

## The essential function of Swc4p – a protein shared by two chromatin-modifying complexes of the yeast *Saccharomyces cerevisiae* – resides within its N-terminal part

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The Swc4p protein, encoded by an essential gene, is shared by two chromatin-remodeling complexes in *Saccharomyces cerevisiae* cells: NuA4 (nucleosome acetyltransferase of H4) and SWR1. The SWR1 complex catalyzes ATP-dependent exchange of the nucleosomal histone H2A for H2AZ (Htz1p). The activity of NuA4 is responsible mainly for the acetylation of the H4 histone but also for the acetylation of H2A and H2AZ. In this work we investigated the role of the Swc4p protein. Using random mutagenesis we isolated a collection of *swc4* mutants and showed that the essential function of Swc4p resides in its N-terminal part, within the first 269 amino acids of the 476-amino acid-long protein. We also demonstrated that Swc4p is able to accommodate numerous mutations without losing its functionality under standard growth conditions. However, when *swc4* mutants were exposed to methyl methanesulfonate (MMS), hydroxyurea or benomyl, severe growth deficiencies appeared, pointing to an involvement of Swc4p in many chromatin-based processes. The mutants' phenotypes did not result from an impairment of histone acetylation, as in the mutant which bears the shortest isolated variant of truncated Swc4p, the level of overall H4 acetylation was unchanged.

**Keywords:** chromatin remodeling complexes, histone acetylation, mutagenesis, mutant phenotype

### INTRODUCTION

Chromatin modifications and remodeling are reversible processes that serve to regulate DNA accessibility within chromatin fibers in eukaryotic cells (Chakravarthy *et al.*, 2005; Kouzarides, 2007). Two classes of highly conserved enzymes are responsible for this type of regulation. One class includes ATP-dependent chromatin-remodeling enzymes which disrupt histones–DNA interactions (Cairns, 2007). The second class consists of enzymes that catalyze covalent post-translational modifications of histones, such as acetylation, methylation, phosphorylation and ubiquitylation (Shilatifard, 2006; Shahbazian & Grunstein, 2007). Such specific modifications of his-

tone tails may affect the level of chromatin compactness or create sites to which other proteins can bind *via* distinct functional domains (Luger & Hansen, 2005; Ruthenburg *et al.*, 2007; Taverna *et al.*, 2007). Both classes of enzymes function as subunits of large complexes (Lall, 2007). Sometimes subunits are shared by multiple complexes, although in many instances the role of a particular subunit within a given complex is not known, and for this reason it is also unclear whether the same subunit performs always the same function or whether it can have different functions in different complexes.

NuA4 (nucleosome acetyltransferase of H4) and SWR1 represent such subunit-sharing complexes in the yeast *Saccharomyces cerevisiae*. The SWR1 com-

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**Abbreviations:** aa, amino acid(s); DMSO, dimethylsulfoxide; 5-FOA, 5-fluoroorotic acid; HU, hydroxyurea; MMS, methyl methanesulfonate; ORF, open reading frame; *ts*, temperature-sensitive.

plex catalyzes ATP-dependent exchange of the nucleosomal histone H2A for H2AZ (Htz1p) (Krogan *et al.*, 2003; Kobor *et al.*, 2004; Mizuguchi *et al.*, 2004). The activity of NuA4 is responsible mainly for the acetylation of histone H4 but also of H2A and H2AZ (Smith *et al.*, 1998; Clarke *et al.*, 1999; Allard *et al.*, 1999; Koegh *et al.*, 2006). SWR1 and NuA4 have four common subunits: Act1p, Arp4p, Yaf9p and Swc4p. Yaf9p and Swc4p are specific for NuA4 and SWR1, whereas Act1p and Arp4p are also present in the INO80 complex (Shen *et al.*, 2000; Kobor *et al.*, 2004; Mizuguchi *et al.*, 2004).

In this work we investigated the role of the essential protein Swc4p. Using random mutagenesis we isolated a collection of *swc4* mutants and showed that the essential function of Swc4p resides within its N-terminal part. We also provide evidence that Swc4p is involved in many different chromatin-based processes.

## MATERIALS AND METHODS

**Strains, media and growth conditions.** Yeast strains used in this work are listed in Table 1. Yeast cells were grown on standard rich YP medium with 2% glucose as a carbon source (YPD) or on minimal YNB medium (0.67% yeast nitrogen base without amino acids) supplemented with all the required amino acids and nucleotides, and 2% glucose as a carbon source (YNBD) (Sherman, 2002). Ura<sup>-</sup> derivatives of the analyzed strains were obtained by selection with 0.1% 5-fluoroorotic acid (5-FOA) added to minimal medium (Boeke *et al.*, 1984). To assess the mutants' growth deficiencies the media were supplemented with a range of concentrations of the appropriate chemicals added after sterilization. Cells were spotted at 10-fold dilutions and grown at 28°C (or at the indicated temperatures) for 3–4 days.

**DNA manipulations.** Routine DNA manipulations: plasmid preparation, subcloning, *Escherichia coli* transformation and agarose gel electrophoresis were carried out as described (Sambrook *et al.*, 1989). Yeast transformations were performed by the lithium acetate procedure (Gietz & Woods, 1998). Plasmid DNA from yeast, used subsequently to transform *E. coli*, was isolated as described (Robzyk & Kassir, 1992). DNA sequencing reactions were analyzed on an ABI310 Genetic Analyzer (Perkin-Elmer).

**Cloning of the *SWC4* gene.** A 1974 bp DNA fragment comprising 1431 bp of the *SWC4* gene coding sequence with 300 bp of its promoter and 243 bp of its terminator sequence was amplified by PCR using the following primers:

GOD-A: 5'-AAGAGCTCATCGCTGTATATATGAT-CAATGGC-3'

and GOD-B: 5'-AACTICGAGCAGTTGATATTCTATGAGATGACA-3'

to which the *SacI* and *XhoI* restriction sites were added, respectively, and cloned into *SacI*–*XhoI* digested pRS416 and pRS313 vectors (Sikorski & Hieter, 1989) to yield the *SWC4*/pRS416 and *SWC4*/pRS313 plasmids. PCR was performed on genomic DNA of the S288C strain with the "Expand High Fidelity PCR System" (Roche Diagnostics) according to the manufacturer's instructions. The *SWC4* sequence was verified by sequencing.

**Random mutagenesis of the *SWC4* gene.** A library of mutated alleles of *SWC4* was created by random mutagenesis procedures based on hydroxylamine treatment or mutagenic PCR. Hydroxylamine mutagenesis was performed using the *SWC4*/pRS313 plasmid and a standard protocol as described previously (Humphreys *et al.*, 1976).

A compilation of several published methods was used to introduce mutations by PCR techniques (Spee *et al.*, 1993; Fromant *et al.*, 1995; Melnikov & Youngman, 1999; Xu *et al.*, 1999). The mutagenic PCR amplifications were done using the *SWC4*/pRS416 plasmid as a template and the same GOD-A and GOD-B pair of primers as used for *SWC4* cloning. Under the conditions employed mutations affecting each base pair should occur with similar probability. The conditions of four separate mutageneses were optimized to give the intended levels of mutations. These levels were: approx. 1–2, 3–4, 5–6 and 8–10 changes per 1000 base pairs, respectively. Subsequently, PCR products from each reaction were cut with *PfI*MI and *ClaI* which have unique restriction sites within the cloned *SWC4* sequence. The *PfI*MI/*ClaI* cutting resulted in truncation of *SWC4* which became devoid of 62 bp from the 5'-terminus of the ORF and 54 bp from the 3'-terminus, yielding 1315 bp long fragments of the randomly mutagenized *SWC4* gene. The randomly mutagenized and *PfI*MI–*ClaI* restricted DNA was cloned into *PfI*MI–*ClaI*-cut *SWC4*/pRS313 to yield four libraries of differently mutagenized *swc4* alleles.

**Selection of mutant *swc4* alleles.** The AM1 *SWC4*/*swc4*Δ heterozygous diploid strain was derived from BY4743 by inserting the kanMX4 selectable marker into the *SWC4* gene by standard disruption methods (Adams *et al.*, 1997). The AM1 strain was transformed with the *SWC4*/pRS416 plasmid constructed as described above. After sporulation and tetrad dissection, for which standard media and procedures were used, the AM1-1A strain (*swc4*Δ [*SWC4*/pRS416]) was obtained. To screen for mutant alleles of *swc4*, the AM1-1A strain was transformed separately with each of the libraries of the mutated *SWC4* gene on the pRS313 plas-

Table 1. Yeast strains used in this study

Strain	Genotype	
BY4741	<i>MATa, his3Δ1, leu2Δ0, ura3Δ, met15Δ0</i>	E C*
BY4743	<i>MATa/ MATα, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, uraΔ3/uraΔ3, lys2Δ0/LYS2; MET15/met15Δ0</i>	E C
AM1	<i>MATa/ MATα, his3Δ1/his3Δ1, leu2Δ0/leu2Δ0, ura3Δ/ura3Δ, lys2Δ0/LYS2; MET15/met15Δ0, swc4Δ::kMX4/SWC4</i>	This study
AM1-1A	<i>MATa, his3Δ1, leu2Δ0, ura3Δ, met15Δ0, swc4Δ::kMX4 [SWC4/pRS416]</i>	This study
BY <i>swr1Δ</i>	<i>MATa, his3Δ1, leu2Δ0, ura3Δ, met15Δ0, swr1Δ::kMX4</i>	E C
BY <i>htz1Δ</i>	<i>MATα, his3Δ1, leu2Δ0, ura3Δ, met15Δ0, htz1Δ::kMX4</i>	E C
BY <i>yaf9Δ</i>	<i>MATa, his3Δ1, leu2Δ0, ura3Δ, met15Δ0, yaf9Δ::kMX4</i>	E C
BY <i>ops71Δ</i>	<i>MATa, his3Δ1, leu2Δ0, ura3Δ, met15Δ0, ops71Δ::kMX4</i>	E C
BY <i>eaf7Δ</i>	<i>MATa, his3Δ1, leu2Δ0, ura3Δ, met15Δ0, eaf7Δ::kMX4</i>	E C
<i>esa1-1851</i>	<i>MATα, esa1-1851</i>	Bird <i>et al.</i> , 2002

\*Euroscarf strains collection

mid, plated on YNBD medium supplemented with lysine, methionine and leucine, and incubated at 23°C for 7–10 days. His<sup>+</sup> transformants were replica-plated on SD plates supplemented with lysine, methionine and leucine and additionally with uracil and 0.1% 5-FOA to counter-select against the *URA3*-based *SWC4*/pRS416 plasmid and to remove the wild-type copy of *SWC4*. This procedure was repeated twice and subsequently all Ura<sup>-</sup> His<sup>+</sup> colonies were replica-plated on YPD and dimethylsulfoxide (DMSO)-containing YPD plates. YPD plates were incubated at 23°, 37° and 39°C, and YPD+DMSO plates at 28°C. Transformants able to form colonies under permissive but not under restrictive conditions were selected. Plasmid DNA from primary transformants obtained from PCR-generated libraries was isolated and used to re-transform the AM1-1A strain. In the case of the hydroxylamine-generated library, mutations resulting in the growth deficiency of the AM1-1A strain could have also occurred within the vector sequence. To eliminate such a possibility, plasmid DNA from the transformants was isolated, *PflMI*-*ClaI* fragments of the potentially mutated *SWC4* were released and cloned into *PflMI*-*ClaI* cut *SWC4*/pRS313. The resulting plasmids were used to re-transform the AM1-1A strain. After plasmid shuffling, the phenotypes of the strains were checked. Mutated *swc4* alleles were sequenced to identify the mutation site(s).

**Integration of the *swc4* alleles into the genomic *SWC4* locus.** The selected *swc4* alleles were cloned into the integrative pRS306 vector (Sikorski & Hieter, 1989) to yield *swc4-x*/pRS306 plasmids. The obtained plasmids were linearized within *SWC4* by *SphI* cutting and used to transform the BY4741 strain. The pop-in pop-out method was used to get the required integration (Adams *et al.*, 1997).

**Construction of plasmids expressing truncated versions of the Swc4p protein.** Plasmids bearing the *swc4-24* and *swc4-25* alleles were used as PCR

templates for constructing plasmids encoding C-terminally truncated forms of Swc4p.

The GOD-A primer was used as the upstream primer in all reactions, and the following primers were used as downstream primers:

**swc4-25c** 5'-TGGATCCTATCACCTATGTTGGGCAAACCT-3'

**swc4-24c** 5'-TGGATCCTATCACTTGGCAGCCATTTTC-3'

**swc4+SANTc** 5'-TGGATCCTATCAATTCCTACAGGTGTAATAAAAATTT-3'

Two STOP codons were present in each of the downstream primer. The amplified sequences contained 300 bp of the *SWC4* promoter and fragments of *SWC4* encoding 332 aa (*swc4-25c* primer), 269 aa (*swc4-24c* primer) and 211 aa (*swc4+SANTc* primer). The resulting PCR products were cut with *Sall* and *BamHI* and cloned into the *Sall*-*BamHI* restricted p415TEF vector (Mumberg *et al.*, 1995). This cloning strategy resulted in the removal of the *TEF1* promoter from the p415TEF plasmid while keeping the *CYC1* terminator, and allowed for the expression of the truncated alleles of *SWC4* from their own promoter.

**Checking the acetylation level of the H4 histone.** Yeast proteins were isolated from logarithmically growing cells as described previously (Briggs *et al.*, 2001), separated on 14% SDS/PAGE and transferred to Immobilon-P membrane from Millipore.  $\alpha$ -AcH4 antibody from Upstate 06-866 was used to detect the hyperacetylated form of H4. Anti-actin C4 antibody from ICN was used for actin, serving as an internal standard for gel loading. Protein levels were quantified by chemiluminescence using FluorChem SP from Alpha Innotech.

**Protein alignment.** An alignment of selected proteins from the P151823 family (SYSTEMS Protein Family) was created using MAFFT v.6 and the L-INS-I strategy. The accession number is ALIGN\_001224 at ftp://ftp.ebi.ac.uk/pub/databases/embl/align.

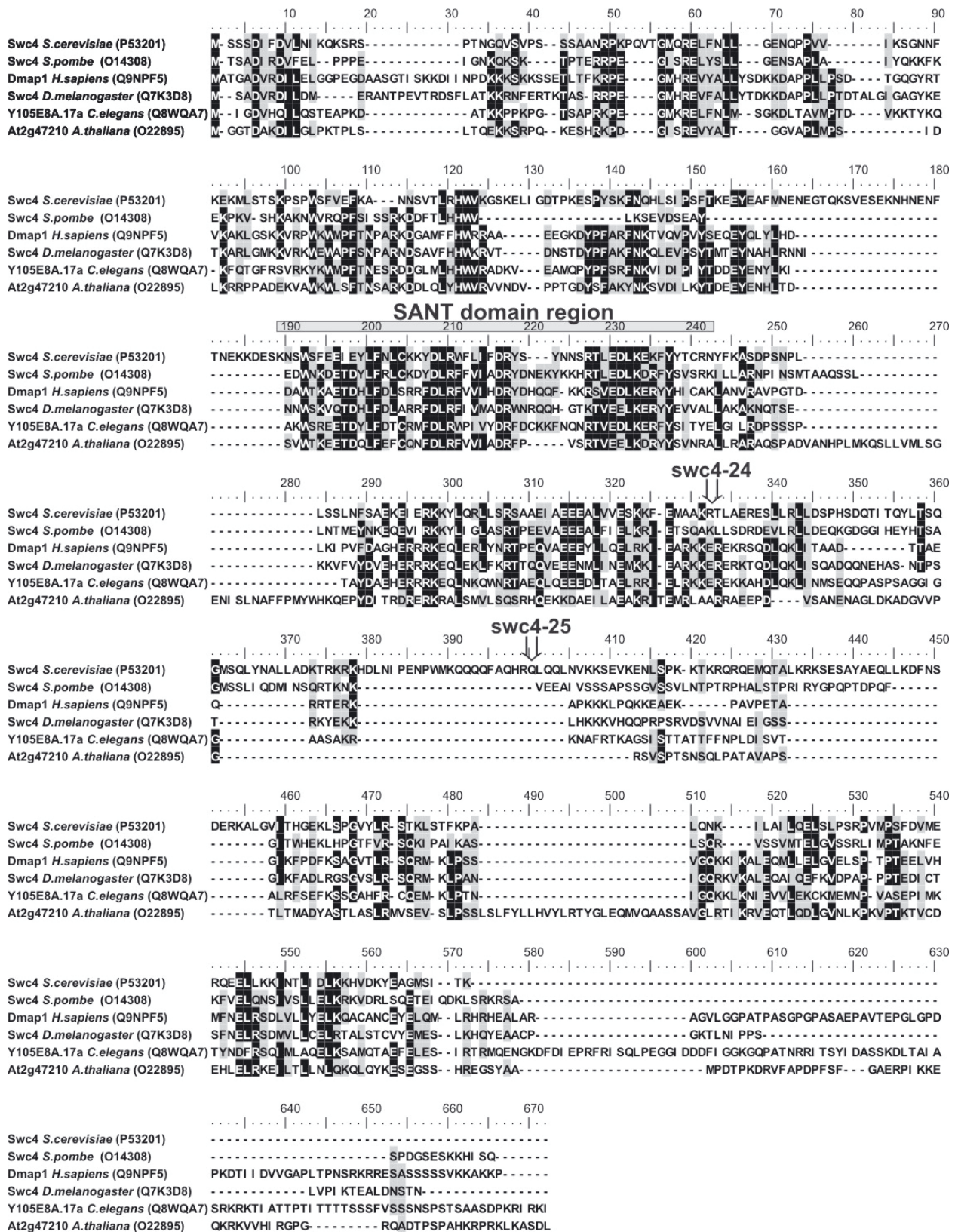


Figure 1. Alignment of selected proteins from the P151823 family (SYSTERS Protein Family).

The region of the SANT domain is marked. The arrows indicate the sites of STOP codon generation in the *swc4-24* and *swc4-25* mutants. The layout was created with the BioEdit software package (Hall, 1999).

## RESULTS

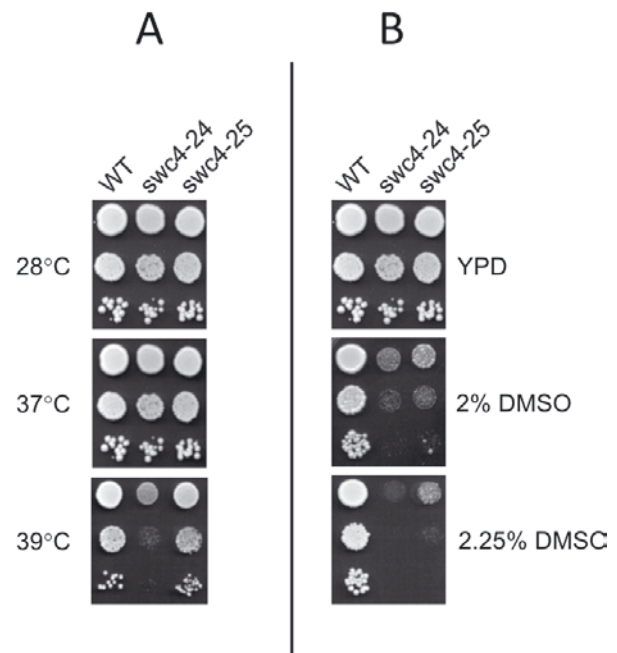
**Swc4p is able to accommodate numerous mutations**

The *SWC4* gene is essential in yeast. Although it may be assumed that Swc4 protein role in the cell is connected with the activity of the complexes in which it has been identified, the mechanism underlying its essential function is not known. Still, no work has been undertaken so far to assess the precise function of Swc4p. A bioinformatical approach allowed the assigning of Swc4p to the protein family P151823 (SYSTERS Protein Family) (Meinel *et al.*, 2005) and identification of proteins similar to Swc4p from other organisms, among them the human protein DMAP1 (Rountree *et al.*, 2000; Doyon *et al.*, 2004) (Fig. 1). All proteins from this family contain the SANT domain, which is thought to act as a binding module for histones or other basic proteins (Boyer *et al.*, 2004). We sought to isolate *swc4* mutant alleles and to analyze the spectrum of their phenotypes in order to pinpoint amino acid changes connected with the appearance of particular phenotypic features. We reasoned that the analysis of such connections in the context of the sequence homology between Swc4p and its counterparts from other organisms would allow us to draw conclusions about the function of the protein.

We performed random mutagenesis of *SWC4* as described in Materials and Methods. The obtained level of mutational changes was as intended and ranged from 1 to around 8–10 changes per 1000 base pairs. The mutagenesis procedure was designed in such a way that mutations appearing within the first 62 base pairs or the last 54 base pairs of the ORF were eliminated, so the number of amino acids prone to changes decreased from 476 (full-length Swc4p) to 438 (see Materials and Methods).

For *swc4* mutant library screening we used the strain AM1-1A (with the genotype *swc4Δ* [*SWC4*/pRS416]), which was obtained by dissecting the heterozygous diploid *swc4Δ*/*SWC4* transformed with the *SWC4*/pRS416 plasmid to allow survival of the resulting *swc4Δ* haploid strains.

AM1-1A was transformed independently with each of the *swc4* mutant allele libraries and a total of approx. 27000 His<sup>+</sup> transformants were obtained: 15000 from hydroxylamine-mutated libraries and 12000 from PCR-mutated libraries. Since *SWC4* is essential in yeast, we were looking for temperature-sensitive (*ts*) *swc4* mutants, as this phenotype is commonly associated with mutations in essential genes. First, the *SWC4*/pRS416 plasmid had to be removed. This was achieved by repeated replica-plating of all transformants on medium containing 5-FOA (Boeke *et al.*, 1984) and growing them at 23°C or 28°C. Growth

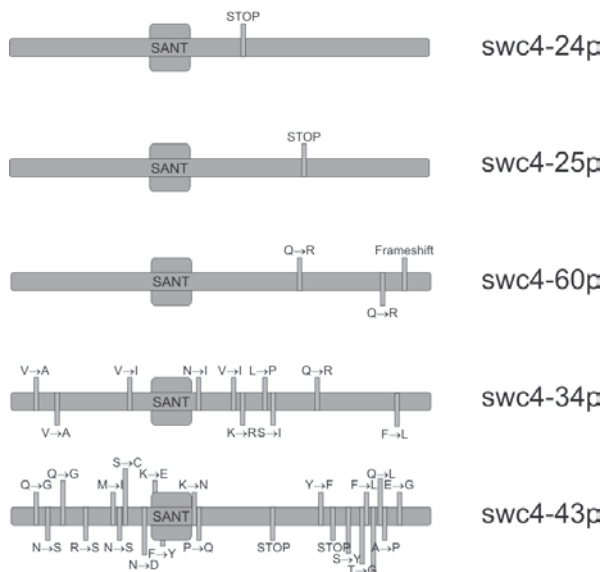


**Figure 2. Phenotypes displayed by the *swc4-24* and *swc4-25* mutant strains grown at different temperatures or in the presence of DMSO.**

The wild-type BY4741 strain and its derivatives *swc4-24* and *swc4-25* were grown overnight in YPD medium. Cells were spun down, resuspended in water to a final concentration of  $2 \times 10^7$  cells/ml and 10-fold serially diluted. Five microliters of each dilution was spotted onto YPD or YPD plus DMSO plates. (A) Strains grown on YPD at 28°C, 37°C or 39°C (B) Strains grown at 28°C on YPD supplemented with 2% or 2.25% DMSO. All plates were incubated for 4 days.

of the resulting His<sup>+</sup> Ura<sup>-</sup> transformants became completely dependent on the plasmids derived from the *swc4* allele libraries. Then, transformants were replica-plated on YPD plates and allowed to grow at 23°C, 28°C or 37°C. To our surprise, no *ts* mutants could be found — not even from the library containing the most intensely mutagenized alleles — unless the temperature was raised to 39°C instead of the 37°C that is routinely used for *ts* screening. For this reason, we applied a second phenotype for the screening of the *swc4* mutant libraries — sensitivity of the His<sup>+</sup> Ura<sup>-</sup> transformants to DMSO. We had previously noticed that sensitivity to DMSO is associated with mutations in many genes encoding subunits of chromatin-modifying complexes (Chelstowska *et al.*, in preparation). Since DMSO sensitivity is a very clear-cut phenotype, it turned out very useful for screening.

Altogether, 140 mutants were isolated and re-screened. Eighteen *swc4* alleles were chosen for sequencing based on their sensitivity to DMSO and/or their lack of growth at 39°C; two examples are presented in Fig. 2. We classified the isolated alleles according to the types of mutations identified; examples of all classes are shown in Fig. 3. All



**Figure 3. Schematic representation of alleles exemplifying all classes of mutants identified in the mutational SWC4 screen.**

*swc4-24*: R270STOP

*swc4-25*: Q338STOP

*swc4-60*: Q330R, Q421R, K454frameshift

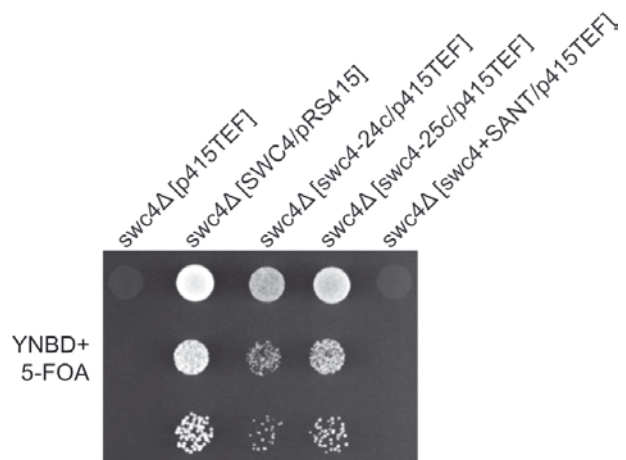
*swc4-34*: V24A, V58A, V142I, N220I, V258I, K269R, L281P, S287I, K359R, F442L

*swc4-43*: Q23G, N33S, E67G, R92S, N132I, N135S, S141C, N154D, K161E, F166Y, K214N, P221Q, Q289STOP, Y377F, S403STOP, S410Y, T415G, F416L, K417Q, Q421L, A426P, E446G

type I mutants, in which only 1–2 nucleotides were changed, contained truncated versions of the Swc4p protein, as a result of mutations generating stop codons. Both *swc4-24* and *swc4-25* belong to this class of mutants. The shortest of the obtained truncations — all of which still maintained the essential function of Swc4p — resulted in the removal of 207 amino acids from the C-terminus of the 476-aa long protein. This result shows that the N-terminal part of Swc4p is sufficient for yeast viability.

Mutants classified as type II carried insertions or deletions that lead to frame-shift mutations; in some cases these were also accompanied by amino acid substitutions. This type of mutants is represented by the *swc4-60* allele. In this mutant, an insertion caused the replacement of 22 C-terminal amino acids by a sequence of 11 different amino acids, and two point mutations caused Q to R changes in positions 330 and 421. Separation of the two substitutions from the frame-shift mutation showed that the frame-shift alone was responsible both for the DMSO sensitivity and for the 39°C *ts* phenotype, whereas the amino acid substitutions were irrelevant for the two assayed phenotypes.

Type III DMSO- and 39°C-sensitive mutants contained 8–17 amino acid replacements distributed



**Figure 4. Truncated versions of Swc4p are able to support growth of the *swc4Δ* strain.**

The AM1-1A strain (*swc4Δ* [SWC4/pR416]) was transformed with plasmids p415TEF, SWC4/pRS415, *swc4-24c*/p415TEF, *swc4-25c*/p415TEF or *swc4+SANTc*/p415TEF. The transformants were grown overnight in selective YNBD medium supplemented with the required amino acids. Cells were spun down, resuspended in water to a final concentration of  $2 \times 10^7$  cells/ml and 10-fold serially diluted. Five microliters of each dilution was spotted onto a YNBD plate supplemented with the required amino acids and 5-FOA. The plate was incubated for 6 days at 28°C.

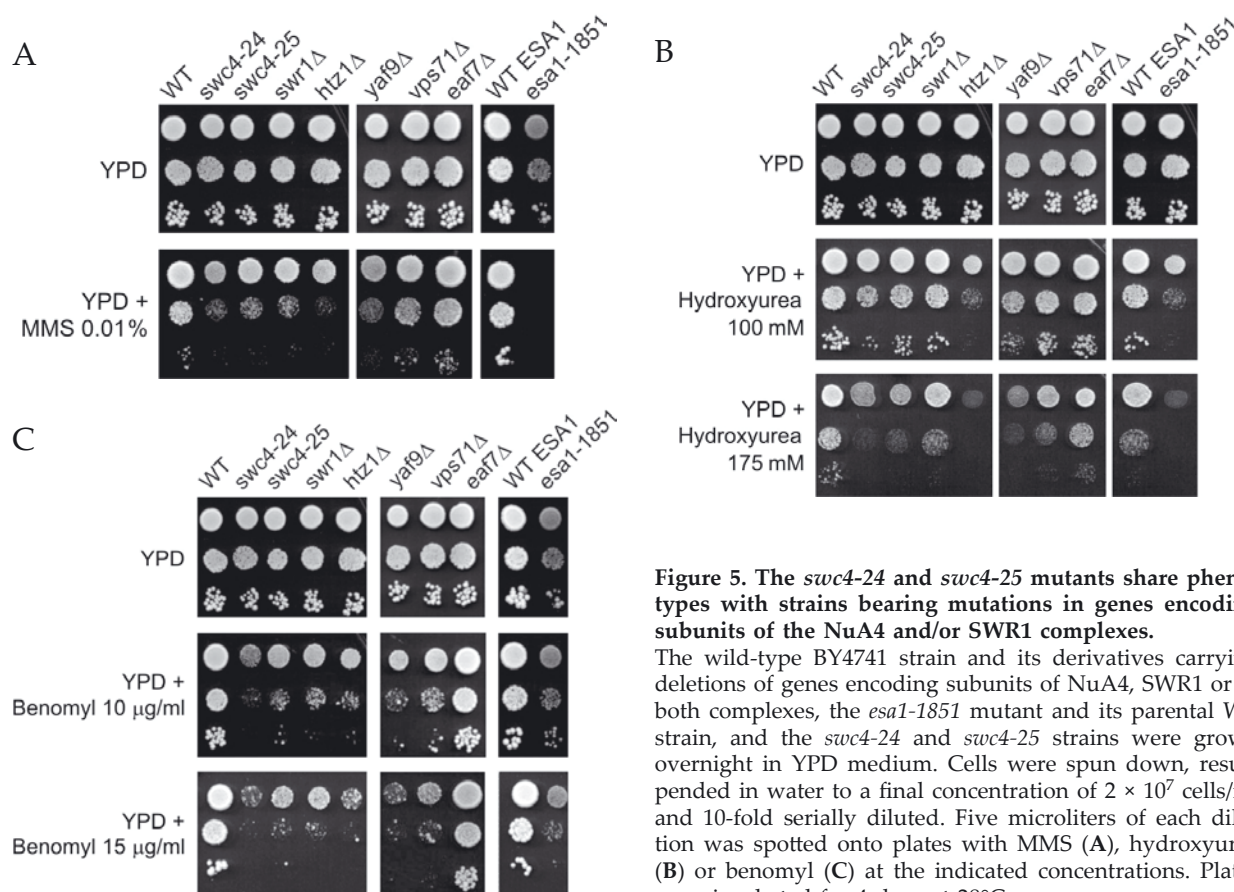
all over the protein sequence. An example of this class of mutants is *swc4-34*, in which ten mutations contribute to the phenotype.

Type IV mutants are those in which a stop codon mutation caused truncation of the C-terminus, and additionally numerous amino acid substitutions in the remaining N-terminal part were present. The most affected is *swc4-43*, which carries 12 amino acid substitutions as well as a novel stop codon that removes 187 amino acids.

#### The essential function of Swc4p is contained within its N-terminal part

The shortest version of Swc4p identified in the screen — still able to fulfill its essential function — is Swc4-24p, missing 207 amino acids from the C-terminal part as a result of a generated stop codon. To eliminate the possibility of stop codon read-through, which would provide a small amount of full-length Swc4p, occurring in the cells, the truncated *swc4-24* and *swc4-25* alleles were amplified from the original library plasmids with primers introducing two in-frame stop codons (see Materials and Methods). The *swc4-24* and *swc4-25* alleles were then expressed under the control of the SWC4 promoter and shown to support viability of the *swc4Δ* strain (Fig. 4).

As can be seen in Fig. 4, on minimal 5-FOA medium the *swc4Δ* [*swc4-24*] and *swc4Δ* [*swc4-25*]



**Figure 5. The *swc4-24* and *swc4-25* mutants share phenotypes with strains bearing mutations in genes encoding subunits of the NuA4 and/or SWR1 complexes.**

The wild-type BY4741 strain and its derivatives carrying deletions of genes encoding subunits of NuA4, SWR1 or of both complexes, the *esa1-1851* mutant and its parental WT strain, and the *swc4-24* and *swc4-25* strains were grown overnight in YPD medium. Cells were spun down, resuspended in water to a final concentration of  $2 \times 10^7$  cells/ml and 10-fold serially diluted. Five microliters of each dilution was spotted onto plates with MMS (A), hydroxyurea (B) or benomyl (C) at the indicated concentrations. Plates were incubated for 4 days at 28°C.

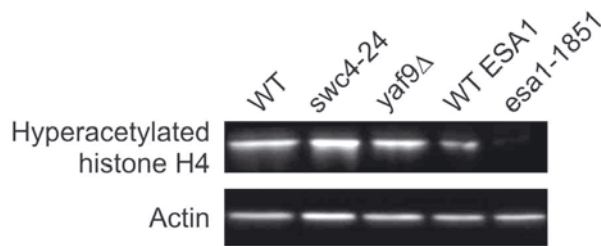
strains grew slower than *swc4Δ* [SWC4]. However, all three strains displayed similar growth rates when assayed on rich YPD medium (Fig. 2). It is worth noting that although no potential domain has been predicted within the 57 amino acids that lie downstream of the SANT domain and are retained in the *swc4-24* mutant, the removal of this fragment from *swc4-24p* yielded a protein that was unable to rescue *swc4Δ* lethality (Fig. 4), despite the presence of the SANT domain.

#### The *swc4-24* and *swc4-25* mutant strains share some phenotypes with other mutants deficient in the activities of NuA4 and/or SWR1

Swc4p is a protein shared by the yeast chromatin-modifying complexes NuA4 and SWR1 (Kobor *et al.*, 2004). We therefore decided to compare the phenotypes of our *swc4* mutants with the phenotypes of strains carrying mutations in other subunits of NuA4, SWR1, or both complexes. To this end, the *swc4-24* and *swc4-25* alleles were integrated into the SWC4 locus (as described in Materials and Methods). The set of analyzed reference mutants contained *esa1-1851* and *eaf7Δ* (mutations in subunits specific for NuA4), *swr1Δ*, *htz1Δ* and *vps71Δ* (specific for SWR1), and *yaf9Δ*, *swc4-24* and

*swc4-25* (affecting both complexes). *ESA1* encodes an essential histone acetyltransferase, the catalytic subunit of NuA4 (Smith *et al.*, 1998). Since the *esa1Δ* strain is inviable, we used the *esa1-1851* strain, generously provided by Dr. M. F. Christman (Bird *et al.*, 2002). Swr1p is a Swi2/Snf2-related ATPase that is the structural component of the SWR1 complex, which exchanges the H2AZ histone variant for the chromatin-bound H2A histone. The *HTZ1* gene encodes the non-canonical H2AZ histone in *S. cerevisiae* (Jackson & Gorovsky, 2000). Vps71p and Eaf7p were identified by protein complex purification as subunits of SWR1 and NuA4, respectively. Vps71p is also required for vacuolar protein sorting (Bonangelino *et al.*, 2002). Yaf9p is a subunit of both the NuA4 histone H4 acetyltransferase complex and the SWR1 complex and was shown to interact directly with Swc4p (Bittner *et al.*, 2004; Wu *et al.*, 2005).

We tested all of the selected mutants for growth phenotypes known to be associated with chromatin-based activities. In these tests we used a DNA single- and double-strand damage-inducing agent — methyl methanesulfonate (MMS), an inhibitor of DNA synthesis — hydroxyurea (HU), and a microtubule-depolymerizing agent — benomyl (Hampsey, 1997).



**Figure 6. The acetylation of histone H4 is preserved in *swc4-24* mutant cells.**

Logarithmically growing YPD cultures of the WT strain and of the indicated mutants were divided into two sets of subcultures. One set was allowed to continue growth at 28°C, the second set was transferred to 39°C. After 2 h the cells were collected and proteins were isolated and separated on 14% SDS/PAGE, followed by detection with specific anti-AcH4 and anti-actin antibodies. The levels of both proteins were quantified by chemiluminescence.

The results of the performed growth tests are presented in Fig. 5. As shown in Fig. 5A, 0.01% MMS was harmful to all mutants except *eaf7Δ*. At this concentration, growth of *vps71Δ* was less inhibited than that of the other mutants, but at 0.02% MMS *vps71Δ* was completely repressed whereas the WT and *eaf7Δ* strains still grew (not shown). The *esa1-1851* mutant turned out to be extremely sensitive, as even the lowest concentration of MMS used in the drop test (0.005%) completely inhibited its growth (not shown). The differences between the mutants were minor and only close examination allowed the identification of *swc4-24*, *yaf9Δ* and *htz1Δ* as the most significantly affected. Hydroxyurea caused similar growth deficiencies of the mutant strains as MMS. As shown in Fig. 5B, when cells were challenged with 100 mM HU, the growth of *esa1-1851* and *htz1Δ* was considerably reduced. Some growth inhibition of *swc4-24* and *yaf9Δ* could also be noticed. Raising the concentration to 175 mM HU was detrimental to all mutants except *eaf7Δ*. Similar growth defects were also observed when the mutants were exposed to benomyl, a compound interfering with chromosome segregation. As shown in Fig. 5C, the *swc4-24* mutant displayed the highest sensitivity to benomyl from the whole set of analyzed strains. The growth of all strains — except *eaf7Δ* — was extremely affected already in the presence of 15 μg/ml of benomyl (Fig. 5C). A further increase in benomyl concentration, up to 20 μg/ml, affected *eaf7Δ* only slightly, while completely blocking the growth of all other mutants (not shown).

The results of the phenotypic tests suggest that Swc4p is involved in DNA repair, replication and segregation.

### The overall histone H4 acetylation is preserved in *swc4-24* mutant cells

Activity of the NuA4 complex is indispensable in yeast. In fact, its catalytic subunit Esa1p is the only essential histone acetyltransferase in *S. cerevisiae*. Mutants in the *ESA1* gene were shown to have a decreased level of overall histone H4 acetylation (Bird *et al.*, 2002; Boudreault *et al.*, 2003). We reasoned that if the *swc4* mutants affect NuA4 function they would also display changes in histone acetylation. To check that we analyzed the overall H4 acetylation in the *swc4-24* strain alongside the *esa1-1851* mutant, known to have a very low level of H4 acetylation. We also included *yaf9Δ* in the analysis, because of the reported physical interaction between Yaf9p and Swc4p (Bittner *et al.*, 2004) and because its role in H4 acetylation had been addressed previously (Le Masson *et al.*, 2003).

As shown in Fig. 6, the overall acetylation of histone H4 is preserved in the *swc4-24* mutant cells. When WT and mutant strains were incubated for 2 h at 39°C, the temperature in which the *swc4-24* mutant's phenotype is manifested, there was no difference in H4 acetylation between both strains. Overall H4 acetylation in *yaf9Δ* cells was the same as in the WT strain under restrictive conditions, as had been reported before (Le Masson *et al.*, 2003). The *esa1-1851* strain, used as a qualitative acetylation control, displayed hardly any detectable level of hyperacetylated H4.

## DISCUSSION

The Swc4p protein encoded by an essential gene in yeast has not been the subject of any detailed study so far. The lack of structural information prompted us to use a random mutagenesis approach to identify amino acids and/or regions crucial for Swc4p function. We also hoped that the isolation of mutant strains would allow us to identify the cellular functions that are disturbed when Swc4p is defective.

Using a broad spectrum of mutagenesis conditions and analyzing a large number of *swc4* mutant alleles should have assured the isolation of different kinds of mutants: ranging from single amino acid substitutions in crucial regions of the protein to large changes in protein architecture (frame-shifts, truncations). The criteria used to distinguish between the mutant and WT phenotypes were reduced growth at 39°C and/or sensitivity to DMSO.

The data obtained suggest that there is no particular region within the Swc4p protein where introducing a single amino acid replacement would result in the appearance of a mutant phenotype. The



smallest number of amino acid substitutions which we identified as necessary to cause the above-described sensitivities was eight. Consequently, all the mutants obtained by low-level mutagenesis carried mutations introducing frame-shifts or creating stop codons.

The shortest isolated version of Swc4p – fully functional under standard growth conditions – missed 207 out of the 476 amino acids. This was surprising in the light of the fact that (1) the C-terminal part of Swc4p was found to be directly involved in the interaction with Yaf9p, another component of both the NuA4 and SWR1 complexes (Bittner *et al.*, 2004), and (2) a detailed study of the SWR1 complex in strains from which nonessential SWR1 subunits were being systematically removed has revealed that deletion of *YAF9* alone leads to a complete loss of Swc4p from the complex (Wu *et al.*, 2005). In both *swc4-24* and *swc4-25* the part of the protein crucial for the interaction with Yaf9p is missing, so it can be assumed that these truncated versions of Swc4p are excluded from the SWR1 complex. Together these data suggest that the truncated mutant protein is functional despite being absent from the SWR1 complex.

The data published recently describe the internal organization of the NuA4 complex (Auger *et al.*, 2008; Mitchell *et al.*, 2008). Swc4p is present in all but one of the Esa1-TAP complexes isolated from strains lacking nonessential subunits of NuA4. The protein which forms a platform for NuA4 assembly and lack of which leads to the complex collapse is Eaf1. In the *eaf1Δ* strain only the minimal Piccolo NuA4 complex containing Esa1, Epl1 and Yng2 is observed. The *eaf1Δ* strain is viable, which indicates that association of Swc4p with Esa1 is not essential. It is possible that the Swc4 protein may be involved in the activity of other complexes which, as opposed to SWR and NuA4, are not amenable to *in vitro* purification. The essential function of the Swc4p could be conveyed by those complexes.

Our results showing that the essential function of Swc4p resides within its N-terminal part are compatible with the data reported recently and obtained by a different approach than random mutagenesis (Auger *et al.*, 2008). We extend these results by showing that Swc4p is able to accommodate numerous mutations as well as undergo further shortening, without losing its essential function.

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