially targeted by SWR1 for exchange with H2A.Z and whether acetylation of phospho-H2A by NuA4 specifically activates the reaction. It is intriguing to note that Arp4, a subunit shared by NuA4 and SWR1 and important for SWR1 histone exchange reaction. It is intriguing to note that Arp4, a subunit shared by NuA4 and SWR1 and important for SWR1 histone exchange activity (26), was shown to interact with H2A C-terminal tail in vitro only when it carries the DNA damage inducible phosphorylation of serine 129 (54). This is certainly related to Tip60 function in the dynamics of γ-H2AX during DNA damage response in mammalian cells (51, 57).

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