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Role of Jade-1 in the Histone Acetyltransferase (HAT) HBO1 Complex*◆

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Regulation of global chromatin acetylation is important for chromatin remodeling. A small family of Jade proteins includes Jade-1L, Jade-2, and Jade-3, each bearing two mid-molecule tandem plant homology domain (PHD) zinc fingers. We previously demonstrated that the short isoform of Jade-1L protein, Jade-1, is associated with endogenous histone acetyltransferase (HAT) activity. It has been found that Jade-1L/2/3 proteins co-purify with a novel HAT complex, consisting of HBO1, ING4/5, and Eaf6. We investigated a role for Jade-1/1L in the HBO1 complex. When overexpressed individually, neither Jade-1/1L nor HBO1 affected histone acetylation. However, co-expression of Jade-1/1L and HBO1 increased acetylation of the bulk of endogenous histone H4 in epithelial cells in a synergistic manner, suggesting that Jade1/1L positively regulates HBO1 HAT activity. Conversely, small interfering RNA-mediated depletion of endogenous Jade resulted in reduced levels of H4 acetylation. Moreover, HBO1-mediated H4 acetylation activity was enhanced severalfold by the presence of Jade-1/1L *in vitro*. The removal of PHD fingers affected neither binding nor mutual Jade-1-HBO1 stabilization but completely abrogated the synergistic Jade-1/1L- and HBO1-mediated histone H4 acetylation in live cells and *in vitro* with reconstituted oligonucleosome substrates. Therefore, PHDs are necessary for Jade-1/1L-induced acetylation of nucleosomal histones by HBO1. In contrast to Jade-1/1L, the PHD zinc finger protein ING4/5 failed to synergize with HBO1 to promote histone acetylation. The physical interaction of ING4/5 with HBO1 occurred in the presence of Jade-1L or Jade-3 but not with the Jade-1 short isoform. In summary, this study demonstrates that Jade-1/1L are crucial co-factors for HBO1-mediated histone H4 acetylation.

Gene for apoptosis and differentiation-1, Jade-1 (PHF17), has been identified as a protein interacting with the von Hippel Lindau gene product by a yeast two-hybrid approach (1). The 509-amino acid Jade-1 protein contains one canonical $Cys_4HisCis_3$ plant homology domain (PHD)[2] followed by a noncanonical extended PHD domain, which are zinc-binding motifs. Most PHD family proteins are localized to the cell nucleus and are associated with chromatin, chromatin-modifying enzymes, and transcription factors (2, 3). We recently reported that endogenous Jade-1 is localized to the cell nucleus and that ectopically expressed Jade-1 possesses intrinsic transcriptional activity (4). Most importantly, we demonstrated that Jade-1 can promote endogenous histone H4 acetylation by associating with a histone H4-specific endogenous HAT. Interestingly, the histone H4-specific HAT, TIP60, interacted with Jade-1 physically and functionally *in vitro* and in live cells.

Gene data base analysis revealed two other Jade-1 homologs, Jade-2 (PHF15) and Jade-3 (PHF16). Jade-3 has been previously identified by screening genes induced by steroid hormones in breast cancer cells (5). Jade-1 mRNA gives rise to two protein products, the full-length Jade-1L consisting of 842 amino acids and its truncated splice variant, missing a large C-terminal fragment of 333 amino acids. Interestingly, although the N-terminal portions of all three full-length Jade polypeptides are most homologous, the C-terminal sequences are variable. The short isoform of Jade-1 is the most described of all Jade family proteins so far.

The Jacques Cote laboratory has been actively characterizing native complexes of the MYST family of HATs (6). Recently, it has been reported that endogenous Jade proteins, including Jade-1L, Jade-2, and Jade-3, co-purify with the histone H4-specific HBO1 complex along with either of the individual PHD zinc finger proteins ING4/5 (7). Interestingly, in contrast to Jade-1L, the truncated version of Jade-1 was not found within the native HBO1 complex or any HAT complexes. These studies identified HAT HBO1 as a native Jade-1L partner and supported Jade-1 association with endogenous histone H4-specific HAT (4).

HBO1 (MYST2, KAT7 (8)) was originally identified using a yeast two-hybrid screen as an origin recognition complex-1 (Orc1)-interacting HAT (9, 10). According to the primary

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[2] The abbreviations used are: PHD, plant homology domain; CAT, chloramphenicol acetyltransferase; HAT, histone acetyltransferase; IP-HAT, immunoprecipitation-HAT; siRNA, small interfering RNA; HA, hemagglutinin; CREB, cAMP-response element-binding protein; HBO1, HAT-binding origin replication factor-1.

sequence, HBO1 is a close homolog of the HAT TIP60. The 611-amino acid HBO1 polypeptide contains a serine-rich zinc finger followed by a 270-amino acid C-terminal MYST homology domain, which is also present in several other known members of this family. Limited information regarding the biological role of HBO1 is available. HBO1 has been implicated in transcriptional regulation by the androgen receptor, progesterone receptor, in replication origin function, lymphomagenesis, and adipogenesis (10–15).

HATs have been studied extensively as regulators of gene transcription (16–19). However, it has become clear that global acetylation of histones, specifically histone H4, occurs and is required in different types of DNA metabolism, including DNA replication, recombination, and repair (9, 10, 20–33). Indeed, since eukaryotic DNA is chromatinized, any process involving DNA in its physiological state will likely involve chromatin assembly and remodeling complexes, including HATs.

More than 300 gene products have been identified to date that contain one or more PHD type zinc fingers. Most characterized PHD proteins are found in the nucleus. The properties of PHD zinc fingers make them good candidates for intracellular protein scaffolds because they are small, stable, and very diverse in sequence. There are several suggested functions for PHD fingers including roles in chromatin remodeling (2, 34, 35).

The extended PHD motif is found in several leukemia-associated proteins, such as AF10, AF17, and the mixed lineage leukemia protein MLL (36). The tandem arrangement of a canonical PHD followed by an extended PHD motif characteristic to Jade-1 is found in AF10, AF17, and BR140 (36). Several reports have provided evidence that PHD fingers may be protein-protein interaction domains (37). It has been reported that the PHD finger of p300 binds isolated nucleosomes *in vitro* (34). The PHD finger was also found to be an integral part of the CREB-binding protein minimal acetyl transferase domain (38, 39).

Recent studies report that a subtype of PHD fingers found in the ING family of proteins and in the bromodomain and PHD finger transcription factor (BPTF) binds a trimethylated lysine residue of the histone H3 tail, H3K4Me3 (40–42). This discovery strongly supports the "histone code" hypothesis (19, 43) and provides a novel mechanism for chromatin-mediated transcriptional regulation and epigenetic control.

We recently reported that Jade-1 is capable of promoting histone H4 acetylation and that this Jade-1 function requires intact PHD fingers. We also examined interactions of Jade-1 with the histone H4-specific HAT TIP60 (KAT5(8)) and reported that the PHD fingers of Jade-1 are dispensable for binding with TIP60 but are required for promoting TIP60-mediated histone acetylation function (4). We hypothesized that intact PHD fingers of Jade-1 provide HAT(s) with nucleosomal histone affinity and thereby promote histone acetylation in the context of chromatin.

We aimed to examine the interactions of Jade-1, Jade-1L, and Jade-3 with HBO1 and ING4/5. We report here that Jade-1 and Jade-1L, but not Jade-3, synergize with HBO1 to acetylate histone H4, whereas PHD proteins ING4/5 are not essential. Our results further support the hypothesis that Jade-1/1L provides HBO1 with specificity toward a nucleosomal histone substrate and that this function fully depends on intact Jade-1 PHD zinc fingers.

EXPERIMENTAL PROCEDURES

Cell Lines and Transfection—293T17 human embryonic kidney cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, glutamine, and penicillin-streptomycin (Invitrogen). Subconfluent cells grown in 35-, 60-, or 100-mm dishes were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Antibodies and Chemicals—Jade-1 antisera was described earlier (1, 4). FLAG M5 and M2 monoclonal antibodies were from Sigma. HA monoclonal antibody was from Santa Cruz Biotechnology. Polyclonal antibodies for histone acetyl-H4 and acetyl-H3 were from Serotec, and total H3, total histone H4, ING4, and fibrillarin antibodies were from AbCam. Goat anti-mouse and anti-rabbit IgG-horseradish peroxidase conjugate were from Bio-Rad. Protein A/G agarose was from Santa Cruz Biotechnology. Protease inhibitor mixture was from Roche Diagnostics.

Constructs—FLAG-tagged and HA-tagged HBO1, ING5, and ING4 cDNAs were described previously (7). Enzyme dead HA-HBO1$^{E/Q}$ mutant with Glu/Gln substitution in residue 508 was generated by PCR-based site-directed mutagenesis and subcloning of mutated HBO1 into pACHA plasmid. FLAG-TIP60, FLAG-Jade-1, and mutants cDNAs were described previously (1, 4). FLAG-Jade-1$_{dd}$ mutant was lacking amino acids 202–253 and 312–371. FLAG-Jade-1$_{dC}$ had a C-terminal deletion of 418–509 amino acids. The FLAG-Jade-1$_{dd}$ was PCR-amplified from HA-Jade-1$_{dd}$ and HA-Jade-1$_{d}$ templates and inserted using HindIII/BglII and BglII/XbaI restriction sites correspondingly. FLAG-Jade-1$_{dC}$ was PCR-amplified using full-length FLAG-Jade-1 as a template and inserted using HindIII/XbaI restriction sites into pFLAG-CMV2 plasmid. HA-Jade-1, HA-Jade-1$_{dd}$, and all other Jade-1 constructs were described previously (1, 4). Myc-tagged Jade-1 and Jade-1 deletion constructs were generated by subcloning into the pCS2+*myc* expression vector (gift from D. Seldin, Boston University School of Medicine). FLAG-Jade-1L cDNA was purchased from Open Biosystems. The apparent molecular mass of the FLAG-tagged protein product of Jade-1L was 92 kDa and was recognized by FLAG antibody and by Jade-1 anti-serum (1, 4) (not shown).The PCR fragment of Jade-3 cDNA (kindly provided by Drs. C. Sonnenschein and P. Geck from Tufts University) was subcloned into the FLAG tag bearing the pCMV5 vector. FLAG-tagged Jade-3 protein was 95 kDa as estimated by SDS-PAGE (see Fig. 6) and was readily recognized by FLAG antibody and rather weakly by Jade-1 anti-serum (not shown). The vectors used as a reporter in the CAT assay contain five GAL4-binding sites cloned upstream of the AdML promoter (gift from Dr. T. Kouzarides, Gurdon Institute, Cambridge, UK). Plasmid pSG424 encodes the DNA-binding domain of Gal4 driven by the SV40 early promoter/enhancer and was described previously (4, 44). The Jade-1 coding sequence was fused in-frame C-terminal to the Gal4 amino acids 1–147, yielding a Gal4-Jade-1 protein.

Endogenous Jade-1 was down-regulated with siRNA by transient transfection. Plasmids for Jade-1 siRNA and control siRNA were purchased from Dharmacon.

Reporter Gene Assay—Transfections were performed as described previously (4). Cells seeded in 60-mm dishes were co-transfected with the indicated amount of either SV40 promoter-driven Gal4 DNA-binding domain or SV40 promoter-driven Gal4-Jade-1 and 2 μg of CAT-reporter plasmid (G5AdML, described above). After 36 h of transfection, cells were washed in phosphate-buffered saline, resuspended in 150 μl of 0.25 M Tris, pH 8.0, and lysed by freezing and thawing three times (liquid nitrogen/37 °C). Supernatants were clarified by centrifugation (5 min, 12,000 × *g*). CAT assays were performed as described before (4). Experiments were repeated at least two times.

Analysis of Endogenous Histones in Nuclear Fraction—Most procedures were done as described earlier (4). We omitted the trichloroacetic acid precipitation step in most experiments as we found that this results in more efficient recovery of histone fractions and allows more uniform loading for analysis by Western blot. Briefly, conditioned medium from cells grown in 60-mm dishes were aspirated, and cell layers were washed with phosphate-buffered saline, lysed for 5 min in 0.5 ml of 10 mM Tris buffer, pH 8.0, containing 0.6% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and supplemented with protease inhibitor mixture. The nuclear fraction was isolated by centrifuging lysates at 1200 × *g* for 5 min and solubilized in 1xSDS sample buffer. Proteins were resolved by SDS-PAGE, and histones expression was analyzed by Western blots. Chicken erythrocyte histone octamers and nucleosomal array preparations (45) were kindly provided by Craig Peterson (University of Massachusetts School of Medicine, Worcester, MA).

Immunoprecipitation-HAT Assay—IP-HAT assay was done essentially as described in (4). The amount of pulled down HBO1 was kept constant in each sample. In some experiments, minor differences in the amount of pulled down HBO1 were quantitated by densitometry and used to calculate HAT specific activities.

Cultured cells grown in 60-mm dishes were lysed in 50 mM Tris, pH 7.8, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and protease inhibitor mixture. HA antibodies were added to 1 ml of lysates precleared by centrifugation and incubated overnight. Protein A-agarose/protein G-agarose (1:1 mix, 15 μl total) was added, and the mixture was rotated slowly for 4 h. After washes, the immune complexes were mixed with 30 μl of HAT reaction mix containing 50 mM Tris, pH 7.8, 10% glycerol, 2 mM $MgCl_2$, 0.5 mM EDTA, 1 mM sodium butyrate, 0.5 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1.0 μg of either core histones or reconstituted nucleosomes, and 1 μl of [^{3}H]acetyl CoA (7.4 Gbq/mmol; PerkinElmer Life Sciences). After 30 min of incubation, the reaction was stopped by the addition of SDS-sample buffer. One-tenth aliquot of each immunoprecipitation sample was used to analyze proteins in immunocomplexes by SDS-PAGE/Western blot with corresponding antibodies. The rest of these samples were used to assess incorporation of [^{3}H]acetyl-CoA into histones. Samples were separated on 15% SDS-PAGE, and core histones were visualized by staining with Coomassie Blue. Bands were excised with a razor blade, and histones were extracted from the polyacrylamide gels by incubating in Solvable reagent (PerkinElmer Life Sciences) according to the manufacturer's instructions. Ultima Gold scintillation mixture was added to the samples, and radioactivity was quantitated with a liquid scintillation analyzer (all from PerkinElmer Life Sciences). Data presented in bar graphs in the legends for Figs. 8–10 are means of at least three experiments ± S.E.

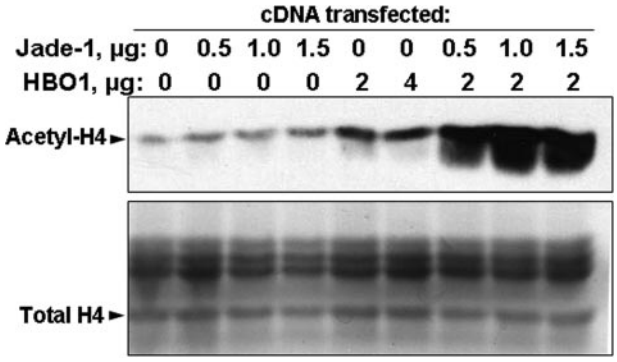

FIGURE 1. **Jade-1 and HBO1 synergize to induce massive acetylation of endogenous histone H4.** The indicated amounts of HA-Jade-1 and FLAG-HBO1 cDNA were co-transfected into 293T cells, core histones were isolated, and the levels of histone H4 acetylation were assessed by Western blot as described under "Experimental Procedures." Jade-1 expression levels were assessed in total cell extracts by Western blot with Jade-1 anti-serum.

Immunoprecipitation and Western Blot—Immunoprecipitations were done as described in Ref. 4. Precipitated proteins were resolved by SDS-PAGE and visualized by Western blots. Quantification of Western blot images was done by densitometry by using ImageJ software (National Institutes of Health).

RESULTS

Jade-1 and HBO1 Synergize to Induce Massive Acetylation of Endogenous Histone H4—We previously demonstrated that the short isoform of Jade-1L, namely Jade-1, is associated with HAT activity and that its overexpression promotes endogenous histone H4 acetylation (1, 4). Our data suggested that Jade-1 might be an essential component of a HAT complex. Recently, it has been shown that stable ING4 and ING5 complexes co-purify with endogenous Jade-1L/2/3 and HBO1 proteins (7). To address the possible role of Jade-1 in regulating HBO1 function, we examined the effects of Jade-1 expression levels on HBO1-dependent acetylation of endogenous histone H4 in intact cells. In this experiment, we transfected cells with low levels (up to 1.5 μg) of Jade-1 expression vector, which induced only slight increases in H4 acetylation.

Strikingly, although neither low amounts of overexpressed Jade-1 nor even high concentrations of HBO1 alone promoted significant histone H4 hyperacetylation, the co-expression of both partners resulted in a dramatic 100-fold hyperacetylation of endogenous histone H4 (Fig. 1, *last three lanes*). To our knowledge, this is the first demonstration of such massive acetylation of any endogenous histone in live cells by the ectopic expression of an individual protein rather than by the addition of a drug, such as an HDAC inhibitor. We next examined whether this cooperativity was dependent on HBO1 enzyme activity. Catalytically inactive HBO1 was expressed using a mutant cDNA encoding a protein with a single amino

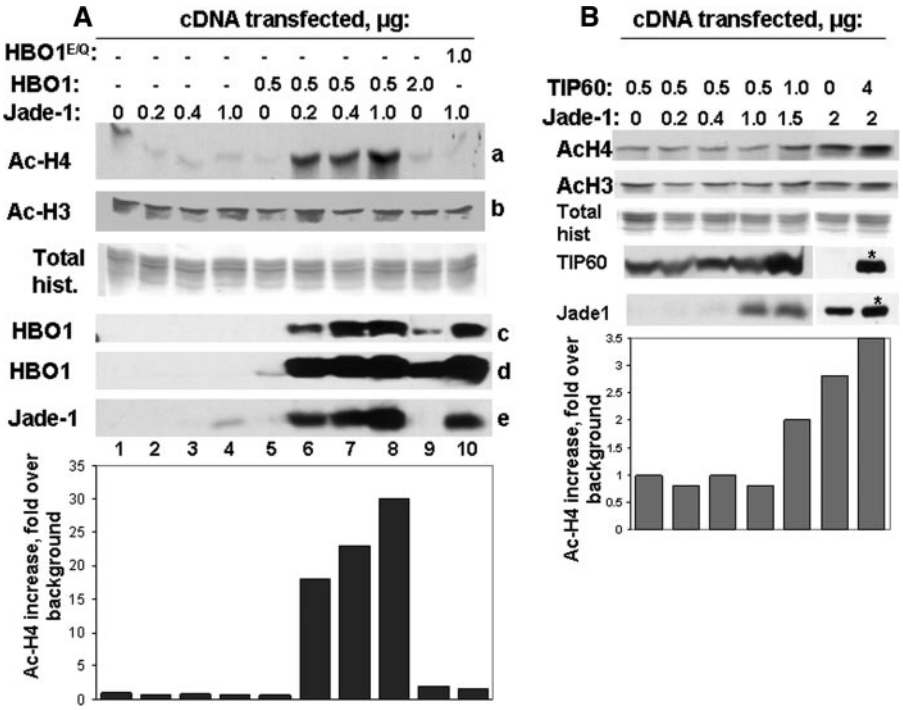

FIGURE 2. **Functional co-operation and mutual stabilization between Jade-1 and HBO1.** *A* and *B*, transfections, preparation of samples, and Western blots were done as described under "Experimental Procedures" and in the legend for Fig. 1. Densitometry was done using ImageJ software (National Institutes of Health). Images of total histones (*Total hist.*) were visualized by staining with Ponceau S and used for normalization of acetyl-H4 band intensity in each sample. The graph represents the -fold increase of acetyl-H4 density over background (control, 0 μg of Jade-1-HBO1). Blots for FLAG-HBO1 and HA-Jade-1 (*panel A*, *lanes c* and *e*) and FLAG-TIP60 (*panel B*, except for the *two last lanes* with *asterisks*) were exposed for 15 s. *, HA-Jade-1 and FLAG-TIP60 blots shown were exposed for 4 s.

acid substitution (Glu → Gln) within the conserved putative active site (see "Experimental Procedures").

Loss of enzyme activity was verified by *in vitro* IP-HAT assay (see Fig. 8*D*). Importantly, the enzyme dead mutant, designated "HBO1$^{E/Q}$," failed to cooperate with Jade-1 in inducing histone H4 acetylation (Fig. 2*A*, *lanes 7*, *8*, and *10*). Therefore, HBO1 catalytic activity is necessary for Jade-1-induced H4 acetylation, suggesting that Jade-1 positively regulates HBO1 HAT activity.

HBO1 reportedly plays a key role in bulk histone H4 acetylation in mammalian cells (7). To test whether endogenous Jade-1 is required for H4 acetylation in cells, we used siRNA to ablate Jade-1 expression. We achieved ~80% reduction of endogenous Jade-1 expression using siRNA (Fig. 3).

Interestingly, down-regulation of endogenous Jade-1 reduced histone H4 acetylation by 60% (Fig. 3). Taken together, our results indicate that Jade-1 is important for global HBO1-mediated histone H4 acetylation in intact cells. We previously reported physical and functional interactions of Jade-1 with the HBO1-related MYST family member TIP60.

We compared the effects of Jade-1 on HBO1- and TIP60-mediated acetylation of endogenous histones H4 and H3. Similar to the results of Fig. 1, HBO1 cooperated with Jade-1 (Fig. 2*A*) to promote acetylation of histone H4 (but not H3). Co-transfection of 0.2 μg of Jade-1 with 0.5 μg of HBO1 led to a 20-fold increase in H4 acetylation. Under identical experimental conditions, co-expression of 0.2 μg of Jade-1 and 0.5 μg of TIP60 did not induce detectable increases in global H4 acetylation. Increasing the levels of transfected plasmids to 1.0 μg (TIP60) and 1.5 μg (Jade-1) only led to a 2-fold increase in global H4 acetylation (although similar high levels of HBO1 and TIP60 were expressed as shown by immunoblot analyses). We conclude that Tip60 is significantly less potent than HBO1 at promoting global histone H4 acetylation (Fig. 2*B*) even with Jade-1 co-expression. Our finding that Jade-1 cooperates more effectively with HBO1 than with TIP60 suggests a predominant role for Jade-1-HBO1 in bulk histone H4 acetylation.

PHD Zinc Fingers, as Well as the C- and N-terminal Domains of Jade-1, Are Required for HBO1-mediated Histone H4 Acetylation—We previously reported that deletion of PHD zinc fingers abolished Jade-1-induced histone acetylation in live cells (but did not affect the interaction of Jade-1 with TIP60 (4)). Therefore, we determined the requirement of the Jade-1 PHD fingers in HBO1-mediated H4 acetylation in intact cells.

As expected, Jade-1$_{wt}$ protein markedly synergized with HBO1 (Fig. 4*A*) to promote acetylation of endogenous histone H4. In contrast, Jade-1 deletion mutants lacking PHD fingers failed to promote H4 acetylation.

We earlier demonstrated that deletion of the C-terminal fragment from the Jade-1 polypeptide yielded a mutant (designated "Jade-1$_{dc}$") that failed to promote histone acetylation when overexpressed (4) (see Fig. 4*B* for the schematics of Jade-1 deletion mutants). Similar to the PHD deletion mutants, Jade-1$_{dc}$ failed to promote HBO1-induced H4 acetylation (Fig. 4*A*).

Finally we examined HAT-associated activity of the Jade-1 mutant missing the entire N-terminal portion of the molecule. The removal of the N terminus also inactivated the ability of Jade-1 to promote histone H4 acetylation as well as to synergize with HBO1 (Fig. 4*A*). In summary, these data demonstrate the importance of Jade-1 in the HBO1 complex and the requirement of intact Jade-1 PHD zinc fingers and N- and C-terminal domains for histone H4 acetylation by HBO1 in live cells.

Ectopically Expressed Jade-1 and HBO1 Mutually Stabilize Each Other—We noted that ectopically co-expressed Jade-1 and HBO1 enhance each other's expression levels (Figs. 1, 2*A*, and 4*A*), strongly suggesting mutually dependent stabilization effects. Similar levels of mutual stabilization were observed when Jade-1$_{dd}$ was co-expressed with HBO1 (Fig. 4*A*). Note that unlike Jade-1-HBO1-induced H4 acetylation, the effect of Jade-1-HBO1 mutual stabilization is independent of the Jade-1 PHD fingers (Fig. 4*A*, compare *lanes 2*, *3*, and *7–9*). These data suggest that in the absence of the PHD zinc finger, even high levels of the HBO1-Jade-1 complex are incapable of acetylating

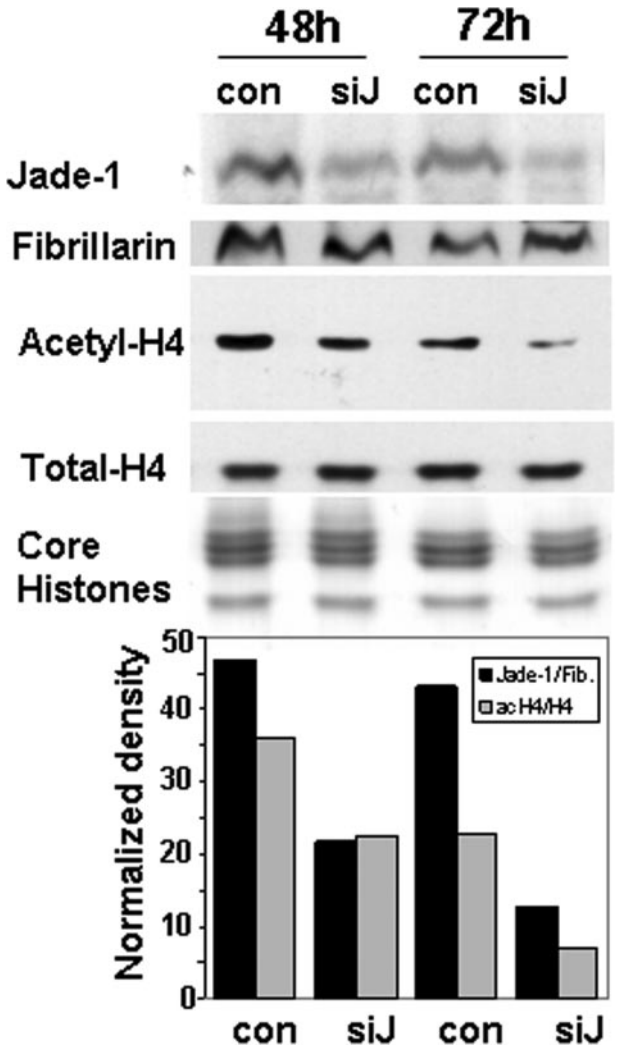

FIGURE 3. **Down-regulation of endogenous Jade-1 decreases bulk histone H4 acetylation.** Parallel samples of 293T cells seeded in 6-well plates were transfected with siRNA control (*con*) or siRNA Jade-1 (*siJ*) as recommended by the manufacturer. After the indicated times, cells were harvested, and the nuclear fraction was analyzed for Jade-1 and acetylated histone H4 expression by Western blot. Blots were stripped and reprobed with fibrillarin and total histone H4 antibody for loading controls.

endogenous histone H4. The stabilization effects were so strong that it was difficult to quantify by densitometry the actual level of HBO1 induction by Jade-1 or Jade-1_{dd} or *vice versa*. In contrast, Jade-1_{dC} and Jade-1_{dN} were significantly less effective than Jade-1_{wt} or Jade-1_{dd} in respect to mutual stabilization (Fig. 4*A*). These data further support Jade-1 and HBO1 interactions.

Physical Interactions between HBO1 and Jade-1—The synergistic acetylation of H4 by Jade1 short isoform and HBO1 and their mutual stabilization when co-expressed suggest physical interactions between these proteins. We performed deletion analysis of Jade-1 to identify domains required for binding with HBO1, histone H4 acetylation, and Jade-1-HBO1 mutual stabilization. Wild type and mutant forms of Jade-1 or HBO1 were immunoprecipitated from cell lysates, and the resulting immune complexes were analyzed by immunoblotting to test for the presence of HBO1 or Jade-1 (Fig. 5, *A* and *B*).

These reciprocal immunoprecipitation experiments showed that wild type Jade-1 associated with HBO1 and *vice versa* (Fig. 5, *A* and *B*). As expected, deletion of the PHD zinc fingers did not affect Jade-1-HBO1 interactions (Fig. 5*B*), demonstrating that Jade-1 PHD fingers are not required for complex formation with HBO1. In contrast, Jade-1 mutants harboring N- and C-terminal deletions failed to interact with HBO1 (Fig. 5*A*).

Note that the pattern of HBO1 physical interactions with wild type and N- and C-terminal deletion mutants of Jade-1 perfectly correlates with that of their functional interactions (Fig. 4*A*). Thus, wild type, but not N- and C-terminal deletion mutants of Jade-1, bind to HBO1 and synergize with HBO1 in mutual stabilization and histone H4 acetylation assays. In contrast, Jade-1_{dd} binds to and is stabilized by HBO1, but fails to promote HBO1-induced H4 acetylation when co-expressed in cultured cells (Figs. 4*A* and 5*B*). Taken together, our data suggest that Jade-1 interacts with HBO1 via N- and C-terminal domains. Although PHD zinc fingers are dispensable for the physical interaction with HBO1, they are essential for the histone H4-directed acetylation activity of the Jade-1-HBO1 complex in live cells.

Functional Interactions of Full-length Jade-1L and Jade-3 with HBO1—Thus far, we have examined interactions of the truncated version (designated as "Jade-1") of full-length Jade-1L lacking the C-terminal domain. The full-length protein products of three Jade genes that were found in the HBO1 complex (namely Jade-1L, Jade-2, and Jade-3) have significant homology, excluding their C-terminal region spanning about 300 residues. In addition, ING4/5 were also found in HBO1 complex (7). We sought to compare the activities of Jade variants and ING4/5 with respect to HBO1 association, stabilization, and activation.

Therefore, an HBO1 expression vector was transfected into 293 cells individually or in combination with plasmids encoding the Jade variants and/or ING4/5. Histone H4 acetylation and expression levels of Jade-HBO1-INGs were determined by immunoblotting. Interestingly, Jade-1L was less potent in stabilizing HBO1 than its truncated variant, yet similarly to Jade-1, Jade-1L strongly induced HBO1-dependent H4 acetylation.

Surprisingly, despite strong homology with Jade-1, co-expression of Jade-3 with HBO1 did not promote histone H4 acetylation (Fig. 6 *A*, *lane 9*). Note that similarly to Jade-1L, Jade-3 also significantly stabilized the HBO1 protein.

In contrast with Jade-1/1L, ING4 and ING5 failed to stabilize HBO1 or stimulate HBO1-induced histone H4 acetylation. Therefore, stabilization of HBO1 by Jade and Jade-induced H4 acetylation are not general properties of PHD-containing proteins. We also examined whether INGs might further enhance Jade-1/Jade-1L-HBO1 synergy in acetylation of histone H4. The addition of either ING4 or ING5 only slightly affected histone acetylation induced by the combination of Jade-1/Jade-1L and HBO1 (Fig. 6 A, *lanes 13–15* and *18–20*).

Physical Interactions of Jade-1, Jade-1L, Jade-3, and ING4 with HBO1—We performed reciprocal co-immunoprecipitation experiments to examine interactions between Jade variants and HBO1. As shown in Fig. 7, all three Jade proteins associated with HBO1, demonstrating interaction between the protein partners. The C-terminal domain of Jade-1L has been suggested to bear the binding site for INGs.[3] We next

[3] J. Cote, unpublished observation.

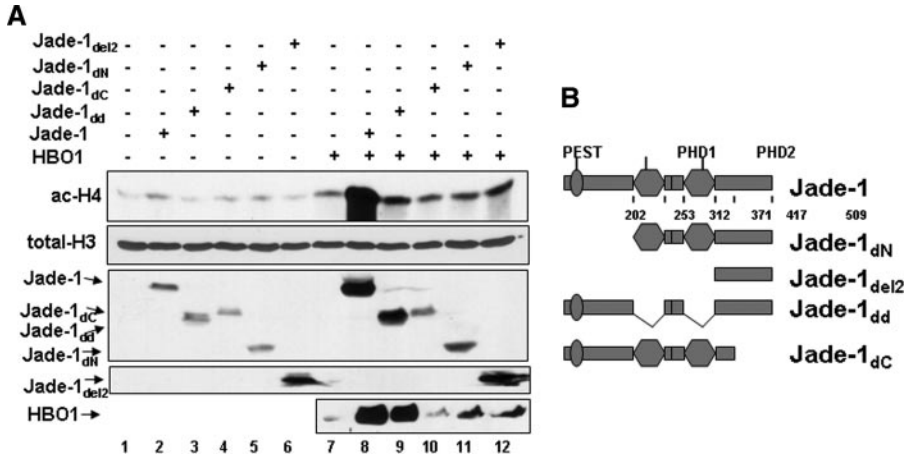

FIGURE 4. **Deletion analysis of Jade-1 synergistic interactions with HBO1.** *A*, six-well dishes of 293T cells were co-transfected with 0.5 μg of Myc-tagged wild type or mutated Jade-1 cDNA plasmids without or with 2.0 μg of FLAG-tagged HBO1. Core histones were isolated, and histone H4 acetylation levels were assessed as described in the legend for Fig. 1. Levels of Jade-1 and mutants in total cell extracts were visualized by Western blots with monoclonal Myc antibody; HBO1 was visualized with FLAG monoclonal antibody. Full-length intact Jade-1 polypeptide is required for HBO1 to acetylate endogenous histone H4. Mutual Jade-1-HBO1 stabilization is independent of the PHD fingers of Jade-1 but requires an intact N- and C-terminal and intra-PHD region. *B*, schematics of Jade-1 mutant constructs are shown.

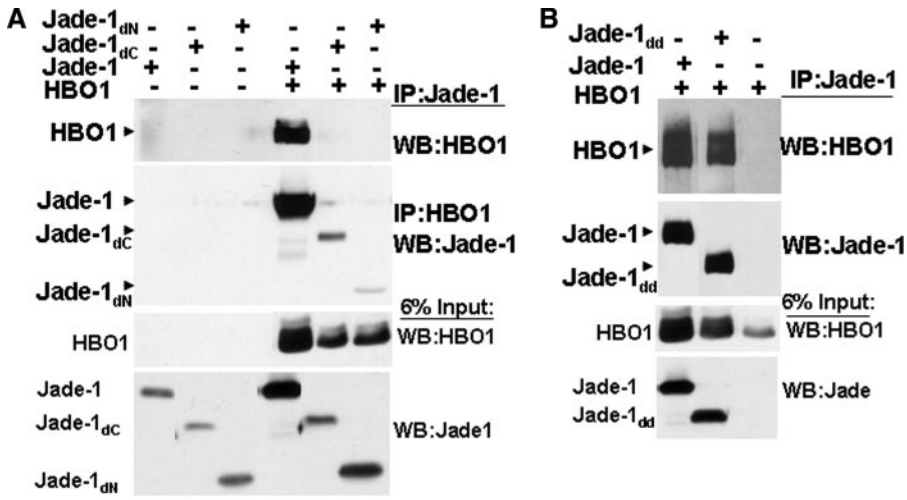

FIGURE 5. **Physical interactions between Jade-1 and HBO1.** *A* and *B*, 60-mm dishes of cells were transfected with 3 μg of Myc-tagged wild type or mutant Jade-1 cDNA without or with FLAG-tagged HBO1 cDNA plasmids. Forty-eight h after transfection, cells were harvested in 1 ml of 50 mM Tris, pH 7.8, 0.5% Nonidet P-40, 150 mM NaCl, 1 mM EDTA, and 5 mM $MgCl_2$ and protease inhibitor mixture. Lysates were divided in two aliquots for a two-way co-immunoprecipitation assay. HA-Jade-1 proteins or FLAG-HBO1 were immunoprecipitated from cell lysates by incubation with anti-HA or anti-FLAG monoclonal antibody followed by incubation with a protein A/G-agarose mix. After washes, proteins were resolved by SDS-PAGE and visualized by Western blots (*WB*) with the indicated antibody. *B*, Jade-1 co-precipitation (*lower panel*) was visualized by stripping and reprobing the *upper panel* blot with Jade-1 anti-serum.

examined whether ING4 co-precipitates with the Jade-HBO1 complex. Both Jade-1L and Jade-3 proteins precipitated ING4 along with HBO1 (Fig. 7). In contrast, the truncated Jade-1 isoform failed to precipitate ING4 protein, demonstrating that the C-terminal portions of full-length Jade-1L and Jade-3 proteins are required for ING4/5 binding and HBO1-Jade-ING complex formation.

Effects of Jade-1, Jade-1L, Jade-3, and ING4/5 on the Ability of HBO1 to Acetylate Soluble and Chromatinized Histones in Vitro—We have shown that Jade-1 enhances the ability of HBO1 to acetylate histone H4 globally in intact cells. The results of Figs. 2*A* (*lanes 6* and *9*), 4*A* (*lanes 7*, *8*, and *9*), and 6*A* (*lanes 8*, *9*, *19*, *20*, and *21*) suggest that Jade-1 enhances the specific HAT activity of HBO1.

However, due to mutual stabilization of HBO1 and Jade-1, it is possible that the stimulatory effect of Jade on H4 acetylation resulted from increased HBO1 levels in Jade-1-transfected cells. Therefore, to determine the effects of Jade proteins on specific HAT activity of HBO1, we performed *in vitro* IP-HAT assays using soluble and nucleosomal core histones as substrates (Fig. 8, *A* and *B*). The amount of HBO1 in each sample was verified by Western blot and normalized (Fig. 8*B*, *inset*).

As shown in Fig. 8*A*, the basal HAT activity of HBO1 immune complexes was significantly (6–7-fold) increased by co-expressed Jade-1 and Jade-1L (Fig. 8*A*). Jade-3 was less effective in promoting HBO1 HAT activity. This was especially evident when the experiment was done with nucleosomal histones as substrates, in which case Jade-3 stimulated HAT HBO1 only by 2–3-fold (Fig. 8*B*).

Therefore, consistent with the stimulatory effect of Jade variants on HBO1-induced H4 acetylation in intact cells, HBO1 HAT activity *in vitro* was stimulated by Jade-1 and Jade-1L. In contrast with Jade-1/1L, ING4/5 failed to promote HBO1 HAT activity. Immune complexes containing the $HBO1^{E/Q}$ mutant lacked detectable HAT activity regardless of Jade-1 expression (Fig. 8, *C* and *D*). Thus, the stimulatory effects of Jade-1 on H4 acetylation *in vitro* were strictly dependent on HBO1 catalytic activity.

Jade-1 PHD Zinc Fingers Are Required for HBO1 to Acetylate Nucleosomal Histones—The results of Figs. 4 and 5*B* showed that Jade-1_{dd} lacking the PHD fingers bound and stabilized HBO1 but failed to promote histone acetylation in live cells. To further examine the role of PHD zinc fingers, we determined the ability of the Jade-1_{dd}-HBO1 complex to acetylate histones *in vitro* (Fig. 9). Jade-1_{dd} failed to augment HBO1 HAT activity toward nucleosomal histones *in vitro*. Surprisingly, however, Jade-1_{dd} readily stimulated HBO1-mediated acetylation of soluble core histones (Fig. 9). Therefore, our data demonstrate a key role of Jade-1 PHD zinc fingers in stimulating acetylation of nucleosomal but not soluble histones by HBO1 complex.

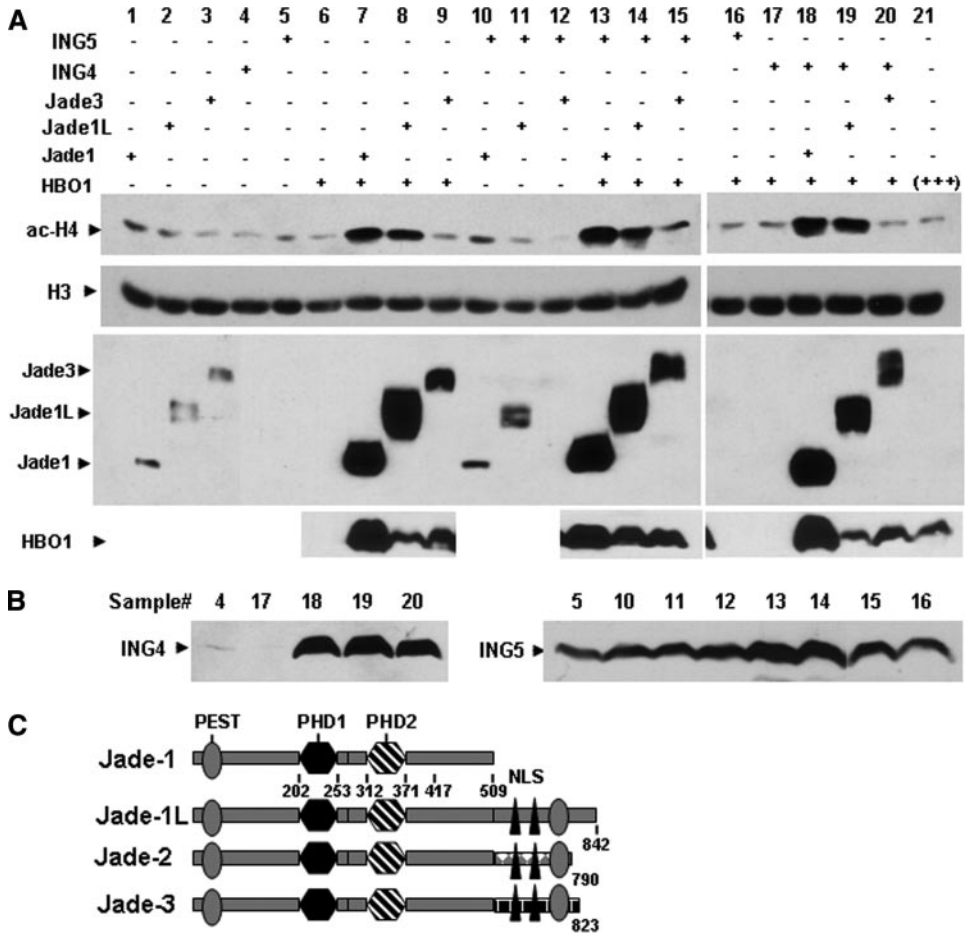

FIGURE 6. **Jade-1 and Jade-1L but not Jade-3 or INGs synergize with HBO1 to acetylate histone H4 in the live cell HAT assay.** *A*, 293T cells were transfected with the indicated DNA, total histone fraction was isolated, and H4 acetylation levels were assessed by Western blot. H3 levels serve as a loading control. Proteins were visualized by Western blot with anti-HA for Jade-1, Jade-1_{dd}, and Jade-1_{dC} and anti-FLAG for Jade-3, HBO1, and ING5. Note that Jade-3 stabilizes but does not synergize with HBO1. *B*, Western blot for ING4 and ING5. +++, excess of HBO1 transfected still failed to acetylate histone H4. *C*, Jade protein family. A schematic representation is shown. *NLS*, nuclear localization sequence.

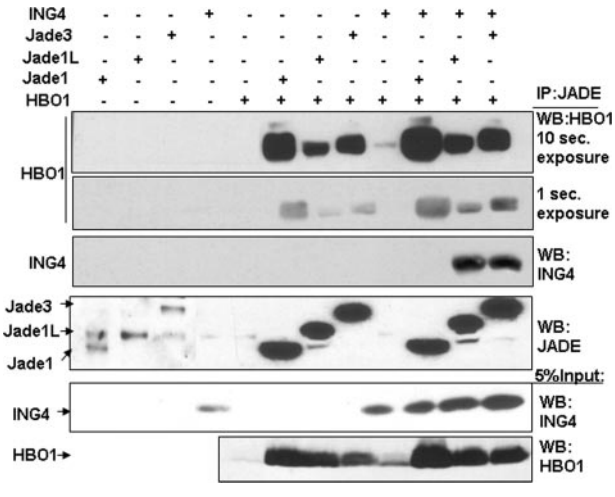

FIGURE 7. **Jade-1/1L/3 proteins and HBO1 physically interact and mutually stabilize each other.** ING4 requires Jade-1L or Jade-3 to complex with HBO1. *Upper panels*, FLAG-tagged Jade proteins were overexpressed and immunoprecipitated as described in the legend for Fig. 5. The presence of HA-HBO1 and HA-ING4 in precipitates was assessed by Western blots. The long and the short exposures of HBO1 Western blots (*WB*) are presented for best visualization. *Lower panels*, input, total cell lysates.

HBO1 Enhances Gal4-Jade-1 Transcriptional Activities—We previously reported that Jade-1 protein possesses intrinsic transcriptional activity (4). In those studies, a Gal4-Jade-1 fusion protein activated transcription of a viral promoter bearing five repeats of Gal4-specific binding sequences. We investigated the effects of HBO1 on Gal4-Jade-1 transcriptional activity.

Increasing amounts of HBO1 cDNA were co-transfected alone or in combination with Gal4-Jade-1, and rates of transcription were evaluated by CAT assays (Fig 10). HBO1 augmented Gal4-Jade-1 activity in a concentration-dependent manner, demonstrating another functional interaction between Jade-1 and HBO1 and suggesting a role for the HBO1-Jade complex in regulating transcription (Fig 10).

Since TIP60 is a homolog of HBO1 and interacts with Jade-1, we also examined the effect of TIP60 on Gal4-Jade-1 transcriptional activity. In contrast with the stimulatory effects of HBO1, TIP60 failed to activate Gal4-Jade-1-induced transcription (Fig 10). Therefore, the effect of HBO1 on Jade-1-induced transcription was relatively specific.

DISCUSSION

We previously demonstrated that PHD zinc finger protein Jade-1 is localized to the nucleus, promotes histone H4 acetylation by associating with a HAT, and possesses intrinsic transcriptional activity when tethered to a viral promoter (4). Recently, it has been reported that HAT HBO1 and three homologs of the Jade protein family, Jade-1L, Jade-2, and Jade-3, co-purify within the stable ING4/5 complex (7). HBO1 belongs to the MYST family of HATs and was discovered several years ago (9). Biological and biochemical roles of HBO1 are not completely understood, although roles in transcription, DNA replication, and DNA damage signaling have been suggested.

We investigated interactions between Jade-1/1L/3, ING4/5, and HBO1 and made several novel observations, uncovering a crucial role for Jade-1 proteins in the HBO1 complex function. Our data show that: 1) Jade-1/1L, but not ING4/5, are required for and synergize with HBO1 to promote histone H4 acetylation; 2) the PHD zinc fingers of Jade-1 are required for HBO1 to promote endogenous histone H4 acetylation in live cells and to acetylate nucleosomal histones *in vitro*; 3) Jade-1/1L and HBO1 strongly stabilize each other in a mutually dependent manner; 4) Jade-1 PHD zinc fingers are dispensable for binding to HBO1 and for Jade-1-HBO1 mutual stabilization; 5) ING4/5 require

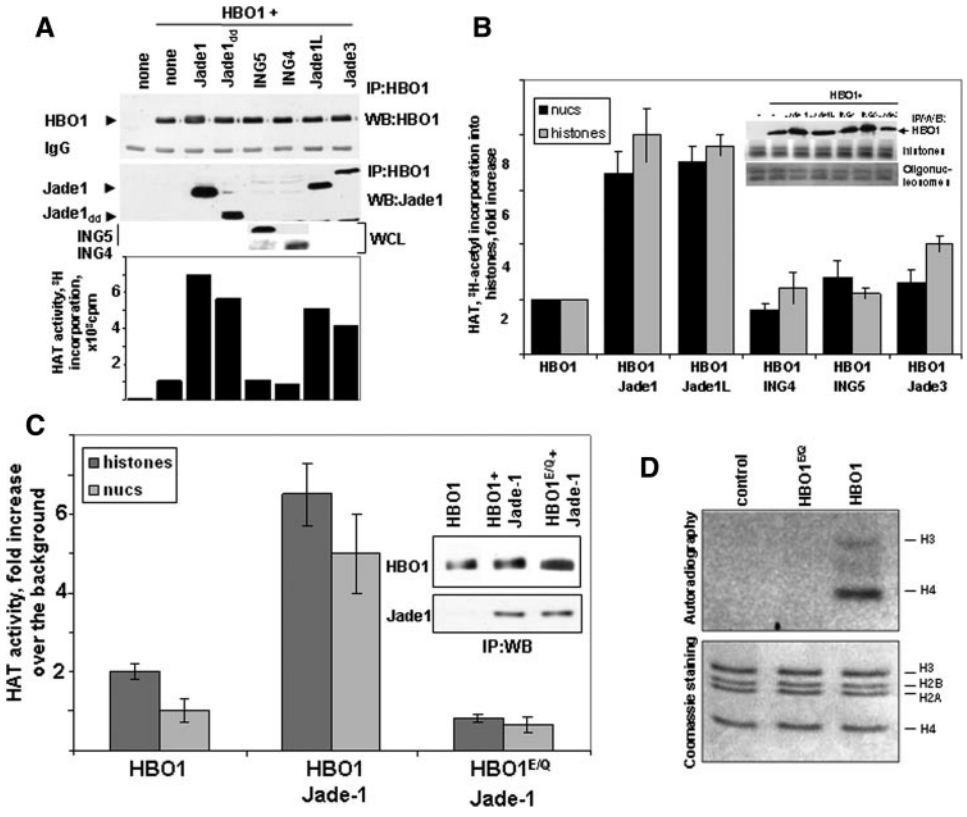

FIGURE 8. **Jade-1/1L promote specific activity of HAT HBO1 *in vitro*.** *A* and *B*, Jade-1 and Jade-1L but not Jade-3 promote specific activity of the HAT HBO1 *in vitro*. An IP-HAT assay is shown. In *A* and *B*, cells were transfected with HBO1 and one of the effectors, Jade-1, Jade-1L, ING4, or ING5. The HA-HBO1 complex was immunoprecipitated with HA-antibody, and the HAT reaction was performed by incubation with soluble core histones (*A*) or recombinant reconstituted oligonucleosomes (*nucs*) (*B*) and [^{3}H]acetyl-CoA. One-tenth aliquots of each sample were used to analyze HBO1 contents and the complex integrity by Western blot (*WB*) (*A*, a representative experiment; B, *inset*). Histones were separated by SDS-PAGE, stained with Coomassie Blue (*B*, *inset*), and excised, and ^{3}H incorporation was quantified by scintillation counting. To calculate HBO1 specific activity, these values were normalized to the amount of pulled down HBO1 determined by densitometry. Each experiment was repeated at least twice. In *B*, *bars* represent the -fold increase over HBO1 basal activity for each of the substrates. Absolute values for tritium incorporation for basal HBO1 activity were 1027 $\pm$ 122 (S.E.) cpm and 250 $\pm$ 45 (S.E.) cpm with soluble and nucleosomal histones, respectively. *C*, functional cooperativity with Jade-1 *in vitro* requires enzymatically active HAT HBO1. See the description of *panel B* and "Experimental Procedures." *D*, HAT enzymatic activity is abolished in the HBO1$^{E/Q}$ mutant. See the descriptions of *panels A* and *B*. After the completion of the IP-HAT reaction, core histones were separated by SDS-PAGE and stained with Coomassie Blue, and gels were dried and exposed to x-ray film.

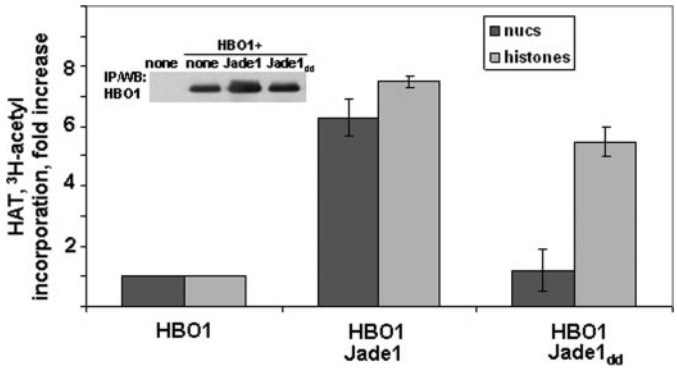

FIGURE 9. **Jade-1 promotion of HBO1 HAT activity is fully dependent on PHD zinc fingers.** Cells were transfected with Jade-1 or Jade-1$_{dd}$ and/or HBO1. The HBO1 complex was immunoprecipitated, and the HAT reaction was performed as described in the legend for Fig. 8 (*panels A* and *B*). *WB*, Western blot; *nucs*, oligonucleosomes.

Jade-1L and Jade-3, but not short Jade-1, to complex with HBO1; and 6) HBO1 enhances Gal4-Jade-1 transcription activity.

The synergistic induction of histone H4 acetylation by Jade-1 and HBO1 is remarkable and novel. Based on our data, it is conceivable that this synergy is at least in part due to the Jade-1-mediated augmentation of specific HBO1 HAT activity and is strictly dependent on two intact PHD zinc fingers of Jade proteins. Although INGs bear a single canonical PHD zinc finger, these proteins failed to synergize with HAT HBO1. Our results suggest specificity of noncanonical tandem PHD zinc fingers found in Jade and indicate the functional diversity of PHD fingers in general.

The biochemical role of PHD zinc fingers has been studied for the past several years. Using an *in vitro* assay, it has been demonstrated that unlike bromodomains, PHD zinc fingers bind to nucleosomes in an acetylation-independent manner (34). We previously demonstrated the association of Jade-1 with histone H4-specific acetyl transferase activity (4). Based on our data and other published studies (34), we proposed that Jade-1 and its PHD fingers might serve to target a HAT to a nucleosomal histone substrate. According to the current study, the removal of two PHD zinc fingers affected neither binding nor mutual Jade-1-HBO1 stabilization but completely abrogated synergy in the nucleosomal histone acetylation function in live cells and *in vitro* in the IP-HAT assay. We hypothesize that removal of PHD fingers reduced the affinity of the Jade-1-HBO1 complex for the nucleosomal substrate. These data agree with previously reported results suggesting that PHD finger deletion results in a dominant-negative phenotype of Jade-1 (4). Considering the results of the current study, it is likely that Jade-1$_{dd}$ binds HBO1, forms a HAT nonfunctional complex incapable of interacting with a substrate, and thereby acts in a dominant negative manner.

Additionally, it is possible that mutual protein stabilization contributes to the synergistic effects of Jade-1-HBO1 on H4 acetylation. Although it is not uncommon for interacting proteins to stabilize each other after co-transfection, the level of HBO1 up-regulation upon co-expression with Jade-1/1L is unusual. However, the following results argue against the notion that Jade-1/1L facilitates the HAT HBO1 by a simple dose effect. First, even the highest levels of transfected HBO1 protein still failed to promote acetylation of histone H4 in live cells (Fig. 6*A*, *lane 21*). Second, high levels of HBO1 stabilized by the presence of Jade-1$_{dd}$ likewise did not induce endogenous

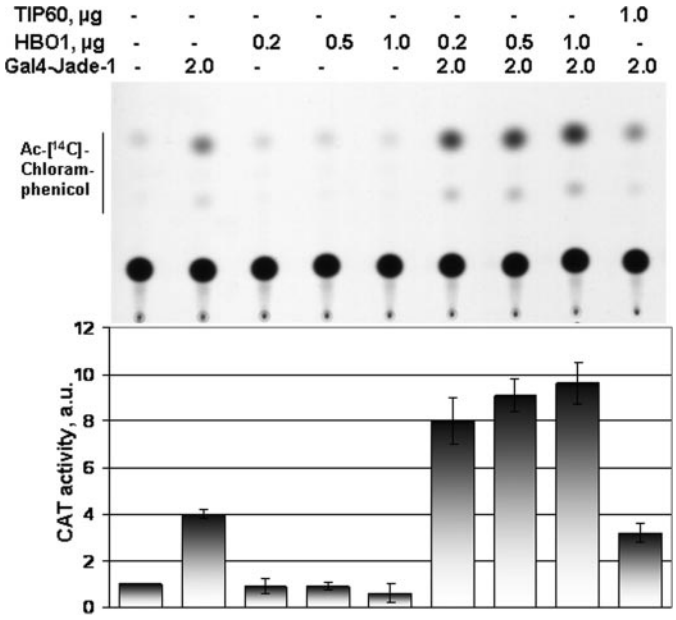

FIGURE 10. **HBO1 enhances Gal4-Jade-1 transcriptional activity.** Sixty-mm dishes of 293T17 cells were co-transfected with 1 μg of AdML promoter-reporter constructs and the indicated amounts of Gal4-Jade-1 without or with HBO1 or TIP60 cDNA constructs. Cells were harvested, and CAT assays were performed as described previously (4) and under "Experimental Procedures." The CAT activity was measured by excising acetylated products from TLC plates and quantifying in a scintillation counter. The experiment was repeated at least twice. *a.u.*, arbitrary units.

histone H4 acetylation (Fig. 4*A*, *lane 9*). Most importantly, the *in vitro* IP-HAT assay experiments demonstrate that the same amounts of immunoprecipitated HBO1 acetylate histones more efficiently in the presence of Jade-1, strongly supporting the idea that the synergy in live cells is due to augmentation of HBO1 specific activity rather than due to a simple dose effect (Figs. 8 and 9).

Determining the precise kinetic and structural mechanism of such functional cooperativity between a HAT, its regulatory proteins and nucleosomes would require more studies and additional approaches, such as, for example, reconstituting recombinant HBO1 complex produced in bacteria. However, the complexity of the acetylation reaction involving multisubunit enzyme and substrate and the absence of a simple reliable assay system can limit functional analysis.

It has been shown that Jade-1L, Jade-2, and Jade-3 co-purify with a stable HBO1 complex and therefore represent components of the native HAT HBO1 complex. Curiously, so far, the short Jade-1 isoform, the focus of our previous and current studies, was not found within the native HBO1 complex, nor any other HAT complexes. Nevertheless, here we demonstrate that both Jade-1 isoforms interact with HBO1 and augment the ability of HBO1 to acetylate histones in live cells and *in vitro* (Figs. 6*A* and 8, *A* and *B*). We previously reported that short Jade-1 interacts physically and functionally with the HBO1 homolog HAT TIP60, although the level of Jade-1 synergy with HBO1 is much higher than with TIP60 (4). We do not exclude the possibility that the short isoform of Jade-1 is functional but not a permanent component of the HBO1 and possibly other HAT complexes.

The inability of Jade-3 to promote histone acetylation or to stabilize HBO1 is rather surprising and requires further investigation. The specific negative phenotype of Jade-3 correlated in both HAT assays, in live cells, and in IP-HAT *in vitro* (Figs. 6*A* and 8, *A* and *B*). The addition of INGs failed to rescue the Jade-3 negative phenotype. This specificity further signifies the role of Jade-1/1L in HAT HBO1 and may be cell type-related.

Our results show that ING4/5 binds Jade-1L and Jade-3 but not the short isoform of Jade-1, strongly suggesting that ING4/5 require the C terminus of Jades for physical interaction (Fig. 7). Interestingly, unlike the rest of the molecule, the primary sequences of the C terminus of full-length Jade homologs are the least conserved regions, and yet they perform a similar function. Expression of ING4/5 does not appear to significantly enhance HBO1-Jade-1L- or HBO1-Jade-3-mediated H4 acetylation, suggesting other functional roles for INGs that are yet to be determined. Alternatively, endogenous INGs might not be limiting factors in this experiment.

Several recent reports identified a novel subset of PHD fingers that directly and selectively binds to the trimethylated lysine 4 or lysine 36 of histone H3 (40, 41, 46). Recognition of methyl-lysine marks within a specific histone tail may allow PHD proteins to translate the histone code by precisely positioning other regulatory proteins and complexes within the epigenome. This finding explains the known correlation between K4 trimethylation and hyperacetylation in histone H3, a pattern characteristic to actively transcribed genes (47–49). Thus, the finding that the PHD finger protein Yng1 in the NuA3 HAT complex interacts with H3K4Me3 uncovers a biochemical link between K4 methylation and hyperacetylation (50).

To date, several reports describe PHD finger interactions with methyl marks within the histone H3 tail. It is unclear whether Jade-1 PHD fingers might have an affinity to H3K4Me3 or H3K36Me3. We do not favor this possibility for several reasons. First, Jade-1 and/or HBO1 do not acetylate nucleosomal histone H3 efficiently (4, 7). Second, (50) yeast strains missing the Jade-1 ortholog Nto1 still retain a robust interaction between Yng1 and H3K4Me3, signifying a role of the PHD fingers of Yng1 (rather than Jade-1 PHD motifs) in recognition of methyl marks by the Yng1 complex.

Whether or not the tandem PHD fingers of Jade-1 recognize any of the histone methyl marks has not yet been studied. Because of predominant histone H4 specificity of Jade-1, it would be interesting to assess whether Jade-1 interacts with known methylated lysine or arginine residues localized to the N terminus of histone H4 and whether Jade-1-HBO1-mediated histone H4 acetylation correlates with the methylation state of histone H4. It is also possible that the noncanonical tandem arrangement of a PHD followed by an extended PHD zinc finger enables Jade-1 recognition of both nucleosomal structure and specific modifications in the histone H4 tail, thus providing unique substrate specificity.

It was proposed that Jade-1 may be involved in epithelial cell differentiation, cell growth control, and embryogenesis (51, 52). Our study classifies Jade-1 as a key functional component of the recently characterized HBO1 complex and implicates the regulatory role of Jade-1 in proper histone/factor acetylation during epithelial cell cycle progression.

Because Jade-1 is absolutely required for HBO1 HAT activity, Jade-1 might participate in cell cycle control via regulation of chromatin acetylation, prereplication complex assembly, and ultimately, DNA synthesis. The ability of Jade-1 to regulate HBO1-mediated histone acetylation also implies a role for Jade-1 in diseases associated with impaired cell cycle control.

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