The Rpd3 histone deacetylase (HDAC) functions in a large complex containing many proteins including Sin3 and Sap30. Previous evidence indicates that the pho23, rpd3, sin3, and sap30 mutants exhibit similar defects in PHO5 regulation. We report that pho23 mutants like rpd3, sin3, and sap30 are hypersensitive to cycloheximide and heat shock and exhibit enhanced silencing of rDNA, telomeric, and HMR loci, suggesting that these genes are functionally related. Based on these observations, we explored whether Pho23 is a component of the Rpd3 HDAC complex. Our results demonstrate that Myc-Pho23 co-immunoprecipitates with HA-Rpd3 and HA-Sap30. Furthermore, similar levels of HDAC activity were detected in immunoprecipitates of HA-Pho23, HA-Rpd3, or HA-Sap30. In contrast, HDAC activity was not detected in immunoprecipitates of HA-Pho23 or HA-Sap30 from strains lacking Rpd3, suggesting that Rpd3 is the HDAC associated with these proteins. However, HDAC activity was detected in immunoprecipitates of HA-Sap30 or HA-Rpd3 from cells lacking Pho23, although levels were significantly lower than those detected in wild-type cells, indicating that Rpd3 activity is compromised in the absence of Pho23. Together, our genetic and biochemical studies provide strong evidence that Pho23 is a component of the Rpd3 HDAC complex, and is required for the normal function of this complex.

Modifications of chromatin by histone acetyltransferases (HATs) and histone deacetylases (HDACs) play important roles in transcriptional regulation (1–4). Many proteins possessing intrinsic HAT activity have been identified from various organisms, and many of these proteins have been shown to be transcriptional coactivators or have other transcription-related functions. Similarly, several HDACs have been identified in different organisms as multiprotein complexes that are associated with transcriptional repressors and co-repressors (5–7). In many cases, HATs and HDACs are targeted to specific promoters through their interaction with DNA-binding transcription factors, suggesting that they regulate transcriptional activity by modifying the local chromatin structure at target promoters (8–10). However, recent reports suggest that HATs also function in an untargeted manner to acetylate histones on a genome-wide scale (11, 12).

Packaging of DNA into chromatin is thought to affect transcription by impeding the access of transcription factors to DNA regulatory sequences. HATs acetylate lysine residues on core histones, thereby neutralizing the positive charge of the histone tails and decreasing their affinity for DNA and/or adjacent nucleosomes in higher order chromosomal structures (7, 13). Such a modification of chromatin is thought to increase the accessibility of DNA to transcription regulatory complexes (14, 15). Thus, in general, hyperacetylation of histones correlates with activation of gene expression, whereas deacetylation represses transcription (16, 17). Consistent with this model, the targeted recruitment of the Gen5 HAT to specific promoters correlates with both transcriptional activation and acetylation of core histones in the vicinity of the promoters, and the HAT activity of Gen5 is required for transcriptional activation of such target genes (8, 12). Also consistent with this model, targeted recruitment of the Rpd3 HDAC complex to specific promoters correlates with both decreased acetylation and transcriptional repression, and Rpd3 catalytic activity is important for transcriptional repression (9, 10, 18). However, in contradiction to this model, transcriptional silencing of centromeric heterochromatin of Drosophila melanogaster and the yeast mating-type loci require acetylation of histone H4 (19, 20) and mutation of Rpd3 enhances silencing at rDNA, telomeric, and mating-type loci (21–25), implying that Rpd3 functions to counteract rather than to establish or maintain silencing. One possible explanation for how acetylation is required for both activation and silencing of genes is that the acetylated states of different regions of chromatin may provide distinct recognition signals for transcriptional activator and repressor protein complexes (25, 26). Loss of Rpd3 may also indirectly enhance silencing of telomeric genes sensitive to histone depletion through an increase in histone gene expression (27).

Genetic and biochemical studies of yeast Rpd3 and the human homologs HDAC1 and HDAC2 first established the role of HDACs in gene regulation. Rpd3 is the catalytic component of a large multiprotein complex that contains Sin3, Sap30, Sds3, and many other proteins (22, 28–32). Some of these papers report the identification of a smaller (~0.6 MDa) HDAC com-

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Pho23 Is Associated with the Rpd3 Histone Deacetylase and Is Required for Its Normal Function in Regulation of Gene Expression and Silencing in Saccharomyces cerevisiae*

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The Rpd3 histone deacetylase (HDAC) functions in a large complex containing many proteins including Sin3 and Sap30. Previous evidence indicates that the pho23, rpd3, sin3, and sap30 mutants exhibit similar defects in PHO5 regulation. We report that pho23 mutants like rpd3, sin3, and sap30 are hypersensitive to cycloheximide and heat shock and exhibit enhanced silencing of rDNA, telomeric, and HMR loci, suggesting that these genes are functionally related. Based on these observations, we explored whether Pho23 is a component of the Rpd3 HDAC complex. Our results demonstrate that Myc-Pho23 co-immunoprecipitates with HA-Rpd3 and HA-Sap30. Furthermore, similar levels of HDAC activity were detected in immunoprecipitates of HA-Pho23, HA-Rpd3, or HA-Sap30. In contrast, HDAC activity was not detected in immunoprecipitates of HA-Pho23 or HA-Sap30 from strains lacking Rpd3, suggesting that Rpd3 is the HDAC associated with these proteins. However, HDAC activity was detected in immunoprecipitates of HA-Sap30 or HA-Rpd3 from cells lacking Pho23, although levels were significantly lower than those detected in wild-type cells, indicating that Rpd3 activity is compromised in the absence of Pho23. Together, our genetic and biochemical studies provide strong evidence that Pho23 is a component of the Rpd3 HDAC complex, and is required for the normal function of this complex.

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§ The abbreviations used are: HAT, histone acetyltransferase; HDAC, histone deacetylase; PHD, plant homeodomain; PCR, polymerase chain reaction; GFP, green fluorescent protein; PBS, phosphate-buffered saline.
complex containing Rpd3 (22, 32); however, it is not clear whether these reports reflect the existence of structurally and functionally distinct Rpd3 complexes in vivo or whether they represent different forms of the same complex. Several components of the Rpd3 HDAC complex, including Rpd3, Sin3, and Sap30, have been highly conserved both structurally and functionally in eucaryotes (30, 31, 33–35). In mammals, the HDAC1,2–Sap30 complex mediates transcriptional repression by interacting with the Mad family of DNA-binding transcriptional repressors (33, 36, 37) and the nuclear hormone receptors N-CoR and SMRT (31, 38–40). Similarly, in yeast, the Rpd3 complex is required for transcriptional repression by Ume6, a zinc finger protein that binds URS1 elements and regulates genes involved in meiosis and arginine catabolism (29). In yeast, Pho23 is closely related to two other yeast proteins, Yng1 and Yng2, and human Ing1, a candidate tumor suppressor (50). The carboxyl-terminal regions of these proteins contain PHD domains, which are found in several proteins implicated in chromatin-mediated gene regulation (51, 52). We have previously shown that Yng1 and Yng2 are associated with specific HAT complexes in yeast (50). In this paper we report evidence that Pho23 is associated with an Rpd3 HDAC complex and that it is required for the normal function of Rpd3 in the silencing of rDNA, telomeric, and mating-type loci.

**EXPERIMENTAL PROCEDURES**

**Yeast Strains and Genetic Analysis**—The genotypes of yeast strains used in this study are listed in Table I. *S. cerevisiae* culture, transformation, mating, tetrad analysis, and other genetic manipulations were performed as described previously (53, 54).

**DNA Manipulation and Analysis**—Procedures used for DNA manipulation and analysis (purification, cloning, electrophoresis, transformation, etc.) were described previously (55). PCR was performed as described previously (56).

**Plasmids**—pAD4H and pUA6 contain the 2-μm origin of replication and ADH1 promoter and encode the HA-epitope and Myc epitope, respectively (57). pADGFPHA was derived by cloning the PCR-derived coding sequence of the enhanced green fluorescent protein (eGFP, CLONTECH) into pAD4H as described previously (50). pADHA-Pho23, pADHA-Rpd3, and pADHA-Sap30 were generated by cloning the PCR-derived open reading frames of Pho23, Rpd3, and Sap30 into pAD4H. pADGFPHA-Pho23 was generated by cloning the PCR-derived coding region of Pho23 in pUA6.

**Immunoprecipitation**—Proteins were isolated from yeast cultures grown in synthetic media to a *A*<sub>600</sub> of ~1.0. Cells from 150 ml of culture were collected by centrifugation, washed once with lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 2 mM EDTA, 0.2% Triton X-100), and resuspended in 2 ml of lysis buffer with protease inhibitors (1 μg/ml pepstatin A, 200 μg/ml phenylmethylsulfonyl fluoride, 500 μg/ml benzamidine HCl, 10 μg/ml aprotinin, 1 μg/ml leupeptin). Cell suspensions were aliquoted into two tubes with 1.5 g of glass beads (425–600 μm, Sigma) and shaken for 5 min in a Mini-BeadBeater (Biospec Products) at 4 °C. Cell debris was removed by centrifugation for 2 min at 2500 rpm and 2 × 5 min at 10,000 rpm. Protein concentrations (typically 10–15 mg/ml) were determined at this point using a Bio-Rad Protein assay. 2 mg of protein was used in 1-ml immunoprecipitation reactions in lysis buffer with protease inhibitors at 4 °C with gentle rotation. Immunoprecipitation reactions were preclared with 40 μl of protein A-Sepharose beads for 20 min. After removal of the beads, 60 μl of protein A-Sepharose beads cross-linked to 12CA5 (anti-HA) was added, and reactions were incubated overnight. Beads were collected by a 1-min centrifugation at 2,200 rpm and washed 5–10 × with 1 ml of lysis buffer. For some experiments, one-third of the beads were removed at this point for HDAC assays. For Western analyses, 20 μl of protein sample buffer was added to the beads, boiled, and separated by SDS-PAGE. Proteins were electrophoretically transferred onto nitrocellulose membranes (1.5 h, 100 V), blocked for 1–12 h in 5% milk in TBS (157 mM NaCl, 2.7 mM

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**TABLE I**

| Strain | Genotype | Source |
|--------|----------|--------|
| BY4742 | Matα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0 | Research Genetics |
| rpd3Δ | BY4742 rpd3Δ::kanMX4 | Research Genetics |
| sap30Δ | BY4742 sap30Δ::kanMX4 | Research Genetics |
| pho23Δ | Matα his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 pho23Δ::kanMX4 | Research Genetics |
| JCC1 | Matα ade8 hist2 leu2 lys2 trp1 ura3 can1 | J. Colicelli, UCLA |
| JS313 | JS311 sin3Δ::kanMX4 | Ref. 24 |
| JS694 | YNB9 rpd3Δ::kanMX4 | This study |
| JS753 | YCB647 sin3Δ::kanMX4 | This study |
| JS754 | YCB647 rpd3Δ::kanMX4 | This study |
| JS755 | YCB647 sap30Δ::kanMX4 | This study |
| JS756 | YNB9 sin3Δ::kanMX4 | This study |
| JS758 | YNB9 pho23Δ::kanMX4 | This study |
| JS767 | JS311 pho23Δ::kanMX4 | This study |
| JS768 | JS311 pho23Δ::kanMX4 rpd3Δ::kanMX4 | This study |
| M475 | JS311 sap30Δ::LEU2 LEU2 leu2::lacZ | Ref. 24 |
| M480 | JS311 rpd3Δ::LEU2::lacZ | Ref. 24 |
| TWY7 | YCB647 pho23Δ::kanMX4 | This study |
| TWY9 | YCB647 pho23Δ::kanMX4 | This study |
| TWY17 | YLS09 pho23Δ::kanMX4 | This study |
| YCD647 | MATα his3Δ1 leu2Δ1 TRP1 lys2Δ202 trp1Δ63 his3Δ52 ADH4::TEL::URA3 | Ref. 62 |
| YLS59 | MATα ade2Δ1 can1Δ100 his3Δ11-15 leu2Δ3-112 trp1Δ1 ura3Δ1 por1Δ1 | Ref. 63 |
| YNB9 | MATα ade2Δ1 hisG his3Δ200 leu2Δ1 lys2Δ3 met15Δ0 ura3Δ167 RDN1::Ty1-MET15, mURA3/HIS3 TELV::ADE2 | This study |
| YSK661 | Matα ura3Δ3-52 lys2Δ800 ade2Δ101 trp1Δ63 his3Δ200 leu2Δ1 rpd3Δ::URA3 | Ref. 44 |
| YSK663 | Matα ura3Δ3-52 lys2Δ800 ade2Δ101 trp1Δ63 his3Δ200 leu2Δ1 | Ref. 44 |
| YSK664 | Matα ura3Δ3-52 lys2Δ800 ade2Δ101 trp1Δ63 his3Δ200 leu2Δ1 hda1Δ::HIS3 | Ref. 44 |
Pho23 Is Associated with Rpd3 HDAC

KCl, 25 mM Tris, pH 7.4), and incubated 1 h with primary antibody (1:2000 12CA5, 1:30 9E10) in TBS + 0.05% Tween 20. After washing, tagged proteins were detected with horseradish peroxidase-conjugated anti-mouse secondary antibodies and ECL reagents (Amersham Pharmacia Biotech).

Histone Deacetyltransferase Assays—HDAC assays were performed as described previously (58, 59), with minor modifications. Immunoprecipitates were rotated at 23 °C for 1 h in a total volume of 200 μl containing 5.5 μg of [3H]acetate-labeled HeLa histones, 40 μl of 5× HDAC buffer (50 mM Tris-HCl, pH 8.0, 0.75 mM NaCl, 50% glycerol), and ±50 μl of 1 M sodium butyrate. The reaction was stopped with the addition of 50 μl of Quench solution (259 μl of HCl, 28 μl of acetic acid, 2,713 μl of H2O) and 600 μl of ethyl acetate. Samples were vortexed and spun for 1 min in a microcentrifuge, and 200 μl of the organic phase was counted to detect the released [3H]acetate.

Preparation of [3H]Acetyl-labeled Histones—[3H]Acetyllsine-labeled histones were prepared from HeLa cells as described previously (60) with minor modifications. 1 liter of HeLa cells were grown to a density of 2 × 10⁸ cells/ml, pelleted (1500 × g), resuspended in 25 ml of PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, and 1.8 mM KH2PO4, pH 7.4) containing 100 μg/ml cycloheximide, 10 mM sodium butyrate, and 0.2 mM/ml [3H]acetic acid, and incubated for 1 h at 37°C. Cells were chilled on ice, washed three times in 10 ml of PBS plus 10 mM sodium butyrate, and then lysed in 8 ml of NIB (1% Nonidet P-40 in IB buffer (10 mM Tris-HCl, pH 7.4, 2 mM MgCl2, 3 mM CaCl2, 10 mM sodium butyrate, and 1 mM phenylmethylsulfonyl fluoride)). Nuclei were harvested, washed twice in 8 ml of NIB, washed once in NIB with 100 mM NaCl, and washed once in 8 ml of IB with 100 mM NaCl. Nuclei were high salt-extracted twice in 8 ml of IB with 400 mM NaCl followed by centrifugation, and the nuclear pellet was extracted twice in 10 volumes of 0.2 M H2SO4, for 90 min on ice and centrifuged (30,000 × g, 25 min). Pooled supernatants were dialyzed extensively against 100 mM acetic acid at 4°C, and the extracted histones were lyophilized and resuspended in H2O (4 mg/ml). 4 ml of histones were dialyzed against PBS; 1 ml of Quench solution and 12 ml of ethyl acetate were added; and the aqueous phase was recovered and dialyzed against 0.5× PBS, yielding prequenched [3H]acetyllsine-labeled histones (1.1 mg/ml, 2 × 10⁶ cpm/mg).

RESULTS

PHO23 Mutants Exhibit Enhanced Silencing of Genetic Loci—Mutants for RPD3, SIN3, and SAP30, which encode components of the Rpd3 HDAC complex, have previously been shown to exhibit derepressed silencing of rDNA, telomeric, and mating-type loci (22–25). We previously identified mutants in a genetic screen that either enhanced or suppressed silencing of rDNA (24). Random Tn3 transposon insertions were generated in a strain that harbored three different polymerase II-transcribed reporter genes in the rDNA: MET15, HIS3, and mURA3 (24, 61). Mutants that strengthened silencing of all three reporters were isolated, and the disrupted genes were named IRS (increased rDNA silencing) (24). Therefore, one of the normal functions of the IRS genes is to antagonize rDNA silencing. RPD3 (IRS2) and SAP30 (IRS8) were both isolated from this screen (24). Also, as predicted, deletion of the SIN3 gene caused an irs phenotype (24, 25).

We now report that one of the IRS genes (IRS3) that we identified from this screen is PHO23. In Fig. 1, the increased rDNA silencing phenotype of a pho23 mutant is compared with the rDNA silencing phenotypes of rpd3, sin3, and sap30 mutants. The increase in silencing of rDNA::HIS3 and mURA3 reporters is indicated by weaker His+ and Ura+ growth of the mutants. All four single mutations increased silencing of mURA3 to similar levels (Fig. 1A). However, using the HIS3
Pho23 Is Associated with Rpd3 HDAC

 reporter, the pho23 and sap30 mutants had a similar level of enhanced silencing, which was not as strong as the rpd3 and sin3 mutants. A pho23 rpd3 double mutant had the same strength of rDNA silencing as the single rpd3 mutant, which is consistent with PHO23 and RPD3 being in the same genetic pathway. The sap30 mutant was already known to have a weaker silencing phenotype than rpd3 or sin3 mutants (25), even though Sap30 is a known subunit of the Rpd3 HDAC complex (30). In terms of rDNA silencing, the PHO23/SIN3 gene can therefore be categorized in the same class as SAP30.

To confirm the above results, the same mutants were tested for silencing of the rDNA:MET15 reporter (Fig. 1B). Using this assay, stronger silencing of MET15 is indicated by a darker colony color (61). The results from this assay were fully consistent with the assays in Fig. 1A. Again, the pho23 and sap30 mutants had the same degree of enhanced silencing as WT but weaker than the rpd3 and sin3 mutants.

Because rpd3, sin3, and sap30 mutations also enhance silencing at telomeres and the silent mating-type loci, we predicted that mutations of pho23 would have the same phenotype. This would be consistent with Pho23 being functionally related to Rpd3, Sin3, and Sap30. We therefore tested the effect of pho23 deletion on telomeric and HMR silencing. Using a telomeric URA3 reporter, rpd3Δ and sin3Δ mutants strongly enhanced silencing, as indicated by dramatically weaker Ura− growth (Fig. 1C). The pho23Δ mutants also strengthened telomeric silencing but less than 5-fold compared with the WT strain (Fig. 1C), which is consistent with the rDNA silencing results. Using a quantitative telomeric ADE2 reporter (62), increased silencing was easily observed for the pho23Δ mutant by its pink colony color (Fig. 1D). We tested for increased silencing at HMR using a weakened allele (hmr2A), which was marked with TRP1. The hmr2A::TRP1 allele is slightly leaky, which allows for some expression of TRP1 and results in Trp− papillae (63). Previous studies with a similar hmrΔA::ADE2 allele have shown that rpd3Δ, sin3Δ, and sap30Δ mutants all enhance silencing, with the sap30Δ-mediated enhancement being the weakest (25). Here we report that deletion of pho23 also enhanced silencing of hmrΔA::TRP1, as indicated by less Trp− papillae (Fig. 1E). Taken together, the genetic results suggest that Pho23 and Rpd3 are functionally related in regulating silencing at rDNA, telomeric, and mating-type loci.

Pho23, Rpd3, Sin3, and Sap30 Mutants Exhibit Other Similar Phenotypes Including Defects in Regulation of PHO5 Expression and Hypersensitivity to Cycloheximide and Heat Shock—PHO23 was originally isolated from a genetic screen for genes involved in repression of PHO5 (49), which encodes a secreted acid phosphatase. Mutations in PHO23 cause derepression of PHO5 in high phosphate growth medium, which is normally repressive for PHO5 expression (Fig. 2A). Mutants for RPD3, SIN3, and SAF3 also exhibit the same derepressed PHO5 phenotype (Fig. 2A), as reported previously (42), further suggesting that these genes are functionally related to PHO23. However, rpd3 mutants exhibit a more severe phenotype than sap30 or pho23 mutants, consistent with the results in Fig. 1. To further explore the functional similarity of these genes, we examined other phenotypes. We found that pho23 mutants, like rpd3, sin3, and sap30 mutants, are hypersensitive to cycloheximide (Fig. 2A). Also, we previously reported that pho23 mutants are hypersensitive to heat shock treatment (50). Here we show that rpd3 and sap30 mutants are also hypersensitive to heat shock at 60 °C compared with wild-type (Fig. 2B), although rpd3 mutants are more sensitive than pho23 or sap30 mutants.

Pho23 Is Associated with Rpd3 and Sap30—Because mutants of Pho23 and Rpd3 exhibit similar phenotypes including enhanced gene silencing, derepressed expression of PHO5, hypersensitivity to cycloheximide, and hypersensitivity to heat shock, we speculated that Pho23 might be a component of the Rpd3 HDAC complex. To investigate this possibility, we performed a series of co-immunoprecipitation assays. To determine whether Pho23 and Rpd3 are physically associated, we co-expressed Myc-Pho23 with either HA-Rpd3 or HA-GFP (as a control) in wild-type yeast strains and performed co-immunoprecipitation assays. Our results show that Myc-Pho23 was undetectable in the control immunoprecipitates, whereas a clearly detectable level of Myc-Pho23 co-immunoprecipitated with HA-Rpd3, indicating that these proteins interact in a complex in vivo (Fig. 3A). We similarly detected Myc-Pho23 in immunoprecipitates of HA-Sap30, providing further evidence that Pho23 is a component of the Rpd3 HDAC complex (Fig. 3B).

Pho23 Is Associated with HDAC Activity That Requires Rpd3—Next, we performed HDAC assays on immunoprecipitated HA-Pho23 from yeast extracts. Our results show that...
HA-Pho23 from wild-type cells was associated with measurable HDAC activity approximately 5-fold higher than control levels (Fig. 4A). These observations strongly suggest that Pho23 is a component of the Rpd3 HDAC complex and is not associated with other protein complexes with measurable HDAC activity.

**Rpd3 Complex Activity Is Reduced in pho23Δ Cells**—We performed additional assays to further explore the role of Pho23 in the Rpd3 HDAC complex. We found that immunoprecipitates of HA-Pho23, HA-Sap30, or HA-Rpd3 from wild-type strains contained similar levels of HDAC activity, but immunoprecipitates of HA-Pho23 or HA-Sap30 from strains lacking Rpd3 had only

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**Fig. 3.** Pho23 associates with Rpd3 and Sap30. **A**, extracts (30 μg of protein) from JC1 cells co-expressing Myc-Pho23 (lanes 3, 4) and either HA-GFP as a control (lanes 1 and 2) or HA-Rpd3 (lanes 5 and 6) were assayed for expression of Myc-Pho23 by Western blot analysis using anti-Myc (9E10) antibody (top panel). Extracts were immunoprecipitated (I.P.) with anti-HA (12CA5) antibody (see “Experimental Procedures”); half of the immunoprecipitate was stained with anti-HA antibody (middle panel), and half was probed with anti-Myc (9E10) antibody (bottom panel). **B**, similarly, extracts from JC1 cells co-expressing Myc-Pho23 (lanes 7–10) and either HA-GFP (lanes 7 and 8) or HA-Sap30 (lanes 5, 6, 9, and 10) were examined by Western blot analysis using anti-Myc (9E10) antibody (top panel). Extracts were immunoprecipitated with anti-HA (12CA5) antibody; half of the immunoprecipitate was probed with anti-HA antibody (middle panel), and half was probed with anti-Myc antibody (bottom panel). Plasmids used to express proteins were pAUD6, pADMyc-Pho23, pADHA-GFP, pADHA-Rpd3, and pADHA-Sap30.

**Fig. 4.** Pho23 associates with an Rpd3-dependent HDAC activity. **A**, extracts from YSK663 (WT), YSK661 (rpd3Δ), or YSK664 (hda1Δ) cells expressing HA epitope or HA-Pho23 were immunoprecipitated with anti-HA (12CA5) antibody. One-third of each immunoprecipitated sample was examined by Western blot analysis using anti-HA antibody (top). One-third of each immunoprecipitated sample was assayed for HDAC activity (bottom) in the presence (+) or absence (−) of sodium butyrate, a HDAC inhibitor (see “Experimental Procedures”). Graphs indicate the average cpm of [3H]acetate released in samples from several independent experiments. **B**, extracts from BY4742 (WT), rpd3Δ, or pho23Δ cells expressing HA epitope, HA-Pho23, HA-Sap30, or HA-Rpd3 were immunoprecipitated with anti-HA (12CA5) antibody. One-third of each immunoprecipitated sample was examined by Western blot analysis using anti-HA antibody (top). HA-Rpd3 migrates at a similar molecular weight as IgG heavy chain (HC). One-third of each immunoprecipitated sample was assayed for HDAC activity (bottom). Plasmids used to express proteins were pAD4H, pADHA-Pho23, pADHA-Sap30, and pADHA-Rpd3.
background levels of HDAC activity (Fig. 4B). These observations suggest that the total HDAC activity associated with Sap30, like that associated with Pho23, requires Rpd3, suggesting that Sap30 is not associated with other Rpd3-independent HDAC complexes. To examine whether Pho23 is required for Rpd3 complex HDAC activity, we examined immunoprecipitates from pho23Δ cell extracts. Our results show that immunoprecipitates of HA-Sap30 or HA-Rpd3 from pho23Δ cells contained measurable levels of HDAC activity, although the levels of activity were consistently reduced by ~35% (Sap30) and 41% (Rpd3) compared with those from wild-type cells (Fig. 4B). These observations suggest that Pho23 is not required for HDAC activity of the Rpd3 complex, but the absence of Pho23 compromises the level of HDAC activity as measured on histones in vitro.

**DISCUSSION**

Previous studies have demonstrated that Rpd3 is the catalytic HDAC component of a large protein complex containing Sin3, Sap30, Sds3, and many other proteins (22, 28, 32). To understand the functions and regulation of this complex it is necessary to define all of the components associated with Rpd3. Our results suggest that Pho23 is a component of the Rpd3 HDAC complex and that Pho23 is important for the normal function of this complex in vitro and in vivo. First, mutants in Pho23, Rpd3, Sin3, and Sap30 exhibit similar phenotypes including derepression of PHO5 expression, cycloheximide sensitivity, heat shock sensitivity, and enhanced silencing of rDNA, telomeric, and mating-type loci. Second, Pho23 physically associates with both Rpd3 and Sap30. Third, Pho23 is associated with HDAC activity in wild-type cells but not in rpd3Δ cells. Fourth, deletion of PHO23 compromises the activity of the Rpd3 HDAC complex in vitro.

The fact that pho23 deletion confers similar phenotypes as rpd3, sin3, and sap30 deletions suggests that they act in the same genetic pathway. A plausible explanation for these observations is that Pho23 is important for the normal function of the Rpd3 HDAC complex in vitro. It is noteworthy, however, that the effects of pho23 mutation are not as strong as rpd3 but are similar to those of sap30 mutants. Thus, Rpd3 function appears to be compromised but not completely impaired in vivo by the absence of Pho23. This finding is consistent with our observations that the HDAC activity of Rpd3, as measured by the ability to deacetylate histones in vitro, is reduced in the absence of Pho23. This decrease in Rpd3 activity in vitro may reflect a similar decrease in vivo, which may account for the observed phenotypes. However, it is also possible that the lack of Pho23 may have other effects such as impaired targeting of the Rpd3 complex to the appropriate promoters. In this regard, it is interesting to note that although loss of Rpd3 leads to a global increase in histone acetylation (22), we did not detect a similar increase in pho23 mutants (50). Thus, Pho23 may be important for deacetylation of specific targeted loci but not for global deacetylation by Rpd3.

Yng1, Yng2, and Pho23 are closely related proteins. We have shown previously that Yng1 and Yng2 are associated with HAT activities in yeast (22). Our evidence indicated that Yng2 is a component of the NuA4 HAT complex, and it is required for the normal acetylation of histone H4 in vivo. Furthermore, our evidence suggests that Yng1 is a component of a distinct HAT complex that has a strong activity and specific histone substrate preference in vitro (50). We also found that Pho23 was associated with a weak and nonspecific HAT activity, but it is not clear whether Pho23 is a bona fide component of a HAT complex in addition to its association with the Rpd3 HDAC complex. Our studies reported here show that Pho23 is important for Rpd3 HDAC activity, but it is not essential for all Rpd3 activity in vitro or in vivo. The presence of these three related proteins in HAT and HDAC complexes suggests that they are playing a novel role that is conserved between these complexes. It is not clear what the roles of Yng1, Yng2, and Pho23 are in HAT and HDAC complexes, but it is possible that they are involved in complex formation or stability, regulation of complex activity, or histone substrate specificity or they may serve as factors that direct HAT and HDAC complexes to their appropriate gene targets.

The conservation of Yng1, Yng2, and Pho23 with mammalian Ing1, and the high conservation of HAT and HDAC complexes between yeast and mammals, suggest the possibility that mammalian Ing1 may have similar functions in HAT and HDAC complexes. Indeed, a recent report indicates that an Ing1 isoform, Ing1b, is associated with the HDAC1-Sin3 complex in HeLa cells (64). However, another Ing1 isoform, Ing1c, does not associate with this HDAC complex, suggesting the possibility that different Ing1 isoforms, like the three yeast homologs, have roles in distinct HAT and HDAC complexes.

The yeast and mammalian Ing1 homologs share strong sequence identity in the PHD domains in their carboxy-terminal regions. PHD domains consist of Cys4-His-Cys5 zinc-binding motifs that are structurally similar to RING and LIM domains (51, 65). They are present in many proteins that have been implicated in chromatin-mediated transcription, and mutations in several of these proteins are associated with human disease, including Williams syndrome (WSFT), hereditary sensory neuropathy (PHF2), x-thalassemia and mental retardation (ATRX), autoimmune polyglandular syndrome type I (AIRE), myeloid leukemias (MLL, CBP, MOZ, and AF10), and immunodeficiency syndrome ICF (DMNT3B) (see Refs. 50 and 52). Also, mutations in the PHD domain of human Ing1 were recently identified in head and neck squamous cell carcinomas (66). Thus, PHD domains appear to play an important role in human disease, yet the precise role of these domains remains unclear. Further studies in yeast may provide a mechanistic basis for understanding the role of the human Ing1 candidate tumor suppressor in regulating cell growth and apoptosis, and they may also provide a similar understanding of the roles of other PHD domain containing proteins in human disease.

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