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Histone chaperone specificity in Rtt109 activation

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Abstract

Rtt109 is a histone acetyltransferase that requires a histone chaperone for the acetylation of histone 3 at lysine 56 (H3K56). Rtt109 forms a complex with the chaperone Vps75 *in vivo* and is implicated in DNA replication and repair. Here we show that both Rtt109 and Vps75 bind histones with high affinity, but only the complex is efficient for catalysis. The C-terminal acidic domain of Vps75 contributes to activation of Rtt109 and is necessary for *in vivo* functionality of Vps75, but it is not required for interaction with either Rtt109 or histones. We demonstrate that Vps75 is a structural homolog of yeast Nap1 by solving its crystal structure. Nap1 and Vps75 interact with histones and Rtt109 with comparable affinities. However, only Vps75 stimulates Rtt109 enzymatic activity. Our data highlight the functional specificity of Vps75 in Rtt109 activation.

The packaging of DNA into chromatin has profound implications for all cellular processes that require access to the DNA substrate. Numerous activities have been identified that make compacted chromatin more amenable to the complex machinery responsible for transcription, replication and repair. These activities include histone chaperone-mediated nucleosome assembly and disassembly, post-translational modifications of histones, incorporation of histone variants and ATP-dependent chromatin remodeling. Evidence is emerging that all of these activities are tightly interwoven and cooperate in complex ways to achieve the delicate balance of chromatin compaction and decompaction.

Histone chaperones have been recognized as important players in regulating DNA accessibility and chromatin fluidity (recently reviewed in refs. 1,2). Many histone chaperones are found in complex with histone modification enzymes or ATP-dependent chromatin-remodeling proteins. For example, human nucleosome assembly proteins 1 and 2 (NAP1 and NAP2) physically interact with p300 (ref. 3); yeast Nap1 and other histone chaperones collaborate with the remodeling factor SWR1 in the replacement of histone H2A with the histone variant H2A.Z (ref. 4), and the putative Nap1 family member Vps75 forms a complex with the newly discovered histone acetyltransferase (HAT) Rtt109 (refs. 4-6).

Yeast Rtt109 acetylates H3K56, a modification that is likely to have a role in DNA replication and in maintaining genome stability in fungi7-10. Recent findings have demonstrated that

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Accession codes. Protein Data Bank: Coordinates for Vps75 have been deposited with accession code 2ZD7.

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Rtt109 also acetylates H3K911. Rtt109 is only the second-known HAT that seems to require a histone chaperone for its activity^{6,12}; the first documented case was the complex of Hat1 (the catalytic component) and Hat2 (the ortholog of the human histone binding protein RBAP48 (ref. 13)). *In vitro*, the reaction catalyzed by recombinant Rtt109 alone is slow and inefficient⁹, and Vps75 serves to activate Rtt109's HAT activity⁶. Vps75 is a bona fide histone chaperone on the basis of its ability to bind histones and to assemble chromatin *in vitro* and to associate with chromatin *in vivo*¹⁴. Vps75 has approximately 24% sequence homology with yeast Nap1, a multifunctional histone chaperone with pleiotropic roles in chromatin metabolism and cell-cycle regulation (reviewed in refs. 15,16). The unrelated histone chaperone Asf1 also stimulates Rtt109 activity *in vitro*^{6,7}. Notably, a deletion of *VPS75* has only minor effects on global H3K56 acetylation (H3K56ac) in yeast cells, also supporting redundant functions *in vivo* for the chaperones and Rtt109 stimulation^{12,14}. Asf1 may be a good candidate for these redundant functions, because deletion of *ASF1* does lead to loss of H3K56ac, but it also leads to loss of H3K9ac, a modification that is performed by a different HAT, Gcn5 (ref. 17). Moreover, Asf1 is not a member of the Nap1 family. Thus, the relationships between these chaperones and their roles in Rtt109 stimulation are unclear.

Here we describe the crystal structure of *Saccharomyces cerevisiae* Vps75 and compare its structural and functional properties to those of *S. cerevisiae* Nap1. Both chaperones (the only two known Nap1 family members in yeast) bind histones with similarly high affinities, and both proteins stably interact with Rtt109; however, only Vps75 is capable of stimulating Rtt109 HAT activity. In addition, deletion of *VPS75* results in dramatic and diverse mutant phenotypes, in contrast to the lack of effects observed for the deletion of *NAP1*. The flexible C-terminal domain of Vps75 is important for the *in vivo* functions of Vps75 and modulates Rtt109 activity *in vitro*. Together, our data demonstrate a remarkable specialization of Vps75 for the interaction with and stimulation of Rtt109.

RESULTS

Vps75 is a distinct member of the NAP1 family

The 24% sequence identity between yeast Nap1 and yeast Vps75 does not suggest a strong degree of structural homology between the two proteins (Fig. 1a). We determined the crystal structure of yeast Vps75 to a resolution of 1.85Å. Attempts to identify a molecular replacement solution with the previously published Nap1 structure as a search model¹⁸ were unsuccessful, and we therefore used selenomethionine (SeMet) derivatives for MAD phasing. The electron density throughout almost the entire amino acid sequence was of excellent quality (Supplementary Fig. 1a online).

Vps75 is a homodimer with two chains in the asymmetric unit. This is consistent with sedimentation velocity data that demonstrate the presence of a single species with an *S*(20,w) (the sedimentation coefficient corrected for water at 20 °C) of 3.8 (Supplementary Fig. 2a online). To confirm unequivocally that Vps75 is a dimer in solution, we performed sedimentation equilibrium of a version of Vps75 that lacks the C-terminal acidic domain (see below, Vps75_{1–223}, with a calculated molecular mass of 28,527 Da) under similar solution conditions and over a 16.2-fold range of concentrations. Details are given in Supplementary Figure 2b and Supplementary Methods online. Vps75_{1–223} is best described as a homogenous population of Vps75 dimers under these conditions. Given the near-identical behavior of full-length and Vps75_{1–223} in sedimentation velocity experiments, we conclude that full-length Vps75 is also a dimer in solution.

Vps75 shares several structural features with Nap1 (Fig. 1b). Both proteins are obligate homodimers that are held together through the antiparallel pairing of the long α 2 helices of two monomers. The pronounced curvature of this helix, previously observed for Nap1, is

maintained in Vps75 and is caused by the presence of a proline approximately two-thirds into the $\alpha 2$ helices (Fig. 1a, asterisk). Many hydrophobic residues are conserved between the two proteins. Like Nap1, Vps75 is an acidic protein with a pI of 4.64. The charge distribution for Vps75 is uneven, with a relatively random distribution of acidic, basic and neutral amino acids on the upper side of the dome-shaped dimer, whereas most of the acidic residues are clustered at the underside, especially in the cavity formed by the two β -fold domains (Supplementary Fig. 1b).

The Vps75 structure differs from that of Nap1 in several important aspects. First, Vps75 has neither an N-terminal tail nor the equivalent of the accessory domain previously defined in Nap1 (ref. 18) (Fig. 1a, yellow). Vps75 contains a C-terminal acidic domain (CTAD); however, this region has fewer negative charges (16 compared to 28) and a short stretch of hydrophobic amino acids that is not present in Nap1 (Fig. 1a, underlined). This region of the CTAD is clearly visible in the Vps75 electron-density map (Supplementary Fig. 1c) because it is involved in crystal contacts with a neighboring Vps75 dimer. Second, the N-terminal end of the long $\alpha 2$ helix is almost completely covered by the accessory domain in Nap1, whereas it is solvent exposed in Vps75 (Fig. 1c). The accessory domain is absent in Vps75. Third, the region of Nap1 that contains the nuclear localization sequence (NLS) assumes different conformations in the two structures (Fig. 1b,d). In Nap1, the NLS is part of an extended β hairpin ($\beta 5$ - $\beta 6$) that is responsible for oligomerization of Nap1 dimers¹⁹. In Vps75, this region forms a helix-loop-helix conformation. As is the case with Nap1, this region in Vps75 harbors eight basic residues (Fig. 1a) and is the most positively charged region in the entire protein (Supplementary Fig. 1b). Fourth, the region equivalent to the Nap1 $\alpha 1$ helix is absent in Vps75 (Supplementary Fig. 1d). In Nap1, this helix forms an acute angle to $\alpha 219$ and its presence restricts the orientation of the penultimate $\alpha 7$ and $\alpha 8$ helices. In Vps75, $\alpha 7$ and $\alpha 8$ form a single, uninterrupted helix, presumably allowing a wider range of motion for the C-terminal domain in solution.

Using gel-shift assays, we demonstrate that full-length Vps75 forms well-defined supershifts upon addition of increasing amounts of either H2A-H2B dimer or (H3-H4)₂ tetramer (Fig. 2a). H2A-H2B and H3-H4 do not enter the gel under these conditions because of their strong positive charge. We determined the binding affinities of the various Vps75—histone complexes by monitoring a change in fluorescence of a fluorophore attached to either H3 or Vps75 upon incubation with H2A-H2B dimer or (H3-H4)₂ tetramer (Fig. 2b). Both histone complexes bind Vps75 with low nanomolar affinity. This value is comparable to the affinity between Asf1₁₋₁₆₈ and H3-H4, which was measured under the same conditions (Table 1). The values were the same irrespective of the location of the fluorescent label (25 nM and 27 nM for labeled Vps75 or labeled H3-H4, respectively; Table 1). H3 binds Vps75 in the absence of H4, but with reduced affinity. As is the case for Nap1 (ref. 20), the C-terminal domain of Vps75 does not contribute to histone binding (Fig. 2b,c, Table 1). The stoichiometry of a Vps75–H3–H4 complex was also determined using a fluorescence assay (Fig. 2d): one Vps75 dimer binds two molecules of H3–H4. At this stage, it is unknown whether Vps75 binds one (H3–H4)₂ tetramer or two half-tetramers (H3–H4). We believe that the presence of higher-order gel shifts (as seen in Fig. 2a,c) represent lower-affinity complexes of Vps75 and histones, possibly also multimers of histone-bound Vps75. Eventually, these higher-order (low-affinity) complexes become too large (or too positively charged) to enter the gel, a phenomenon that is commonly observed during native gel electrophoresis.

Nap1 can remove histone H2A–H2B dimers from assembled nucleosomes *in vitro*²⁰ and we therefore tested whether Vps75 has this same ability. Nucleosomes were assembled with the fluorescently labeled mutant H2A–T112C and then incubated with equivalent amounts of Vps75 or Nap1 (Supplementary Fig. 3 online). Under conditions where the addition of Nap1 leads to the formation of a repositioned nucleosome species and H2A–H2B dimer—depleted nucleosomes, Vps75 had no discernible effect on nucleosome composition. These results

suggest that the two chaperones are functionally distinct, despite their similar structure and histone binding properties.

The C-terminal acidic domain of Vps75 is required for *in vivo* function(s)

To further investigate the functional differences between Vps75 and Nap1, we compared the effects of gene disruptions *in vivo* (Fig. 3a and Table 2). Previous work has shown that the deletion of *NAP1* in yeast has no obvious phenotype under various conditions²¹, and this was confirmed in our analyses. In contrast, a strain in which *VPS75* was deleted showed sensitivity to UV radiation, hydroxyurea, methyl methanesulfonate (MMS) and methotrexate (Fig. 3a). All of these agents are known to induce DNA damage²². The deletion of **VPS75** also resulted in temperature sensitivity (slow growth at 38 °C). We also observed slow growth on 1 M NaCl and in the absence of inositol (Table 2). The observed phenotypes are not reflective of overall growth defects, as the strain containing the deletion of **VPS75** grows normally on 1 M sorbitol (an inducer of osmotic stress) and H₂O₂ (an inducer of oxidative stress).

All of the phenotypes resulting from a deletion of *VPS75* were rescued with a shuttle vector encoding full-length Vps75 (Fig. 3b). However, a shuttle vector encoding a version of Vps75 that is missing the CTAD (Vps75_{1–223}) was, at most, partially able to revert any of the effects of *VPS75* deletion on growth under the conditions tested.

Notably, under *in vitro* conditions, the CTAD did not contribute quantitatively to the interaction of Vps75 with histones (Table 1). Thus, the lack of phenotype rescue in the absence of the CTAD suggests previously unknown *in vivo* functional activities for this domain.

The CTAD of Vps75 contributes to stimulation of Rtt109 HAT activity

In vivo, a substantial portion of Vps75 is found in complex with the HAT Rtt109. To test the possibility that the Vps75 CTAD functions through mediating the interaction with Rtt109, we compared the ability of full-length and truncated Vps75 to interact with recombinant, purified Rtt109 *in vitro*. Vps75 (either full-length or Vps75_{1–223}) was incubated with increasing amounts of Rtt109 (Fig. 3c). Rtt109 has a pI of 9.5 and does not enter the gel on its own (lanes 5 and 10). In contrast, both versions of Vps75 form clearly defined bands (lanes 1 and 6). Upon addition of Rtt109, supershifts of Vps75 were observed at comparable concentrations, demonstrating that the Vps75 CTAD is not required for the interaction with Rtt109.

To further verify the composition of the complexes in the shifted bands, we took two approaches. First, we excised the bands from the native gel and analyzed them by SDS-PAGE (Supplementary Fig. 4 online). Both Vps75 and Rtt109 were present in all excised bands we analyzed. Furthermore, the two proteins were present at apparently equal stoichiometries (Supplementary Fig. 4, lanes 4–6 and 8–10). To further investigate the homogeneity of the Vps75–Rtt109 complexes, we compared the solution state of a Vps75–Rtt109 complex at a 1:1 molar ratio to that of Vps75 (full-length and Vps75_{1–223}) and Rtt109 alone. Under our experimental conditions, Vps75 and Rtt109 are both monodisperse, and they sediment with an *S*-value of 3.1 and 3.8, respectively. A 1:1 complex of Vps75 (or Vps75_{1–223}) and Rtt109 sediments with an *S*-value of 6.1, with a portion of the material still in the unassociated form (Supplementary Fig. 2a). Given the relatively low *K_d* of the complex (Table 1), it is to be expected that some of the protein remains unassociated under the experimental conditions of 8 μM. Under our experimental conditions, we observed no higher-order complexes. Together, these data indicate that both full-length and truncated Vps75 form defined and uniform complexes with Rtt109.

It may be that the CTAD of Vps75 acts to modulate the HAT activity of Rtt109. We incubated recombinant yeast (H3-H4)₂ tetramer with preformed Rtt109–Vps75 or Rtt109–

Vps75_{1–223} complexes and probed for H3K56ac (Fig. 3d). The ability of Rtt109 to acetylate H3K56 was increased about 30-fold upon the addition of full-length Vps75 (Fig. 3e). Comparatively, Vps75_{1–223} was clearly not as efficient in promoting Rtt109-mediated H3K56 acetylation, especially under conditions where Vps75 is limiting (substoichiometric amounts of Vps75 to Rtt109). The addition of polyglutamate (40–100 kDa) had no effect on Rtt109 HAT activity, indicating that the function of the Vps75 CTAD goes beyond merely adding negative charge. The most straightforward explanation for our results (quantified in Fig. 3e) is that the CTAD of Vps75 contributes to the stability of the interaction between Vps75 and Rtt109 (Table 1), because acetylation levels reach near wild-type levels at higher concentrations of Vps75_{1–223}.

We next tested whether the CTAD of Vps75 contributes to the specificity of Rtt109-mediated acetylation. We analyzed the end products of the HAT reaction by acid-urea gels (Fig. 3f). This method allows for the separation of H3 molecules with one or more acetyl groups. This confirmed that in the absence of Vps75, no acetyl group is added to either H3 or H4, and that the stimulatory effect of Vps75_{1–223} is much weaker than that of full-length Vps75 (Fig. 3f, above and below, respectively). No additional bands of acetylated histones were detected in the presence of Vps75_{1–223}.

To determine the affinity of Rtt109 for histones, we used fluorescently labeled (H3-H4)₂ tetramer and measured the changes in fluorescence upon Rtt109 addition. Rtt109 binds H3-H4 with an affinity of 150 nM (Table 1). In contrast, the interaction between Rtt109 and Vps75 (in the absence of histones) is much lower (~5 μ M; Table 1). Both values argue against a simple role of a histone chaperone in increasing the affinity of Rtt109 for its histone substrate.

Nap1 binds Rtt109 but does not stimulate its enzymatic activity

As a deletion of *VPS75* has no effect on overall levels of H3K56ac *in vivo*, we asked whether Nap1 could functionally replace Vps75 in Rtt109 activation. We used gel-shift assays to demonstrate that Nap1 interacts robustly with Rtt109 (Fig. 4a). The presence of defined and similar supershifts obtained upon addition of increasing amounts of Rtt109 to either Vps75 or Nap1 indicates that Rtt109 forms equivalent complexes with either chaperone. The interaction between Nap1 and Rtt109 was also demonstrated through co-immunoprecipitation assays *in vivo* in a strain carrying a deletion of *VPS75* (Supplementary Fig. 5a online). This interaction persists through extensive washing steps at 500 mM NaCl.

The addition of Nap1 to Rtt109 does not result in a detectable stimulation of H3K56 acetylation, as tested by western blot analysis (Fig. 4b,c). Only minor levels of acetylation were observed by urea acid gels on H3 in the presence of the Rtt109–Nap1 complex, even when a large excess of Nap1 over Rtt109 was used (Fig. 4d). Whereas Vps75 stimulated efficient Rtt109-mediated acetylation of H3 (in the absence of H4), Nap1 showed no such effect on Rtt109 (Supplementary Fig. 5b).

DISCUSSION

Numerous homologs of Nap1 have been identified in metazoans, many with ill-defined functions in transcription regulation and other cellular functions (reviewed in refs. 2,15,16). In contrast, only two Nap1 family members exist in yeast: Nap1 and Vps75. The structural homology between Vps75 and Nap1 is immediately apparent from our studies, despite the low degree of sequence conservation. Vps75 also shares several structural characteristics with the Nap1 family member SET, a human oncoprotein with pleiotropic nuclear functions². Both proteins lack the extended N-terminal tail as well as the accessory domain previously identified in Nap1, and both proteins lack the nuclear export sequence and are thus predominantly nuclear in location. On the basis of this observation, it can be argued that Vps75 is a structural homolog

of SET in yeast. SET is involved in histone and nucleosome metabolism, interacts with various transcription factors and has been found as a part of a complex that inhibits the acetyltransferase activity of p300/CBP and PCAF23. Thus, like Vps75, it seems to have specialized in noncanonical histone-chaperone functions.

Vps75 binds histones with high affinity, but does not dissociate nucleosomes

Vps75 binds histones with high affinity *in vitro*. Low nanomolar affinities for various histone complexes seem to be a hallmark of the histone chaperones tested to date—Vps75, Asf1 and Nap1 from various species (this study, and A.J.A. and K.L., unpublished observations). Previously published studies (including ours) have used glutathione *S*-transferase (GST) pull-down assays to establish a preference of the three chaperones for H3-H4^{15,24,25}. Our quantitative binding studies presented here suggest that H2A-H2B binds Vps75 with higher affinity than H3-H4. Thus, under the conditions tested, Vps75 is not an exclusive H3-H4 histone chaperone but is capable of binding both types of histone complexes with high affinity. One dimer of Vps75 binds two molecules of H3 and H4, either in the form of a (H3-H4)₂ tetramer or as two half- tetramers. The CTAD of Vps75 does not contribute to binding affinity. As is always the case with *in vitro* studies, we cannot exclude the possibility that the proteins may interact differently *in vivo*.

As Vps75 and Nap1 bind histones with similar affinities, the inability of Vps75 to disassemble nucleosomes under conditions where Nap1 disrupts them was unexpected. We have previously shown that the Nap1 CTAD is required for efficient nucleosome disruption *in vitro*²⁰, whereas the Vps75 CTAD (which differs in amino acid composition compared to Nap1) seems to contribute to Rtt109 stimulation.

VPS75 and NAP1 deletions cause different phenotypes in yeast

Further indications for nonoverlapping functions of Vps75 and Nap1 and for the importance of the CTAD of Vps75 *in vivo* come from gene-knockout studies. Whereas no growth defects were observed for the *NAP1* deletion, our results suggest a role for Vps75 in DNA damage repair (see also ref. 10). Published work (as well as our own unpublished data) on the deletion of *RTT109* demonstrates similar hydroxyurea and MMS mutant phenotypes¹⁰, confirming the functional link between Vps75 and Rtt109. There is controversy in the field about the mutant phenotypes of a *VPS75* deletion strain^{7,10,14}. We have found that the *VPS75* deletion strain is extremely vulnerable to spontaneous suppression of growth phenotypes. Whether this, strain background or the actual experimental growth conditions are involved in these discrepancies is unclear at this time.

If H3K56ac levels remain unchanged in a *VPS75* deletion strain^{7,14}, why then does this strain have phenotypes that are consistent with a role of Vps75 in DNA repair? Vps75 may have other roles in DNA damage repair that may result in the Rtt109-dependent acetylation of targets other than histones, or that are unrelated to Rtt109 activity. Of note, a global protein-expression profiling study in yeast showed that Vps75 was one of 157 proteins whose level increased by more than three-fold upon treatment with MMS²⁵. Notably, Rtt109 was not listed in this group of proteins. It is possible that Vps75 is functionally redundant with another protein, such as Asf1, with respect to Rtt109 activity. We have demonstrated that another logical candidate, Nap1, is capable of interaction with Rtt109 both *in vivo* and *in vitro*, but fails to stimulate acetylation.

It is surprising that two structurally unrelated histone chaperones (Vps75 and Asf1) with seemingly different histone binding properties (but similar affinities for histones) are capable of stimulating the enzymatic activity of Rtt109 to a similar extent *in vitro*⁶. It has been argued that Vps75 either increases the binding affinity of Rtt109 for H3-H4 or that it helps provide

specificity for H3K5612. However, the affinity of Rtt109 for H3-H4 in the absence of chaperone is certainly tighter than most reported K_m 's of other HATs. *In vitro* data support the hypothesis that Asf1 functions to present H3-H4 to an Rtt109—Vps75 complex, especially at limiting concentrations of Rtt109—Vps7512. Consistent with this interpretation, we observe a saturation of HAT activity at roughly equimolar concentrations of Rtt109 and Vps75, making it unlikely that Vps75 acts as part of the substrate. Rather, Vps75 could function through (i) a direct stimulation of the enzymatic activity, (ii) modulating the affinity of Rtt109 for either the Asf1—H3-H4 substrate or for the product of the enzymatic reaction, or (iii) through correctly positioning H3K56 in the active site. Any of these functional models requires the CTAD of Vps75.

Structural information for the chaperone–histone interaction is available for only Asf1 (ref. 26). Given that there are no structural or sequence similarities between Vps75 and Asf1, it is unlikely that the two proteins bind histones and Rtt109 in a similar manner; however, both can stimulate HAT activity to similar extents *in vitro*. Notably, Nap1, a structural homolog of Vps75 with similar histone binding properties and the demonstrated ability to form a complex with Rtt109, does not stimulate HAT activity. The differences in sequence composition of the Nap1 and Vps75 CTAD may explain why Nap1 has no effect on Rtt109 activity. Together, these results suggest that Vps75 and Nap1 have distinct functions although they retain structural homology and a high affinity for histone complexes.

METHODS

Expression and purification of recombinant proteins

Details are given in the Supplementary Methods.

Structure determination

Recombinant Vps75 was crystallized by sitting-drop vapor diffusion at 16 °C from drops consisting of an equal mixture of protein (15 mg ml⁻¹) and reservoir solution (32% (v/v) PEG400, 50 mM NaCl and 25 mM HEPES, pH 7.5). Crystals (average size 0.05 × 0.30 × 0.03 mm) were obtained after 7 d. We flash-cooled crystals in liquid nitrogen directly from the well solution before data collection at beamline 4.2.2 at the Advanced Light Source (ALS). Data were processed and reduced with d*TREK27. We derived phases using MAD with data collected from SeMet mercury derivative crystals. The model was built with O28, and refined with CNS29. The final model contains one dimer in the asymmetric unit. Diffraction data, refinement statistics and model parameters are given in Table 3. Structure superpositions were carried out using LSQMAN30.

Electrophoretic mobility shift assays

Protein complexes were analyzed by electrophoretic mobility shift assays (EMSAs) under native conditions. EMSAs were performed by incubating 10 μM Vps75 or Nap1 in a 10 μl reaction with the indicated concentrations of histones or Rtt109 at 4 °C for 16 h with 10 mM Tris, pH 7.5, 100 mM NaCl, 1 mM EDTA and 1 mM DTT. The samples were loaded onto a 5% acrylamide, 0.2× Tris-borate with EDTA (TBE) gel, electrophoresed for 50 min at 150 V and stained with Coomassie brilliant blue.

Binding-affinity measurements

Fluorescence titrations were used to determine the binding affinity of 0.2–0.4 nM Alexa-546 or Alexa-488 (H2A-H2B only)—labeled proteins (Vps75, H2A-H2B-T112C, or H3-H4 (E63C)) to histone chaperones or Rtt109 in 300 mM NaCl, 0.5 mM EDTA, 1 mM DTT and 20 mM Tris-HCl, pH 7.5, using an AVIV model ATF105 spectrofluorometer. Labeled protein

was added to both the sample and the reference cuvette, with nonlabeled protein added to the sample cuvette and buffer added to the reference. We normalized the ratio of the fluorescence signal from the sample cuvette to the reference cuvette using equation (1):

$$f.c.\text{obs} = \frac{R_{\text{obs}} - R_i}{R_{\text{max}} - R_i} \quad (1)$$

where $f.c.\text{obs}$ is equal to the fraction change for each concentration X added, R_{obs} is equal to the ratio at concentration X , the R_{max} is equal to the ratio at saturating protein, and R_i is the ratio where the protein concentration added is equal to zero. The binding affinity (K_d) of the various complexes was determined by fitting the $f.c.\text{obs}$ as a function of protein added (P_i) fit using equation (2) with the Kaleididagraph software:

$$f.c. = \frac{f.c.\text{max} * [P_i]}{[P_i] + K_d} \quad (2)$$

Stoichiometry

We determined stoichiometries by fluorescence titrations as above with the labeled protein concentration increased to more than ten-fold higher than the K_d . The fluorescence ratio was plotted as a function of the ratio of protein titrated to labeled protein. Under these conditions, the protein ratio at which the fluorescence ratio levels off is equal to the stoichiometry.

GST pull-down assay

0.5 nmoles of GST-tagged Rtt109 was immobilized on 50 μl of glutathione Sepharose 4B resin (GE Healthcare). The resin was then mixed with or without 1 nmol histone chaperone in the presence of various amount (5–500 pmol) of recombinant (H3-H4)₂ tetramer and incubated for 3 h. We removed unbound H3-H4 by washing three times with HEPES buffer (20 mM HEPES, 0.5 mM EDTA, 10% (v/v) glycerol, 0.05% (v/v) Nonidet p-40, 5 μM ZnSO₄ and 2.5 mM MgCl₂) at 450 mM KCl. To detect bound H3-H4, we used Alexa 488—labeled histone (H3-H4)₂ tetramer (labeled on H4-T71C). GST-tagged Rtt109 with Vps75 (full length), Vps75_{1–223} or Nap1 (full length) interaction was tested separately in the same high-salt buffer condition. The results were analyzed by 15% SDS-PAGE and Storm (Amersham Biosciences). Minor nonspecific binding of H3-H4 was observed only at low-salt conditions (0–200 mM).

Yeast strains, plasmids and media

Yeast strain BY4741 (MATa his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0) was used for all investigations. We used yeast standard laboratory methods and techniques. Deletion mutants in the BY4741 background were purchased from Open Biosystems. BY4741 cells were grown for 48 h on rich media containing 2% (w/v) glucose. A shuttle vector encoding genomic *VPS75* was transformed into the *vps75* Δ mutant strain. The shuttle vector (pRS316) contained a full-length copy of the genomic DNA sequence for *VPS75* as well as a selectable marker URA3. The Vps75 shuttle vector was transformed into mutant *vps75* Δ cells and plated on selective media (CAA-U). Site-directed mutagenesis was used to generate pRS316 Vps75_{1–223} and confirmed by sequencing before use. The shuttle vector pRS316 Vps75_{1–223} was transformed into a *vps75* Δ mutant strain and grown on selective media (CAA-U). The final yeast strain containing pRS316-Vps75_{1–223} was confirmed by PCR amplification of the *VPS75* locus and DNA sequencing. The ability of the truncated shuttle vector to recover phenotypes associated with the *vps75* Δ mutant strain was compared to that of the full-length *VPS75* shuttle vector. Cells were grown in media and then diluted to an absorbance at 600 nm (A_{600}) of 0.1. We made ten-fold serial dilutions of each culture and spotted them onto plates as indicated in Table 2. Cells

were allowed to grow for 48–72 h at 30 °C or 38 °C. The MMS plate was made within 24 h of use. The yeasts were grown in liquid YPD medium (1% yeast extract, 1% bactopectone and 2% glucose) at 30 °C. Plates for DNA damage assays contained YPD with or without 0.025% MMS or 0.2 M hydroxyurea and were photographed after 3 d of growth at 30 °C. For UV-sensitivity assays, cell cultures were diluted to an A_{600} of 0.2, along with ten-fold serial dilutions, and these were spotted on YPD plates. UV irradiation at 254 nm was performed with a Stratalinker 2400 (Stratagene) at 75 J m^{-2} . Duplicate sets of plated cells were exposed to UV irradiation and incubated at 30 °C for 3–4 d. All of our studies have been done in 8–16 replicate cultures.

Histone acetyltransferase assays

Reactions were performed in 50 mM Tris-HCl, pH 7.5, and 0.5 mM DTT, 100 mM NaCl using 1 mM acetyl CoA and analyzed by immunoblotting and acid urea gel. 10 μl reactions were incubated at 20 °C for 1 h, 2 h or 3 h and stopped by freezing in liquid nitrogen.

Western blot analysis

We resolved his-tone proteins by 15% SDS-PAGE for 30 min at 30 mA and transferred to nitrocellulose. Blots were probed with antibodies against H3K56ac (Upstate) or H3 (Abcam). Membranes were incubated at 4 °C for 10 h in TBST (5 mM Tris, pH 7.4, 27.4 mM NaCl, 0.5 mM KCl and 0.1% (v/v) Tween 20) with antibodies against either H3K56ac (1:6,000) or H3 (1:1,000). We diluted primary and secondary antibodies (a horseradish peroxidase—conjugated anti-rabbit IgG secondary antibody) in TBS containing 0.1% (v/v) Tween 20 and 5% (v/v) milk. Western blots were developed with an ECL detection kit (Amersham Biosciences). Secondary antibodies conjugated to horseradish peroxidase were detected using a Storm phosphorimager (Amersham Biosciences). To quantify modifications on his-tones, the intensity of the H3K56ac bands was analyzed by ImageQuant v5.1 (Amersham Biosciences). Data were reported as average values with s.d.; with a few exceptions, data points were derived from at least three independent gels. The gels were probed with antibodies against unmodified H3 to provide for a loading control. All data from the H3K56ac bands were normalized to unmodified H3.

Acid urea gel

We analyzed the total amount of H3 acetylation by acid urea gel electrophoresis. We used 15% polyacrylamide gels containing 5% (v/v) acetic acid and 6 M urea to separate modified from unmodified histones on the basis of differences in their charge³¹. Proteins were denatured in 6 M urea. Gels containing urea were prepared freshly to prevent nonspecific carbamylation of histone. The gel was run for 3 h at 150 V in 5% (v/v) acetic acid buffer and stained with Coomassie brilliant blue. Acetylated H3 was clearly separated from unmodified histones.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Vps75 is a distinct member of the Nap1 family. **(a)** Structure-based amino acid sequence alignment of yeast Nap1 and Vps75. Blue, yellow, green and red boxes indicate subdomains A, B, C and D, respectively, as previously designated in the Nap1 structure¹⁸. The alignment was generated using LSQMAN. Secondary-structural elements are shown above and below the corresponding sequences in both Nap1 and Vps75. Proline residues that induce helix curvature are indicated by asterisks. **(b)** Superposition of the Vps75 (blue) and Nap1 (yellow) structures. **(c,d)** Ribbon diagram of Nap1 and Vps75. Color coding is the same as in **a**. Secondary-structural elements are as defined in **a**. **(c)** A detailed comparison of the accessory domain in the same view as in Supplementary Fig. 1c. **(d)** Ribbon diagram of the region

encompassing the nuclear localization signal (NLS) in Nap1 and the equivalent region in Vps75.

Figure 2.

Vps75 binds histones with high affinity. **(a)** Vps75 dimer (5 μ M) was incubated with recombinant yeast H2A-H2B dimer (lanes 1–5 show 0 μ M, 1 μ M, 2 μ M, 3 μ M and 4 μ M, respectively) or recombinant yeast (H3-H4)₂ tetramer (lanes 6–10 show 0 μ M, 1 μ M, 2 μ M, 3 μ M and 4 μ M, respectively) at 4 °C for 10 h. Complex formation was analyzed by 5% native PAGE and stained with Coomassie brilliant blue. The position of Vps75–histone dimer complexes is indicated. **(b)** Affinity measurement of the Vps75–H3-H4 interaction. The normalized fluorescence change (Norm. fluoro. change) upon binding of Rtt109 to H3-H4 and of Vps75 or Vps75_{1–223} to Alexa-546-labeled H3-H4 measured by fluorescence titration. The binding curve for Vps75–Rtt109 is also shown. A standard binding isotherm (Methods) was fit to these data to determine the K_d for each interaction (see Table 1 for K_d values). **(c)** As in **a**, except that Vps75_{1–223} was used instead of full-length Vps75. **(d)** Stoichiometry of H3-H4–Vps75 complexes. Normalized fluorescence change in labeled (H3-H4)₂ (where the labeled protein concentration is five times the K_d) as a function of ratio of (H3-H4)₂ to Vps75 dimer.

Figure 3.

The C-terminal acidic domain of Vps75 contributes to *in vivo* and *in vitro* functions. **(a)** The *VPS75* deletion strain is sensitive to genotoxic agents, unlike a *NAP1* deletion strain. Ten-fold serial dilutions of wild-type (WT) and deletion strains were applied as spots to plates with rich media containing either glucose (YPD), hydroxyurea (HU 200 mM) or methane methylsulfonate (MMS 0.025%) and incubated at 30 °C. The deletion of *RAD26* (which is involved in transcription-coupled repair) is used as a control for the plates. **(b)** The C-terminal tail of Vps75 is required for restoring growth defects. Serial dilutions of the indicated strains were plated on YPD, HU (200 mM) and MMS (0.025%). **(c)** The ability of Vps75 to form a complex with Rtt109 was tested using gel shifts (5% polyacrylamide in 0.2× TBE). We

combined 10 μ l of 10 μ M Vps75 dimer with increasing amounts of Rtt109 (lanes 1–4 or lanes 6–9: 0 μ M, 1 μ M, 2 μ M and 3 μ M, respectively; lanes 5 and 10 show a control of 3 μ M Rtt109 which, owing to its strong positive charge, does not enter the gel). **(d)** Vps75 is required for the HAT activity of Rtt109. Purified Vps75 or Vps75_{1–223} dimer (lanes 2–5 or lanes 6–9: 0.1 μ M, 0.2 μ M, 0.4 μ M and 0.8 μ M, respectively) and Rtt109 (0.5 μ M) were incubated with 5 μ M yeast (H3-H4)₂ tetramer in the presence of 1 mM acetyl-CoA for 3 h. The level of H3K56ac was detected by western blot analysis. Total protein H3 on the blot was detected by anti-H3 antibody. The activity of full-length (Vps75(FL), lanes 2–5) and C-terminal truncated Vps75_{1–223} (above, lanes 6–9) were compared. Vps75_{1–223} showed a reduction in H3K56ac compared to full-length Vps75. **(e)** Normalized HAT activity curves (obtained by monitoring changes in intensity of the western blot) are shown to the right. Error bars represent s.d. **(f)** The same reactions as in **d** were analyzed by acetic urea gel to identify other reaction products. Acetylation on H3 causes a reduction in gel mobility. Polyglutamate (Poly-glu) has no effect on acetylation.

Figure 4.

Nap1 does not stimulate Rtt109 HAT activity. **(a)** The ability of Nap1 to form a complex with Rtt109 was tested by gel-shift analysis. 10 μM Nap1 or Vps75₁₋₂₂₃ was incubated with Rtt109 (lanes 1–5 or lanes 6–10: 0 μM, 1 μM, 2 μM, 3 μM and 4 μM, respectively) at 4 °C for 10 h, and complex formation was analyzed by 5% native PAGE. **(b,c)** Nap1 does not enhance Rtt109-dependent H3 acetylation. The HAT activity of Rtt109 in the presence of Vps75 (lanes 2–5) or Nap1 (lanes 6–9) was tested using western blotting **(b,c)**. 0.5 μM Rtt109 and 5 μM yeast (H3-H4)₂ tetramer were incubated in the presence of 1 mM acetyl-CoA and histone chaperone for 2 h **(b)**. The amount of histone chaperones (0–8 μM) is indicated. The gels were probed with an anti-H3K56ac antibody, and the normalized densitometer scans of three independent experiments (obtained by monitoring changes in intensity of western blot) are shown in **c**. Note different scale of *x* axis compared to Figure 3e. **(d)** The same samples as in **b** were analyzed on an acid urea gel to detect modifications on residues other than H3K56.

Table 1

Affinity measurements of protein-protein interactions

Interaction	Protein titrated	Protein labeled	K_d (M)
Vps75—H3-H4	Vps75	H3-H4	$2.5 \pm 0.3 \times 10^{-8}$
	H3-H4	Vps75	$2.7 \pm 0.1 \times 10^{-8}$
	Vps75 ₁₋₂₂₂	H3-H4	$1.3 \pm 0.1 \times 10^{-8}$
Vps75—H3	H3	Vps75	$1.2 \pm 0.1 \times 10^{-7}$
Vps75—H2A-H2B	Vps75	H2A-H2B	$4.5 \pm 0.3 \times 10^{-9}$
Vps75—Rtt109	Rtt109	Vps75	$>5 \times 10^{-6}$
Rtt109—H3-H4	Rtt109	H3-H4	$1.5 \pm 0.1 \times 10^{-7}$
Asf1—H3-H4	Asf1	H3-H4	$1.0 \pm 0.1 \times 10^{-8}$

All measurements were performed in 20 mM Tris-HCl, pH 7.5, 0.5 mM EDTA, 1 mM DTT, 0.1 mg ml⁻¹ BSA and 300 mM NaCl. Labeled proteins were kept at 0.2–0.4 nM or <<five-fold the K_d . Standard errors from curve fitting are listed. See Methods for details.

Table 2

Phenotypic comparison of yeast deletion strains

	BY4741	<i>Δnap1</i>	<i>Δvps75</i>
30 °C	+++	+++	+++
38 °C	+++	+++	–
UV (75 J)	+++	+++	+
HU (200 mM)	+++	+++	–
MMS (0.025%)	+++	+++	–
MTX (50 μg ml ^{−1})	+++	+++	+
NaCl (1 M)	+++	+++	++
Minus inositol	+++	+++	++
Sorbitol (1 M)	+++	+++	+++
H ₂ O ₂	+++	+++	+++

Ten-fold serial dilutions of strains were applied as spots to the media indicated (see also Fig. 4). +++, robust growth, comparable to a wild-type strain; + to ++, intermediate values; –, no growth. HU, hydroxyurea; MMS, methyl methanesulfonate; MTX, methotrexate. Unless specified in Methods, the plates contained YPD medium.

Table 3
Vps75 data collection, phasing and refinement statistics

Native		SeMet	Hg
Data collection			
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$
Cell dimensions a, b, c (Å)	80.7, 84.6, 86.2	80.6, 84.8, 86.2	80.5, 84.5, 86.3
		Peak	Peak
		Inflection	Inflection
		Remote	Remote
Wavelength	1.1	0.9793	0.9640
Resolution (Å)	50–1.85	50–2.0	50–2.0
R_{merge}^a	0.081 (0.421)	0.105 (0.433)	0.074 (0.414)
$I/\sigma I^a$	11.4 (4.1)	9.7 (3.2)	11.4 (4.1)
Complete (%) ^a	90 (93)	90 (93)	90 (93)
Redundancy ^a	8 (7.6)	8 (7.8)	8 (8)
Refinement			
Resolution (Å)	50–1.85		
No. reflections ^b	92,802		
$R_{\text{work}} / R_{\text{free}}$	0.23 / 0.25		
No. atoms			
Protein	3,727		
Water	266		
B -factors			
Protein	41.9		
Water	41.6		
r.m.s. deviations			
Bond lengths (Å)	0.006289		
Bond angles (°)	1.13951		

^aValues in parentheses are for the highest-resolution shell.

^bReflections of $|F_0| > 1.0$.