

# Histone Deacetylases and SAP18, a Novel Polypeptide, Are Components of a Human Sin3 Complex

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## Summary

An important event in gene expression is the covalent modification of histone proteins. We have found that the mammalian transcriptional repressor Sin3 (mSin3) exists in a complex with histone deacetylases HDAC1 and HDAC2. Consistent with the observation that mSin3-mediated repression of transcription involves the modification of histone polypeptides, we found that the mSin3-containing complex includes polypeptides that tether the mSin3 complex to core histone proteins. In addition, two novel mSin3-associated polypeptides, SAP18 and SAP30, were identified. We isolated a cDNA encoding human SAP18 and found that SAP18 is a component of an mSin3-containing complex *in vivo*. Moreover, we demonstrate a direct interaction between SAP18 and mSin3. SAP18 represses transcription *in vivo* when tethered to the promoter, consistent with the ability of SAP18 to interact with mSin3.

## Introduction

In a eukaryotic cell, genes are complexed with chromosomal proteins, particularly histones. Thus, a central question is how RNA polymerases and transcription factors gain access to DNA tightly packed in chromatin. In the last few years, factors capable of altering the structure of chromatin have been identified (for review, see Kingston et al., 1996). One important event is the acetylation of lysine residues of the core histones (Brownell and Allis, 1996; Wolffe and Pruss, 1996). Biochemical studies have revealed a correlation between the level of histone acetylation and transcriptional activity (Hebbes et al., 1988; Braunstein et al., 1993). Hyperacetylation of core histones correlates with gene activation, while hypoacetylation of core histones correlates with gene repression (Wolffe and Pruss, 1996). It is believed that histone acetylation makes chromatin less condensed and thereby facilitates the access of transcription factors to DNA (Lee et al., 1993; Garcia-Ramirez et al., 1995). Recently, factors containing histone acetyltransferase activity have been identified. Significantly, several of these are transcriptional coactivators, such

as Gcn5, p300/CBP, and TAF250 (Bannister and Kouzarides, 1996; Brownell et al., 1996; Mizzen et al., 1996; Ogryzko et al., 1996; for review, see Wade and Wolffe, 1997). Since histone acetylation can be reversed by histone deacetylase, it is likely that activation or repression may result from the targeting of a histone acetyltransferase or deacetylase to a particular gene. However, studies describing the participation of histone deacetylases/acetylases in transcription activation/repression, respectively, have also been reported (De Rubertis et al., 1996). The recent identification and cloning of yeast and human histone deacetylases HDA1 (Rundlett et al., 1996), HDAC1 (Taunton et al., 1996) and HDAC2 (Yang et al., 1996) allow this model to be examined.

The histone deacetylase HDAC1 is a 55 kDa polypeptide highly homologous (60% identical) to the yeast global transcriptional regulator Rpd3p (Vidal and Gaber, 1991; Taunton et al., 1996). HDAC2 was isolated as a protein interacting with the activator/repressor YY1 and is 85% identical in amino acid sequences to HDAC1 (Yang et al., 1996). Neither a DNA-binding domain nor a protein interaction domain has been identified in these polypeptides. However, HDAC1 copurified with the Rb-associated protein RbAp48 (Qian et al., 1993; Taunton et al., 1996). RbAp48 is apparently not required for the enzymatic activity of HDAC1 (Taunton et al., 1996). Interestingly, RbAp48 is a subunit of the human and *Drosophila* chromatin assembly factor CAF-1 (Tyler et al., 1996; Verreault et al., 1996). Although it is not clear whether RbAp48 is necessary for CAF1 activity, RbAp48 can interact with histone H4 (Verreault et al., 1996). In addition, an RbAp48-related protein, Hap2p, has been shown to be a subunit of a yeast B-type histone H4 acetyltransferase (Parthun et al., 1996). Therefore, the RbAp48 protein appears to function as a molecular bridge between histone metabolic enzymes and core histones (Parthun et al., 1996; Verreault et al., 1996).

Genetic studies suggest that Rpd3p is functionally related to the global transcriptional repressor Sin3p/Rpd1p. Sin3p was isolated originally as an antagonist of Swi5p (Nasmyth et al., 1987; Sternberg et al., 1987). The *SIN3* gene encodes a protein with four putative paired amphipathic helix (PAH) domains believed to be involved in protein-protein interactions (Wang et al., 1990). Although Sin3p does not possess DNA-binding activity, it can repress transcription when tethered to a promoter through a heterologous DNA-binding domain (Wang and Stillman, 1993). Therefore, Sin3p has been postulated to repress transcription by interacting with sequence-specific DNA-binding proteins (Wang and Stillman, 1993). The isolation of a mammalian homolog of yeast Sin3p (mSin3) demonstrated that this is indeed the case (Ayer et al., 1995; Schreiber-Agus et al., 1995). The PAH2 domain of mSin3 was found to interact with the N-terminal regions of the DNA-binding proteins Mad, Mxi1, and Mnt. This interaction is required for the repression activity of the Max-Mad/Max-Mxi1/Max-Mnt heterodimers (Ayer et al., 1995; Schreiber-Agus et al., 1995; Hurlin et al., 1997). Recently, it was demonstrated that mSin3, when tethered to DNA through the N terminus

of Mad or Mnt, can repress c-Myc-mediated transcriptional activation and c-Myc-induced cell transformation (Ayer et al., 1996; Harper et al., 1996; Roussel et al., 1996; Hurlin et al., 1997).

Sin3p/Rpd1p and HDAC1/Rpd3p regulate the same set of genes and appear to be involved in the same regulatory pathway (Vidal et al., 1991). Since both yeast and mammalian Sin3 proteins can interact with DNA-binding proteins (Wang and Stillman, 1990; Ayer et al., 1995; Schreiber-Agus et al., 1995), it has been postulated that Sin3-mediated repression of transcription may involve recruitment of the histone deacetylase HDAC1/Rpd3p through protein-protein interactions (Wolffe, 1996).

In the present studies, we have immunopurified a Sin3-containing complex from human cells. We have found that mSin3 is associated with histone deacetylases HDAC1 and HDAC2, as well as with RbAp48, RbAp46, and two novel polypeptides. We demonstrate that the polypeptides in this complex are functionally important for mSin3-mediated repression of transcription.

## Results

### Mammalian Sin3 Interacts with the Histone Deacetylase HDAC1

To gain insight into the molecular mechanism of mSin3-mediated transcriptional repression, we purified mSin3 from human cells, using Western blots to follow its chromatographic behavior. We found that mSin3 exists in large complexes and copurifies with histone deacetylases HDAC1 and HDAC2, as well as with RbAp48 and a related polypeptide, RbAp46 (Qian et al., 1993; Qian and Lee, 1995) (data not shown; see below).

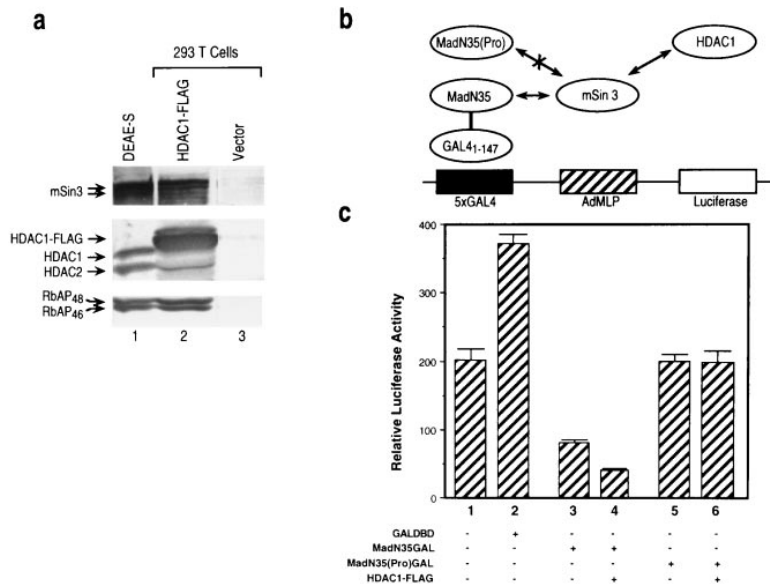
To analyze whether mSin3, HDAC1, and the Rb-associated polypeptides interact *in vivo*, cells were transfected with an expression vector encoding a C terminus FLAG-tagged HDAC1 (Taunton et al., 1996). Cells transfected with the HDAC1 expression vector, or an empty FLAG tag vector, were lysed, and antibodies against the FLAG tag were used to immunoprecipitate HDAC1-FLAG. The transient expression of HDAC1-FLAG and the possible coimmunoprecipitation of mSin3 and the Rb-associated polypeptides were analyzed by Western blot, using antibodies specific to the polypeptides. The results demonstrate that the anti-FLAG antibodies immunoprecipitated HDAC1-FLAG, mSin3, and both the RbAp46 and RbAp48 polypeptides (Figure 1a, lane 2). In contrast, cells transfected with the empty vector did not show significant levels of the polypeptides (lane 3). Therefore, we conclude that mSin3, HDAC1, and the RbAPs interact *in vivo*.

To investigate whether HDAC1 plays a role in mSin3-mediated repression of transcription *in vivo*, cells were transfected with an HDAC1 expression vector. HDAC1 was tethered to the promoter through its ability to interact with mSin3. mSin3 was directed to the promoter through its ability to interact with the N-terminal domain of Mad (Ayer et al., 1995) (see Figure 1b). The luciferase reporter, driven by the adenovirus major late promoter with five Gal4 sites upstream of the TATA motif, was cotransfected with plasmid encoding a fusion protein

composed of the Gal4 DNA-binding domain (amino acids 1–147) and the N-terminal 35 amino acids of Mad, the mSin3-interacting domain (SID, MadN35Gal; see Ayer et al., 1996). Specificity was assessed by cotransfecting a plasmid encoding a mutant SID domain that disables the interaction between Mad and mSin3 [Mad(Pro)N35Gal, Ayer et al., 1996] (see Figure 1b). The MadN35Gal plasmid has been shown previously to be able to repress transcription in a mSin3-dependent manner (Ayer et al., 1996). Consistent with previous results, transfection of the plasmid encoding the Gal4 DNA-binding domain (amino acids 1–147) alone resulted in stimulation of luciferase activity (Figure 1c, column 1 versus 2). This effect is likely due to the cryptic activation domain present in Gal4<sub>1–147</sub>. Nonetheless, transfection of a plasmid encoding MadN35Gal resulted in repression of transcription, as the levels of luciferase activity were reduced approximately 5-fold (column 3). The observed repression was specific, as a reporter lacking the Gal4 site was not affected (data not shown). Moreover, the effect was due to the tethering of mSin3 to the promoter, since transfection of a plasmid encoding the mutant SID domain has reduced effect (Figure 1c, columns 2 and 3 versus 2 and 5). The small effect observed with the mutant is most likely caused by a weak interaction between mSin3 and Mad(Pro)N35Gal. Importantly, cotransfection of a plasmid encoding HDAC1 resulted in an increase in the mSin3-mediated repression of transcription (column 3 versus 4). This effect, while modest, was specifically mediated through mSin3, as no effect was observed when a plasmid encoding HDAC1 was cotransfected with the SID mutant (column 5 versus 6). In addition, cotransfection of the HDAC1 expression vector together with a reporter lacking the Gal4 sites was without effect (data not shown). The modest effect observed by cotransfecting HDAC1 is not surprising and can be attributed to high levels of the intracellular concentrations of HDAC1 and HDAC2 (see below), or, alternatively, to the observation that the transiently transfected plasmid did not assemble into “normal” chromatin (Archer et al., 1992; Bunker and Kingston, 1996). The fact that the effect of HDAC1 was dependent on the ability to tether mSin3 to the promoter, together with the results presented above demonstrating an interaction between mSin3 and HDAC1 *in vivo*, and *in vitro* (data not shown, see below), strongly suggests that the interaction between mSin3 and HDAC1 is important for the mSin3-mediated repression of transcription.

### Affinity Purification of mSin3-Containing Complexes

The results described above suggested that mSin3, HDAC1, and the Rb-associated polypeptides may be components of a complex, and therefore, we sought to isolate such a complex. An affinity column with antibodies against the PAH2 domain of mSin3 cross-linked to protein A-agarose beads was used to immunoaffinity purify the mSin3-containing complex(es). As controls, we used antibodies directed against GST or Cdk8, a component of the RNAPII complex (Maldonado et al., 1996). Western blot analysis of the eluates derived from each column (Figure 2a, lanes B) demonstrates that the anti-mSin3 column retained mSin3, HDAC1, and HDAC2



**Figure 1. HDAC1 Is Functionally Associated with mSin3 In Vivo**

(a) mSin3 and HDAC1 are associated in vivo. 293T cells plated on 100-mm dishes were transfected with 10  $\mu$ g of either an empty FLAG-containing vector (lane 3) or plasmids encoding C-terminal FLAG-tagged HDAC1 (lane 2). Whole cell lysates were incubated with anti-FLAG M2 affinity gel. Immunoabsorbed materials were washed as described (Yeung et al., 1994), eluted, and analyzed for mSin3, HDAC1, and RbAPs by Western blot. The HDAC1 polypeptide appears as a doublet due to the fact that the antibodies used recognized HDAC1 and HDAC2 (see text for details). Lane 1 contains 10  $\mu$ l of DEAE-Sepharcel pool, which serves as a control for monitoring mSin3 and HDAC1/HDAC2.

(b) Schematic representation of the assay used to analyze repression of transcription in an mSin3/HDAC1-dependent manner.

(c) mSin3-mediated transcription repression is enhanced by histone deacetylase HDAC1. 293T cells plated in 100 mm plates were transfected with 2  $\mu$ g of reporter plasmid and

8  $\mu$ g of MadN35Gal or Mad(pro)N35Gal, together with 4  $\mu$ g of HDAC1-FLAG plasmids where indicated (+). Forty-eight hours after transfection, cells were harvested and cell extracts were assayed for luciferase activity. Transfection efficiencies were normalized using  $\beta$ -galactosidase assay. Transfections were repeated at least two times in duplicate. Presence and absence of the effector plasmid are indicated by (+) and (-), respectively.

(lane 6), as well as RbAp46 and RbAp48 (data not shown, see below), whereas the anti-Cdk8 column retained RNAPII and cyclin C (lane 9). The above polypeptides were not detected in the eluate from the anti-GST column (lane 3). Recent studies have indicated that HDAC2 interacts with YY1 (Yang et al., 1996); however, YY1 was absent in the eluate derived from the anti-mSin3 column (data not shown).

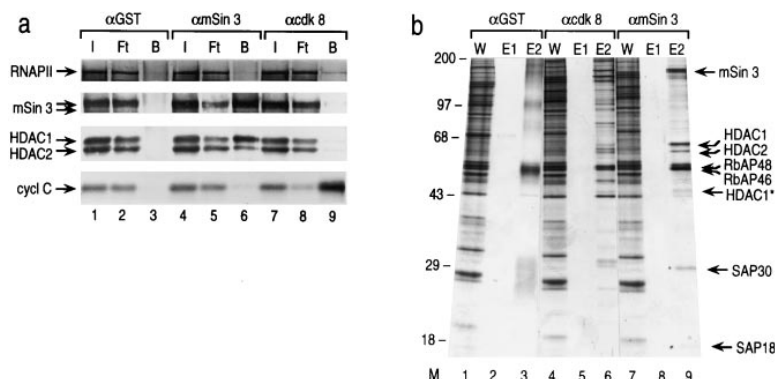
Silver staining of a SDS-PAGE gel containing the eluates derived from each column revealed no specific polypeptides in the anti-GST-derived fraction (Figure 2b, lane 3). Consistent with the demonstration that Cdk8 is an integral component of the yeast (Koleske and Young, 1995) and human (Maldonado et al., 1996) RNAPII complexes, and with the observations that these complexes contain many undefined proteins, the anti-Cdk8-derived fraction was heterogeneous (Figure 2b, lane 6). In contrast, only about 10 predominant polypeptides were

present in the anti-mSin3-derived fraction (Figure 2b, lane 9).

### Characterization of the Polypeptides Present in the Anti-mSin3 Affinity Purified Fraction

Western blot analysis, together with microsequencing of isolated polypeptides, was used to characterize each of the polypeptides present in the anti-mSin3 affinity purified fraction. Western blot analysis demonstrated the presence of mSin3 in the immunopurified complex (Figure 2). This was expected, since mSin3-antibodies were used to immunoaffinity purify the complex.

Microsequencing of peptides derived from the second largest polypeptide identified it as histone deacetylase HDAC1 (Figure 3a). Western blot analysis confirmed this result and revealed that the third largest polypeptide was also immunoreactive to anti-HDAC1 antibodies



**Figure 2. Isolation of mSin3-Associated Polypeptides**

(a) Western blot analysis of the fractions derived from the different affinity columns. Samples derived from the different columns, as indicated above, were analyzed by Western blot. The blot was probed with different antibodies, as indicated on the left side of the panel. (I) represents input (DEAE-Sepharcel pool), (Ft) represents flow-through, and (B) represents bound material. The HDAC1 polypeptide appears as a doublet because the antibodies used recognized HDAC1 and HDAC2 polypeptides.

(b) Silver staining of an SDS-PAGE gel containing the samples derived from the different

affinity columns. For each column (0.5 ml bed volume), an aliquot of wash (W), first elution (E1), and second elution (E2) were resolved on a 10% SDS-PAGE for silver staining. The positions of protein size markers are indicated. The identity of the major bands in lane 9 was attributed based on the results from Western blot analysis and/or peptide sequencing. SAP30 and SAP18 represent mSin3-associated peptides p30 and p18.

(Figures 2a and 2b). Microsequencing of a peptide derived from this polypeptide revealed it to be the recently isolated histone deacetylase HDAC2 (Figure 3a). Interestingly, microsequencing of a peptide derived from the polypeptide, migrating slightly above the 43 kDa marker (see HDAC1\* in Figure 2b), demonstrated that it was derived from HDAC1 and/or HDAC2 (Figure 3a). However, mass fitting of other peptides derived from HDAC1\* demonstrated it to be HDAC1 (data not shown). Therefore, we conclude that this polypeptide is either a proteolyzed or a differentially spliced form of HDAC1.

Western blot analysis demonstrated that the doublet migrating with an apparent mass of approximately 50 kDa (Figure 2b) corresponds to the Rb-associated polypeptides (data not shown). Microsequencing of six peptides derived from these proteins confirmed the Western blot results, as two peptides specific for RbAp46 and two other peptides specific for RbAp48 were found (Figure 3a). In addition, two peptides shared by RbAp46 and RbAp48 were also obtained (Figure 3a). Based on the UV absorbance (OD<sub>214</sub>) of the HPLC-separated tryptic peptides specific to each polypeptide, the molar ratio of RbAp46 to RbAp48 was estimated to be 4:1 (data not shown). We noticed that there are proteins in the anti-GST and the anti-Cdk8 columns that appear to comigrate with the RbAp46 and RbAp48 (Figure 2b, lanes 3 and 6). These polypeptides were not immunoreactive to antibodies recognizing RbAp46 and RbAp48 (data not shown), and most likely represent non-cross-linked immunoglobulin heavy chain.

Microsequencing of the two smaller mSin3-associated polypeptides, SAP30 (data not shown) and SAP18 (Figure 3b), revealed that they are novel proteins. Two peptide sequences derived from the SAP18 polypeptide were obtained and used to isolate a full-length cDNA clone encoding this polypeptide (see Experimental Procedures). The nucleotide sequence encoding SAP18 predicts a long open reading frame encoding a polypeptide of 153 amino acids with a calculated molecular mass of 17.56 kDa. This value is in close agreement with the apparent mass of SAP18 (Figure 3b). The predicted amino acid sequence of the SAP18 cDNA clone contains the two derived peptide sequences (Figure 3b). A search of the GenBank database identified a *C. elegans* SAP18 homolog (GenBank accession number Z46787) that is 66% similar and 49.3% identical to its human counterpart (Figure 3b). A search of the yeast genome database identified a potential yeast SAP18 homolog (GenBank accession number Z49705) that is 36.9% similar and 16.1% identical to its human counterpart (Figure 3b). The sequence of SAP18 does not give any insights into its function. However, like mSin3, it is ubiquitously expressed in all tissues analyzed (data not shown).

**SAP18 Interacts with mSin3 and Enhances the Ability of mSin3-Mediated Repression of Transcription**

To analyze whether the presence of SAP18 in the mSin3 affinity purified sample was specific, we analyzed whether SAP18 interacts with mSin3 and HDAC1. Toward this end, a GST-SAP18 fusion protein was purified and used to pull down mSin3 and HDAC1 that were radiolabeled in an in vitro transcription-translation system.

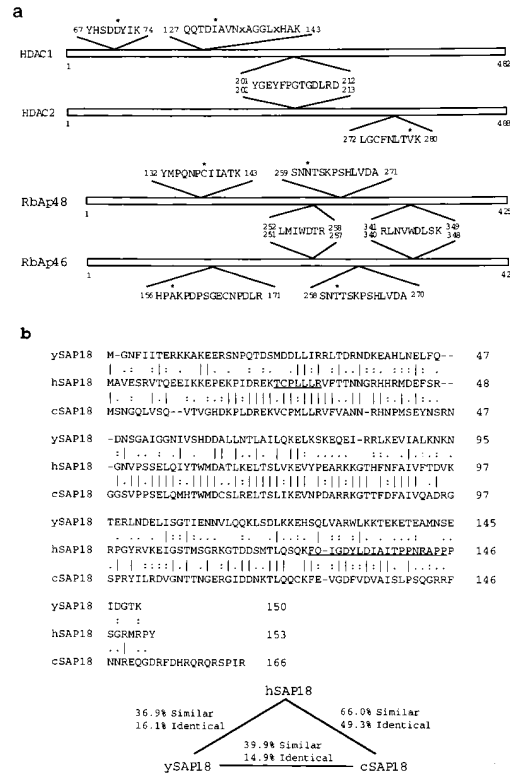
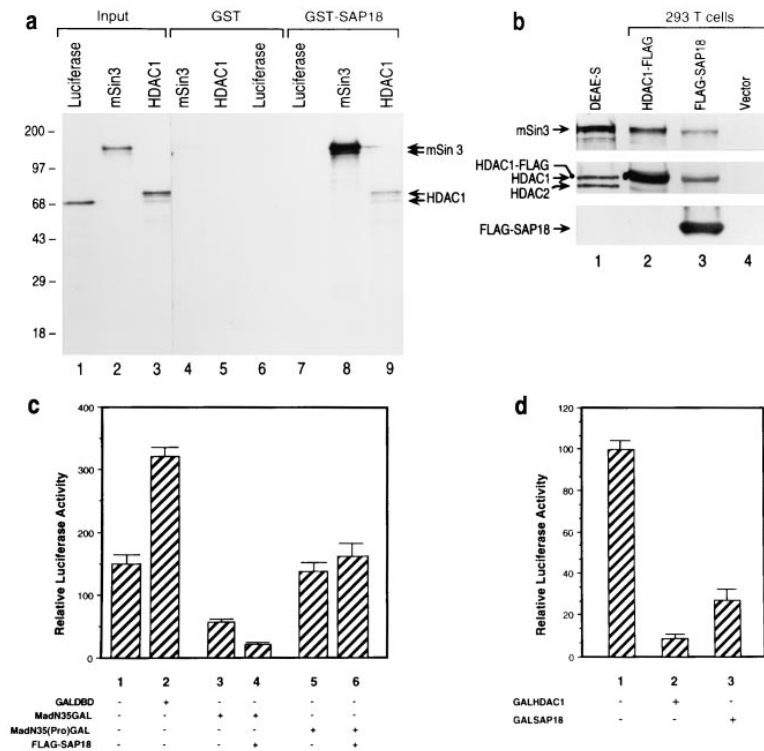


Figure 3. Amino Acid Sequence of Polypeptides Present in the mSin3-Affinity-Purified Sample

(a) Schematic representation of the HDAC1, HDAC2, RbAp46, and RbAp48 polypeptides. Peptide sequences obtained from microsequencing of the HDAC1, HDAC2, and HDAC1\* bands (Figure 2b, lane 9) are shown on the top, as indicated on the panel. The peptide sequence shared by HDAC1 and HDAC2 was obtained from the polypeptide labeled (HDAC1\*) in Figure 2b. The peptide sequence obtained from the bands labeled (RbAp46) and (RbAp48) (Figure 2b, lane 9) are shown. Of the 6 sequenced peptides from the doublet, 2 are unique to RbAp48 (correspond to amino acids 132–143 and 259–271), 2 are unique to RbAp46 (correspond to amino acids 156–171 and 258–270), and 2 are common to both. The amino acids unique to each polypeptide are indicated by an asterisk.

(b) Amino acid sequence alignment of human SAP18 (hSAP18) with its *C. elegans* homolog (cSAP18) and potential *S. cerevisiae* homolog (ySAP18). Sequence alignment was performed using Gap of the GCG program (University of Wisconsin-Madison). Peptide sequences obtained from microsequencing of the band labeled SAP18 in Figure 2b are underlined. The *C. elegans* homolog was identified by BLAST nonredundant GenBank database (accession number Z46787). The potential *S. cerevisiae* homolog was identified by BLAST Saccharomyces Genome Database (Stanford University, accession number Z49705). Vertical lines represent identical amino acids; dashes represent gaps; dots represent similar amino acids. Percentages of similarities and identities are shown at the bottom.

Equal amounts of radiolabeled proteins (Figure 4a) were incubated with either GST (lanes 4–6) or GST-SAP18 (lanes 7–9) that were attached to glutathione-agarose beads. No radiolabeled protein was pulled down by the GST protein alone (lanes 4–6). However, under the same conditions, mSin3 was efficiently pulled down by GST-SAP18 (lane 8). HDAC1 could also be pulled down by the GST-SAP18 (lane 9). However, the pull-down was not as efficient as with mSin3 (compare lanes 8 and 9). The interaction was specific to both



**Figure 4. SAP18 Functionally Interacts with mSin3 In Vitro and In Vivo**

(a) In vitro interaction between SAP18 and mSin3. Equal amounts of in vitro translated and labeled mSin3 (lanes 4 and 8), HDAC1 (lanes 5 and 9), and luciferase (lanes 6 and 7) were incubated with 2  $\mu$ g of GST or GST-SAP18 proteins in the presence of glutathione-agarose beads. Proteins were synthesized and labeled in vitro using rabbit reticulocyte lysate and [<sup>35</sup>S]-methionine according to the instructions of the manufacturer (Promega). Fusion proteins were bound to 10  $\mu$ l of glutathione-agarose beads and incubated with equal amounts of in vitro translated protein in 0.5 ml of buffer containing 20 mM Tris-HCl (pH 7.5), 0.1 M NaCl, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, and 0.2% NP-40 at 4°C overnight. The beads were washed three times with the same buffer, and the bound proteins were eluted with 20  $\mu$ l of SDS-PAGE loading buffer. Eluted proteins were resolved by electrophoresis and were visualized by fluorography. Amounts of about 1/10 of each protein were used as input (lanes 1–3). The positions of protein size markers are indicated.

(b) SAP18 is associated with both mSin3 and HDAC1 in vivo. 293T cells plated in 100-mm dishes were transfected with 10  $\mu$ g of empty FLAG-containing vector (lane 4), or plasmids encoding FLAG-tagged HDAC1 (lane 2), or

SAP18 (lane 3). Immunoprecipitation and Western blot analysis were performed as described in Figure 1a. The top strip was probed with antibodies against mSin3. The middle strip was probed with antibodies that recognize HDAC1 and HDAC2. The bottom strip was probed with anti-FLAG antibodies. Lane 1 is the DEAE-Sephacel pool, which serves as a control for monitoring mSin3 and HDAC1/HDAC2.

(c) SAP18 enhances mSin3-mediated transcription repression. 293T cells were transfected with 2  $\mu$ g of reporter plasmid (see Figure 2) together with 8  $\mu$ g of MadN35Gal or MadN35(Pro)GAL in the presence or absence of 4  $\mu$ g of FLAG-SAP18. Forty-eight hours after transfection, cells were harvested and cell extracts were assayed for luciferase activity. Transfection efficiencies were normalized using  $\beta$ -galactosidase assay. Transfections were repeated at least two times in duplicate. Presence and absence of effector plasmids are indicated by (+) and (–), respectively.

(d) SAP18 can function as a transcriptional repressor when brought to the promoter. Transfections were performed using the TK promoter with five Gal4 sites, as described above and indicated in the figure. Transfection efficiencies were normalized using  $\beta$ -galactosidase assay. Transfections were repeated at least three times in duplicate.

polypeptides, as GST-SAP18 failed to pull down luciferase (lane 7). It is likely that SAP18 directly interacts with mSin3. However, we cannot rule out the possibility that other polypeptides present in the rabbit reticulocyte lysate mediate the interaction.

Having established an in vitro interaction, we next asked whether SAP18 interactions could be demonstrated in vivo. Toward this end, cells were transfected with plasmid encoding either an N-terminal FLAG epitope-tagged SAP18, or a C-terminal FLAG epitope-tagged HDAC1. Cells transfected with these plasmids, or with an empty FLAG vector plasmid, were lysed, and antibodies against the FLAG tag were used to immunoprecipitate FLAG-SAP18, HDAC1-FLAG, and interacting polypeptides. The results demonstrate that the anti-FLAG antibodies immunoprecipitated FLAG-SAP18 as well as mSin3 and HDAC1 from extracts derived from cells transfected with the FLAG-SAP18 plasmid (Figure 4b, lane 3). In contrast, no specific polypeptides were immunoprecipitated from the cells transfected with the empty vector (lane 4). In agreement with the results presented above (Figure 1a), the anti-FLAG antibodies immunoprecipitated HDAC1-FLAG and mSin3 from cells transfected with the HDAC1-FLAG expression vector (Figure 4b, lane 2). The results strongly suggest that

SAP18 exists in a complex with mSin3 and HDAC1 in vivo.

To analyze the role of SAP18 in mSin3-mediated repression of transcription in vivo, 293T cells were cotransfected as described in Figure 1b, but the HDAC1 expression vector was substituted by an SAP18 expression vector (Figure 4c). In agreement with previous studies, and with the studies described above (Figure 1c), transfection of an expression vector encoding the Gal4 DNA-binding domain alone (Gal4<sub>1–147</sub>) resulted in stimulation of transcription from the reporter containing Gal4 sites (Figure 4c, column 1 versus 2). In agreement with the results presented in Figure 1c, transcription was severely reduced by transfection of a vector encoding the Gal4 DNA-binding domain fused to the SID domain of Mad (Figure 4c, column 3). Transfection of an expression vector containing Gal4<sub>1–147</sub> fused to a mutant SID domain resulted in reduced repression (Figure 4c, column 5). Importantly, cotransfection of an expression vector encoding SAP18 resulted in a further decrease in the levels of repression observed with the fusion protein containing a wild-type SID domain (column 4). The SAP18 effect was specific, as cotransfection of a plasmid encoding SAP18 with a plasmid containing a mutation in the SID domain was without effect (Figure 4c,

column 5 versus 6). Moreover, the effect observed by cotransfecting SAP18 was dependent on the presence of the Gal4 sites in the reporter, as SAP18 was without effect on a reporter lacking the Gal4 sites (data not shown).

Since we found that mSin3 interacts with SAP18, we speculated that tethering SAP18 to the promoter would result in the formation of an mSin3-containing complex, and therefore, would result in repression of transcription. As shown in Figure 4d, tethering HDAC1 or SAP18 to the promoter through the Gal4 DNA-binding domain resulted in transcription inhibition. The observed repression requires SAP18/HDAC1 to be tethered to the reporter, as no repression was observed from a reporter without Gal4 sites (data not shown) or when an expression vector encoding SAP18 or HDAC1 alone, in the absence of the Gal4 DNA-binding domain, was transfected (data not shown, see Figures 1c and 4c). Thus, the results collectively demonstrate that SAP18 is a component of the mSin3-repressing complex.

## Discussion

The studies presented here, using a combination of conventional and affinity chromatography, and transfection studies, demonstrate that mSin3 exists in a functional complex with histone deacetylases *in vivo* and *in vitro*. We have isolated a mammalian Sin3-containing complex and have characterized its constituent polypeptides. Consistent with the suggestion that mSin3-mediated repression of transcription involves the modification of core histones, we found that the mSin3-containing complex includes polypeptides with the ability to tether the mSin3 complex to core histones, such as the Rb-associated polypeptides p46 and p48. However, the Rb polypeptide was absent in the complex (data not shown). In addition, two novel mSin3-associated polypeptides, SAP18 and SAP30, were identified. We isolated a cDNA encoding human SAP18 and found that SAP18 is a component of an mSin3-containing complex *in vivo*. Moreover, we have demonstrated a direct and functional interaction between SAP18 and mSin3. Consistent with the ability of SAP18 to interact with mSin3, we found that a fusion protein between the Gal4 DNA-binding domain and SAP18 repress transcription of a reporter containing Gal4-binding sites, but not of a reporter lacking the Gal4 sites. The novel SAP30 polypeptide present in the affinity purified mSin3-containing complex was not analyzed in the present studies. However, SAP30 most likely represents a true component of the mSin3 complex, since independent studies using the yeast two-hybrid system identified SAP30 as an mSin3-binding protein (D. Ayer, personal communication). Thus, the polypeptides present in the mSin3 affinity purified complex represent proteins involved in mSin3 function *in vivo*.

Our studies provide evidence that mSin3 and HDAC1 are present in the same complex *in vitro* and *in vivo*. However, the chromatographic behavior of mSin3 suggests that mSin3 may form different protein complexes (data not shown). This is supported by the finding that two histone deacetylases, HDAC1 and HDAC2, are present in the mSin3 affinity purified complex. In addition,

the Rb-associated polypeptides were not stoichiometric, with RbAp46 being more abundant. Importantly, we found that RbAp46 and RbAp48, together with mSin3, were coimmunoprecipitated by antibodies immunoprecipitating HDAC1-FLAG (Figure 1a). However, we also observed that antibodies immunoprecipitating FLAG-SAP18 coimmunoprecipitated mSin3 and HDAC1, but not HDAC2 (Figure 4b and data not shown).

Why are the Rb-associated proteins p46 and p48 present in the affinity purified mSin3 complex? Both RbAp46 and RbAp48 were originally isolated from HeLa cell extracts as proteins that bound to immobilized Rb fragment (Qian et al., 1993; Qian and Lee, 1995). The two proteins are 90% identical and belong to the WD-repeat protein family that is involved in highly diverse cellular processes (Neer et al., 1994). RbAp48 was found to be a subunit of human histone deacetylase HDAC1 (Taunton et al., 1996). Although it is not required for histone deacetylase activity, it was postulated that RbAp48 targets HDAC1 to core histone proteins (Taunton et al., 1996). A p46/p48 homolog from *S. cerevisiae*, termed Hat2p, was recently found to be a subunit of the B-type histone H4 acetyltransferase Hat1p (Parthun et al., 1996). Hat2p is required for high affinity binding of the histone acetyltransferase Hat1p to histone H4 (Parthun et al., 1996). In addition, RbAp48 is a subunit of the chromatin assembly factor CAF1 (Tyler et al., 1996; Verreault et al., 1996). Importantly, RbAp48 interacts with histone H4 (Verreault et al., 1996). It is therefore likely that RbAp48 and/or RbAp46 function to target histone-modifying enzymes to core histones.

We have demonstrated that SAP18 exists in the same complex with mSin3 and HDAC1 (Figure 4), and that SAP18 enhances mSin3-HDAC1-mediated transcriptional repression. Importantly, we observed that SAP18, when tethered to the promoter, can direct the formation of a repressive complex (Figure 4). A key question is the function of these polypeptides in Sin3-mediated repression of transcription. The availability of an mSin3 complex and its recombinant polypeptides, together with a chromatin reconstituted RNAPII transcription system (G. Orphanides, G. LeRoy, and D. R., unpublished data), is likely to define the role of the different polypeptides in mSin3 repression of transcription.

## Experimental Procedures

### Purification of mSin3-Containing Complexes

mSin3-containing complexes were purified from HeLa nuclear extract using Western blots as an assay. Proteins derived from HeLa cell nuclear extracts were fractionated by following the procedure implemented for the purification of the human RNAPII complex (Maldonado et al., 1996). A detailed protocol for the conventional purification of mSin3-containing complexes will be published elsewhere. Affinity purification of mSin3-associated polypeptides was performed, using affinity purified antibodies against the PAH2 domain of mSin3. Antibodies were coupled to 0.5 ml of protein A-agarose beads (Repligen) as described (Harlow and Lane 1988). The resin was incubated overnight at 4°C with the DEAE-Sepharose pool (2 ml, 0.6 mg/ml; see Maldonado et al., 1996) in buffer containing 20 mM Tris-HCl (pH 7.9), 0.2 mM EDTA, 1 mM DTT, 0.2 mM PMSF, 10% glycerol, 100 mM KCl, and 0.1% NP-40. Immunoabsorbed complexes were then washed extensively with the same buffer, except that the salt was raised to 700 mM KCl. The bound proteins were eluted with 0.1 M glycine (pH 2.6). The column was neutralized with

0.1 M Tris-HCl (pH 8.0) and equilibrated with the above binding buffer before it was reused. Eluates derived from the immunoaffinity column steps were concentrated on a micro MonoS column using the SMART system. Fractions were analyzed by SDS-PAGE, followed by Western blot analysis and silver staining.

#### Peptides Sequencing and SAP18 Cloning

Ponceau S-stained bands were excised, in-situ tryptic-digested, and fractionated by reverse-phase HPLC using an 0.8-mm Vydac C18 column (Lui et al., 1996). Selected peak fractions were analyzed by a combination of matrix-assisted laser desorption time-of-flight mass spectrometry (Voyager-RP; PerSeptive Biosystems, Framingham, MA) and automated Edman sequencing (477A; Applied Biosystems, Foster City, CA) (Tempst et al., 1994). When matches with a known protein were found (HDAC1, HDAC2, RbAp46, and RbAp48), exhaustive mass-spectrometric analysis was carried out and the results compared to the predicted tryptic peptides of published sequences by mass fitting. In the case of SAP18, the obtained peptide sequences match several EST cDNA clones (GenBank accession numbers W49739, AA025356, and N74746). Analysis of the EST sequences indicated many putative sequencing frameshift errors. From all of these clones, a hybrid sequence encoding a 153 aa protein with a M<sub>r</sub> of 17,562 Da was constructed. The hybrid sequence was confirmed by sequencing the EST cDNA clones obtained from Research Genetics.

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#### GenBank Accession Number

The accession number for the hSAP18 nucleotide sequence reported in this paper is U96915.