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Faculty of Science and Technology

Institute of Bioengineering

Krista Mehaņikova

Interactions between *Saccharomyces cerevisiae* YEATS domain-containing Yaf9 and Swc4 proteins

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Supervisor(s):

Researcher, PhD Henel Jürgens

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Interactions between *Saccharomyces cerevisiae* YEATS domain-containing Yaf9 and Swc4 proteins

Abstract

The YEATS domain is a conserved histone mark reader implicated in the regulation of chromatin structure and transcription. In *Saccharomyces cerevisiae*, the YEATS domain-containing protein Yaf9 is a subunit of SWR1 and NuA4 chromatin remodelling complexes. In both, it directly interacts with a shared subunit Swc4. This study investigated the Yaf9-Swc4 interaction and possible Yaf9 role outside of its complex-associated functions. A series of Yaf9 deletion mutants truncated at different regions of the protein were constructed using overlap extension PCR and were introduced into yeast. Protein interactions were examined using co-immunoprecipitation, and mutant strains were phenotyped via stress assays involving formamide exposure. The results revealed that deletions in the C-terminal region of Yaf9, as well as within the 160-168 amino acid region – located in the YEATS domain, but outside of the domain's primary reader region – disrupted interaction with Swc4 and phenocopied a full *YAF9* deletion under stress. Additionally, results of this work show that Yaf9 likely does not act independently of both complexes. These findings highlight regions of Yaf9 required for Swc4 association and enhance understanding about the protein's function. Moreover, these data provide a basis for protein studies in higher eukaryotes.

Keywords: Yaf9, Swc4, YEATS domain-containing proteins, *Saccharomyces cerevisiae*

CERCS: T490 Biotechnology

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Research group: Chromatin research group

YEATS domeeni sisaldava Yaf9 ja Swc4 valkude vahelised interaktsioonid pagaripärmis *Saccharomyces cerevisiae*

Lühikokkuvõte

YEATS domeen on evolutsiooniliselt kõrgelt konserveerunud histooni modifikatsioone äratundev domeen, millel on oluline roll kromatiini struktuuri ja transkriptsiooni regulatsioonis. Pagaripärmis *Saccharomyces cerevisiae* sisaldab YEATS domeeni valk Yaf9, mis kuulub kromatiini remodelleerivate valgukomplekside SWR1 ja NuA4 koosseisu. Yaf9 interakteerub otseselt Swc4 valguga, mis samuti kuulub mõlemasse kompleksi. Käesoleva töö eesmärgiks oli uurida Yaf9 ja Swc4 valkude vahelist interaktsiooni ning Yaf9 võimalikku iseseisvat rolli

väljaspool nimetatud valgukomplekse. Töö käigus konstrueeriti mutantsed pärmitüved, mis sisaldasid Yaf9 valgu eri piirkondade deletsioone. Yaf9 ja Swc4 valkude omavahelisi interaktsioone tuvastati immunopresipitatsiooni abil ning mutantseid fenotüüpe hinnati stressitundlikkuse analüüsiga, kasutades formamiidi. Tulemused näitasid, et Yaf9 C-terminaalse ning YEATS domeeni lõpus oleva piirkonna (aminohapped 160-168) deletsioonid on vajalikud interaktsiooniks Swc4-ga ja omasid stressitingimustes *yaf9*Δ tüvega sarnast fenotüüpi. Töö tulemustest selgus, et Yaf9 on funktsioonaalne vaid SWR1 ja NuA4 komplekside koosseisus. Antud töös tuvastati piirkonnad Yaf9 valgus, mis on olulised Swc4-ga seostumiseks ning aitavad paremini mõista selle valgu funktsiooni. Lisaks annavad töös saadud andmed väärtusliku lähtepunkti homoloogsete valkude uurimiseks kõrgemates eukarüootides.

Võtmesõnad:

Yaf9, Swc4, YEATS domeeni sisaldavad valgud, *Saccharomyces cerevisiae*

CERCS: T490 Biotehnoloogia

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TERMS, ABBREVIATIONS AND NOTATIONS

Co-IP – co-immunoprecipitation

DDT – dichlorodiphenyltrichloroethane

DMSO – dimethyl sulfoxide

EDTA – ethylenediaminetetraacetic acid

HAT – histone acetyltransferase

HDAT – histone deacetyl transferase

LB – lysogeny broth

PCR – polymerase chain reaction

PTM – posttranslational modification

rpm – revolutions per minute

SC – synthetic complete

SDS – sodium dodecyl sulfate

TBS – tris-buffered saline

TE – tris, EDTA

WT – wild type

YPD – yeast extract peptone dextrose

INTRODUCTION

Eukaryotic genomes are organised into a complex called chromatin, the basic unit of which is a nucleosome, comprised of ~146 base pairs of DNA wrapped around a histone protein octamer. At its most condensed form in mitosis, chromatin forms the chromosome. The change in chromatin structure is also an epigenetic regulator of gene expression, as the lightly packed euchromatin is mostly accessible for transcription, whereas heterochromatin is not. The structure of chromatin can be regulated by posttranslational modification of histone tails, such as addition of methyl- and acetyl groups, ubiquitination, sumoylation and others.

Proteins recognising these modifications are known as reader proteins. One of the less studied reader protein domains is the YEATS domain, which, among other less characterised roles, recognises acetylated and crotonylated lysine residues. YEATS domain is highly conserved and can be found in organisms ranging from fungi to humans. YEATS domain proteins in humans, such as ENL, AF9 and Gas41, have been linked to cancers and pathologies.

Due to the conserved nature of these proteins, *Saccharomyces cerevisiae* can be used as a model for studying them, and the findings can be applied to similar proteins in higher organisms, especially concerning their role in disease. There are three YEATS domain-containing proteins in *S. cerevisiae* – Taf14, Sas5 and Yaf9.

This work aims to investigate the interaction between *S. cerevisiae* YEATS domain protein Yaf9 and its interaction target Swc4. Both proteins are a part of two chromatin remodelling complexes in yeast, SWR1 and NuA4, and in both complexes they have been shown to directly interact with one another. Several Yaf9 mutants with deletions of different parts of the gene were constructed, and a co-immunoprecipitation assay was performed to identify the regions important for the protein interaction. The obtained mutant strains were also phenotyped by carrying out a stress resistance assay. The findings of this work shed light on the specific regions of Yaf9 necessary for its interaction with Swc4, as well as help determine if Yaf9 has any function independently of its role in protein complexes.

1 LITERATURE REVIEW

1.1 BAKER'S YEAST *SACCHAROMYCES CEREVISIAE*

Saccharomyces cerevisiae, also known as baker's yeast, is a unicellular fungus and one of the most widely used eukaryotic model organisms (Karathia et al., 2011). Some of the reasons for this are its simple cultivation conditions, rapid growth and ease of genetic modification (Johnston, 1996). In 1996, *S. cerevisiae* strain S288C was the first eukaryote to have its genome completely sequenced. It showed that the yeast's genome is made up of approximately 6000 genes organised in 16 chromosomes (Goffeau et al., 1996). When the genomes of other organisms, such as mouse and human, were sequenced a few years later, new opportunities arose for using yeast as a model for studying higher organisms (Mouse Genome Sequencing Consortium, 2002; Venter et al., 2001). The average genome-wide amino acid identity between baker's yeast and human is 32% (Kachroo et al., 2015), and there are 1953 protein ortholog groups between the two species [data from InParanoid] (Persson & Sonnhammer, 2023). Moreover, a study surveying 414 essential *S. cerevisiae* genes has shown that about half of them can be successfully replaced by their human orthologs (Kachroo et al., 2015).

1.2 CHROMATIN

There are several challenges to fitting the large eukaryotic genomes into cell nuclei. One of the most obvious is that the length of DNA molecules far exceeds the length of eukaryotic cells (Petes et al., 1973). For example, each human cell contains approximately 2 meters of DNA, which must be compacted enough to fit in its ~10 μm nucleus (Crapo et al., 1982). And yet, this compacted DNA still must remain accessible to cellular processes such as transcription, replication, DNA repair and meiotic recombination (Rossetto et al., 2012). DNA is negatively charged due to its phosphate backbone, thus another obstacle to DNA compaction is the DNA-DNA electrostatic repulsion (Dueva et al., 2019). To overcome these challenges, a complex called chromatin, containing by weight nearly equal amounts of DNA and protein, is formed, and goes through several levels of precise packing to form the chromosome (Kornberg, 1974).

Basic, positively charged proteins called histones are involved in the first level of DNA packing (Dueva et al., 2019). Four core histones – H2A, H2B, H3, and H4 – each repeated twice, make up the histone octamer (Kornberg, 1974). About 146 bp of DNA wrap around the histone octamer in 1,65 turns of a left-handed superhelix to form the nucleosome core (Luger et al., 1997). Each histone protein consists of a globular domain and disordered amino- and carboxyl-terminal histone tails that protrude from the nucleosome core (see Figure 1) (Lorch et al., 2023). The nucleosome core functions as a repressor that prevents all transcription except that which

is induced by positive regulatory mechanisms (Lorch et al., 2023). The DNA between nucleosome cores is called linker DNA (Huang et al., 2018). A fifth histone, H1, is not a part of the histone octamer, but it binds linker DNA and therefore is called linker histone (Kornberg, 1974). The nucleosome core with ~165 bp of DNA together with the linker histone is called the chromatosome, and the chromatosome with the additional linker DNA makes up a nucleosome (Simpson, 1978). Colloquially, and sometimes in educational literature, the nucleosome core is often referred to as the nucleosome (McGinty & Tan, 2015; Reece et al., 2014). Nucleosomes are a part of the next level of chromatin structure. They make up a fibre 10-11 nm in diameter that has also been termed "beads on a string" due to its appearance, the linker DNA being the "string" and nucleosome cores being the "beads" (Reece et al., 2014).

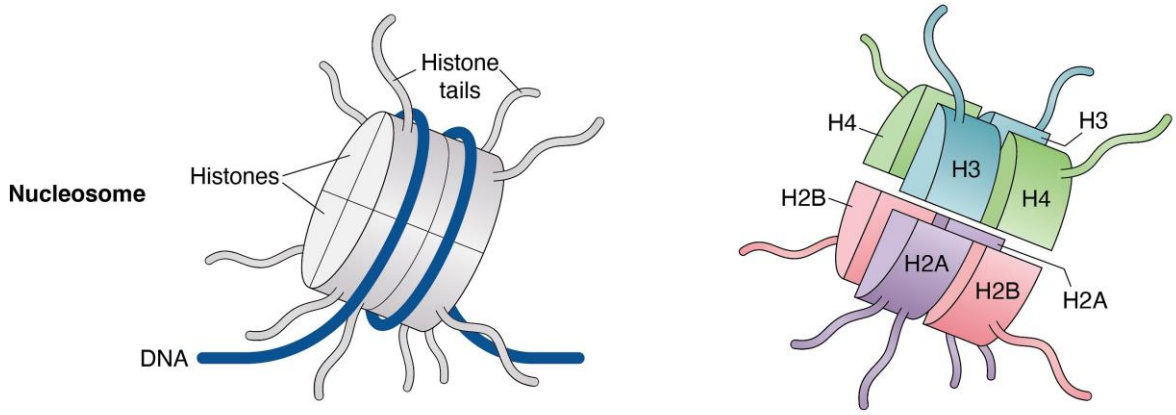

Figure 1. The structure of the histone octamer. Each histone (H2A, H2B, H3 and H4) is repeated twice with H2A-H2B and H3-H4 forming heterodimeric pairs. Histone tails protrude from the nucleosome. Figure adapted from Hegazy et al., 2025.

The next level of chromatin organisation is formed when the extended "beads on a string" formation chromatin folds or coils, creating the 30 nm fibre (see Figure 2). The 30 nm fibre further organizes into looped domains, which are anchored to a protein-based chromosome scaffold, forming a 300-nm fibre. Further coiling produces a chromosome, with the width of one chromatid being 700 nm (Reece et al., 2014).

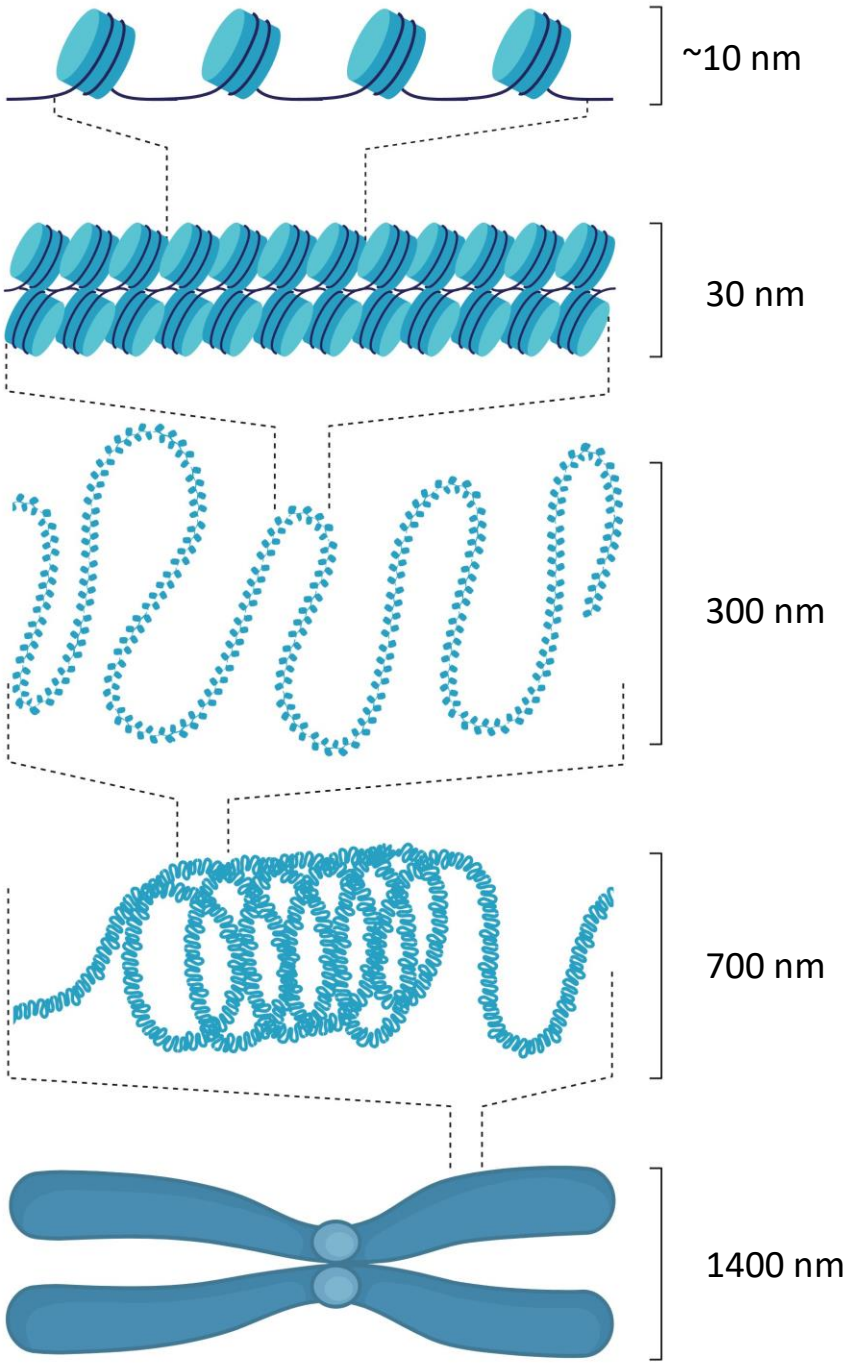

Figure 2. The structure of chromatin. DNA wraps around the histone octamer to form the 10 nm "beads on a string" structure. The "beads on a string" fold and coil to form the 30 nm fibre. It then creates loops which make up the 300 nm fibre. Further condensation creates the mitotic chromosome structure. Figure created in biorender.com, adapted from Jansen & Verstrepen, 2011.

Chromatin goes through the aforementioned changes during different phases of the cell cycle. The condensed chromosome as shown in Figure 2 can be seen during cell division in metaphase. In interphase, chromatin is much less condensed; however, some levels of organisation are still

observed. Some of the chromatin appears to be in the unfolded 10 nm "beads on a string" formation, while some is present in a more compact state, the 30 nm fibre, which in some regions can further be condensed to form the looped structures. This condensed chromatin is termed heterochromatin, and the unfolded form – euchromatin (Reece et al., 2014). This difference in chromatin organisation can be observed because modifying the level of chromatin condensation affects gene expression. The DNA in euchromatin is mostly open for transcription machinery, whereas heterochromatin is largely inaccessible (Morrison & Thakur, 2021).

1.3 HISTONE MODIFICATIONS

As described previously, chromatin is a rapidly changing structure, and the interactions between DNA and proteins are not permanent. Posttranslational modifications (PTMs) of histone protein tails are a way of dynamically regulating the structure of chromatin and can either promote or inhibit transcription epigenetically (Lorch et al., 2023). The accessible tails of histones are subject to covalent modifications, notable examples of which are acetylation and methylation.

1.3.1 Histone acetylation

Histone acetylation was first identified in the 1960s, and it involves adding acetyl groups to lysine residues of the protruding histone tails (Allfrey et al., 1964). This neutralises the positive charge of the lysine residues, weakening the interaction between histones and DNA, which loosens up the chromatin (Bannister & Kouzarides, 2011). Generally, acetylation is correlated to transcription activation, whereas deacetylation is correlated to transcription repression (Brownell et al., 1996). Acetylation is regulated by two opposite families of enzymes – histone acetyltransferases (HATs) and histone deacetylases (HDATs) (Bannister & Kouzarides, 2011). In the acetylation reaction, HATs add an acetyl group to the lysine residue using acetyl coenzyme A as a cofactor (see Figure 3A). Histone deacetylases remove the acetyl group from histone tails, releasing acetate as a byproduct.

Another relatively recently discovered lysine acylation is crotonylation. Similarly to acetylation, it neutralizes positive charge but uses crotonyl coenzyme A as a cofactor. Both can be recognized by chromatin reader domains (Tan et al., 2011).

1.3.2 Histone methylation

Histone methylation occurs by adding a methyl group to the side chains of lysines and arginines, however, unlike acetylation, it does not change the charge of the histone protein (Bannister & Kouzarides, 2011). In *S. cerevisiae*, histone methylation is found in histones H3 and H4 (Garcia et al., 2007). Methylation at lysines and arginines may be in one of the different forms: mono-, di-, or trimethyl for lysines and mono- or di- (asymmetric or symmetric) for arginines

(Kouzarides, 2007). Methylation is correlated with either activation or repression depending on the site and degree of methylation. Thus, it is important which residue exactly is methylated, as the effect does not rely on the presence of a methyl group alone. Generally, methylations of H3 at lysine 4, 36 and 79 are implicated in transcription activation, whereas methylations of H3 at lysine 9 and 27 and H4 at lysine 20 are associated with transcription repression (see Figure 3B) (Kouzarides, 2007).

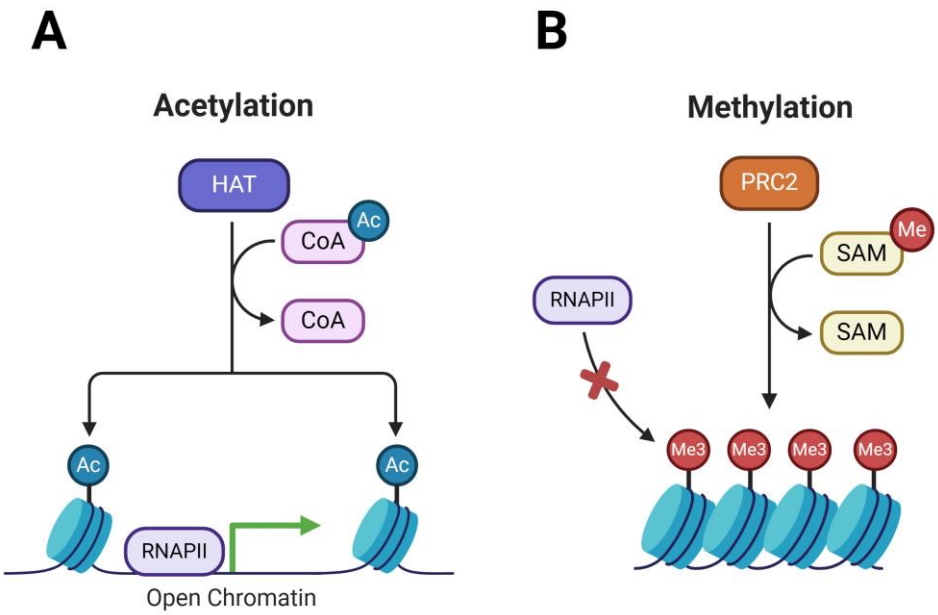

Figure 3. Examples of the mode-of-action of A) acetylation and B) methylation processes. B) shows transcription repression by trimethylation of histone H3 at lysine 27 (H3K27) in humans by Polycomb Repressive Complex 2 (PRC2) (Shi et al., 2017). This complex is absent in *S. cerevisiae*. Figure created in biorender.com.

Methylation is maintained by histone methyltransferases and demethylases which have a higher site specificity than HATs and HDATs. Three methyltransferases have been well characterised in baker's yeast: Set1, Set2 and Dot1 (Briggs et al., 2001; Fingerman et al., 2007; Strahl et al., 2002). Up until the beginning of this century it was thought that methylation is an irreversible modification, however, several demethylases have been identified since (Kim & Buratowski, 2007; Ryu & Ahn, 2014).

1.3.3 Other posttranslational modifications

Though methylation and acetylation are the most familiar PTMs, other modifications have been discovered and are also present in *S. cerevisiae*. All four core histone tails contain sites which can be phosphorylated. Histone phosphorylation occurs on serine, threonine and tyrosine residues and is carried out by protein kinases, but dephosphorylation – by phosphatases. Histone

phosphorylation is implicated in DNA damage response and gene expression (Rossetto et al., 2012). Another important PTM is ubiquitination at H2BK123. It has been found that this modification functions as a signal for methylation of H3K4 and H3K79 (Dover et al., 2002; Lee et al., 2007). However, methylation of H3K4 and H3K79 is not solely dependent on this, as mutants unable to be ubiquitinated are still shown to possess these PTMs (Foster & Downs, 2009). All four core histones can also be sumoylated (Nathan et al., 2006).

1.3.4 Histone variants

The core histones that make up the histone octamer can be replaced by histone variants. In baker's yeast, there are two histone variants – Htz1 (also termed H2A.Z in mammals, H2A.F in birds and H2A.F/Z in sea urchin), which is a variant of the canonical H2A, and Cse4 (Cenp-A in mammals), a variant of the canonical H3 (Pusarla & Bhargava, 2005). They differ from their canonical counterparts in both sequence and functions. For instance, H2.AZ (Htz1) has only a 60% sequence identity compared to canonical H2A (Kawano et al., 2011).

Canonical histones are produced in the S phase of the cell cycle and are incorporated in the DNA in a replication dependent manner; however, histone variants are synthesized throughout the cell cycle and are incorporated in chromatin independently of DNA replication (Kawano et al., 2011). Histone variants are also specifically located. For example, Cse4 is a centromere-specific histone variant that is involved in proper kinetochore formation and chromosome segregation (Camahort et al., 2009). It is believed that Cse4 is recruited to the centromere by a protein called Scm3 (Camahort et al., 2007).

The functions of Htz1 include specifying the euchromatic regions of the genome, controlling the spread of heterochromatin and preventing gene silencing (Kobor et al., 2004). H2A replacement with Htz1 is carried out by a protein complex called SWR1 (Mizuguchi et al., 2004).

1.4 READERS OF HISTONE MODIFICATIONS

The execution of chromatin modifications involves recruitment of various proteins, which can be broadly categorized into three groups: writers, which add modifications, erasers, which remove them, and readers, which recognize existing modifications and can recruit additional proteins or protein complexes to further modify the chromatin. Reader proteins contain evolutionarily conserved PTM recognizing domains (Yun et al., 2011). Some of the best studied reader domains include bromodomains, double PHD finger domains and YEATS domains, which, among other modifications, recognise acetylated lysine residues (Dhalluin et al., 1999; Lange et al., 2008; Shanle et al., 2015).

1.4.1 YEATS domain proteins

The YEATS domain is one of the lesser studied reader protein domains. Its name is derived from the first proteins in which it was discovered – Yaf9, ENL, AF9, Taf14, Sas5 (Le Masson et al., 2003). YEATS domain binds to acetylated and crotonylated lysine residues (Li et al., 2014, 2016).

YEATS domain proteins in humans have been implicated in several types of cancers. For example, the transcriptional activator ENL, a YEATS domain protein, is essential for leukaemia cell survival but not for normal hematopoietic stem and progenitor cells, suggesting a possibility for treatment targeting ENL, as it binds disproportionately to promoters of leukaemia related genes (Erb et al., 2017). Recently, a study has identified ENL and AF9 YEATS domain binding probe and showed that inhibiting the ENL YEATS domain disrupts ENL-dependent transcription and leukaemia growth, confirming its relevance in disease (Garnar-Wortzel et al., 2021).

Another human YEATS protein Gas41, is frequently overexpressed in cancer cells. Gas41 and *S. cerevisiae* protein Yaf9 are the most similar YEATS family proteins in terms of size, YEATS domain and C-terminal domain structure (Le Masson et al., 2003). Gas41 is often amplified in gliomas (Fischer et al., 1996). A study shows that a deletion of the Gas41 C-terminal coiled-coil and knockdown of Gas41 by siRNA induce activation of the p53 tumour suppressor pathway (Park & Roeder, 2006).

There are three YEATS proteins in *S. cerevisiae* – Taf14, Sas5 and Yaf9 (Le Masson et al., 2003). While they are nonessential individually, a deletion of all three is lethal (Zhang et al., 2004).

1.4.2 Yaf9

In *S. cerevisiae*, Yaf9 is a YEATS domain-containing subunit of two complexes involved in chromatin alteration. One is the 13-subunit-containing acetyltransferase complex NuA4 (nucleosome acetyltransferase of H4) that acetylates histones H4, H2A and H2A.Z (Htz1 in yeast) and regulates transcriptional and DNA repair program (Allard, 1999; Le Masson et al., 2003). The other is the aforementioned 14-subunit SWR1 (Mizuguchi et al., 2004). Yaf9 is a yeast homolog of the human protein AF9, hence its name (Yeast AF9) (Bittner et al., 2004; Zhang et al., 2004).

The protein is 226 amino acids long and its molecular weight is ~26 kDa (*Saccharomyces Genome Database*, 2025). Studies with *yaf9*Δ strains have shown that Yaf9 has a role in transcriptional regulation, histone acetylation and DNA repair (Klein et al., 2018). *YAF9* is

nonessential in yeast and *yaf9*Δ mutants show little to no growth inhibition at 30 ℃ (Le Masson et al., 2003). However, *yaf9*Δ strains show increased sensitivity to a number of stress inducing agents and growth conditions compared to the wild type strain. The *yaf9*Δ mutants grow slower than wild type at 16 ℃ and are more heat sensitive at 37 ℃ (Le Masson et al., 2003). They are also sensitive to fungicide benomyl, which acts by depolymerizing microtubules, and an ionizing solvent formamide (Aguilera, 1994; Ogbede et al., 2021).

NuA4 acetylation of lysine residues on histone tails enhances binding of SWR1 and H2A replacement with Htz1 (Kobor et al., 2004). As a part of its function in SWR1, Yaf9 recognizes and preferentially binds acetylated H3K27 through its YEATS domain, leading to histone replacement (Klein et al., 2018).

In both complexes, the direct interaction target for Yaf9 is a protein called Swc4. A study with an SWR1 complex bearing an Swc4 deletion also observed a complete loss of Yaf9 and Bdf1, another SWR1 subunit, indicating an association between them (Lin et al., 2017).

1.4.3 Swc4

SWR1 and NuA4 share four subunits, and Yaf9 and Swc4 are two of them (Liu et al., 2020). Swc4 is 476 amino acids long and its molecular weight is ~55 kDa (*Saccharomyces Genome Database*, 2025). It contains an N-terminal SANT domain, which is essential for its function, and a C-terminal domain through which it interacts with Yaf9 (Bittner et al., 2004; Zhou et al., 2010). Like Yaf9, it is also considered nonessential, however, unlike Yaf9, *swc4Δ* mutants show severe growth defects in normal growth conditions. Deletion of *SWC4* significantly reduces cell viability and reduces telomere length in viable cells; notably, its telomere length regulating function has been shown to be independent of its functions in SWR1 and NuA4 (Liu et al., 2020). Deletion strains also exhibit aneuploidy and chromosome segregation errors (Pan et al., 2023).

2 THE AIMS OF THE THESIS

The goal of this study was to investigate the association between YEATS-containing protein Yaf9 and its interaction target Swc4 and to discover whether Yaf9 functions independently from its associated complexes. For this the following aims were set:

- to construct different Yaf9 deletion mutant strains;
- to detect with co-immunoprecipitation (Co-IP) whether truncated Yaf9 proteins associate with Swc4;
- to phenotype the mutant strains by testing their sensitivity to stress.

3 EXPERIMENTAL PART

3.1 MATERIALS AND METHODS

3.1.1 Strains and plasmid

All but two *S. cerevisiae* strains used in this work were derived from strain W303. A list of all strains is given in the appendix (Table S1).

Incubations of all yeast strains were done at 30 °C unless mentioned otherwise. Liquid cultures were incubated with shaking at 200 rpm.

The plasmid used in the work was pBlueScript-ADE2_YAF9_3xFLAG carrying an *ADE2* marker gene and 3xFLAG-tagged *YAF9*, obtained from laboratory collection.

3.1.2 Media

The media used in this work are given in Table 1.

Table 1. Growth media used in this work.

Medium	Components
YPD	Agar 20 g/l, yeast extract 10 g/l, peptone 20 g/l, glucose 20 g/l
LB	Agar 20 g/l, yeast extract 5 g/l, tryptone 10 g/l, sodium chloride (NaCl) 10 g/l
SC	Agar 20 g/l, YNB (yeast nitrogen base) 6,7 g/l, glucose 20 g/l, adenine 20 µg/ml, uracil 20 µg/ml, tryptophan 40 µg/ml, histidine 40 µg/ml, leucine 80 µg/ml, lysine 80 µg/ml
Amp	100 µg/ml of ampicillin added to LB medium
-ADE	Agar 20 g/l, YNB (yeast nitrogen base) 6,7 g/l, glucose 20 g/l, uracil 20 µg/ml, tryptophan 40 µg/ml, histidine 40 µg/ml, leucine 80 µg/ml, lysine 80 µg/ml
-TRP	Agar 20 g/l, YNB (yeast nitrogen base) 6,7 g/l, glucose 20 g/l, adenine 20 µg/ml, uracil 20 µg/ml, histidine 40 µg/ml, leucine 80 µg/ml, lysine 80 µg/ml
-ADE -TRP	Agar 20 g/l, YNB (yeast nitrogen base) 6,7 g/l, glucose 20 g/l, uracil 20 µg/ml, histidine 40 µg/ml, leucine 80 µg/ml, lysine 80 µg/ml
G418	2 µl/ml of G418 from 100 mg/ml stock added to YPD medium
-AA	Agar 20 g/l, YNB (yeast nitrogen base) 6,7 g/l, glucose 20 g/l

For liquid media agar was omitted.

3.1.3 Deletion mutation introduction

Four Yaf9 mutants were constructed in this work by introducing deletion mutations in different parts of the gene. These mutants were: Δling (119-141), Δ160-168 (helix at the end of YEATS domain), Δ169-185 (linker between helixes), Δ186-226 (C-terminal helix) (see Figure 4). Mutant Δ2-10 had been constructed in the laboratory previously following the same procedure as described in this work and was tested together with the other strains.

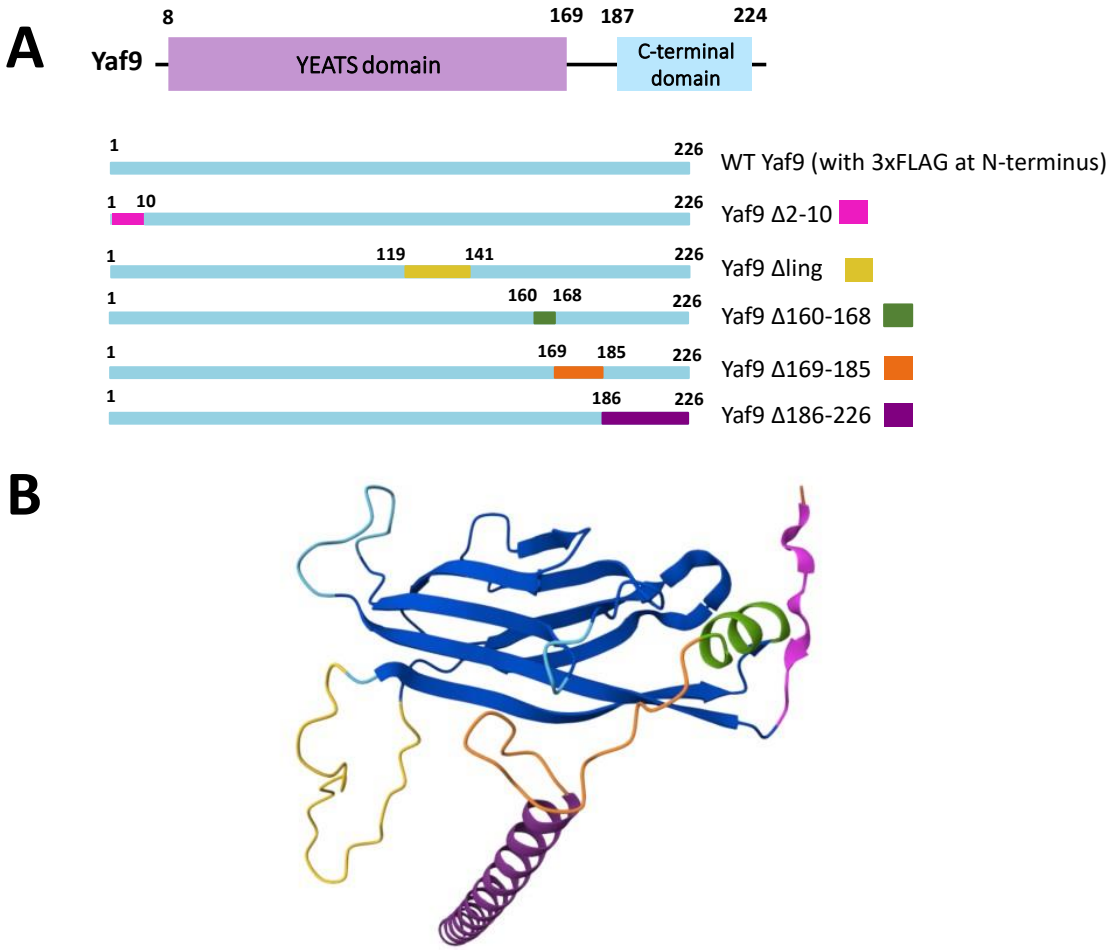

Figure 4. Overview of deletion mutations in the Yaf9 gene. A) Schematic representation of the gene with marked locations of introduced deletions. B) Corresponding 3D structure of the protein, with coloured regions matching the deletion sites in A). Structure from AlphaFold Protein Structure Database, alphafold.ebi.ac.uk. PDB ID: P53930.

Deletion mutations in the Yaf9 gene were introduced by overlap extension PCR. For each mutant, in total four PCR reactions were performed to obtain a plasmid carrying a truncated gene. In the first and second reaction, two fragments that flank the deletion site were synthesized (see Figure 5). Primers used for reactions 1 and 2 are given in Table 2.

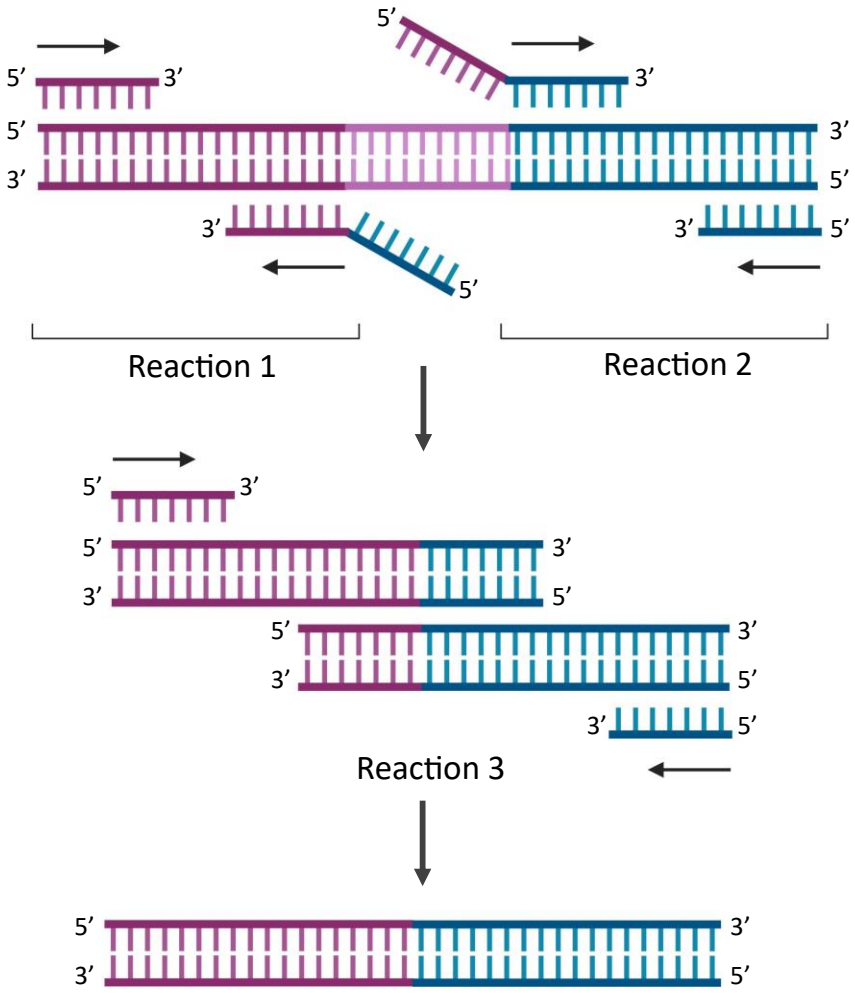

Figure 5. Overview of PCR reactions for truncating the *YAF9* gene. Figure created in biorender.com.

Table 2. Primer pairs used in reactions 1 and 2.

Deletion	Reaction 1	Reaction 2
Δ2-10	Yaf9_D2-10_R + Yaf9_prom_F	Yaf9_D2-10_F + Yaf9_term_R
Δling (119-141)	Yaf9_ling_del_R + Yaf9_prom_F	Yaf9_ling_del_F + Yaf9_term_R
Δ160-168	Yaf9_D160-168_R + Yaf9_prom_F	Yaf9_D160-168_F + Yaf9_term_R
Δ169-185	Yaf9_D169-185_R + Yaf9_prom_F	Yaf9_D169-185_F + Yaf9_term_R
Δ186-226	Yaf9_D186-226_R + Yaf9_prom_F	Yaf9_D186-226_F + Yaf9_term_R

All primer sequences are given in the appendix (Table S2). The reaction mix for the first two reactions are given in Table 3.

Table 3. Reaction mix for reactions 1 and 2.

Component	Volume
5x FIREPol PCR master mix	5 µl
pBlueScript-ADE2_YAF9_3xFLAG plasmid DNA	1 µl
Primer 1	1 µl
Primer 2	1 µl
H_2O	17 µl
Total	25 µl

In Reaction 3, both fragments flanking the deletion and containing complimentary overlaps are ligated and a single truncated fragment is obtained (see Figure 5). The reaction mix is given in Table 4.

Table 4. Reaction mix for reaction 3.

5x FIREPol PCR master mix	5 µl
Yaf9_prom_F	1 µl
Yaf9_term_R	1 µl
Reaction 1 DNA product (concentrations ranging from 276 to 296 ng/µl)	3 µl
Reaction 2 DNA product (concentrations ranging from 245 to 280 ng/µl)	3 µl
H_2O	12 µl
Total	25 µl

Amplification settings for reactions 1, 2 and 3 were: initial denaturation for 5 minutes at 95 °C, 30 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 58 °C, extension for 30 seconds at 72 °C, and a final extension for 7 minutes at 72 °C.

The final reaction was a whole plasmid PCR using the fragment obtained in the previous reaction as a primer. The reaction mix is given in Table 5.

Table 5. Reaction mix for whole plasmid PCR.

pBlueScript-ADE2_YAF9_3xFLAG plasmid DNA	2 µl
Reaction 3 DNA product (concentrations ranging from 380 to 1170 ng/µl)	10 µl
Buffer (5x Phusion Green HF Buffer, Thermo Scientific)	10 µl
dNTPs 2mM	5 µl
$MgCl_2$ 25 mM	5 µl
DMSO	1,5 µl
DNA polymerase (Phusion DNA Polymerase, 2 U/µl; Thermo Scientific)	0,5 µl
H_2O	16 µl
Total	50 µl

Amplification settings for whole plasmid PCR were: initial denaturation for 5 minutes at 98 °C, 18 cycles of denaturation for 30 seconds at 98 °C, annealing for 30 seconds at 58 °C, extension for 16 minutes at 68 °C, and a final extension for 16 minutes at 68 °C.

The PCR products were stored at -20 °C. PCR products of reactions 1, 2, 3 and other reactions where mentioned, were visualised using gel electrophoresis for which 1 % agarose gel was used. To prepare the gel, 1x TAE buffer (40 mM Tris-acetate; 1 mM EDTA) was mixed with agarose and heated until the agarose had dissolved. The solution was cooled, and 0,5 µg/ml of ethidium bromide were added. This was either used immediately or stored in an incubator at 65 °C until use. GeneRuler 1 kb DNA ladder (Thermo Scientific) was used as a marker for all gel electrophoreses. DNA from reactions 1, 2 and 3 was precipitated. To each PCR product 1/10 volume of sodium acetate and three volumes of ice cold 96 % ethanol were added. This mix was centrifuged for 15 minutes at maximum speed. After centrifugation, supernatant was

removed, and precipitate pellet was washed with three volumes of 70 % ethanol. This was centrifuged again at maximum speed for 5 minutes, supernatant was removed, and the DNA pellet was dried at 37 °C. When dried, pellet was resuspended in 50 µl of distilled H_2O. DNA concentration was determined using NanoDrop spectrophotometer (NanoDrop 1000 Spectrophotometer, Thermo Scientific). The concentrated and purified DNA was also stored at -20 °C.

3.1.4 Bacterial transformation

The plasmids constructed in the previous step were transformed to *Escherichia coli*. The day before the transformation, plasmid DNA was treated with DpnI. For this, 2 µl of DpnI (10 U/ul, Thermo Scientific) were added to 20 µl of PCR product from the previous PCR step and incubated at 37 °C overnight.

Before transformation, selective Amp plates were put in the 37 °C incubator.

Competent *E. coli* DH5α cells were thawed on ice. 100 µl of the cells were added to a 1,5 ml tube together with 10 µl of DpnI treated plasmid DNA. The mixture was incubated on ice for 10 minutes. After that, the suspension was heat shocked at 37 °C for one minute and cooled on ice. Then, 1 ml of LB medium was added, and the suspension was incubated at 37 °C with shaking for one hour to recover. It was then centrifuged for one minute at 5000 rpm and the supernatant was removed, leaving approximately 100 µl to resuspend the cells for plating. Cells were plated on Amp plates and incubated at 37 °C until visible colonies had grown.

3.1.5 Plasmid control

A control PCR was performed after the colonies had grown. Reaction components are given in Table 6.

Table 6. Reaction mix for control PCR after bacterial transformation.

5x FIREPol PCR master mix	2 µl
Yaf9-tag-cntr nr 1066_0	0,25 µl
Yaf9_term_R	0,25 µl
Yaf9-D160-168-cntr-R_0	
H_2O	7,5 µl
Total	10 µl

Yaf9-D160-168-cntr-R_0 was used as a reverse primer for Δling and Δ160-168 colony control, Yaf9_term_R was used for Δ169-185 and Δ186-226 control.

Cells from a bacterial colony were picked up with a sterile toothpick and added to the reaction mix. PCR was then carried out using the following settings: initial denaturation for 5 minutes at 95 °C, 30 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 58 °C, extension for 30 seconds at 72 °C, and a final extension for 7 minutes at 72 °C. PCR products were visualised using agarose gel electrophoresis as described previously.

After the plasmid purification step in 3.1.6, the DNA fragments were sequenced using Sanger sequencing at the Institute of Genomics Core Facility for additional confirmation.

3.1.6 Plasmid purification

Bacterial colonies which were proven by control PCR to contain correct plasmids were cultivated in 50 ml of LB medium with ampicillin (100 µg/ml) at 37 ℃ and 180 rpm overnight for plasmid purification. The culture was purified using NucleoBond Xtra Midi Kit following manufacturer's protocol. The extracted plasmid DNA was quantified using NanoDrop spectrophotometer and stored at -20 °C.

3.1.7 Yeast transformation

Yeast transformation was carried out using the lithium acetate (LiAC) method. On the day before, yeast strain AKY2732 was inoculated in 15 ml of liquid YPD media and grown overnight.

Plasmid linearisation was also performed before the transformation. A 30 µl reaction mix consisting of mutant plasmid, 3 µl, 10x Orange buffer, 3 µl, AflII/BspTI restriction enzyme (10 U/µL, Thermo Scientific), 2 µl and H_2O, 22 µl was prepared and incubated at 37 °C overnight.

The next day 1,5 ml of cell culture at optical density of ~ $1x10^7$ cells/ml were collected in 1,5 ml tubes by centrifuging for 2 minutes at 5000 rpm. Supernatant was removed and cells were resuspended in 500 µl of LiAc + TE (10 mM Tris-HCl, 1 mM EDTA-NaOH) solution (10 mM LiAc + 1x TE) and collected by centrifuging for 2 minutes at 5000 rpm. Supernatant was removed and cells were resuspended in 40 µl of LiAc + TE to which 7 µl of denatured carried DNA (denatured at 95 °C for 6 min, cooled on ice) were added. Next, 10 µl of the linearised plasmid DNA were added to the mixture. This was kept at room temperature for 5 minutes before adding 300 µl of LiAc + PEG4000 (10 mM LiAc + 1x TE + 40 % PEG4000). This was vortexed and incubated for 15 minutes at room temperature. Next, 15 µl of DMSO were added, mixture was vortexed and heat shocked at 42 °C for 10 minutes. Afterwards, cells were cooled

on ice for one minute and collected by centrifugation for 3 minutes at 3000 rpm. Supernatant was removed, and cell pellet was resuspended in 150 µl of sterile H_2O. Cells were plated on -ADE agar plates and incubated until colonies appeared.

3.1.8 Yeast colony control

To control for correct colonies after transformation, DNA was extracted from colonies which had grown on the selection plates.

For this, a small amount of yeast cells was added to a 1,5 ml tube containing 100 µl LiAc + SDS solution (0,2 M LiAc + 1 % SDS). It was vortexed and incubated at 70 °C for 10 minutes. Then, 300 µl of 96 % EtOH were added, and tube was vortexed. Cells were collected by centrifugation at 13000 rpm for 5 minutes. Supernatant was removed and the cell pellet was dried at 42 °C for approximately 20 minutes. When dried, pellet was suspended in 100 µl of TE, short-spun down and the supernatant was used for PCR. The reaction mix is given in Table 7.

Table 7. Reaction mix for yeast colony PCR.

5x FIREPol PCR master mix	2 µl
Ade2-cut-ctrl1-AFI1-F	0,25 µl
Ade2-cut-ctrl2-RGA1-R	0,25 µl
Extracted DNA	1 µl
H_2O	6,5 µl
Total	10 µl

PCR was carried out using the following settings: initial denaturation for 5 minutes at 95 °C, 30 cycles of denaturation for 30 seconds at 95 °C, annealing for 30 seconds at 58 °C, extension for 30 seconds at 72 °C, and a final extension for 7 minutes at 72 °C. PCR products were visualised using agarose gel electrophoresis as described previously.

The correct colonies were tested further by Western blot analysis. The colonies proven by Western blot to express correct Yaf9 protein were streaked on YPD plates and incubated. After colonies had grown, stocks were prepared in 50 % glycerol and stored at -80 °C.

3.1.9 Sample and gel preparation for Western blot

To prepare samples for Western blot analysis, yeast strains were inoculated in 10 ml of liquid YPD medium and grown overnight.

The next day 1 ml of ~1x10^7 cells/ml culture was added to 1,5 ml tubes and cells were collected by centrifuging at 6000 rpm for one minute and supernatant was removed. Cells were then washed once in 1 ml of ice-cold water and afterwards suspended in 1 ml of ice-cold 2M LiAc and incubated on ice for 5 minutes. Centrifugation step was repeated, and cells were suspended in 1 ml of 400mM sodium hydroxide (NaOH) and incubated on ice for 5 minutes. Centrifugation step was repeated, and cells were suspended in 50-150 µl of 2xSDS loading buffer. The suspension was incubated at 95 °C for 5 minutes. Then it was vortexed and centrifuged for 3 minutes at 13000 rpm. The supernatant was collected in a different tube and stored at -20 °C until use.

The components for SDS-polyacrylamide gels are given in Table 8.

Table 8. Composition of SDS-polyacrylamide gels for Western blot analysis.

	12 % separating gel	**5 % stacking gel**
H_2O	4,9 ml	2,7 ml
30 % acrylamide mix	6 ml	670 µl
1,5 M Tris (pH 8,8)	3,8 ml	-
1,5 M Tris (pH 6,8)	-	500 µl
10 % SDS	150 µl	40 µl
10% NaPs (sodium persulfate)	150 µl	40 µl
TEMED (tetramethylethylenediamine)	6 µl	4 µl
Total	15 ml	4 ml

3.1.10 Western blot

The gels were assembled in the Bio-Rad Mini-PROTEAN Tetra Cell and 1xSDS running buffer (0,5 % SDS; 0,125M Tris-HCl pH 6,8; 0,96 M glycine) was added into the tank. 4 µl of protein ladder (Precision Plus Protein Dual Color Standards, Bio-Rad) were loaded in the first well and

10 µl of protein samples in the remaining wells. The electrophoresis was run at 120 V during the stacking gel part and at 170 V during the separating gel.

For protein transfer from gel to blotting membrane, the stacking gel was removed, and the separating gel was soaked in transfer buffer (48 mM Tris; 39 mM glycine; 0,037 % SDS; 20 % methanol) for approximately 15 minutes. The blotting membrane (PVDF transfer membrane, 0,2 µm, Thermo Scientific) was first soaked in methanol and then also soaked in transfer buffer for approximately 15 minutes. Whatman cellulose filter papers were also briefly soaked in transfer buffer. Proteins were transferred from gel to membrane using Bio-Rad Trans-blot SD semi-dry transfer cell. The filter papers, gel and membrane were assembled, and the transfer was run at 15 V for one hour.

After the transfer, the gel was immersed in Coomassie stain and stained for approximately 20 minutes on the rocker. Then it was washed with destaining solution (50 % methanol, 10 % acetic acid, 40 % H_2O) until the background was clear. Meanwhile, the membrane was covered with blocking buffer (5 % milk solution in 1xTBS+0,05 % Tween20) for approximately one hour with gentle rocking. Blocking prevents non-specific binding of antibodies which are used later. Then, the primary antibody (either ANTI-FLAG M2, Sigma-Aldrich, or Mouse mAb to BPV type 1 E2 protein, Icosagen) was added to the blocking buffer in 1:10000 dilution. Membrane was covered with primary antibody in blocking buffer and incubated at 4 °C with gentle rocking overnight.

The next day the membrane was washed for 5 minutes in 1xTBS+0,05 % Tween20 three times. Then it was transferred to blocking buffer with secondary antibody (Goat anti-Mouse IgG (H+L) Secondary Antibody, HRP, Invitrogen) added in 1:10000 dilution and incubated at room temperature for one hour on the rocker. Washing steps were then repeated as described before.

For protein visualization, 1,8 ml of ECL solution (Immobilon Western Chemiluminescent HRP Substrate, Merck) were prepared at a 1:1 ratio and added to the blot. It was incubated for approximately 5 minutes, dried and then visualized using the Bio-Rad ChemiDoc XRS+ System and Image Lab 6.0 program.

3.1.11 Co-immunoprecipitation

Co-immunoprecipitation (Co-IP) was performed in order to check for association between the truncated Yaf9 proteins and Swc4. Four buffers were prepared for the assay. Buffers and their components are given in Table 9.

Table 9. Co-immunoprecipitation buffers prepared for 8 samples.

2x buffer G	**Lysis buffer**
5 ml 1M HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) buffer (pH 7,5)	10 ml 2x buffer G
3 ml 5M NaCl	0,5 ml 20 % Triton X-100
0,2 ml 0,5M EDTA	4 ml 50 % glycerol
Distilled H_2O for a total volume of 50 ml	10 µl 1M DDT
	200 µl 100x protease inhibitor cocktail (cOmplete, Mini Protease Inhibitor Cocktail, Roche)
	Distilled H_2O for a total volume of 20 ml
Wash buffer 1 (WB1)	**Wash buffer 2 (WB2)**
12,5 ml 2x buffer G	5 ml 2x buffer G
0,625 ml 20 % Triton X-100	1 ml 50 % glycerol
2,5 ml 50 % glycerol	Distilled H_2O for a total volume of 10 ml
12,5 µl 1M DDT	
Distilled H_2O for a total volume of 25 ml	

Yeast strains were grown in 50 ml of YPD medium overnight. The cultures were transferred to 50 ml tubes, centrifuged at 3000 rpm for 3 minutes to collect the cells. Supernatant was removed and cells were washed with distilled H_2O. The cell pellets were resuspended in 1 ml of ice-cold lysis buffer and divided between two 1,5 ml tubes. Glass beads were added, and tubes were shaken at 4 °C for 15 minutes to lyse the cells. The lysates were then transferred to new tubes and centrifuged at 13000 rpm for 5 minutes, supernatants were transferred new tubes and 1,5 µl of antibody (Mouse mAb to BPV type 1 E2 protein, Icosagen) were added to each tube. These were put on a rocker at 4 °C for two hours.

Next, 20 µl of ProteinA Sepharose Fast Flow beads (GE Healthcare Bio-Sciences AB) per sample were added to 1,5 ml tubes and centrifuged at 5000 rpm for one minute, supernatant was removed. Beads were then washed with lysis buffer five times: 1 ml of buffer was added,

centrifuged, supernatant removed. After washing, the beads were suspended in 45 µl of lysis buffer per sample and this mixture was divided to eight 1,5 ml tubes (65 µl of beads + buffer per tube). The cell lysates that were previously incubated together with the antibody were added to the beads + buffer and incubated on the rocker at 4 °C for one more hour.

Afterwards, the cell lysate and bead suspensions were centrifuged at 5000 rpm for one minute. Supernatant was removed and 1 ml of WB1 was added to wash the beads. This was centrifuged, supernatant was removed and the wash with WB1 was repeated two more times. The last wash in the same manner was done using WB2. Supernatant was removed and 30 µl of 2x SDS buffer were added to the beads. This was incubated at 95 °C for 5 minutes and afterwards vortexed for 10 seconds. Lastly, tubes were centrifuged at 5000 rpm for one minute, supernatant was transferred to new tubes for storage. These samples were stored at -20 °C and later analysed through a Western blot. In Western blot, ANTI-FLAG M2 antibody (Sigma-Aldrich) was used for detection.

3.1.12 Yeast crossing and spore dissection

To remove the wild type *YAF9* copy from the genome, a cross with a *yaf9*Δ strain was performed to segregate the wild type *YAF9* gene. This method was chosen over transformation to minimize the risk of unintended mutations and off-target effects.

Strains AKY2736, AKY2779, AKY2780 and AKY2781 were crossed with strain AKY1820 on YPD agar plates and incubated to allow conjugation and diploid formation. After visible growth could be observed, the YPD plates were replica plated on -ADE -TRP plates to select for the diploid strains and incubated. After diploids had grown, they were streaked on sporulation plates and incubated until spores appeared.

The spores were treated with zymolyase (5 U/µl, ZYMO RESEARCH) to degrade the spore wall. After treatment, the solution was dropped onto a YPD plate and tetrad dissection was performed using Singer Instruments MSM 400 tetrad dissection microscope. Plates were incubated until visible colonies had grown. They were then replica plated on YPD, -ADE, -TRP and G418 plates to determine colonies which carried the correct markers. The correct colonies (growing on all replica plates) were then crossed on YPD plates with strains DC14 (*MAT*a) and DC17 (*MAT*α) to determine their mating type. After grown, these plates were replica plated on -AA plates and incubated to select for the colonies that had mated and created diploids. Thus, the mating type of each colony is opposite the mating type with which it had mated and created a diploid.

A colony of each mating type for each new strain was chosen and streaked on YPD and incubated. When grown, a Western blot was performed to confirm mutant Yaf9 expression protein and size. After confirmation, stocks were prepared in 50 % glycerol and stored at -80 °C.

3.1.13 Spot test assay

A spot test assay was performed to observe cell growth under formamide stress. Formamide is an ionizing solvent that destabilizes noncovalent bonds such as hydrogen bonds within DNA, and sensitivity to it is a conditional phenotype in yeast (Aguilera, 1994).

For this, small amounts of cells were taken with a sterile toothpick and added to 1,5 ml tubes containing 400 µl of sterile distilled water. The cell suspensions were vortexed, and optical densities were measured using NanoDrop spectrophotometer. Then the suspensions were adjusted to cell density of 10000 cells/µl and were used further to carry out tenfold serial dilutions. 5 µl from each dilution were plated onto solid SC media with and without 2,5 % formamide added. Plates were incubated for at least two days until visible colonies had formed. Results were visualised with EPSON Perfection 1200 (Seiko Epson Corporation).

3.2 RESULTS

3.2.1 Yaf9 mutant yeast strain construction

In *Saccharomyces cerevisiae*, Yaf9 is part of two complexes – SWR1 and NuA4 (Zhang et al., 2004). Studies have shown that Yaf9 is associated with the complexes through interaction with another subunit, Swc4 (Lin et al., 2017; Ji et al., 2022). One of the aims of the study was to analyse the interaction between Yaf9 and Swc4 proteins. To achieve this, various Yaf9 deletion mutant strains were constructed to determine which regions of the protein are important for their interaction. The deletions in *YAF9* gene were introduced by overlap extension PCR, in which the first steps were two PCRs to amplify the fragments flanking the desired deletions, termed Reactions 1 and 2 in subsection 3.1.3. The products of these reactions are visualised in Figure 6.

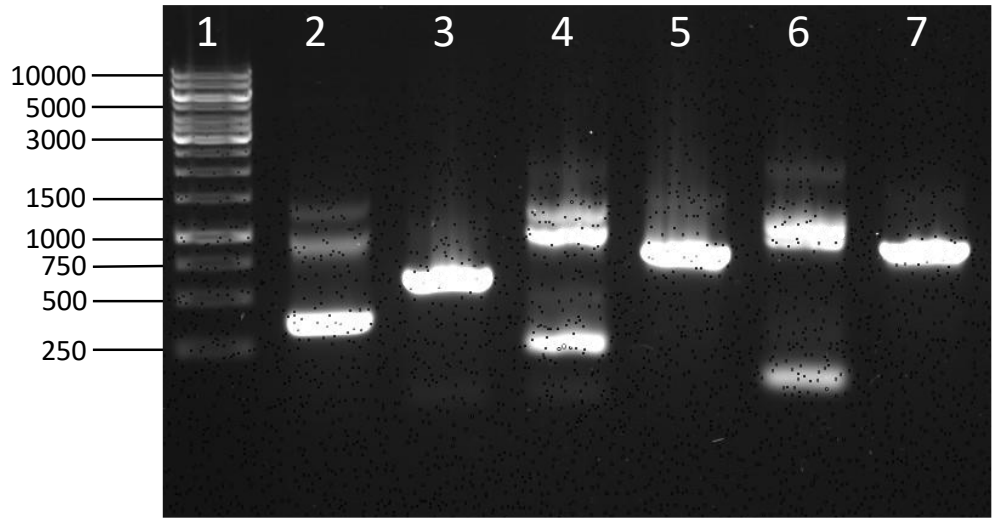

Figure 6. Gel electrophoresis image of PCR-amplified *YAF9* fragments flanking the deletion sites. Lane 1 contains GeneRuler 1 kb DNA ladder (Thermo Scientific), lane 2 – fragment downstream of Δling (expected size 338 bp), lane 3 – fragment upstream of Δling (exp. size 571 bp), lane 4 – fragment downstream of Δ169-185 (exp. size 207 bp), lane 5 – fragment upstream of Δ169-185 (exp. size 721 bp), lane 6 – fragment downstream of Δ186-226 (exp. size 83 bp), lane 7 – fragment upstream of Δ186-226 (exp. size 772 bp).

The obtained PCR products corresponded to their expected sizes and were used in the third PCR, which was to ligate the fragments flanking the deletions. The products of this reaction were expected to be about 850-950 bp in length depending on the size of the deletion and were used as primers for the whole plasmid PCR (see Figure 7).

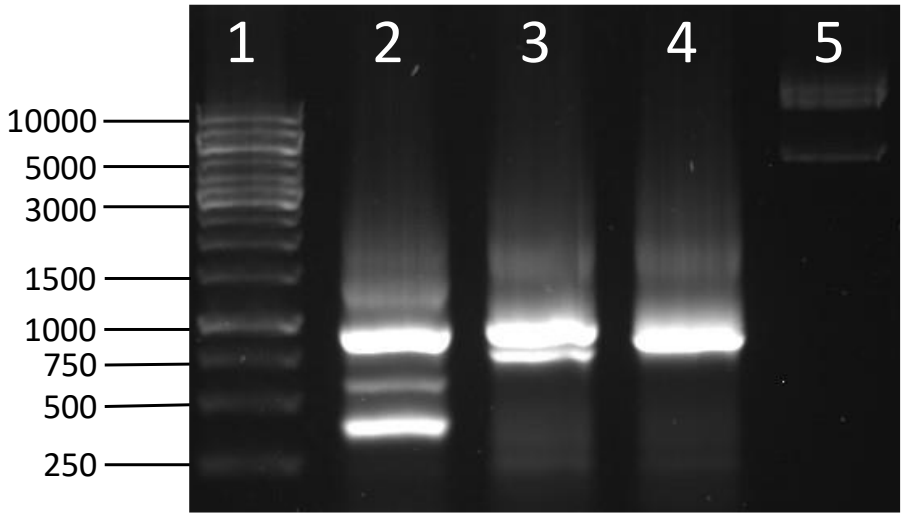

Figure 7. Gel electrophoresis image of *YAF9* truncation primers constructed for whole plasmid PCR. Lane 1 contains GeneRuler 1 kb DNA ladder (Thermo Scientific), lane 2 – Δling primer, lane 3 – Δ169-185, lane 4 – Δ186-226, but lane 5 contains pBlueScript-ADE2_YAF9_3xFLAG plasmid DNA as a control. Δ160-168 not pictured.

After whole plasmid PCR, the products were treated with DpnI restriction enzyme overnight to digest the template DNA. Then they were transformed in *E. coli*. Positive colonies were controlled by PCR (see Figure 8). Expected size of amplified fragments for Δling was 213 bp, for Δ160-168 – 255 bp, for Δ169-185 – 464 bp, but for Δ186-226 – 392 bp.

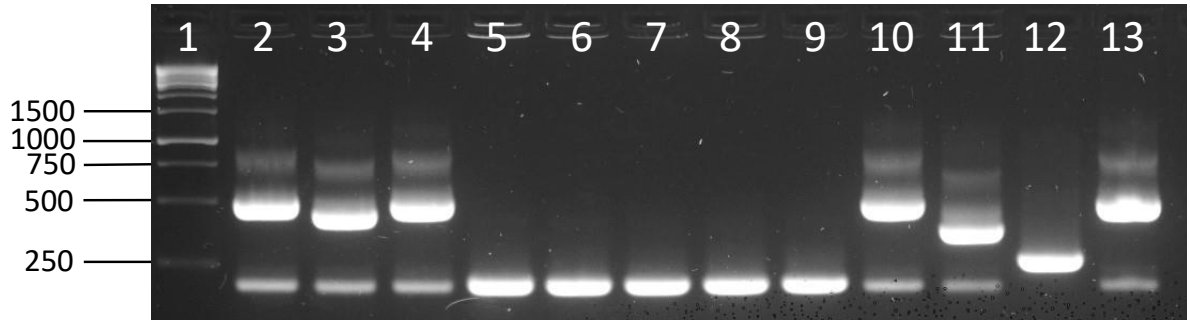

Figure 8. Gel electrophoresis image of bacterial colony control PCR. Lane 1 contains GeneRuler 1 kb DNA ladder (Thermo Scientific), lanes 2-4 – Δ169-185, lanes 5-9 – Δling, lanes 10 and 11 – Δ186-226. Lane 12 contains a control for Δling (a fragment of WT gene amplified without deletion), but lane 13 – control for Δ169-185 and Δ186-226. Δ160-168 not pictured.

Based on the expected fragment size, colonies from lanes 3, 5 and 11 were chosen to be cultivated as a liquid culture to extract and purify plasmid DNA. Mutant Δ160-168 also had one positive colony, this was also cultivated, and plasmid DNA was extracted and purified. The plasmids were then controlled by sequencing. All of the plasmids contained correct deletions and could be transformed in yeast. Before transformation, the plasmid was linearised with AflII restriction enzyme. This way, through homologous recombination, the yeast replaces the

endogenous *ADE2* gene with the *ADE2* plasmid containing *YAF9* with various deletions. To verify whether the transformation was successful, the cells were plated on -ADE selective medium, where only cells containing the *ADE2* gene are able to grow. Colonies were also controlled with PCR and the correct ones were chosen for Western blot analysis to confirm whether the Yaf9 proteins carrying the deletions are expressed. The samples were separated on a 12 % SDS–polyacrylamide gel, transferred to a PVDF membrane, incubated with the primary antibody ANTI-FLAG M2 (Sigma-Aldrich F3165) and the secondary antibody Goat anti-Mouse IgG HRP (Invitrogen), and visualized (see Figure 9). Strain AKY2727 in lane 2, used as a control, carries a WT Yaf9 protein with a 3xFLAG tag, and its expected molecular weight with the tag is ~ 28,7 kDa (*Saccharomyces Genome Database*, 2025). Strain AKY2732 in lane 3 has a 3x1E2 tagged Swc4 protein, but no tag on Yaf9, strain AKY2736 in lane 4 carries proteins with both tags. All of the truncated proteins were also expressed, and the molecular weights reflected the sizes of the deletions.

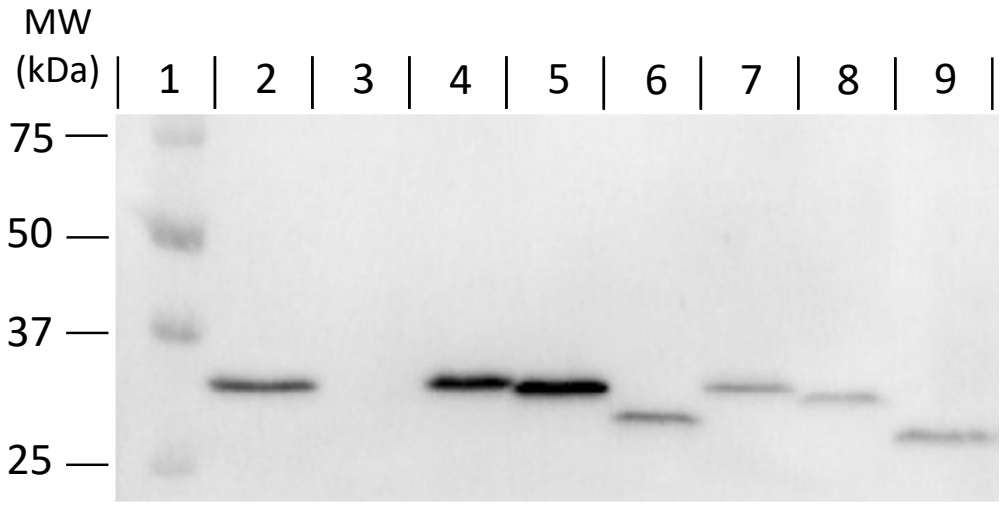

Figure 9. Detection of Flag-tagged truncated Yaf9 proteins via Western blot. The samples were separated on a 12% SDS-polyacrylamide gel. The primary antibody used was ANTI-FLAG M2, and the secondary antibody was Goat anti-Mouse IgG HRP. Lane 1 contains a protein molecular weight (MW) marker (Precision Plus Protein Dual Color Standards, Bio-Rad), lane 2 – strain AKY2727 as a positive control, lane 3 – strain AKY2732 as a negative control, lane 4 – strain AKY2736 as a positive control, lane 5 – AKY2782 (Δ2-10), lane 6 – AKY2778 (Δling), lane 7 – AKY2779 (Δ160-168), lane 8 – AKY2780 (Δ169-185), but lane 9 – AKY2781 (Δ186-226). kDa – kilodalton.

Another Western blot was performed to confirm the presence of 1E2-tagged Swc4. As predicted, Swc4 was expressed in all mutants and the protein was the same size in all (expected size – ~58 kDa) (*Saccharomyces Genome Database*, 2025). The results are given in Figure 10.

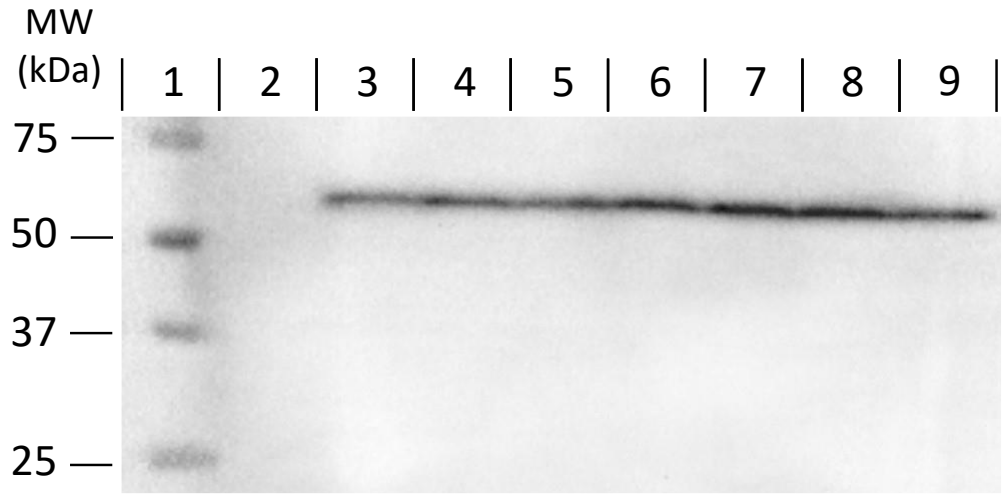

Figure 10. Detection of 1E2-tagged Swc4 protein via Western blot. The samples were separated on a 12% SDS-polyacrylamide gel. The primary antibody used was Mouse mAb to BPV type 1 E2, and the secondary antibody was Goat anti-Mouse IgG HRP. Lane 1 contains a protein molecular weight (MW) marker (Precision Plus Protein Dual Color Standards, Bio-Rad), lane 2 – strain AKY2727 as a negative control, lane 3 – strain AKY2732 as a positive control, lane 4 – strain AKY2736 as a positive control, lane 5 – AKY2782 (Δ2-10), lane 6 – AKY2778 (Δling), lane 7 – AKY2779 (Δ160-168), lane 8 – AKY2780 (Δ169-185), but lane 9 – AKY2781 (Δ186-226). kDa – kilodalton.

3.2.2 Co-immunoprecipitation

After confirming protein expression by Western blot, a Co-IP assay was performed to assess whether the truncated Yaf9 variants associate and coprecipitate with its interaction target, Swc4. Swc4 was captured on beads from a cell lysate using the 1E2 antibody. Flag-tagged Yaf9 mutants, if associated, would also be pulled down and could be identified by Western blot using an ANTI-FLAG antibody. Results are given in Figure 11.

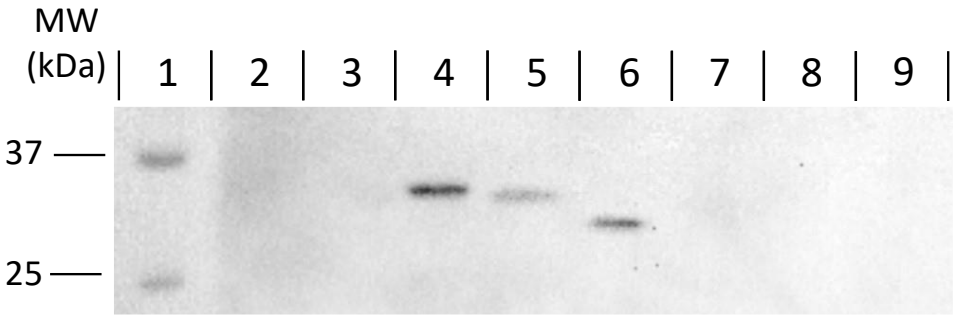

Figure 11. Co-immunoprecipitation assay blot. The assay was performed using the 1E2 antibody to capture Swc4 and its associated proteins on beads, followed by Western blot analysis with an ANTI-FLAG antibody to detect Swc4-associated Yaf9. Lane 1 contains a protein molecular weight (MW) marker (Precision Plus Protein Dual Color Standards, Bio-Rad), lane 2 – strain AKY2727 as a negative control, lane 3 – strain AKY2732 as a negative control, lane 4 – strain AKY2736 as a positive control, lane 5 – AKY2782 (Δ2-10), lane 6 – AKY2778 (Δling), lane 7 – AKY2779 (Δ160-168), lane 8 – AKY2780 (Δ169-185), but lane 9 – AKY2781 (Δ186-226). kDa – kilodalton.

It can be observed that only Δ2-10 and Δling mutants remain associated with Swc4, whereas mutants with deletions located closer to and within the C-terminal domain lose their association.

3.2.3 Formamide stress

Currently, extensive understanding of the mechanisms of Yaf9 contribution to SWR1 and NuA4 complexes is still lacking. Therefore, another aim of this study was to test whether Yaf9 functions only as a subunit of SWR1 and NuA4 or does it possibly have roles independently from these complexes. For this, a formamide stress assay was chosen, since formamide causes growth defects in strains lacking chromatin remodelling genes, including *YAF9* (Ogbede et al., 2021).

To test the Yaf9 mutation effect on stress resistance, the strains obtained in 3.1.7, which were shown by Co-IP to not interact with Swc4 – AKY2779, AKY2780, AKY2781, as well as AKY2736 for control – were crossed with AKY1820, a *yaf9Δ* strain to remove the WT *YAF9* copy from the genome. In these strains Yaf9 is not a stable subunit of SWR1 and NuA4 anymore. Yeasts were crossed, sporulation was induced, and tetrad dissection was performed. The new strains, only possessing a copy of *YAF9* in the *ADE2* locus, were used for stress resistance assay.

For the test, a serial dilution method was used. Cells from each strain were suspended in H_2O, tenfold serial dilutions were prepared and 5 µl of each dilution were plated on SC agar plates with and without formamide. Plates were incubated and the growth was monitored over 3 days. On the third day, plates were photographed (see Figure 12).

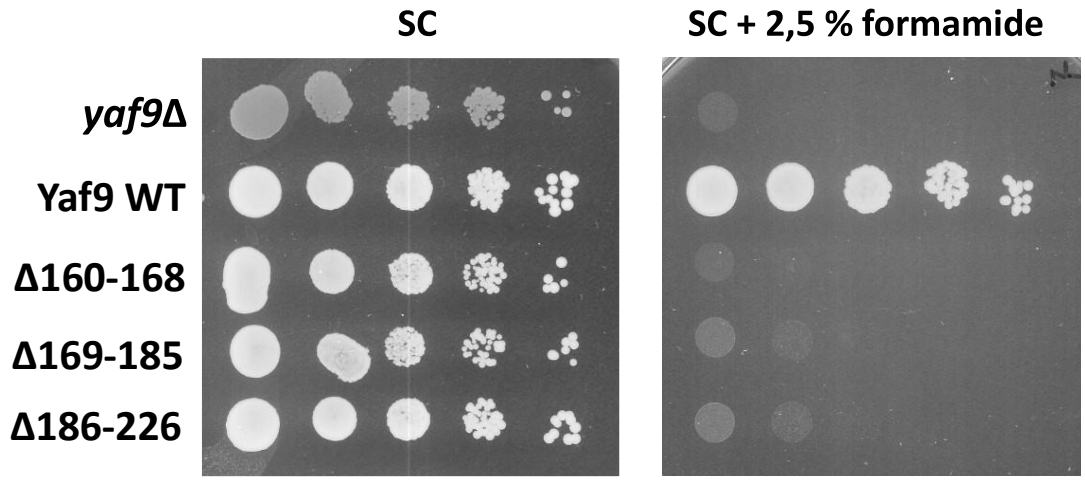

Figure 12. Tenfold serial dilution assay. Δ160-168 (AKY2836), Δ169-185 (AKY2837) and Δ186-226 (AKY2838) compared to Yaf9 WT (strain AKY2835, positive control) and *yaf9Δ* (strain AKY1820, negative control). Results photographed on the third day after plating.

In normal growth conditions all mutant strains, including *yaf9Δ*, showed no growth defect compared to wild type strain. However, exposure to formamide caused severe growth inhibition in all mutant strains. At a concentration of 2,5 %, formamide does not affect the growth of the wild type strain. This suggests that Yaf9 is required in both NuA4 and SWR1 complexes simultaneously, or that it plays a role in coordinating their interaction, rather than functioning independently from them.

3.3 DISCUSSION

Previous studies have largely focused on the deletion of Yaf9 C-terminal domain (221-254 in *Candida albicans*, 186-226 in *Saccharomyces cerevisiae*) and different smaller truncations within YEATS domain (Bittner et al., 2004; Lo et al., 2024). In this work, additional deletions were carried out, which to author's knowledge, had not previously been done. It was observed that deletions of *S. cerevisiae* Yaf9 amino acid regions 160-168 (helix at the end of YEATS domain) and 169-185 (linker between helixes) gave the same phenotype as the C-terminal helix deletion and a full Yaf9 deletion, indicating the importance of these structures in protein-protein interactions.

Interestingly, the 160-168 deletion, which showed a similar phenotype to the null mutant and mutant missing the C-terminal helix, is located within the YEATS domain (*Saccharomyces Genome Database*, 2025). This could lead to expect a similar result as for Δling (119-141), which still showed an association with Swc4, as both deletions are located only 19 amino acids apart. However, since 160-168 is a short helix at the end of the domain, it is not involved in the principal function of YEATS, that is, it does not interact with acetylated or crotonylated lysine residues (Li et al., 2014, 2016). Rather, it is possible that it has a structural function. Ji et al. (2022) have shown the predicted structure of Swc4-Yaf9 complex in NuA4 (see Figure 13).

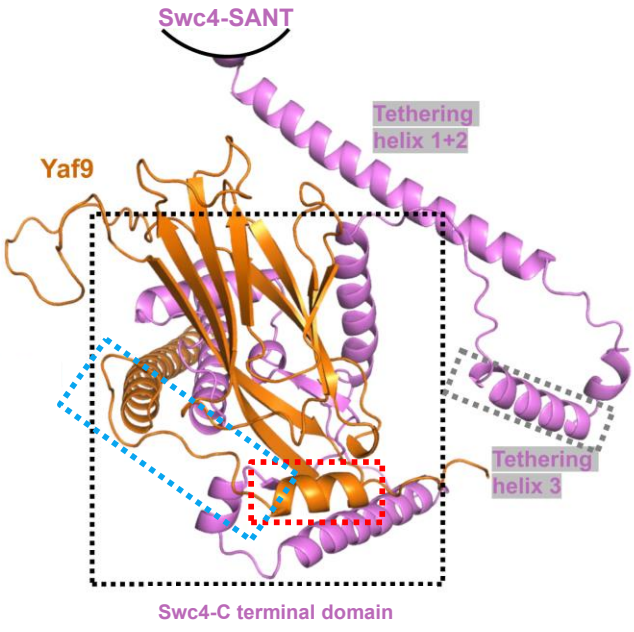

Figure 13. Predicted structure of Swc4-Yaf9 by AlphaFold-Multimer. The black dashed box indicates the location of Swc4 C-terminal domain, red dashed box indicates Yaf9 160-168 helix at the end of YEATS domain, blue dashed box indicates Yaf9 169-185 linker between helixes. Figure adapted from Ji et al., 2022.

The predicted protein-protein structure shows Yaf9 positioned with the Swc4 C-terminal domain. It has been known that both proteins interact through their respective C-terminal

regions (Bittner et al., 2004). The current work shows that, in addition to the C-terminal helix (Δ186-226), this also includes the 160-168 helix at the end of YEATS domain and 169-185 helix linker.

The mutants for stress resistance testing were chosen based on the lack of association between them and Swc4 in Co-IP assay. Additionally, previous works studying the effects of deletions within YEATS or a complete deletion of YEATS domain in yeast proteins have shown either little negative effect or even positive effect regarding stress resistance. For example, a study with Taf14 protein showed that a *taf14Δ* strain performed better in both normal conditions and under different stresses than a strain with partial YEATS deletion or a strain only possessing the YEATS domain part of the protein. The latter was also outperformed by a strain with a partial deletion (Schulze et al., 2010). The article also stresses the importance of the C-terminal domain, rather than YEATS domain, in association of these proteins in their respective complexes. The effect of the loss of Yaf9 C-terminal domain has also been studied in the pathogenic yeast *Candida albicans*. Similarly to this work, the authors found that a deletion of the C-terminal domain of the *C. albicans* Yaf9 protein caused a phenotype that mimics the full deletion mutant. However, they also observed that mutations within the YEATS domain caused little to no effect on stress resistance and virulence of the yeast (Lo et al., 2024). Taken altogether, this led to the conclusion that Δ2-10 and Δling mutants from this study would likely not show any significant growth impairment in the stress assay, so they were omitted. However, to be completely sure that this would be the case, the removal of the wild type gene and stress resistance testing should also be carried out for these mutants.

The results of the stress resistance assay also lead to conclude that Yaf9 functions as a subunit of NuA4 and SWR1, rather than functioning separately from both complexes. The observed phenotypes of Δ160-168, Δ169-185 and Δ186-226 resembled *yaf9Δ*, even though these mutants did not possess any impairment within the YEATS domain required for chromatin modification recognition. This means that, even with a functioning YEATS domain, Yaf9 does not act independently. Similarily, the growth impairment under stress that is seen in *yaf9Δ* and in the aforementioned mutant strains, is then likely due to NuA4 and SWR1 not being recruited to chromatin, which is a function that is typically carried out by Yaf9, associated in the complexes through an interaction with Swc4. Since the findings of this work are similar to Lo et al. (2024), who found that *C. albicans* strains with Yaf9 dissociated from NuA4 and SWR1 and with an intact YEATS domain were sensitive to several stressors, it can be presumed that this property of Yaf9 is not unique to *S. cerevisiae* and could be attributed to this protein in other organisms, at least in fungi.

SUMMARY

This work was carried out to assess interactions between *Saccharomyces cerevisiae* YEATS domain-containing Yaf9 and Swc4 proteins. Several Yaf9 mutant strains with deletions in different parts of the protein were constructed and the association with Swc4 was tested by co-immunoprecipitation assay. It was found that deletions towards the N-terminus of the protein, namely Δ2-10 and Δling (119-141) do not affect the interaction between the two proteins, whereas C-terminal deletions do. Mutants Δ160-168, Δ169-185 and Δ186-226 did not show an association with Swc4. These mutants which were found to not form an association with Swc4 were crossed with a *yaf9Δ* strain to remove the wild type gene from the genome, and the progeny were phenotyped by performing a stress tolerance assay using formamide as the stressor. The mutant strains, same as wild type, showed no growth impairment growing on regular SC media. However, under stress conditions, the mutant phenotypes were akin to a full deletion strain, exhibiting severely impaired growth. The results show 1) which parts of the protein are responsible for maintaining the association with Swc4 and 2) that Yaf9 most likely only functions as a subunit of the NuA4 and SWR1 protein complexes, since the lack of association within the complex, mediated by Swc4, causes a defective growth phenotype, even with the functional domain of the protein remaining intact.

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APPENDIX

Table S1. Yeast strains used in this work

Strain	Description	Genotype	Source
AKY1820	*yaf9Δ* strain used in yeast crossing to remove endogenous *YAF9* gene	W303, MATα, ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-101 lys2Δ can1-100 yaf9::TRP1	Laboratory collection
AKY2732	1E2-tagged WT Swc4	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-101 lys2Δ can1-100 3x1E2-SWC4	Laboratory collection
AKY2727	Flag-tagged WT Yaf9	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-101 lys2Δ can1-100 3xFLAG-YAF9	Laboratory collection
AKY2736	1E2-tagged WT Swc4 and Flag-tagged WT Yaf9	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 ade2-101 lys2Δ can1-100 3x1E2-SWC4 3xFLAG-YAF9	Laboratory collection
AKY2778	Yaf9 Δling	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 lys2Δ can1-100 ade2::3xFLAG-Yaf9Δling-ADE2 3x1E2-SWC4	This work
AKY2779	Yaf9 Δ160-168	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 lys2Δ can1-100 ade2::3xFLAG-Yaf9Δ160-168-ADE2 3x1E2-SWC4	This work
AKY2780	Yaf9 Δ169-185	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 lys2Δ can1-100 ade2::3xFLAG-Yaf9Δ169-185-ADE2 3x1E2-SWC4	This work
AKY2781	Yaf9 Δ186-226	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 lys2Δ can1-100 ade2::3xFLAG-Yaf9Δ186-226-ADE2 3x1E2-SWC4	This work
AKY2782	Yaf9 Δ2-10	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 lys2Δ can1-100 ade2::3xFLAG-Yaf9Δ2-10-ADE2 3x1E2-SWC4	Laboratory collection
AKY2835	WT Yaf9 complemented strain	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 lys2Δ can1-100 ade2::3xFLAG-Yaf9-ADE2 yaf9::TRP1 3x1E2-SWC4	This work
AKY2836	Yaf9 Δ160-168 with endogenous *YAF9* gene deletion	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 lys2Δ can1-100 ade2::3xFLAG-Yaf9Δ160-168-ADE2 yaf9::TRP1 3x1E2-SWC4	This work
AKY2837	Yaf9 Δ169-185 with endogenous *YAF9* gene deletion	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 lys2Δ can1-100 ade2::3xFLAG-Yaf9Δ169-185-ADE2 yaf9::TRP1 3x1E2-SWC4	This work
AKY2838	Yaf9 Δ186-226 with endogenous *YAF9* gene deletion	W303, MATa, ura3-1 leu2-3,112 his3-11,15 trp1-1 lys2Δ can1-100 ade2::3xFLAG-Yaf9Δ186-226-ADE2 yaf9::TRP1 3x1E2-SWC4	This work
DC14	Used for mating type determination	MATa	Brad Cairns/Fred Winston
DC17	Used for mating type determination	MATα	Brad Cairns/Fred Winston

Table S2. Primer sequences used in this work.

Primer	Sequence
Yaf9_D2-10_F	5' atcgattacaaggatgacgatgacaagAAAACTCTCTCCGTGAGCAGACCA 3'
Yaf9_D2-10_R	5' tggtctgctcacggagagagttttCTTGTCATCGTCATCCTTGTAATCGAT 3'
Yaf9_ling_del_R	5' tacacagaactaacttctgcatcATGAAGTCGTAATCTATGGT 3'
Yaf9_ling_del_F	5' accatagattacgacttcatGATGCAGAAGTTAGTTCTGTGTA 3'
Yaf9_D160-168_R	5' aggtaacaaatttcctggtcgATTTGGCTCATTGAATACAATTTCAT 3'
Yaf9_D160-168_F	5' atgaaattgtattcaatgagccaaatCGACCAGGAAATTTGTTACCT 3'
Yaf9_D169-185_F	5' tgaagagtttttcaaaattttaatgagCAAGCAATTAGAACAGGAAGAAATTGA 3'
Yaf9_D169-185_R	5' tcaatttcttcctgttctaattgcttGCTCATTAAAATTTTGAAAAACTCTTCA 3'
Yaf9_D186-226_F	5' caaaactgatgattgtgtatactccTAGGATCACTGCACCCGGAA 3'
Yaf9_D186-226_R	5' ttccgggtgcagtgatcctaGGAGTATACACAATCATCAGTTTTG 3'
Yaf9-term-R	5' TTGTGAGCGCCGACCTTTCAA 3'
Yaf9-prom-F	5' TTCGGATGACGTGATGACGCT 3'
Ade2-cut-ctrl1-AFI1-F	5' GAACTTGACTAGCGCACTACC 3'
Ade2-cut-ctrl2-RGA1-R	5' GGGAACTACAAAGATATCCTGACG 3'
Yaf9-tag-cntr nr 1066_0	5' GAACTGACTGAAACAGGGTGGGG 3'
Yaf9-D160-168-cntr-R_0	5' TGAAGGTAACAAATTTCCTGGTCG 3'

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Krista Mehaņikova
20/05/2025