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TOM1p, A Yeast Hect-domain Protein which Mediates Transcriptional Regulation Through the ADA/SAGA Coactivator Complexes

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The hect-domain has been characterized as a conserved feature of a group of E3 ubiquitin ligases. Here we show that the yeast hect-domain protein TOM1p regulates transcriptional activation through effects on the ADA transcriptional coactivator proteins. Null mutations of *tom1* result in similar defects in transcription from *ADH2* and *HIS3* promoters, and enhanced transcription from the *GAL10* promoter as do null mutations in *ngg1/ada3*. Strains with disruptions of both *ngg1* and *tom1* have the same phenotype as strains with a disruption of only *ngg1* implying that these genes are acting through the same pathway. In the absence of TOM1p, the normal associations of the ADA proteins with SPT3p and the TATA-binding protein are reduced. The action of TOM1p is most likely mediated through ubiquitination since mutation of Cys3235 to Ala, corresponding residues of which are required for thioester bond formation with ubiquitin in other hect-domain proteins, results in similar changes in transcription as the null mutation. A direct role for TOM1p in regulation of ADA-associated proteins is further supported by the finding that SPT7p is ubiquitinated in a TOM1p-dependent fashion and that TOM1p coimmunoprecipitates with the ADA proteins.

Keywords: ADA complex; coactivator; TOM1p; transcription; ubiquitin ligase

Introduction

Regulated transcription by eukaryotic RNA polymerase II requires four classes of proteins: subunits of RNA polymerase II, basal transcription factors, sequence-specific activator proteins and a group of factors which collectively are known as coactivators (Orphanides *et al.*, 1996). Coactivators enhance the function of activator proteins either by providing a regulatory interface with the basal machinery and/or facilitating the activity of the transcription machinery on its chromatin template. As these are two key steps in transcriptional initiation, it is not surprising that as well as having a positive effect on gene induction, coactivators can also play significant roles in repression (for examples see Song *et al.*, 1996; Li *et al.*, 1996).

The ADA genes were identified in *Saccharomyces cerevisiae* based on their requirement for the regulated activation and repression of transcription (Guarente, 1995). Initial studies identified three genes *ADA2*, *NGG1/ADA3* (referred to as *NGG1* for simplicity) and *GCN5* which as single or double disruptions resulted in both decreased transcriptional activation by GCN4p and GAL4p-VP16 fusions (Berger *et al.*, 1992; Pina *et al.*, 1993; Marcus *et al.*, 1994; Georgakopoulos & Thireos, 1992) and increased transcriptional activation by GAL4p and PDR1p (Brandl *et al.*, 1993; 1996; Martens *et al.*, 1996). The phenotype of double disruptions as well as affinity chromatography and coimmunoprecipation suggested that these proteins act in a complex (Pina *et al.*, 1993; Marcus *et al.*, 1994; Georgakopoulos *et al.*, 1995; Horiuchi *et al.*, 1995; Brandl *et al.*, 1996; Candau & Berger, 1996; Saleh *et al.*, 1997). Of this group of proteins, GCN5p is a histone acetyltransferase, implying that at least one role of the ADA proteins is in regulating nucleosome function (Brownell *et al.*, 1996; Kuo *et al.*,

Abbreviations used: HA, hemagglutinin.

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1996; Wang *et al*., 1997; Candau *et al*., 1997; Grant *et al*., 1997; Pollard & Peterson, 1997).

The regulatory functions of the ADA proteins arise as the result of their presence in multiple high molecular mass complexes containing additional components. Analysis of ADA2p and NGG1p in native yeast extracts revealed that these proteins are associated in four complexes, two with approximate sizes of greater than 2 MDa, and others of 900 kDa and 200 kDa (Saleh *et al*., 1997). The finding that a comparable 1.8 MDa ADA complex contains SPT3p, SPT7p, and SPT20p/ADA5p defines at least in part, the composition of one of these complexes, the SAGA-complex (Grant *et al*., 1997). The absence of the SPT proteins in some of the other ADA containing complexes along with the overlapping but non-identical phenotypes of the different null alleles suggests that the SPT and ADA proteins also act independently (Marcus *et al*., 1996; Roberts & Winston, 1996, 1997; Grant *et al*., 1997). The same is likely true for ADA1p which associates with ADA2p, GCN5p and NGG1p but whose disruption results in broader effects than those seen for the other *ADA* genes (Horiuchi *et al*., 1997).

The connection between the *ADA* and *SPT* genes is particularly intriguing because the class of *SPT* genes including *SPT3*, *SPT7*, *SPT8* and *SPT20* have been functionally linked to the TATA-binding protein (TBP; SPT15p; Winston *et al*., 1984, 1987; Eisenmann *et al*., 1989, 1994; Gansheroff *et al*., 1995). In fact, SPT3p interacts directly with TBP (Eisenmann *et al*., 1989, 1992). The link between the TBP class of SPT proteins and *ADA* gene products suggests that the ADA proteins likely affect the basal transcriptional machinery. A role in modulating the activity of TBP had also been predicted based on the ability of the ADA proteins to interact with TBP (Barlev *et al*., 1995; Saleh *et al*., 1997; Roberts & Winston, 1997).

To identify factors that associate with or perhaps regulate the ADA proteins, we previously performed a yeast two-hybrid analysis using the amino-terminal 373 amino acids of NGG1p (Martens *et al*., 1996). This screen identified four genes, including the ubiquitin protease UBP3p which functions to remove ubiquitin moieties from substrate proteins (Baker *et al*., 1992). The interaction between UBP3p and NGG1p suggested that UBP3p may be part of an ADA complex or that a ubiquitinated component of an ADA complex, either NGG1p or a factor(s) associated with NGG1p is ubiquitinated and recognized by UBP3p as a substrate.

Ubiquitination is a key element in the regulation of transcription as it is for other cellular processes (reviewed by Jentsch, 1992; Hochstrasser, 1995, 1996; Wilkinson, 1995). In some instances this regulation is through the direct signalling for rapid degradation by the 26 S proteasome of a modified activator protein (eg GCN4p; Kornitzer *et al*., 1994; see reviews by Ciechanover, 1994; Jentsch & Schlenker, 1995). In other cases ubiquitination may signal events other than direct protein degradation (Hochstrasser, 1996).

Ubiquitin is joined to lysine residues of acceptor proteins *via* a thioester cascade catalyzed by three classes of enzymes: E1 ubiquitin-activating enzymes, E2 ubiquitin-conjugating enzymes and E3 ubiquitin ligases (Chen & Pickart, 1990; Haas *et al*., 1991; van Nocker & Vierstra, 1991; Banerjee *et al*., 1993; Scheffner *et al*., 1995). The E3 proteins play a major role in determining the substrate specificity of the system and are involved in the final transfer of ubiquitin to the target protein.

Characterization of the mechanism by which E6 proteins of papilloma viruses promote degradation of p53 led to the identification of the E3 ubiquitin ligase, E6-AP (Scheffner *et al*., 1993). There are several eukaryotic proteins, including five yeast open reading frames, that show similarity to E6-AP over its carboxyl-terminal 350 amino acids (Huibregtse *et al*., 1995). One of the yeast hect-domain (homologous to E6-AP carboxyl-terminus) proteins, RSP5p (NPI1p; Hein *et al*., 1995; MDP1p; Zolladek *et al*., 1997), was identified by the Winston laboratory as a suppressor of mutations in *SPT3* (cited by Huibregtse *et al*., 1995) and has been found necessary for full transcriptional activation by human steroid receptors in yeast (Imhof & McDonell, 1996). Two of the other yeast hect-domain proteins have been characterized. UFD4p is involved in the ubiquitin fusion degradation pathway (Johnson *et al*., 1995) while TOM1p (Trigger Of Mitosis) was identified based on its involvement in the G2/M transition (Utsugi *et al*., 1995). Interestingly, UreB1, a rat hect-domain protein of 310 amino acids, may have a role in transcriptional regulation through direct DNA binding (Gu *et al*., 1994).

Here we show that the hect-domain protein TOM1p has a role in transcriptional regulation. The effects of TOM1p are likely mediated through the ADA complexes since single and double mutations in *tom1* and *ngg1* result in similar changes in transcription from *ADH2, HIS3* and *GAL10* promoters. Ubiquitination appears essential for the regulation by TOM1p since a cysteine residue conserved among hect-domain proteins and found necessary for thioester bond formation with ubiquitin (Scheffner *et al*., 1995; Huibregtse *et al*., 1995), is required for transcriptional regulation by TOM1p. Furthermore, TOM1p associates with the ADA proteins and is required for the ubiquitination of a 210 kDa protein, likely SPT7p, that co-immunoprecipitates with the ADA components. A mechanism for TOM1p action is suggested by the finding that in the absence of TOM1p the ability of the ADA proteins to interact with SPT3p and TBP is reduced.

Results

TOM1p has a role in transcriptional activation

The interaction of UBP3p with NGG1p in a two-hybrid analysis (Martens *et al*., 1996) suggested

Table 1. Yeast strains used in this study

Strain	Genotype	Reference
CY922	*MATa ade2-101 gal4-Δ gal80-Δ his3-Δ200 leu2-3,2 trp1-Δ901ura3-52 LYS2::GAL1-HIS3*	Brandl *et al.* (1996)
FY1093	*MATa spt7 Δ402::LEU2 his917δlys2-173R2 leu2Δ1 ura3-52 trp1 Δ63 ade8*	Gansheroff *et al.* (1995)
JY335	Isogenic to CY922 except *ngg1-1*	Martens *et al.* (1996)
CY1004	Isogenic to JY335 except *tom1::URA3*	This study
CY1005	Isogenic to CY922 except *tom1Cys3235-Ala*	This study
SY11	Isogenic to CY922 except *tom1Δ244-2952*	This study
SY12	Isogenic to SY11 except *HA-NGG1*	This study
SY13	Isogenic to CY922 except *HA-NGG1*	This study
SY14	Isogenic to CY922 except *HA-ngg1 Δ273-307*	This study

that NGG1p itself, or a protein associated with NGG1p, as part of an ADA complex, may be ubiquitinated. In turn, this predicted that an E3 ubiquitin ligase may be involved in regulation of the ADA complexes. With the availability of the yeast genome sequence, we could analyze yeast E3 ubiquitin ligases for a role in regulation of the ADA proteins. TOM1p (SachDB ORF: YDR457W) was a strong candidate since it was suggested to be involved in nuclear processes, contains a potential nuclear localization signal from amino acids 199 to 210 (Dingwell & Laskey, 1991), and like the ADA proteins, is not essential for viability (Utsugi *et al.*, 1995). *TOM1* encodes a 3268 amino acid protein (374 kDa) and although not previously characterized as an E3 ubiquitin ligase, does have a high degree of sequence similarity to the hect-domain E3 ubiquitin ligases (Huibregtse *et al.*, 1995).

To determine if TOM1p has a role in transcriptional activation related to the ADA proteins, we compared transcription from promoters activated by ADR1p (*ADH2*), GCN4p (*HIS3*) and GAL4p (*GAL10*) in the wild-type strain CY922, an isogenic *tom1* disruption strain, SY11, and in an isogenic strain JY335 which carries a non-functional allele of *ngg1* (see Table 1 for yeast strains). *ADH2*, *HIS3* and *GAL10* promoters were introduced into these strains as *lacZ* fusions. The strains were also transformed with plasmids expressing the appropriate activator protein or grown under relevant inducing conditions to achieve expression of the activator (see Materials and Methods). Disruption of *ngg1* resulted in a decrease in induction of the *ADH2* promoter (Figure 1A) and *HIS3* promoter (Figure 1B) of approximately 12-fold and twofold, respectively. Similarly, disruption of *tom1* in SY11 (*NGG1 tom1*) resulted in a decrease in transcription of *ADH2* and *HIS3* of 13-fold and twofold. As shown previously transcription from the *GAL10* promoter in glucose media is enhanced threefold in a *ngg1* background (Brandl *et al.*, 1993; Figure 1C). Again, disruption of *tom1* resulted in a comparable induction of the *GAL10* promoter. Western blots for GAL4p indicated that increased expression from the *GAL10* promoter was not due to an increase of GAL4p in SY11 as compared to

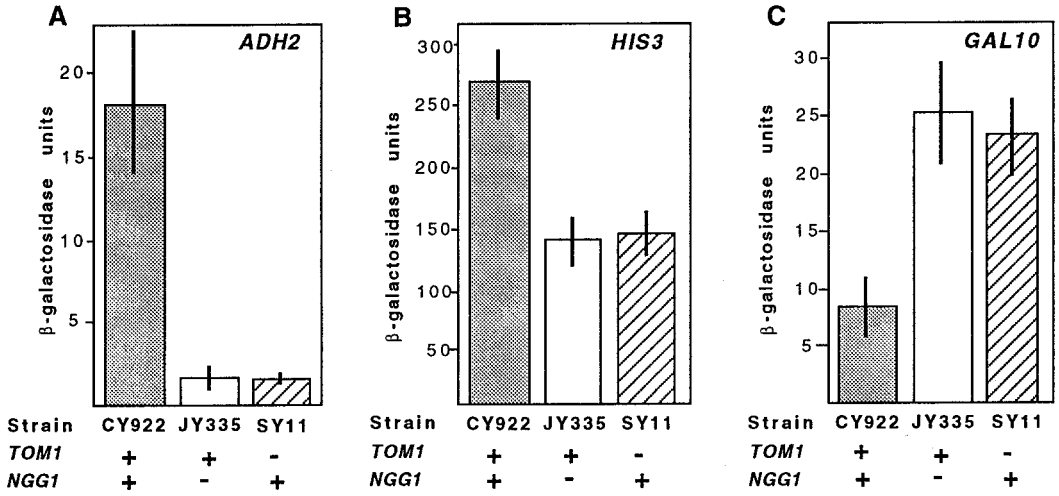

Figure 1. Transcription from *ADH2*, *HIS3* and *GAL10* promoters in *wild-type* (CY922), *tom1* (SY11) and *ngg1* (JY335) yeast strains. A, Transcription from the *ADH2* promoter. The indicated strains containing the *ADH2* promoter as a *lacZ* fusion were grown in 3% ethanol 0.05% glucose. β-galactosidase activity was assayed as described by Ausubel *et al.* (1990) and standardized to cell density. B, Transcription from the *HIS3* promoter. β-Galactosidase activity for strains containing a *HIS3-lacZ* fusion and GCN4p expressed from the *DED1* promoter were determined after disruption of cells with glass beads and standardized to total protein. C, Transcription from the *GAL10* promoter. Strains were transformed with a *GAL10-lacZ* reporter plasmid and a plasmid expressing GAL4p. Cells were grown in 2% glucose and β-galactosidase activity determined as for the *ADH2* promoter. Measurements are the averages of eight trials.

the wild-type strain CY922 (not shown). These experiments show that disruptions of *tom1* and *ngg1* have very similar effects on transcriptional activation and thus suggest that there is a link between TOM1p and the ADA proteins. Further parallels between *TOM1* and *NGG1* were suggested by the similar slow growth phenotype and temperature sensitivity of strains with disruptions of *tom1* and *ngg1* (not shown).

To analyze whether NGG1p and TOM1p act through the same pathway, transcription from *ADH2* and *GAL10* promoters was determined in CY1004, a strain carrying a double disruption of *tom1* and *ngg1* (Table 2). Transcription from the *ADH2* and *GAL10* promoters in the double disruption background was virtually identical to that in each single disruption background. This result suggests that TOM1p is regulating transcriptional activation through the same pathway as the ADA proteins.

An active site cysteine is required for TOM1p's function as a transcriptional regulator

TOM1p contains a hect-domain at its carboxyl terminus related in sequence to those found in E6-AP and RSP5p. The hect-domain is characterized by having a conserved Cys residue that is essential for the formation of a thioester linkage with ubiquitin (Scheffner *et al.*, 1995; Huibregtse *et al.*, 1995). To determine if the role of TOM1p in transcription is dependent on this conserved cysteine residue, we constructed yeast strain CY1005, carrying a derivative of TOM1p with a Cys to Ala substitution at amino acid 3235. $TOM1p_{Cys3235\text{-}Ala}$ was expressed at a level virtually equivalent to the wild-type protein as determined by Western blotting (not shown). Transcription from the *ADH2* promoter was determined in CY1005 and similar to that in the *tom1* deletion strain SY11, was ninefold less than in the wild-type strain CY922 (Figure 2A). Transcription from the *GAL10* promoter was also compared in CY1005, SY11 and CY922 (Figure 2B). In this trial, deletion of *tom1* resulted in a fivefold increase in expression of *GAL10* as compared to the wild-type. The presence of $TOM1p_{Cys3235\text{-}Ala}$ resulted in slightly less of an increase, approximately 3.5-fold. These results suggest that the effect of TOM1p on transcription is principally mediated through its hect-domain.

Table 2. Transcription of *ADH2* and *GAL10* promoters in *tom1* and *ngg1* yeast strains

Promoter	Strain	β-Galactosidase units CY922	JY335	SY11	CY1004
	TOM1	+	+	−	−
	NGG1	+	−	+	−
ADH2		18	1.5	1.4	1.3
GAL10		8	25	23	27

Transcription from *ADH2* and *GAL10* promoters in wild-type, *tom1, ngg1 and tom1 ngg1* yeast strains. The indicated strains containing the *ADH2* or *GAL10* promoters as *lacZ* fusions were assayed for activation by their appropriate transactivator protein (ADR1p and GAL4p, respectively). β-Galactosidase activity was measured as described by Ausubel *et al.* (1990) and standardized to cell density. Measurements are the averages of eight trials.

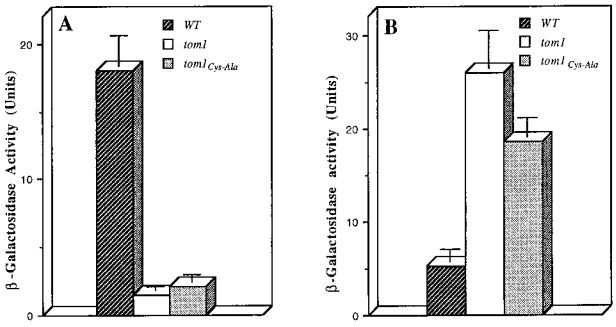

Figure 2. Transcription from the *ADH2* and *GAL10* promoters in the presence of $TOM1p_{Cys3235\text{-}Ala}$. A, Transcriptional activation by ADR1p of a *ADH2-lacZ* fusion promoter in wild-type (CY922), *tom1* (SY11) and $tom1_{Cys\text{-}Ala}$ (CY1005) strains. β-Galactosidase activity was measured as in Figure 1. Measurements are the averages of assays performed on five individual transformants. Standard errors were less than 20% of the means. B, Transcriptional activation by GAL4p of a *GAL10-lacZ* fusion in CY922, SY11, and CY1005.

The two-hybrid interaction of UBP3p and NGG1p requires *TOM1*

In a two-hybrid analysis, the carboxyl-terminal 180 amino acids of the yeast ubiquitin-specific protease, UBP3p, associates with amino acids 1 to 373 of NGG1p (Martens *et al.*, 1996). We believe that this interaction is not directly between $GAL4_{DBD}$-$NGG1p_{1\text{-}373}$ and UBP3p, but rather mediated by additional factors that assemble with $GAL4_{DBD}$-$NGG1p_{1\text{-}373}$ because the endogenous copy of ADA2p is required to observe the interaction (A.S. & C.J.B., unpublished result). One model would then suggest that UBP3p may recognize a ubiquitinated protein in an ADA complex as a substrate. If this component is ubiquitinated in a TOM1p-dependent manner, then the UBP3p-NGG1p two-hybrid interaction may be absent in a *tom1* deletion strain.

In the wild-type background (CY922) the two-hybrid interaction between $NGG1p_{1\text{-}373}$ and $UBP3p_{733\text{-}912}$ is observed as a sevenfold increase in expression of the *GAL10-lacZ* reporter when $GAL4_{DBD}$-$NGG1p_{1\text{-}373}$ is coexpressed with $GAL4_{AD}$-$UBP3p_{733\text{-}912}$ as compared to coexpression with $GAL4_{AD}$-SNF4p (Figure 3). This two-hybrid interaction was absent in the *tom1* disruption strain (SY11) and in the strain containing $TOM1p_{Cys3235\text{-}Ala}$ (SY1005). Absence of the interaction did not result from reduced expression of $GAL4_{DBD}$-$NGG1p_{1\text{-}373}$ in the *tom1* strains, as demonstrated by Western blotting (not shown). As a control for potential non-specific effects of the

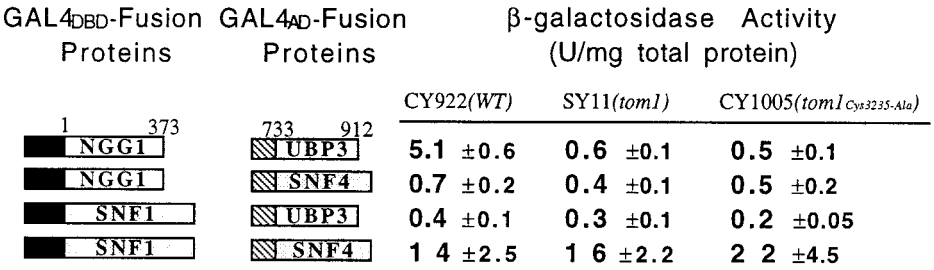

Figure 3. Loss of interaction between NGG1p and UBP3p in the absence of TOM1p. Plasmids encoding the indicated pairs of GAL4p DNA binding domain ($GAL4_{DBD}$; black box) and GAL4p activation domain ($GAL4_{AD}$; striped box) fusion proteins were introduced into CY922 (wild-type), SY11 (*tom1*) and CY1005 ($tom1_{Cys3235\text{-}Ala}$). Cells were grown in minimal media containing 2% glucose and assayed for expression of a *GAL10-lacZ* reporter introduced on a *ADE2* centromeric plasmid. β-Galactosidase assays were performed after glass bead disruption of cells and standardized to protein concentration. Each value represents the average from five independent transformants.

tom1 disruption, increased expression of *GAL10-lacZ* resulting from the interaction between $GAL4_{DBD}$-SNF1p and $GAL4_{AD}$-SNF4p, was found to be similar in wild-type and *tom1* strains. These results demonstrate that the two-hybrid association of UBP3p with NGG1p requires TOM1p and in turn suggests that there may be a substrate for TOM1p in one or more of the ADA complexes.

TOM1p is required for ubiquitination of a protein that immunoprecipitates with HA-NGG1p

To determine if a component of an ADA complex is ubiquitinated in a TOM1p-dependent manner, ubiquitination of ADA components was examined in wild-type and *tom1* backgrounds by Western blotting with anti-ubiquitin antibody. Whole cell extracts were prepared from yeast strains CY922 (*NGG1 TOM1*), SY12 (*HA-NGG1 tom1*), and SY13 (*HA-NGG1 TOM1*). HA-NGG1p and the associated ADA components were immunoprecipitated with anti-HA antibody, separated by SDS-PAGE, and Western-blotted with anti-ubiquitin antibody (Figure 4A). A major band at approximately 210 kDa was detected by the anti-ubiquitin antibody in the SY13 immunoprecipitate (lane 3). This band was specifically immunoprecipitated with the ADA proteins as it was absent from CY922 which lacks HA-NGG1p (lane 1). Furthermore the band was absent from the *tom1* deletion strain SY12 (lane 2), thus indicating that its ubiquitination is dependent on TOM1p and that this function cannot be replaced by other potential ubiquitin ligases. To establish that the absence of the ubiquitinated proteins from SY12 was not due to variations in the amount of immunoprecipitated HA-NGG1p, the same filter was reprobed with anti-HA antibody (Figure 4B). The intensity of the 116 kDa band, corresponding to HA-NGG1p, in immunoprecipitates from SY13 and SY12 was almost identical. Thus an ADA-associated protein is ubiquitinated in a TOM1p-dependent fashion. This agrees with the prediction based on sequence

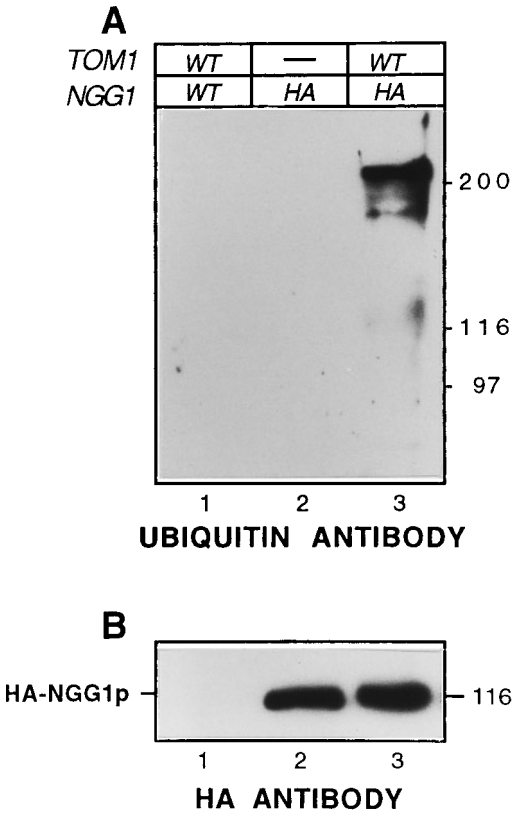

Figure 4. Ubiquitination of an ADA-associated protein requires TOM1p. A, 70 mg of whole cell extract prepared from yeast strains CY922 (*TOM1 NGG1*; lane 1), SY12 (*tom1 HA-NGG1*; lane 2), and SY13 (*TOM1 HA-NGG1*; lane 3) were incubated with anti-HA antibody and Protein A Sepharose. After washing, the immunoprecipitated ADA complex was eluted at 65°C in SDS loading buffer, separated on a 5.5% SDS-polyacrylamide gel, and analyzed by Western blotting with anti-ubiquitin antibody. The principal ubiquitinated band within the ADA complex migrates with an approximate mass of 210 kDa (lane 3) in comparison to protein standards shown on the right. B, The same membrane was reprobed with anti-HA antibody to ensure that equivalent amounts of HA-NGG1p were immunoprecipitated from SY12 and SY13 extracts (lanes 2 and 3, respectively).

comparisons that TOM1p is an E3 ubiquitin ligase. In addition, this result suggests that the transcriptional effects of TOM1p, which closely parallel those of the ADA proteins, could be mediated through its ubiquitination of a component(s) of an ADA complex(es).

SPT7p is ubiquitinated in a TOM1p-dependent fashion

SPT7p which migrates with an estimated mass of approximately 205 kDa on SDS-PAGE (Gansheroff *et al.*, 1995) and associates with at least one form of ADA complex (SAGA-complex; Grant *et al.*, 1997), was an obvious candidate for the ubiquitinated protein. To determine if SPT7p is ubiquitinated in a TOM1p-dependent fashion, SPT7p was isolated by immunoprecipitation after whole cell extracts from wild-type and *tom1* deletion strains were boiled in 1.5% SDS to dissociate protein complexes. The immunoprecipitates were fractionated by SDS-PAGE and Western blotted with anti-ubiquitin and anti-SPT7p antibody. As shown in Figure 5, SPT7p was immunoprecipitated from both *TOM1* (CY922) and *tom1* (SY11) strains. The identity of this band as SPT7p was verified by its absence in the immunoprecipitate from the *spt7 Δ402::LEU2* strain, FY1093 (generously provided by Fred Winston; lane 3). SPT7p was ubiquitinated in the wild-type *TOM1* background but not in the *tom1* deletion background. Reprobing the blot with a polyclonal antibody to NGG1p revealed that no NGG1p was present in the immunoprecipitates, thus helping exclude the possibility that other proteins may associate with SPT7p under the denaturing conditions (not shown).

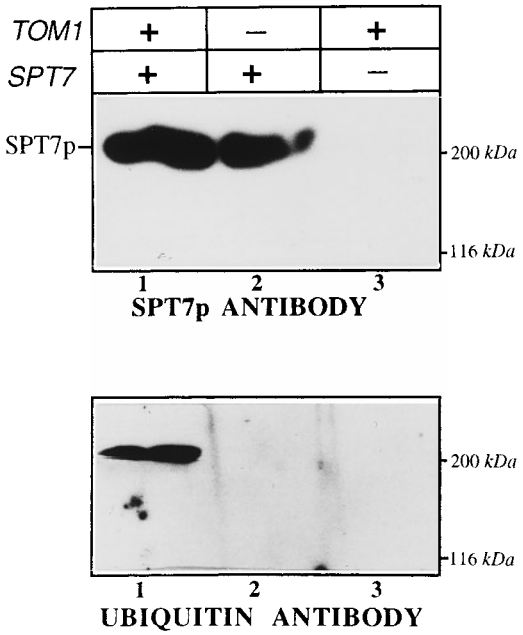

Figure 5. SPT7p is ubiquitinated in a TOM1p-dependent fashion. 10 mg of whole cell extract from yeast strains CY922 (wild-type; lane 1), SY11 (*tom1*; lane 2) and FY1093 (*spt7*; lane 3) was boiled in extraction buffer containing 1.5% SDS and diluted 15-fold in RIPA buffer minus SDS. The samples were immunoprecipitated with anti-SPT7p antibody, separated by SDS-PAGE in a 5.5% gel and Western blotted with anti-SPT7p and anti-ubiquitin antibody.

Coimmunoprecipitation of TOM1p with NGG1p

If the action of TOM1p is direct, that is an ADA-associated protein is a substrate for TOM1p, it might be possible to detect TOM1p in immunoprecipitates of the ADA complexes. ADA-associated proteins were immunoprecititated with anti-HA antibody from 75 mg of whole cell extracts prepared from SY13 (*HA-NGG1 TOM1*), CY922 (*NGG1 TOM1*), and SY12 (*HA-NGG1 tom1*). Immunoprecipitates were probed with antibody directed against a 15 amino acid peptide found within TOM1p. As shown in Figure 6A, TOM1p specifically immunoprecipitated with HA-NGG1p (compare lanes 1 and 2). The identity of the high molecular weight protein as TOM1p was confirmed by the absence of this band in the immunoprecipitate from SY12 (*HA-NGG1 tom1*; lane 3). To ensure that equal amounts of HA-NGG1p were

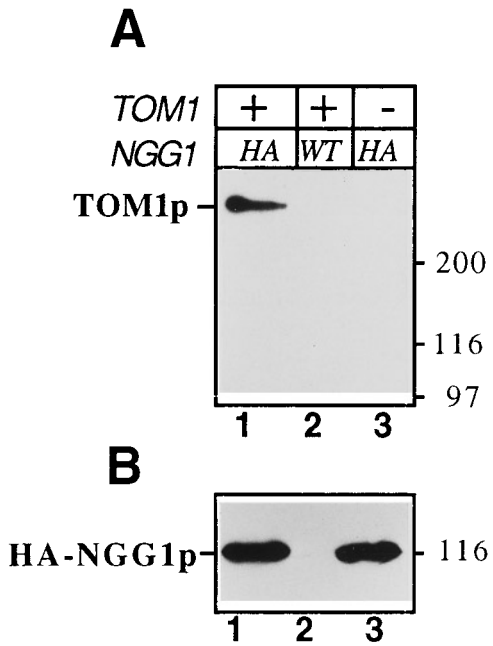

Figure 6. Coimmunoprecipitation of TOM1p with NGG1p from yeast whole cell extracts. A, 75 mg of whole cell extract prepared from yeast strain SY13 (*TOM1 HA-NGG1*; lane 1), CY922 (*TOM1 NGG1*; lane 2) and SY12 (*tom1 HA-NGG1*; lane 3) were incubated with anti-HA antibody and Protein A Sepharose. Protein was eluted at 65°C in SDS loading buffer, separated by SDS-PAGE, and analyzed by Western blotting with affinity purified polyclonal antibody to a 15 amino acids peptide within the carboxyl terminus of TOM1p. The position of TOM1p (lane 1) is labeled. Relevant molecular mass protein standards (kDa) are indicated on the right. B, The same membrane was reprobed with anti-HA antibody to detect immunoprecipitated HA-NGG1p.

immunoprecipitated from extracts of the different strains, the filter was reprobed with anti-HA antibody to detect HA-NGG1p (Figure 6B; compare lanes 1 and 3). These results support the idea that TOM1p acts directly to ubiquitinate an ADA-associated protein. The amount of extract required to visualize the interaction between TOM1p and HA-NGG1p was substantially more than that required to see a core ADA component such as ADA2p. This suggests that TOM1p is not likely an integral component of the ADA complexes but rather is involved in transient associations with the ADA proteins or is restricted to a subset of the complexes.

To confirm the interaction between TOM1p and NGG1p, the reciprocal experiment was performed in which TOM1p was immunoprecipitated from whole cell extracts and the presence of HA-NGG1p assayed by Western blotting (Figure 7A). HA-NGG1p$_{\Delta 274\text{-}307}$, a stably expressed deletion derivative of NGG1p which associates with a subset of the ADA and SPT proteins (Brandl *et al*., 1996; Saleh *et al*., 1997), was also examined to determine if the essential 274 to 307 region of NGG1p is required for interaction with TOM1p. HA-reactive bands of 116 kDa and 110 kDa, corresponding to HA-NGG1p and HA-NGG1p$_{\Delta 274\text{-}307}$, were found in immunoprecipitates from the wild-type*TOM1* strains SY13 and SY14, respectively, but not in immunoprecipitates from the *tom1* deletion strain, SY12 (compare lanes 1 and 4 with lane 2). The identity of HA-NGG1p and HA-NGG1p$_{\Delta 274\text{-}307}$, were confirmed by their absence in the immunoprecipitate from CY922 (*NGG1 TOM1*; lane 3). As shown in Figure 7B, approximately equal amounts of TOM1p were immunoprecipitated from the *TOM1*-containing strains. As well as confirming the interaction of TOM1p and NGG1p, this experiment indicates that the central region of NGG1p from amino acids 274 to 307 is not required for interaction with TOM1p.

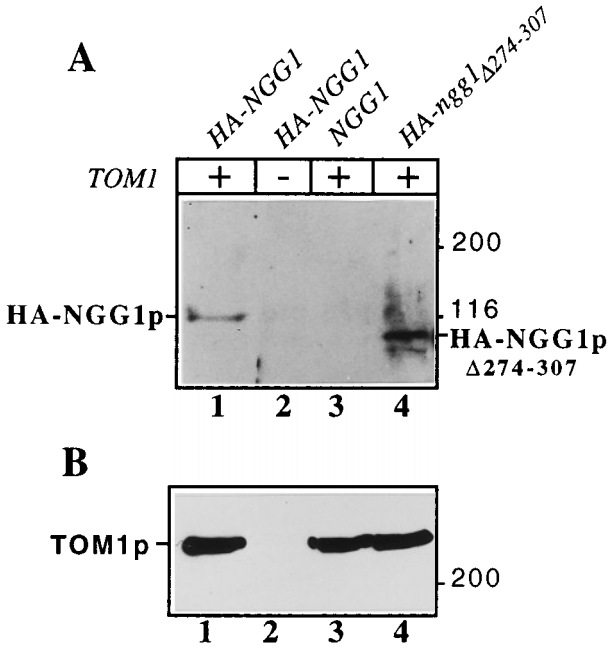

Figure 7. Coimmunoprecipitation of NGG1p with TOM1p from yeast whole cell extracts. A, 75 mg of whole cell extract prepared from yeast strains SY13 (*TOM1 HA-NGG1*; lane 1), SY12 (*tom1 HA-NGG1*; lane 2), CY922 (*TOM1 NGG1*; lane 3) and SY14 (*TOM1 HA-NGG1*$_{\Delta 274\text{-}307}$; lane 4) were incubated with polyclonal antibody directed to the carboxyl terminus of TOM1p followed by incubation with Protein A Sepharose. Bound protein was eluted at 65°C in SDS loading buffer, separated on a 5.5% SDS-polyacrylamide gel, and analyzed by Western blotting with anti-HA antibody as a probe for HA-tagged derivatives of NGG1p. The indicated bands at approximately 116 and 110 kDa represent HA-NGG1p (lane 1) and HA-NGG1p$_{\Delta 274\text{-}307}$ (lane 4), respectively. The migration of molecular mass protein standards are indicated on the right. B, The same membrane was reprobed with the anti-TOM1p antibody.

TOM1p is required for the association of the ADA components with SPT3p and TBP but not with SPT7p

One mechanism by which TOM1p could influence the activity of the ADA proteins would be to alter the protein-protein associations within the complexes. This could occur by targeting components of the complex for degradation or by signaling conformational changes. As the ADA proteins associate with the TBP class of SPT proteins and SPT7p is ubiquitinated in a TOM1p-dependent fashion, we chose to analyze if TOM1p alters the protein-protein interactions between the ADA proteins and SPT7p, SPT3p or SPT15p (TBP). We immunoprecipitated HA-NGG1p from cell extracts of SY13 (*HA-NGG1 TOM1*), SY12 (*HA-NGG1 tom1*) and as a negative control CY922 (*NGG1 TOM1*). As shown in Figure 8 the immunoprecipitation of HA-NGG1p was equally efficient from SY12 and SY13. Likewise, the amount of SPT7p associated with HA-NGG1p was virtually identical in both strains. This suggests that ubiquitination of SPT7p is not required for its association with the ADA components. In contrast, the amount of both SPT3p and TBP associated with HA-NGG1p was reduced to undetectable levels in the *tom1* deletion background. This was not due to a general reduction of SPT3p and TBP in SY12 as determined by Western blotting of the crude extracts (not shown). These results suggest that TOM1p may exert its effects on transcription by altering the associations of the ADA/SAGA complexes with TBP.

Histone acetyltransferase activity of the ADA complexes is unchanged in the absence of TOM1p

The ADA complexes have histone acetyltransferase activity mediated through ADA4p/GCN5p. To determine whether TOM1p may additionally affect

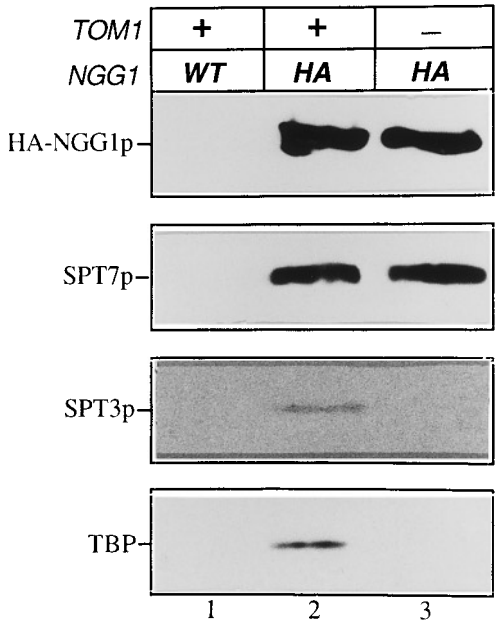

Figure 8. Association of the TBP class of SPT gene products in the presence and absence of TOM1p. 15 mg of whole cell extract prepared from yeast strains CY922 (*TOM1 NGG1*; lane 1), SY13 (*TOM1 HA-NGG1*; lane 2) and SY12 (*tom1 HA-NGG1*; lane 3) were incubated with anti-HA antibody and Protein A Sepharose. After washing, protein was eluted by heating at 65°C in SDS loading buffer, separated in a 6% gel and analyzed by Western blotting with anti-HA antibody to detect HA-NGG1p. The same filter was reprobed with anti-SPT7p antibody. The indicated bands at approximately 116 and 210 kDa represent HA-NGG1p and SPT7p, respectively. Similarly, 30 mg of whole cell extract was incubated with anti-HA antibody covalently coupled to Sepharose 4B. Bound protein was separated in a 12.5% gel and analyzed by Western blotting with a polyclonal antibody to TBP. The same filter was probed with a polyclonal antibody to SPT3p. The immunoreactive bands at approximately 35 and 27 kDa represent SPT3p and TBP, respectively.

transcription by altering the histone acetyltransferase activity of the ADA complexes, we immunoprecipitated HA-NGG1p and associated proteins from the yeast strains CY922 (*NGG1 TOM1*), SY12 (*HA-NGG1 tom1*), SY13 (*HA-NGG1 TOM1*) and SY14 (*HA-ngg1*$_{\Delta 274\text{-}307}$ *TOM1*) with anti-HA antibody. These complexes were assayed for histone acetyltransferase activity using oligonucleosomes as substrates (Figure 9). ADA-associated histone acetyltransferase activity can be identified by its preference for histone H3 and to a lesser extent histone H2B (Wang *et al.*, 1997; Grant *et al.*, 1997; Ruiz-Garcia *et al.*, 1997). No ADA-associated histone acetyltransferase activity was apparent in the control immunoprecipitates from CY922 extracts (lanes 1 and 2). The amount of ADA-associated histone H3 acetyltransferase activity was similar in immunoprecipitates from the *tom1* deletion strain SY12 and the wild-type *TOM1* strain SY13 (compare lane 3 with lane 5 and lane 4 with lane 6). Histone H3 acetylation was calculated for *tom1* and *TOM1* immunoprecipitates by densitometric scanning of five independent experiments performed with 2 and 5 mg of protein. When standardized to the concentration of HA-NGG1p in the extracts, the H3 acetyltransferase activity in immunoprecipitates from the *tom1* strain was found to be 90% of that in the wild-type *TOM1* strain. By comparison, the histone acetyltransferase activity in immunoprecipitates from SY14 (*HA-ngg1*$_{\Delta 274\text{-}307}$) was 10% that found in the wild-type (compare lanes 7 and 8 with lanes 5 and 6, respectively). This experiment indicates that disruption of *tom1* has little influence on the histone acetyltransferase activity of the ADA complexes, and contrasts with a dramatic loss in acetyltransferase activity seen in strains containing the *ngg1*$_{\Delta 274\text{-}307}$ allele.

Discussion

TOM1p regulates the function of the ADA complexes through ubiquitination

Here we show that the hect-domain protein TOM1p is required for the function of the ADA regulatory proteins. A *tom1* deletion strain has the same slow-growth phenotype, temperature sensitivity, and defects in transcription as a *ngg1* deletion strain. Enhanced transcription of promoters activated by ADR1p (*ADH2*) and GCN4p (*HIS3*), both of which require ADA proteins for full activity (Georgakopoulos & Thireos, 1992; Pina *et al.*, 1993; Marcus *et al.*, 1994; Chiang *et al.*, 1996), also require TOM1p. A *tom1* deletion displays a similar relief of transcriptional repression on the *GAL10* promoter as a *ngg1* deletion. In addition, the slow growth and altered transcriptional phenotypes are no more severe in *ngg1 tom1* double mutants than in either single mutant.

Regulation of the ADA complexes by TOM1p is likely direct and dependent upon the predicted E3 ubiquitin ligase function of this protein. First, TOM1p can be immunoprecipitated with HA-NGG1p. Second, TOM1p is required for the ubiquitination of a protein that associates with the ADA proteins, SPT7p. Third, mutation of a cysteine residue within the hect-domain of TOM1p, corresponding to a residue essential for ubiquitination by E6-AP and RSP5p (Scheffner *et al.*, 1995; Huibregtse *et al.*, 1995), results in a strain with changes in transcription from *ADH2* and *GAL10* promoters which are similar to strains with the *tom1* null mutation. This suggests that the role of TOM1p in transcription is mediated through its ubiquitination function and contrasts with the observation from RSP5p in which the active site cysteine was not required to potentiate transcriptional activation

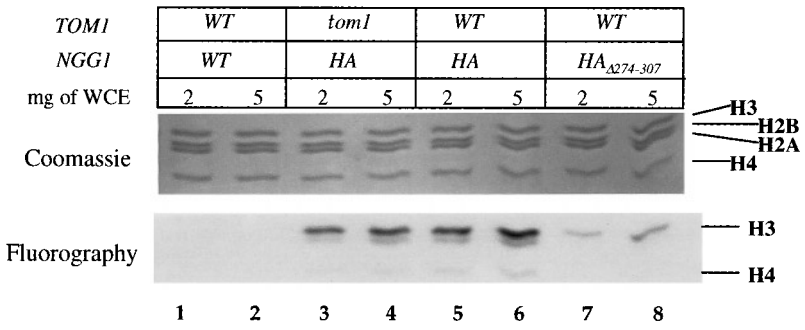

Figure 9. Histone acetyltransferase activity of ADA complexes in the presence and absence of TOM1p. HA-NGG1p-containing complexes were immunoprecipitated from 2 mg (lanes 1, 3, 5 and 7) or 5 mg (lanes 2, 4, 6, 8) of whole cell extract prepared from yeast strains CY922 (*TOM1 NGG1*; lanes 1 and 2), SY12 (*tom1 HA-NGG1*; lanes 3 and 4), SY13 (*TOM1 HA-NGG1*; lanes 5 and 6) and SY14 (*TOM1 HA-NGG1p*$_{\Delta 274-307}$; lanes 7 and 8). Liquid histone acetyltransferase assays were performed using HeLa H1-depleted oligonucleosomes (Cote' *et al.*, 1995) and [^{3}H]acetyl-CoA. Products of the reaction were separated by electrophoresis on a 18% SDS-polyacrylamide gel. The gels were stained with Coomassie brilliant blue to ensure equivalent loading of histone in each lane, destained and subjected to fluorography. The positions to which histones migrate are indicated.

by human steroid receptors in yeast (Imhof & McDonell, 1996).

Ubiquitinated component(s) of the ADA complexes

The finding that the two-hybrid interaction between the carboxyl terminus of the ubiquitin protease UBP3p and NGG1p requires TOM1p suggested that TOM1p is required to ubiquitinate a component(s) of the ADA complexes. We found a protein of 210 kDa that immunoprecipitates with HA-NGG1p, to be ubiquitinated specifically in the presence of TOM1p. Proteins of the TBP-class of SPTs, including SPT7p, SPT3p and ADA5p/SPT20 associate with the ADA proteins in at least one high molecular mass complex called the SAGA-complex (Grant *et al.*, 1997; Roberts & Winston, 1997). Based on its estimated size (Gansheroff *et al.*, 1995; Roberts & Winston, 1997), SPT7p was an obvious candidate to be the ubiquitinated protein. As an initial step to determine if SPT7p is ubiquitinated, we found that SPT7p immunoprecipitated from a denatured yeast extract is ubiquitinated in a TOM1p-dependent fashion. At this time our data strongly support the view that SPT7p is the 210 kDa ubiquitinated protein; we do note the possibility, that ubiquitinated SPT7p is not associated with the complex and a second ADA-associated protein of 210 kDa is ubiquitinated in a TOM1p-dependent fashion.

The coimmunoprecipitation of TOM1p and NGG1p indicates that TOM1p associates with the ADA proteins; however, the amount of input protein required to visualize the association of HA-NGG1p and TOM1p was more consistent with a transient association of the proteins or association in a small subset of complexes rather than with TOM1p being an integral member of one or more of the principal ADA complexes. This agrees with our finding that the fractionation profile on both Mono Q and Superose 6, for ADA complexes prepared from *tom1* deletion and wild-type strains are virtually unchanged (not shown).

What is the role of ubiquitination of ADA component(s)?

Several lines of evidence suggest that the regulation of the ADA/SAGA complexes by TOM1p may not involve protein degradation. The amount of SPT7p associated with the ADA proteins was not increased in the absence of TOM1p as would be expected if ubiquitination of SPT7p was a signal for its degradation. In addition, neither the levels of NGG1p, ADA2p, nor the overall levels of the ADA complexes as judged after their fractionation on gel-filtration and ion-exchange columns, were affected by the absence of TOM1p (not shown). To further support that TOM1p is not involved in general protein turnover, we find that the growth of *tom1* strains is not affected by the presence of 1.5 μg/ml of the amino acid analog canavanine (Seufert & Jentsch, 1990). While these observations do not eliminate the possibility that a minor population or a component of one of the ADA complexes is degraded as the result of ubiquitination, they are consistent with ubiquitination of an ADA component leading to alteration in function in a way similar to those described for other protein modifications. Similar roles for ubiquitination not directly involving protein turnover have been proposed (Arnason & Ellison, 1994; Spence *et al.*, 1995; Chen *et al.*, 1996; Hicke & Riezman, 1996).

The above model suggests that ubiquitination of an ADA complex component alters one of the activities of the complex. The ADA proteins associate with both TBP and activator proteins (Silverman *et al.*, 1994; Barlev *et al.*, 1995; Martens *et al.*, 1996; Melcher & Johnston, 1995; Saleh *et al.*, 1997; Chiang *et al.*, 1996). The role of the ADA proteins in regulating transcription may, at least in part, arise through these interactions. Our finding that TOM1p is required for the association of the ADA components with SPT3p and TBP suggests that ubiquitination of one of the ADA/SAGA components may regulate the interaction with the basal transcriptional machinery. Indeed, the loss of the association of TBP with the complex in the absence of TOM1p is consistent with the TOM1p-

dependent ubiquitination of one of the TBP-class of SPT proteins.

A second function ascribed to the ADA proteins is based on the identification of GCN5p/ADA4p as a histone acetyltransferase (Brownell *et al.*, 1996). The ADA/SAGA complexes are capable of acetylating nucleosomal histone H3 (Grant *et al.*, 1997; Ruiz-Garcia *et al.*, 1997) and recently, SIN1p has been shown to be a substrate (Pollard & Peterson, 1997). Although we cannot exclude that ubiquitination by TOM1p may alter targeting of the acetyltransferase or its substrate specificity, the activity of the acetyltransferase as demonstrated by its acetylation of histone H3 was virtually unchanged in a *tom1* deletion background. This contrasted with the effects of NGG1p$_{\Delta 274\text{-}307}$ in which acetylation of histone H3 was reduced tenfold. This result is particularly noteworthy since the *NGG1*$_{\Delta 274\text{-}307}$ allele results in similar transcriptional defects as disruptions of *tom1* yet gives rise to an ADA complex which is defective in both histone acetylation and interaction with TBP (Brandl *et al.*, 1996; Saleh *et al.*, 1997).

The finding that an E3 ubiquitin ligase regulates the activity of the ADA proteins supports a possible functional significance for our observation that UBP3p associates with NGG1p (Martens *et al.*, 1996). UBP3p could play a role with TOM1p in cycling one or more of the ADA complexes from an active ubiquitinated form to an inactive deubiquinated form. It is interesting to note that a role for UBP3p in regulating silencing of the yeast mating type loci has recently been described (Moazed & Johnson, 1996).

Materials and Methods

DNA constructs

lacZ reporter constructs were cloned as *his3-lacZ* fusions in the *LEU2* centromeric plasmid YCp87 (Brandl *et al.*, 1993) or transferred as *Bam*HI to *Sal*I fragments into the *ADE2* centromeric vector pASZ11 (Stoltz & Linder, 1990). The *HIS3* promoter was from *his3*-189 (Hill *et al.*,1986). The *GAL10* promoter region contains *GAL1-10* sequences from 299 to 649 (Johnston & Davis, 1984) fused to a TATA element sequence of TATAAA at −25 relative to the *his3* transcriptional start site (*his3*-G25, Brandl *et al.*, 1993). Similarly, the *ADH2* promoter contains sequences from 759 to 1052 as defined by Yu *et al.* (1989), including the regions required for ADR1p activation, fused at −25 of *his3*.

Yeast strains, media and growth conditions

Yeast strain CY922 (relevant genotype, *MAT a ade2-101 gal4-Δ gal80- Δ his3-Δ200 leu2-3,112 trp1-Δ901ura3-52 LYS2::GAL1-HIS3*; see Table 1), is a derivative of Y190 (Durfee *et al.*, 1993). JY335 is isogenic to CY922 but contains *ngg1-1* (Brandl *et al.*, 1993; Martens *et al.*, 1996). A Tn10LUK disruption of the coding region of *TOM1* from amino acids 244 to 2952 was introduced in CY922 and JY335 by double strand gene replacement to generate SY10 and CY1004, respectively. *Ura*$^+$ colonies were selected and verified for disruption by PCR and Western blotting. SY11 was generated from SY10 by selecting for loss of *URA3* on 5-fluoroorotic acid (Boeke *et al.*, 1984). YIplac211-*HA-NGG1* (Saleh *et al.*, 1997) was digested with *Kpn*I and integrated into SY11 to generate SY12. Similarly, YIplac211-*HA-NGG1* and YIplac211-*HA-ngg1*$_{\Delta 274\text{-}307}$ were integrated into yeast strain CY922 to generate SY13 and SY14.

To construct the Cys-Ala mutation at amino acid 3235 of TOM1p, a 1.1 kilobase-pair *Hin*dIII fragment containing *URA3* was cloned in the terminal *Kpn*I fragment of *TOM1*, downstream of the TOM1p coding region. The mutation was introduced by site-directed mutagenesis (Kunkel, 1985) using the oligonucleotide 5′-cagttgattgaaaGCggtatgtgatgat-3′. This allele was introduced into yeast strain CY922 by double strand gene replacement to generate CY1005. *URA*$^+$ colonies were verified for introduction of the mutation by PCR analysis and the incorporation of a novel *Aci*I restriction site.

Yeast strains were grown at 30°C in YPD broth (1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) glucose) or in minimal media (0.67% (w/v) yeast nitrogen base without amino acids, 2% glucose and supplemented with amino acids as required).

Two-hybrid analysis

GAL4$_{DBD}$-*NGG1*$_{1\text{-}373}$ contains a *Nde*I-*Nsi*I (blunt) fragment of *NGG1*, encoding the initiator ATG to codon 373 into the *Nde*I-*Sma*I site of pAS1 (Durfee *et al.*, 1993; Martens *et al.*, 1996). Yeast strains CY922, SY11 and CY1005 containing the plasmid pAS1-*GAL4*$_{DBD}$-*NGG1*$_{1\text{-}373}$ and a *GAL10-lacZ* reporter on an *ADE2*-containing centromeric plasmid (Martens *et al.*, 1996) were transformed with a cDNA fragment of *UBP3* encoding amino acids 733 to 912 (Baker *et al.*, 1992) fused to the GAL4p activation domain in pACT (Durfee *et al.*, 1993).

β-Galactosidase assays

Yeast strains were grown in minimal media to an $A_{600\text{ nm}}$ of 1.0 to 1.5. For the induction of ADR1p, starter cultures grown in glucose were washed twice with water and diluted tenfold into media containing 3% (v/v) ethanol and 0.05% glucose. Cells were pelleted, washed, concentrated in *lacZ* buffer (Guarente, 1983) and β-galactosidase activity determined after disruption with glass beads (Himmelfarb *et al.*, 1990). Activity was standardized to protein concentration. For yeast strains expressing potent activator proteins, β-galactosidase activity was assayed as described by Ausubel *et al.* (1990) and standardized to cell density.

To ensure the presence of GCN4p for the analysis of the *HIS3* promoter, *GCN4* was expressed from the *DED1* promoter on the centromeric plasmid YCp88 (Hope & Struhl, 1986). GAL4p expressed from its native promoter was introduced into yeast strains on the *TRP1* centromeric plasmid YCplac22 (YCPG4trp, kindly provided by Ivan Sadowski).

Analysis of histone acetyltransferase activity: Isolation of ADA complexes

Whole cell extracts from one liter cultures of CY922, SY12, and SY13 grown to an absorbance of 1.6 to 1.8 at 600 nm were prepared by grinding in liquid nitrogen (Schultz *et al.*, 1991; Saleh *et al.*, 1997). Extracts were solu-

bilized in 25 mM Tris-HCl (pH 7.5), 15 mM EDTA, 10% glycerol, 140 mM NaCl, 1.0 mM dithiothreitol, 0.1% NP40 and protease inhibitors. After adjusting the pH to 7.5 and removing cellular debris by centrifugation at 40,000 *g*, 25 mg of protein was incubated with 200 µl of Sepharose CL-4B and rotated at 4°C for 30 minutes. Unbound protein was measured as was the HA-NGG1p in each extract by analyzing serial dilutions on a Western blot. 10 µl of anti-HA antibody (12CA5, 12 mg/ml) was incubated for two hours with 2 and 5 mg of protein in a total volume of 1.0 ml. Immune complexes were isolated by a second two hour incubation with 50 µl of Protein A Sepharose that had been incubated with 20 µg of rabbit anti-mouse IgG and IgM (1.0 mg/ml). Beads were washed four times with 1.5 ml of extraction buffer followed by two washes in 40 mM Tris-HCl (pH 7.5), 40 mM NaCl, 10% glycerol, 0.1 mM EDTA, 0.08% NP40 and protease inhibitors.

Liquid histone acetyltransferase assays were performed as described by Grant *et al.* (1997). 1.0 µg of HeLa H1-depleted oligonucleosomes (Cote *et al.*, 1995) was incubated with each immunoprecipitated fraction (6 µl of beads) and [^{3}H]acetyl-CoA (0.25 µCi) in 30 µl of HAT buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 5% glycerol, 0.1 mM EDTA, 1.0 mM DTT, 1.0 mM PMSF, 10 mM sodium butyrate) at 30°C for 60 minutes with frequent mixing. One-half of the reaction (15 µl) was separated by electrophoresis on a 18% SDS-polyacrylamide gel. The gels were stained to ensure loading of equivalent amounts of histone in each lane, destained and fluorographed with Enhance (DuPont NEN).

Immunoprecipitation of NGG1p and TOM1p

For immunoprecipitation of HA-NGG1p, whole cell extracts were prepared from yeast strain SY12, SY13 and CY922 in 50 mM Hepes (pH 7.4), 100 mM NaCl, 16 mM magnesium acetate, 1 mM EGTA, 0.1% NP40, 0.5 mM dithiothreitol, 10% glycerol, and protease inhibitors (IP buffer). 75 mg of extract was rotated for one hour at 4°C with 0.5 ml of Sepharose CL-4B (Pharmacia). Unbound protein was incubated with 10 µg of monoclonal antibody 12CA5 (Wilson *et al.*, 1984) directed against the hemagglutinin (HA) epitope and rotated at 4°C for one hour then added to 75 µl of Protein A Sepharose beads (Pharmacia Biotech Inc.), and rotated for three hours at 4°C. Beads were washed five times with 1.5 ml of IP buffer and bound protein was eluted at 65°C for five minutes in SDS gel loading buffer. For immunoprecipitates that were to be probed with anti-TBP or anti-SPT3p antibodies, the HA-antibody was covalently coupled to cyanogen bromide activated Sepharose 4B (Pharmacia) at a concentration of 8.0 mg/ml, with a volume of 40 µl used per immunoprecipitation.

A similar procedure using Protein A Sepharose was used for the immunoprecipitation of TOM1p from SY12, SY13, SY14 and CY922 extracts, substituting an equivalent of 30 µg of affinity purified polyclonal antibody raised against the peptide sequence CKFSIHRDFGSSERL, located from amino acids 3215 to 3229 at the carboxyl terminus of TOM1p. This antibody was purchased from the Western Immuno Technology Service. The extracts were incubated with antibody for two hours at 4°C followed by rotating the mixture with Protein A Sepharose for three hours.

The denaturing immunoprecipitation for SPT7p was performed by a procedure adapted from Ausubel *et al.* (1990). Whole cell extracts were prepared from yeast strains CY922, SY11 and FY1093 in 50 mM Tris-HCl (pH 7.5), 50 mM NaCl and protease inhibitors. Sodium dodecyl sulfate (final concentration 1.5%) was added to 10 mg of protein and the samples placed in a boiling water for ten minutes. The extracts were transferred to ice for 20 minutes then debris pelleted by centifugation at 20,000 *g* for 15 minutes. The supernatant was diluted 15-fold in RIPA buffer (50 mM Tris-HCl (pH 7.5), 140 mM NaCl, 1% Na-deoxycholate, 1 mM DTT, 1% (v/v) NP40, 1% (w/v) BSA and protease inhibitors) and centrifuged at 12,000 *g* for 20 minutes. 50 µg of polyclonal antibody to SPT7p was added to the supernatant, which was then rotated for three hours at 4°C, followed by the addition of 75 µl of Protein A Sepharose and rotation for two hours. The beads were washed five times with 2 ml of RIPA buffer, then twice with RIPA buffer without DTT, Na-deoxycholate and bovine serum albumin. Bound protein was eluted by incubation at 65°C for five minutes in SDS gel loading buffer and Western blotted as described below using anti-ubiquitin antibody (Research Diagnostics, Inc.)

Western blot analysis of proteins

Western blotting with a primary antibody from Ascites fluid derived from the 12CA5 cell line using PVDF membrane has been described (Brandl *et al.*, 1996). Immunoprecipitations processed for Western blots with anti-TOM1p antibody were separated on 5% SDS gels and transferred to PVDF membrane by semi-dry blotting at 2.0 mA/cm^2 for 2.5 hours. After blotting, membranes were incubated in Tris-buffered saline (TBS: 20 mM Tris (pH 7.5); 0.3 M NaCl) overnight at 4°C, and then blocked in a TBS containing 5% (w/v) casein for two hours at room temperature. Membranes were incubated with anti-TOM1p polyclonal antibody diluted 1:200 in 0.5 × TBS containing 0.5% (v/v) Tween 20 for two hours at room temperature followed by six 20 minutes washes in 20 mM Tris-HCl (pH 7.5), 75 mM NaCl, 0.5% Tween 20 and 0.04% sodium dodecyl sulfate. The Western blot with the anti-ubiquitin antibody was carried out as described by Avantaggiati *et al.* (1996) with minor modifications. After electroblotting, membranes were incubated in 6 M guanidine-HCl, 20 mM Tris-HCl (pH 7.5), 5 mM β-mercaptoethanol, 1 mM PMSF for 30 minutes at 4°C, then washed extensively in phosphate buffered saline (PBS). Anti-ubiquitin antibody (Sigma) was diluted to a concentration of 5 mg/ml in PBS and further diluted 1:100 in blocking solution. Probing with rabbit polyclonal antibodies to the GAL4p DNA binding domain (kindly provided by Ivan Sadowski), the amino-terminal 198 amino acids of SPT3p, SPT7p (kindly provided by Lisa Pacella and Fred Winston) and to TBP (Upstate Biotechnology, Inc.) were performed at dilutions of the primary antibody of 1:1000, 1:3000, 1:500 and 1:2000. With the exception of the SPT3p blots the secondary antibody was goat anti-rabbit IgG (Promega) conjugated to horse radish peroxidase and used at a dilution of 1:4000. For the SPT3p blots, detection was through the use of secondary goat anti-rabbit antibodies conjugated to alkaline phoshatase (BioRad) used at a dilution of 1:3000. When the same membrane was probed sequentially with different antibodies, the membrane was stripped according to the manufacturer's instructions and reprobed with secondary antibody to verify that the first antibody was removed.

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