理學博士學位論文

*Candida albicans*의 Ssn6에 대한 Rpd31과 Rpd32의 조절 작용

Regulatory effect of Rpd31 and Rpd32 on Ssn6 in *Candida albicans*

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Regulatory effect of Rpd31 and Rpd32 on Ssn6 in *Candida albicans*

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ABSTRACT

Candida albicans is one of the most common opportunistic fungal pathogens in humans. Its pathogenicity is directly linked to its ability to undergo a morphological transition from the yeast-form to filamentous hyphae. This morphological change is induced in response to a variety of environmental signals such as serum, proline, N-acetylglucosamine, and different carbon sources. From signal induction to morphological transition, there are complex networks of signal transduction pathways.

Ssn6 is known as a general transcriptional repressor, which exists as a complex with the transcriptional repressor Tup1 through its TPR motifs and regulates the genes of many cellular processes including nutrient utilization, osmotic stress, meiosis, mating, and sporulation in *Saccharomyces cerevisiae*. In *C. albicans*, Ssn6 also functions as a complex with Tup1, which is a distinct repressor for morphogenesis. However, our previous data revealed that Ssn6 is a critical factor for filamentous growth and virulence independently of Tup1.

Despite many studies focusing on the Ssn6-Tup1 complex in *S. cerevisiae* and Tup1 in *C. albicans*, there was not much in-depth understanding of how Ssn6 can regulate the morphological transition and virulence of *C. albicans* in Tup1-independent manner has not been well understood yet. Therefore, the interaction partner of Ssn6 through tandem affinity purification (TAP) was screened and histone deacetylase Rpd31 was identified as a partner interacting with Ssn6 in Tup1-independent manner in this study. The morphological and transcriptional analyses revealed that Rpd31 is one of the critical factors regulating for morphogenesis and virulence as Ssn6 in *C. albicans*. It was also

found that deletion of *SSN6* and *RPD31* induced the development of elongated filaments, but caused to be defective in filament extension. Surprisingly, chromatin immunoprecipitation (ChIP) assays of Ssn6 and Rpd31 showed different patterns of enrichment at the promoter of metabolic gene *INO1* and hyphal extension gene *UME6*. These results suggest that Ssn6 has dual functions as a repressor and/or an activator depends on its target genes and participation of Rpd31 for regulation, which is the critical point determining the roles of Ssn6.

Furthermore, the paralogue of Rpd31 and Rpd32 were also investigated for the regulation of filament-development in *C. albicans*. Rather than that assemble in one complex, Rpd31 and Rpd32 are associated into the different complex, respectively to Ssn6 or Sin3. Two different complexes would regulate the same genes competitively or cooperatively depending on intracellular condition or their target genes. Interestingly, Sds3, which homologue is the component of Rpd3L complex in *S. cerevisiae*, is partially involved in this differential regulation. In the repression of genes such as *INO1* cooperatively by Rpd31 and Rpd32, Sds3 was synchronized with the regulatory actions of Rpd31 and Rpd32. However, in the opposite regulation of genes such as *UME6* by Rpd31 and Rpd32, Sds3 was associated only with the active regulation by Rpd31 but not with repressive regulation by Rpd32. This complicated regulatory system might contribute the flexible pathogenicity of *C. albicans*.

Key words: Tandem affinity purification, *SSN6*, *RPD31*, *RPD32*, *Candida albicans*

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List of Abbreviations

BCIP	5-bromo-4-chloro-3-indoyl phosphate
bps	base pairs
cAMP	cyclic adenosine monophosphate
CBP	calmodulin binding peptide
DHase	dehydrogenase
EDTA	ethylene diamine tetraacetic acid
GlcNAc	N-acetyl glucosamine
HDAC	histone deacetylase
IPTG	isopropyl-β-D-thiogalactopyranoside
LiAc	lithium acetate
MALDI	matrix assisted laser desorption ionization
MAPK	mitogen activated protein kinase
MBP	maltose binding protein
NBT	nitro blue tetrazolium
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonyl fluoride
PKA	protein kinase A
SDS	sodium dodecyl sulfate
TAP	tandem affinity purification
UV	ultraviolet

I. INTRODUCTION

1. Morphogenesis in *Candida albicans*

1.1. Factors involved in morphogenesis of *C. albicans*

1.1.1. Environmental cues inducing hyphal growth

The most important features of *Candida albicans* are morphological plasticity and virulence. Its pathogenicity is linked to its ability to undergo a morphological transition from budding yeasts or hyphal forms. Between two extreme morphology, *C. albicans* also develop another filamentous forms referred to as 'pseudohyphae'. In these intermediate forms, the daughter cell elongates like true hyphae but remains attached to the mother cell, resulting in the filaments composed of elongated cells with constriction at the septa. Although the pseudohyphae have innumerable forms with various width and length of filaments, the distinct morphological differences between true hyphae and pseudohyphae is the width of filaments and constriction at septa. The true hyphae have constant-width and narrow filaments with no constriction at the septa. But, the pseudohyphae have relatively thick filaments with irregular width and constriction at the septa (Sudberry *et al.*, 2004).

Several factors are known that influence on morphological changes. As shown in Table I-1, morphological switching from yeast to hyphal forms can be induced by a wide range of environmental conditions (Odds, 1985). Hyphae are reliably induced from yeast cells by the addition of serum, the most powerful inducer of germ tube formation and a growth temperature at 37 °C. Some kinds of culture conditions such as a temperature above 35 °C, neutral pH, poor source of carbon and nitrogen, some amino acid mixture (Lee's medium), and nutrient broth (spider medium) also induce a hyphal development.

Furthermore, it has been also reported that exogenous human hormones

such as oestradiol and progesterone also induce a hyphal growth. Thus, it is plausible that hyphal induction could be modulated by the other host factors (Bramley *et al*., 1991; Brown and Gow, 1999). In spite of a number of factors that can activate morphological switching in *C. albicans*, no receptors have been reported so far (Brown and Gow, 1999).

1.1.2. Signal transduction pathways

The multiple environmental conditions trigger the complicated signaling pathways which induce the development of filaments in *C. albicans* (Fig. I-1). There are two major signaling pathways referred to as Cph1-mediated MAPK pathway and Efg1-mediated cAMP pathway.

1.1.2.1. MAPK cascade

The MAPK cascade in *S. cerevisiae* is related to develop pseudohyphae, likewise, similar functions of MAPK cascade in *C. albicans* have been identified so far. In *S. cerevisiae*, the signals involved in pseudohyphal pathway and the mating (STE) pathway share common component, the transcription factor Ste12p (Liu et al., 1993). The *C. albicans* Ste12p homolog, Cph1p, complements both mating defect in haploids and pseudohyphal defect in diploids of ste12 mutants (Liu et al., 1994; Lebeter et al. 1996; Csank et al., 1998).

1.1.2.2. cAMP-PKA pathway

In *S. cerevisiae*, nitrogen starvation results in the formation of elongated buds termed pseudohyphae, which is depends on the signals driven from cAMP-PKA pathway (Gimeno *et al*., 1992; Kronstad *et al*., 1998; Lengeler *et*

Table I-1. The environmental conditions affect morphogenesis in *C. albicans* (Biswas *et al*., 2007).

Yeast	Pseudohyphae	Hyphae	Filamentation not well characterized
Growth below 30 °C	High phosphate (up to 600 mM)	N-acetyl-D-glucosamine	Spider medium
Low pH (pH 4.0)	Nitrogen-limited growth on solid medium (SLAD)	Lee's medium 37 ℃	Growth in agar matrix
Inoculum $>10^7$ cells ml^{-1}	pH 6.0, 35 ℃	pH 7.0	Mouse kidneys
		Serum, >34 ℃	Iron deprivation
		Proline	

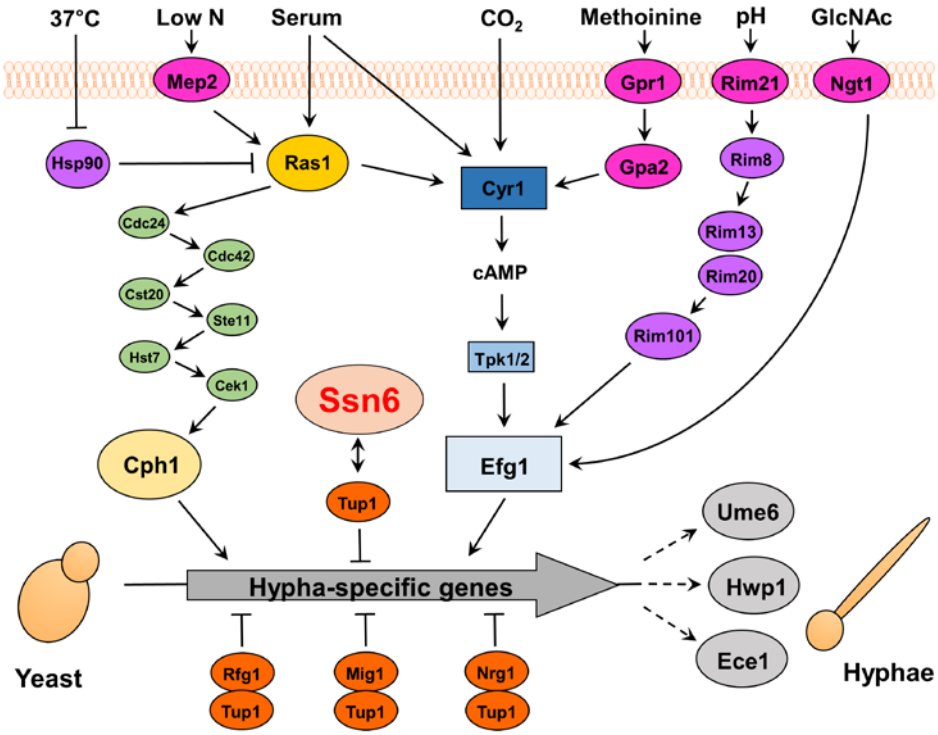


Fig. I-1. Regulation of dimorphism in *C. albicans* by multiple signaling pathway (adapted from Sudbery, 2011; Hwang *et al.*, 2003). Multiple environmental conditions trigger filamentous signaling pathways in *C. albicans*. Two major signaling pathways, Cph1-mediated MAPK pathway and Efg1-mediated cAMP pathway activate filamentous growth. Ssn6-Tup1 complex or Tup1 alone was recruited by DNA-binding proteins such as Nrg1, Mig1, Rfg1 for repression of filament-specific genes.

al., 2000). An increased level of cAMP induces a yeast-to-hypha transition, and inhibition of cAMP phosphodiesterase also induces this transition in *C. albicans* (Sabie and Gadd, 1992). In *C. albicans*, PKA is encoded by two paralogues, *TPK1* and *TPK2*. Both isoforms activate a true hyphae formation with a different manner on hyphal development. For instance, *tpk1* mutants are defective in hyphal formation on solid media but are less affected in liquid medium. However, hyphal formation in *tpk2* mutants is partially affected on solid medium but is hindered in liquid medium. The downstream component of the PKA pathway, Efg1 is a *C. albicans* homolog of *S. cerevisiae* Phd1. Overexpression of *TPK2* showed inability to suppress the mutant phenotype of efg1, whereas overexpression of *EFG1* can overcome the defect of filamentation in a *tpk2* mutant (Sonneborn *et al*., 2000).

The *C. albicans efg1* mutants strain has a moderate defect in hyphal growth in response to many kinds of environmental condition (Lo *et al*., 1997). Even in the medium containing a hyphal growth inducer, serum or GlcNAc, *efg1* mutant is completely abolished in hyphal formation. However, under microaerophilic or agar-embedded condition, hyphal formation is slightly activated. These results indicate that Efg1 may act as both activator and repressor for different subset of genes depending on environmental condition.

Interestingly, overexpression of *EFG1* increases the levels of a smaller 2.2 kb transcript, whereas the levels of the endogenous 3.2 kb major *EFG1* transcripts decrease drastically (Tebarth *et al*., 2003). Sequential deletion analysis of the promoter region revealed that the promoter region containing TATA box is required for an autoregulation of *EFG1*. It seems that Tpk1 and Tpk2 as well as Efg1 can down-regulate the major *EFG1* promoter. This negative autoregulation and PKA-mediated down-regulation are likely

mediated via Sin3-Rpd3-containing HDAC complex (Srikantha *et al*., 2001). Furthermore, Efg1 is involved in control of white-opaque phenotypic switching by modulating its expression level. For example, high expression is required for switching to and for maintenance of the white cell form whereas low expression induces and maintains the opaque cell form (Sonneborn *et al*., 1999).

1.1.3. Response to the environmental pH

The pathway controlling pH-responsive gene expression is well characterized in filamentous fungi, *Aspergillus nidulans*. In this pH regulation, zinc finger transcription factor *PacC* has a central role. *PacC* is synthesized in an inactive form, whose activity is relieved by proteolytic processing event of the C-terminus at alkaline pH (Mingot *et al*., 1999). The activated form of *PacC* induces the expression of alkaline pH-expressed genes and represses acidic pH-regulated genes (Tilburn *et al*., 1995). The *PacC* homolog in *S. cerevisiae*, Rim101 was initially identified as a function of controlling meiosis and haploid invasiveness (Li and Mitchell, 1997; Su and Mitchell, 1993). Like *S. cerevisiae*, there are two major regulators in *C. albicans*, *PHR1* and *PHR2*. The *PHR1* is strongly expressed under condition of alkaline pH and the *PHR2* is expressed at pH below 5.5 (Saporito-Irwin *et al*., 1995). The activity of *C. albicans* Rim101 also controlled by proteolytic cleavage like other species. In the low pH, Rim101 sustained its full integrity. However, under alkaline pH, a C-terminal region is processed to yield its active form (Denison *et al*., 1995; Li and Mitchell, 1997). This proteolysis is controlled by pH via a number of proteins, including Rim20, Rim8, Rim13, and Rim9 (Denison *et al*., 1995; Li and Mitchell, 1997).

1.1.4. Repressors related to hyphal growth

1.1.4.1. *TUP1*

In *S. cerevisiae*, the Tup1 act as a global repressor that regulates over 60 genes involved in glucose-repressed genes, oxygen-repressed genes, haploid specific genes, DNA damage-induced genes and a-specific mating genes (DeRisi *et al*., 1997). Similar to *S. cerevisiae*, *C. albicans* Tup1 homolog contains seven WD40 repeats at the C-terminus, which likely mediate an interaction with specific DNA-binding proteins. And N-terminal region seems to be involved in interacting with Ssn6 (Keleher *et al*., 1992; Komachi and Johnson, 1997). The *tup1* mutant grows in filamentous form regardless of culture condition; mostly pseudohyphae but true hyphae also appeared in some medium. Furthermore, *NRG1* and *RFG1* act as a repressor of filamentous growth by recruiting Tup1 to target genes (Braun *et al*., 2001; Kadosh and Johnson, 2005).

1.1.4.2. *SSN6*

In budding yeast, Ssn6 functions as a general repressor in concert with Tup1 that regulates genes involved in a variety of action such as sporulation, mating, DNA repair and hypoxic gene regulation (Keleher *et al*., 1992; Williams *et al*., 1991). A null mutation of *SSN6* results in constitutive expression of invertase synthesis and other glucose-repressed genes. One third amino-terminal of Ssn6, which includes the TPR motifs, is important for Ssn6 function, although only four of ten TPR repeats essential; the C-terminal region can be phosphorylated but seems to be dispensable (Tzamarias and Struhl, 1994). Neither Ssn6 nor Tup1 has motifs for DNA binding, it is plausible that both proteins were recruited to the promoter region of genes by a gene-specific

DNA binding proteins. In *C. albicans*, *ssn6* mutant showed a stubby pseudohyphal growth and reduced expression of filament specific genes (Hwang *et al*., 2003). Recently in addition, it has been reported that newly generated *ssn6* mutant stains displayed a phenotypic instability (Garcia-Sanchez *et al*., 2005). They also showed that both Ssn6 and Tup1 were required for the Nrg1-mediated repression through a lacZ reporter containing minimal *ADH1* promoter fused with an artificial Nrg1 response element.

1.1.5. *UME6*

Initiation of meiosis in *S. cerevisiae* is regulated by mating type and nutritional conditions that restrict meiosis to diploid cells grown under starvation conditions (Kassir *et al*., 2003). Ume6 represses early meiotic gene transcription by recruiting the Rpd3 histone deacetylase and chromatin-remodeling proteins. Also, Ume6 acts as a negative regulator of *ATG8* that leads to an increase of autophagic activity by cooperate with histone deacetylase complex including Sin3 and Rpd3 (Bartholomew *et al*., 2012). However, in *C. albicans*, *CaUME6* has been shown to be required for hyphal elongation. The *ume6* mutant strain showed a complete defect in hyphae formation under all the growth conditions tested. *UME6* was repressed by the Nrg1-Tup1 repressor in yeast-form cells but *NRG1* was not repressed by Ume6p under hyphal growth conditions. Ectopic *UME6* expression in *efg1*, *cph1* and *ras1* mutant cells rescued the hyphal defects of these mutants under some hyphal growth conditions. Thus, *UME6* is a common downstream target of regulators promoting hyphal development. Ectopic *UME6* expression promoted both germ tube formation and hyphal elongation (Zeidler *et al*., 2009).

1.2. Factors related to virulence in *C. albicans*

C. albicans is found in the normal gastrointestinal flora and the oral mucosa of most healthy humans (Soll, 2002). However, immuno-compromised patients who suffering from AIDS or undergoing immunosuppressive treatment, bloodstream infections often cause serious candidiasis such as *Candida*-due mycocarditis and acute disseminated *Candida* septicemia (Cutler, 1991). The ability that responsible for virulence in *C. albicans* depends on two kinds traits including survival traits dividing in the host environment and virulence traits invading new tissues, evading phagocytic cells, causing symptoms of infections (Gow and Gooday, 1987; Mitchell, 1998).

1.2.1. Adhesins

Candida albicans cell wall is important for growth and interaction with the environment. Biomolecules that promotes the adherence of *C. albicans* to host cells or ligands of host cells is often referred as adhesins. So far, several types of adhesins have been identified in *C. albicans* and these proteins include agglutinin-like proteins, transglutaminase, and integrin-like proteins.

1.2.1.1. *ALS* family

In *C albicans*, *ALS1* (Agglutinin-like sequence) encodes a protein, which is a member of a family with homology to the *S. cerevisiae* α-agglutinin that is involved in cell to cell recognition during a mating. The *ALS* family is capable of secretion and has hydrophobic C-terminus that likely functions as a glycosylphosphatidylinositol (GPI) anchor. And, N-terminal region is thought to be involved in ligand binding. In *C albicans*, both Als1 and Als5 function as an adhesin (Fu *et al.*, 1998; Gaur and Klotz, 1997). The *als1* mutant showed

significant reduction in adherence and failed to form filaments on hyphal inducing solid medium (Fu *et al*., 2002). And they also demonstrated that Als1 is a downstream regulator of the *EFG1*-mediated filamentation pathway. Pga1, the other glycosylphosphatidylinositol-anchored cell wall protein, is also required for a proper adhesion and a biofilm formation (Hashash *et al*., 2011).

1.2.1.2. *HWP1*

During the searching for hyphal- and germ tube-specific gene, *HWP1* (Hyphal wall protein 1) was isolated by differential screen (Staab *et al*., 1996). Hwp1 is an outer surface mannoprotein that is found on surfaces of germ tube, but not yeast in *C. albicans*. Hwp1 is known as a substrate for the mammalian transglutaminase. The *hwp1* mutant strains were avirulent whereas a disruptant in single allele or wild-type strains were lethal in a hematogenously disseminated murine model (Sundstrom *et al*., 2002).

1.2.1.3. Integrin-like protein, Int1

C. albicans INT1 gene was isolated in pursuit of genes that encode integrin-like proteins involved in binding to host extracellular protein. Int1 has a positive role in filamentation, because *C. albicans int1* mutant strain showed a medium-dependent filamentation defect. Expression of *INT1* from a strong promoter in *S. cerevisiae* causes formation of elongated projections similar in appearance to germ tube that adhered to human epithelial cell. This elongated form was not blocked by mutation on component of MAPK pathway, thus it is likely that Int1 acts through the Efg1 pathway (Calderone, 1998; Gale *et al*., 1996; Gale *et al*., 1998). Similar to *hwp1* mutant strain, the *int1* mutants were less virulent, adhered less readily to an epithelial cell line and also showed

deficiencies in filamentation on medium inducing hyphal formation.

2. Identification of protein complex using a mass spectrometry

The completion of genome project in several organisms provides plenty of information to analyze the functions of their genes. Mass spectrometry (MS) has been used to rapidly identify and analyze proteins in a variety of biological samples. To overcome the problems in yeast two hybrid and protein chip analysis, mass spectrometry approach has been combined with dual tag purification method to identify the novel interacting partners of a protein of interest.

2.1. Mass identification

In general, the mass spectrometry was coupled with an isolation steps to purify a protein complex of interest. When proteins were purified by an adequate approach-mainly affinity chromatography, individual proteins were then stained with appropriate dye such as coomassie brilliant blue or silver nitrate. The digested several pieces of peptides can be identified by mass analysis. Generally, mass spectrometer composed of at least five components, inlet, ion source, mass analyzer, ion detector, and data analyzer. Solid, liquid or gaseous states of samples were ionized by ion source then sorted by according to the mass to charge ratio. Resultant ions were detected by ion detector and then analyzed by mass analyzer. So far, several informatics algorithms have been developed to matching the mass data of proteins with that of amino acid sequence. Resulting mass spectrum is compared to theoretical spectra generated

from protein sequences in an appropriate data base by peptide sequencing software such as SEQUEST, and MASCOT (Nesvizhskii and Aebersold, 2004).

2.2. Tandem affinity purification

The dual purification strategy, termed tandem affinity purification (TAP), was first developed by Seraphin and colleagues (Rigaut *et al*., 1999). The strategy for TAP involves C- or N-terminally appending dual tags separated by specific protease cleavage site to a protein of interest. Previously, large scale yeast two hybrid screening has been used for the identification of interacting partners. However, false-positive and false-negative results awaking to the need for additional strategies to easily detect protein complex. The mass spectrometry of protein complexes purified tandem affinity steps enables analysis of samples in very low concentration-even 100 fmol of a protein sample-on proteome-wide scale (Gavin et al., 2002; Ho et al., 2002). Prior to TAP purification, C-terminal or N-terminal TAP tag is introduced in-frame with the coding region of the protein of interest in host cell. Also, to get optimal results, it is preferable to maintain expression of the fusion protein at its natural level via its own promoter. When it is expressed ectopically, it has been reported that some kinds of heat shock proteins, proteasomes are associated with protein complex as contaminants (Swaffield *et al*., 1995). In yeast, high efficiency of homologous recombination enables to bypass the need to construct a plasmid to fuse the TAP tag to the protein of interest. About 100 bp of flanking region in PCR fragments is sufficient for integrating the TAP tag into the target gene (Baudin *et al*., 1993; Gola *et al*., 2003). Generally, most widely used TAP tag consisted of two IgG binding domains of *Staphylococcus aureus* protein A and a calmodulin binding peptide separated by a TEV protease

cleavage site. The overall steps for TAP purification are illustrated in Fig. I-2.

3. Histone deacetylase

In eukaryotes, DNA is packaged into chromatin contains nucleosomes as a basic structural unit. Five major classes of histones are found in all eukaryotes,- H1, H2A, H2B, H3, H4- differing in molecular weight and amino acid composition. Each nucleosome contains eight histones, two copies each of H2A, H2B, H3, and H4 and is assembled onto DNA in repeating arrays to yield 'bead-on-a-string' structure. Histone deacetylases are a class of enzymes that remove acetyl groups from a ε-N-acetyl lysine amino acid on a histone, allowing the histones to wrap the DNA more tightly. The acetylation and deacetylation mechanism of histone in nucleosomes play an important role in gene regulation activity.

3.1. Histone deacetylases (HDACs) in yeast

In yeast, there are three different classes of HDACs have known well studied. The class I histone deacetylase, Rpd3 functions as a global repressor through associated with specific DNA binding proteins in gene regulation. Class II histone deacetylase, comprising Hda1 and Hos1-3, are large proteins that molecular weight up to 130 kDa which are involved in cellular proliferation and differentiation. Class III histone deacetylase, Sir2, has a NAD^+-dependent HDAC activity that concerned with chromatin silencing, cellular metabolism and aging.

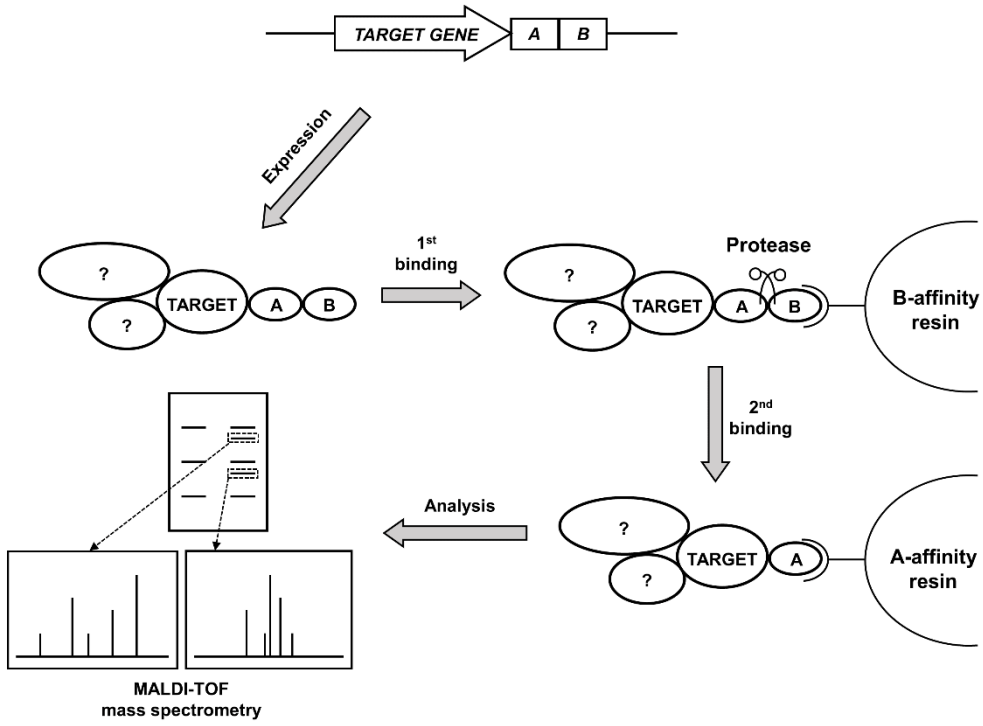


Fig. I-2. Schematic view of Tandem Affinity Purification (TAP) method

3.1.1. *RPD3*

It has been reported that *S. cerevisiae RPD3* mediates signals involved in cell longevity. When *RPD3* was deleted, resulted extended life span and transcriptional silencing at the silent mating type (*HM*) and rDNA locus were increased (Kim *et al*., 1999). Recently, two distinct complexes of Rpd3 have been identified, a small complex of 0.6 MDa (Rpd3S) and a large one of 1.2 MDa (Rpd3L) (Carrozza *et al*., 2005; Keogh *et al*., 2005). Rpd3 itself does not have DNA binding motif so it is recruited to promoters by DNA-binding proteins. For example, Rpd3 is recruited by Ume6 to repress the expression of *INO1*, a gene related to inositol biosynthesis (Kadosh and Struhl, 1997). Also, repression by Ash1 at the *HO* promoter is mediated by Rpd3 recruitment at these sites (Carrozza *et al*., 2005). Recently, it has been reported that Rpd3 seemed to activate transcription of the osmoresponsive gene *HSP12* when recruited to the promoter in response to activation by the mitogen-activated protein kinase Hog1 (De Nadal *et al*., 2004). This finding is somewhat interesting, because Rpd3 was thought to be a repressor in gene regulation. In *C. albicans*, there are two genes of *RPD3* homolog which has known *RPD31* and *RPD32*. The HDAC activities and cellular properties of both proteins in *C. albicans* little had been studied yet. It has only reported that *rpd3* (similar to *RPD32* rather than *RPD31* by ORF number) mutant resulted in an increase in the frequency of switching in both directions in *C. albicans* WO-1, whereas the deletion of *HDA1* resulted in an increase in the frequency of switching from the white phase to the opaque phase direction (Srikantha *et al*., 2001).

3.1.2. *SIR2*

In *S. cerevisiae*, the transcriptional silencing leads to gene inactivation and

transcriptional inhibition by altering a specialized chromatin structure. The deletion of *SIR2* (Silent information regulator) caused increasing acetylated levels in histone H3 and H4 within rDNA region. It also reported that *SIR2* control the replication timing program by modulating the ability of repeated origins (Yoshida *et al*., 2014). In *C. albicans*, *SIR2* causes phenotypic switching and chromosome stability by organizing chromatin structure (Perez-Martin *et al*., 1999).

3.1.3. *SIN3*

SIN3 was first isolated in genetic screens by investigating mating-type switching in *S. cerevisiae* and was simultaneously introduced to the scientific community as SIN3 (SWI independent) (Sternberg *et al*., 1987). In *S. cerevisiae*, Rpd3 exists in a large protein complex that contains the Sin3 corepressor. This complex is recruited to certain promoters where it deacetylates the adjacent histone to repress or activate transcription of the target gene. Deletion of the histone deacetylase *RPD3* or its interacting partner *SIN3* caused early activation of late origins at internal chromosomal loci (Aparicio *et al*., 2004).

3.1.4. *SDS3*

SDS3 (suppressor of defective silencing 3) was identified in a screen for mutations that cause increased silencing of a crippled HMR silencer in a *rap1* mutant background. The *sds3* mutants have phenotypes very similar to those seen in *sin3* and *rpd3* mutants, suggesting that it functions in the same genetic pathway. It has been demonstrated that Sds3 is an integral subunit of a high molecular weight Rpd3 complex in *S. cerevisiae* (Lechner *et al*., 2000).

3.2. Interaction between HDACs and Ssn6-Tup1 complex in *S. cerevisiae*

Transcriptional repression occurs via several mechanisms, including interaction with hypoacetylated N termini of histones, recruitment of histone deacetylases (HDACs), and interactions with the RNA polymerase II holoenzyme. In *S. cerevisiae*, Tup1 binds to underacetylated histone tails and requires multiple histone deacetylases (HDACs) for its repressive functions. It has been reported that the global repressor Tup1 directly interacts with the N-terminal tail domains of histone H3 and H4. Also, some histone mutation abolished Tup1 binding and thereby reduced the repression of multiple Tup1-regulated reporter genes (Edmondson *et al*., 1996). These result provided a clue that links histone deacetylase with Ssn6-Tup1 in *S. cerevisiae*. The direct interaction between Ssn6-Tup1 and histone deacetylases had been reported *in vivo* and *in vitro* (Davie *et al*., 2003; Watson *et al*., 2000; Wu *et al*., 2001). It was found that Tup1 utilize Hda1 to deacetylate histone at Tup1-regulated genes and Tup1 and Ssn6 interact with Hos2 and Rpd3.

3.3. Rpd3 L- and Rpd3 S- complex

Rpd3 (L) and Rpd3(S) are distinct multisubunit complexes containing the Rpd3 histone deacetylase. The smaller complex, Rpd3C(S), shares Sin3 and Ume1 with Rpd3C (L) but contains the unique subunits Rco1 and Eaf3. Disruption of the *GCN5* histone acetyltransferase gene shows a strong synthetic phenotype when combined with either an sds3 mutation affecting only the Rpd3 (L) complex or an rco1 mutation affecting only Rpd3(S) (Biswas *et al*., 2008). Furthermore, Rpd3C(S) mutants exhibit phenotypes remarkably similar to those

of Set2, a histone methyltransferase associated with elongating RNA polymerase II. The chromodomain of Eaf3 recruits Rpd3C(S) to nucleosomes methylated by Set2 on histone H3 lysine 36, leading to deacetylation of transcribed regions (Keogh *et al*., 2005).

4. Aims of this study

In *C. albicans*, Ssn6 also functions as a complex with Tup1, which is a distinct repressor for morphogenesis, but previous data revealed that Ssn6 is a critical factor for filamentous growth and virulence independently of Tup1. Despite many studies focusing on the Ssn6-Tup1 complex in *S. cerevisiae* and Tup1 in *C. albicans*, how Ssn6 can regulate the morphological transition and virulence of *C. albicans* in Tup1-independent manner has not been well understood. Therefore, the interaction partner of Ssn6 through tandem affinity purification (TAP) was screened and histone deacetylase Rpd31 was identified as a partner interacting with Ssn6 in Tup1-independent manner. The morphological and transcriptional analyses revealed that Rpd31 is one of the critical factors regulating for morphogenesis and virulence as Ssn6 in *C. albicans*. It was also found that deletion of *SSN6* and *RPD31* induced the development of elongated filaments, but caused to be defective in filament extension. Surprisingly, chromatin imuunoprecipitation (ChIP) assays of Ssn6 and Rpd31 showed different patterns of enrichment at the promoter of metabolic gene *INO1* and hyphal extension gene *UME6*. From the present results suggest that Ssn6 has dual functions as a repressor or an activator depends on its target genes and participation of Rpd31 for regulation, which is the critical point determining the roles of Ssn6. Furthermore, the paralogue of Rpd31 and Rpd32 were investigated for the regulation of filament-development

in *C. albicans*. Rather than that assemble in one complex, Rpd31 and Rpd32 are associated into the different complex of which representative is Ssn6 or Sin3. The Sds3 which was co-purified with Rpd31 as the Ssn6 complex was also investigated in relation to filament-development. The Sds3 is known as a component of Rpd3L HDAC complex in *S. cerevisiae*, which is required for its structural integrity and catalytic activity. The deletion of *SDS3* showed similar phenotype of *rpd31* mutant that indicates related to *RPD31* rather than *RPD32* in hyphal development. However, the Sds3 was also associated functionally both with Rpd31 and Rpd32 for the repression of metabolic genes such as *INO1*. Overall, two different complexes of Rpd31 and Rpd32 with Sds3 would regulate the same genes competitively or cooperatively depending on intracellular condition or their target genes.

II. MATERIALS AND METHODS

1. Strains and culture conditions

1.1. *Candida albicans* strains and culture conditions

The Clinical isolate SC5314, *Ura3*$^{-}$ auxotroph CAI4, and *Ura3*$^{-}$ *Arg4*$^{-}$ *His1*$^{-}$ auxotroph BWP17 were used for parental wild-type strains. *C. albicans* cells were routinely grown in YPD (1% yeast extract, 2% peptone and 2% dextrose). Cells containing plasmids or cassettes for disruption were cultured in minimal defined medium (modified synthetic-dextrose; SD) consisted of 0.17% yeast nitrogen base without amino acids (Difco), 2% dextrose, 0.5% ammonium sulfate and appropriate supplements. *Ura*$^{-}$ auxotroph transformants were selected on minimal defined medium supplemented with 625 mg 5-fluoroorotic acid and 25 mg uridine per liter (FOA medium). In order to select, cells were plated on YPD plate and incubated at 28 °C for 48 hours. Individual colonies were taken from the plate and suspended in sterile distilled water. About ~5 x 10^6 cells of suspension was spread on FOA medium and incubated for 4 days at 28 °C. Solid media were prepared by adding 1.8% micro agar (Duchefa) to a liquid media. For hyphal-growth, pre-cultured cells in YPD were inoculated into YPD supplemented with 10% Fetal bovine serum (FBS) or Spider (1% mannitol, 1% nutrient broth and 0.2% K_2HPO_4) and grown at 37 °C. All of the C. *albicans* strains used in this study are listed in Table II-1.

1.2. Bacterial strains and culture conditions

Escherichia coli DH5α was used for DNA manipulation. *E. coli* strains were grown at 37 °C in Luria-Bertani (LB, 0.5% yeast extract, 1% tryptone and 1% NaCl) medium supplemented, where it is necessary required, with the following antibiotics at final concentrations: ampicillin, 50 μg/ml; tetracycline

Table II-1. Strains used in this study

Strain	Genotype	Source
E. coli strains		
DH5α	F$^-$*ΔlacU169(ϕ80lacZΔM15)endA1 rec1hsdR17 deoR supE44 thi-1* λ^-*gyrA96 relA1*	(Hanahan, 1983)
***C. albicans* strains**		
SC5314	Wild type	Clinical isolate
CAI4	*ura3Δ::imm434/ura3Δ::imm434*	(Fonzi and Irwin, 1993)
BWP17	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG/arg4Δ::hisG his1Δ::hisG/his1Δ::hisG*	(Wilson *et al*., 2000)
Bca2-9	*ura3Δ::imm434/ ura3Δ::imm434 tup1Δ::hisG/tup1Δ::hisG*	(Braun and Johnson, 1997)
CH403	*ura3Δ::imm434/ura3Δ::imm434 ssn6Δ::hisG/ssn6Δ::hisG-URA3-hisG*	(Hwang *et al*., 2003)
CH404	*ura3Δ::imm434/ura3Δ::imm434 ssn6Δ::hisG/ssn6Δ::hisG*	(Hwang *et al*., 2003)
JO103	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG/arg4Δ::hisG his1Δ::hisG/his1Δ::hisG*	This study

	rpd31Δ::HIS1/rpd31Δ::ARG4	
JO1031	*RPS10::RPS10-URA3*-pGEM-T Easy in JO103	This study
JO103R	*rpd31Δ::RPD31-URA3*-pGEM-T Easy in JO103	This study
JO105	*Δura3::imm434/ Δura3::imm434 (*pYPB1-*ADHpt)*	This study
JO106	*Δura3::imm434/ Δura3::imm434 (*pJO-2*)*	This study
JO107	*Δura3::imm434/ Δura3::imm434 (*p*SSN6)*	This study
JO203	*ssn6Δ::hph*/*ssn6Δ::hph-URA3-hph* in JO103	This study
JO303	*ura3Δ::imm434*/*ura3Δ::imm434 arg4Δ::hisG*/*arg4Δ::hisG his1Δ::hisG*/*his1Δ::hisG rpd32Δ::HIS1/rpd32Δ::ARG4:: RPS10::RPS10-URA3*-pGEM-T Easy	This study
JO403	*rpd32Δ::hph*/*rpd32Δ::hph-URA3-hph* in JO303	This study
JO500	*ura3Δ::imm434*/*ura3Δ::imm434 SSN6::CBPTAP-URA3*	This study
JO603	*ura3Δ::imm434*/*ura3Δ::imm434 arg4Δ::hisG*/*arg4Δ::hisG his1Δ::hisG*/*his1Δ::hisG sds3Δ::HIS1/sds3Δ::ARG4:: RPS10::RPS10-URA3*-pGEM-T Easy	This study
JO700	*ura3Δ::imm434*/*ura3Δ::imm434 RPD31::CBPTAP-URA3*	This study

JO710	*ura3Δ::imm434/ura3Δ::imm434 ssn6Δ::hisG/ssn6Δ::hisG RPD31::CBPTAP-URA3*	This study
JO720	*ura3Δ::imm434/ura3Δ::imm434 arg4Δ::hisG/arg4Δ::hisG his1Δ::hisG/his1Δ::hisG rpd31Δ::HIS1/rpd31Δ::ARG4 SSN6::CBPTAP-URA3*	This study
JO730	*SSN6::CBPTAP-ARG4 RPD31::HPM TAP-HIS1* in BWP17	This study
JO740	*tup1Δ::hph/tup1Δ::hph SSN6::CBPTAP-ARG4 RPD31::HPM TAP-HIS1* in BWP17	This study

15 μg/ml.

2. General genetic manipulation methods

2.1. Plasmids used in this study

General techniques for isolation and manipulation of DNA in *E. coli* were as described previously (Sambrook *et al*., 1989). pGEM-T Easy vector was used for cloning and sequencing of a PCR product. The plasmids of pJS-HPM53H and PBS1479 were used for TAP purification. pYPB-*ADH*pt was used for overexpressing appropriate genes in *C. albicans*. pQF181 and pQF182 were used for disruption of *C. albicans* gene. All of the plasmids used in this study were listed in Table II-2.

2.2. Polymerase chain reaction (PCR)

DNA fragment amplification was performed according to the method recommended by Taq polymerase manufacturer (Promega, Madison, WI) with a slight modification. For the reactions, 100 pmol of degenerate oligonucleotide primers, 200 ng of genomic DNA or 10 ng plasmid DNA and 0.5 units of Taq polymerase were combined in a final volume of 50 μl with reaction buffer (50 mM KCl, 1.2 mM MgCl2, 10 mM Tris-HCl, pH 8.4 and 0.01% gelatin) containing 50μM dNTPs. The mixture was subjected to 30 cycles of 1 min denaturation at 94 ℃, 1 min annealing at 55 ℃ and appropriate time of a gene length for extension at 72 ℃.

2.3. Sequencing of cloned vectors

Table II-2. Plasmids used in this study

Plasmid	Description	Sources
pBS1479	Shuttle vector containing CBPTAP tag	(Puig *et al*., 2001)
pJO-1	pBS1479 replaced *SkTRP1* to *CaURA3*	This study
pJS-HPM53H	Shuttle vector containing HPMTAP tag	(Graumann *et al*., 2004)
pHPM53U	pJS-HPM53H replaced Sc*HIS3* to *CaURA3*	This study
p*ADH*	pGEM T-Easy vector containing *ADH* promoter	This study
pRPDREV1	pGEM T-Easy vector containing *RPD31* ORF region	This study
pRPDREV2	pGEM T-Easy vector containing *RPD31* ORF region with *URA3* marker for reintegration	This study
pSSN-1441	pGEM T-Easy vector containing *SSN6* ORF	This study
pSSN-2	pGEM T-Easy vector containing *SSN6* HPMTAP	This study
pQF181	Disruption vector containing *hph-URA3-hph* cassettes (*URA3*:5'→3' direction)	(Hwang *et al*., 2003)
pQF182	Disruption vector containing *hph-URA3-hph* cassettes(*URA3*:3'←5' direction)	(Hwang *et al*., 2003)
pSSN-181	*hph-URA3-hph* cassettes containing	This study

	homologous recombination region of *SSN6* in pQF181	
pSSN-182	*hph-URA3-hph* cassettes containing homologous recombination region of *SSN6* in pQF182	This study
pR32-181	*hph-URA3-hph* cassettes containing homologous recombination region of *RPD32* in pQF181	This study
pR32-182	*hph-URA3-hph* cassettes containing homologous recombination region of *RPD32* in pQF182	This study
p*RP10-URA3*	pGEM T-Easy vector containing *RPS10-CaURA3*	This study

The cloned plasmids were isolated and cleaned by using a DNA-spin plasmid DNA purification kit (Intron Biotech, Korea). The plasmids were subjected to ALF express DNA sequencer Pharmacia Biotech., Upsala, Sweden).

2.4. Transformation of *C. albicans* by LiAc

Transformation of *C. albicans* cells was performed according to the protocol (Gietz *et al*., 1995) with some modifications (Walther and Wendland, 2003). *C. albicans* cells were grown until an A600 of 0.5. The cell suspension was centrifuged at 8,000 rpm for 5 min at 4 °C, washed once with 10 ml of TE buffer (10 mM Tris-HCl; pH 7.5, 1mM EDTA). After washing, the cells were resuspended in 100 µl of LiAc/TE buffer (100 mM LiAc, 10 mM Tris-HCl; pH 7.5). For transformation, 50 µl of cell suspension was mixed with 300 µl of PEG/LiAc/TE buffer (40% of PEG 3350, 100 mM LiAc, 10 mM Tris-HCl; pH 7.5), 5 µl of carrier DNA and plasmid DNA then incubated at 28 °C for overnight. Transformation was performed by heating the mixture at 42 °C for 15 min. Resulting cells were then plated onto a minimal media supplemented with appropriate amino acids.

2.5. Southern blot analysis

Southern blot hybridization was performed by standard methods (Sambrook *et al*., 1989). *C. albicans* genomic DNA (20µg per a lane) was digested with restriction enzymes (KOSCHEM) and separated on 0.8% agarose gel in running buffer (40 mM Tris-acetate, pH 8.0, and 1 mM EDTA). Blot was transferred to positively charged nylon membrane (Boehringer Mannheim) using an alkaline transfer method. Blots were prehybridized with 10 ml prehybridization buffer for 1 hour at 42 °C and hybridized with an appropriate

probe for overnight at 42 °C. Final washes were done at 65 °C in 0.2 x SSC containing 0.1% sodium dodecyl sulfate (SDS). For hybridization probes, a 542, 270, 550 and 450 bp DNA fragments of *RPD31*, *RPD32*, *TUP1* and *SSN6* were amplified by PCR using following primers in Table II-3. The PCR fragments were labeled with DIG DNA labeling kit (Boehringer Mannheim) as described by a manufacturer. The membrane was incubated with DIG antibody for 1 hour and developed with BCIP and NBT.

2.6. Northern blot analysis

40 μg of total RNA was separated on a 1% (w/v) agarose gel containing formaldehyde, and blotted onto a nylon membrane (Osmonics). The coding region of various genes were PCR amplified and labeled with [α-32P]-dATP. UV-cross linked blots were prehybridized in prehybridization buffer (Pharmacia) for 1 hour at 65 °C, hybridized with 5 min boiled probe for 2 hours at 65 °C. Final washes were done at 65 °C in 0.2 x SSC containing 0.1% sodium dodecyl sulfate (SDS). Primers used for production of indicated probes were as follows Table II-3.

2.7. Western blot analysis

SDS-PAGE was performed on 10% polyacrylamide-denaturing gel. Electro-transfer of proteins from the gel to nitrocellulose membrane (Protran BA83, Schleicher & Schuell) was carried out according to the method proposed by Towbin *et al* (Towbin *et al*., 1992). Nonspecific space on the transferred membrane was blocked by three changes of Tris-buffered saline (TBS; 10 mM Tris-HCl, pH 7.5 and 150 mM NaCl) containing 5% Skim milk, 0.02% sodium

azide for 15 min at 25 °C. This membrane was then incubated in TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl and 0.05% Tween20) with primary antibody for overnight at 25 °C. After washing for three changes of TBST for 15 min, the membrane was incubated for 60 min with secondary antibody solution (anti-mouse IgG antibody-alkaline phosphatase, diluted to 1:10000 in TBST). The membrane was then washed for 15 min with three changes of TBST and rinsed with alkaline phosphatase buffer (100 mM Tris-HCl, pH 9.5, 100 mM NaCl and 10 mM MgCl2). The TBST washed membrane was developed in 20 ml of alkaline phosphatase buffer containing BCIP and NBT at 25 °C.

2.8. *Real-time RT-PCR*

Wild-type and mutant cells from an overnight culture were diluted to an A_{600} of 0.5 in pre-warmed YPD supplemented with 10% FBS and grown at 37 °C. Cells were harvested at the indicated time points (30 min) from triplicate samples. RNA was isolated from a beadbeated cell lysate with an RNAiso Plus (TAKARA) Reagent. The 5 μg of RNA was used to generate cDNA by SuperScript® III Reverse Transcriptase. The qPCR was performed and detected using SYBR premix EX TaqTM (TAKARA) and 7300 Real-Time PCR systems (Applied Biosystems). All primers indicated Table II-3.

3. Tandem Affinity Purification (TAP)

3.1. Strategy for Tagging Ssn6p with TAP tag

3.1.1. Tagging HPM TAP tag

The HPM TAP tag consisted of 9×histidine-myc that had been separated by prescission protease was fused with c-terminus of *SSN6* as following. First,

Table II-3. Primers used in this study

Name	Primer sequences	Usage
RpdF4	5’-GACCACCACAGAACTGAA-3’	Southern probe (*RPD31*)
RpdR4	5’-TAAGATTATATGTCTGTG-3’	Southern probe (*RPD31*)
R32F5	5’-GTTGCGAGAACATGGGCA-3’	Southern probe (*RPD32*)
R32R5	5’-ATCTTCATCTACATCACC-3’	Southern probe (*RPD32*)
TuSqF2	5’-GATAGGTTGGTTTCAGGC-3’	Southern probe (*TUP1*)
Tup6	5’-TCTCGAGTTATTTTTTGGTCCAT-3’	Southern probe (*TUP1*)
Ssn10	5’- CATGTT GAGAATGGTGAAC-3’	Southern probe (*SSN6*)
SqR2	5’-AGCATGATGAGGATTAAT-3’	Southern probe (*SSN6*)
ECE1-1F	5'-ATGAAATTCTCCAAAATT-3'	Northern blot analysis
ECE1-1R	5'-TAAGCTTTTCCG AAATAT-3'	Northern blot analysis
HWP1-1F	5'-ATGAGATTATCAACTGCT-3'	Northern blot analysis
HWP1-1R	5'-CTGTAGTAGTAGATGGAG-3'	Northern blot analysis

INO1-1F	5'-CAGCCTCAATCGTCCTCGG CATC A-3'	Northern blot analysis
INO1-1R	5'-CGAAGCGTCTTGCAAGTTTTGTAA-3'	Northern blot analysis
EFG1-1F	5'-GTCAGAGATATTAAACGAGTGATTCAAACC-3'	Northern blot analysis
EFG1-1R	5'-TCAATGACTGAACTTGGGGTGATTGG-3'	Northern blot analysis
ACT1-1F	5'-ATGTGTAAAGCCGGTT-3'	Northern blot analysis
ACT1-1R	5'-AATTGGAACAACGTGA-3'	Northern blot analysis
INO1-2F	5'-CTAGACTCACAATCGACACGAC-3'	ChIP assay
INO1-2R	5'-CGAATACTGAAAGCATACACAGTTT-3'	ChIP assay
UME6-2F	5'-GCTTTGCTTTACATAATTGGTGATAGG-3'	ChIP assay
UME6-2R	5'-AGAACGACTGTATGGTAATCACTTT-3'	ChIP assay
UME6-3F	5'-CCCAGCACTGCTACTGGATCT-3'	RT-qPCR
UME6-3R	5'-GGTTGGGATTGTGCTTGTTGT-3'	RT-qPCR
ACT1-3F	5'-AGCTTTGTTCAGACCAGCTGATT-3'	RT-qPCR
ACT1-3R	5'-AGTTGAAAGTGGTTTGGTCAATACC-3'	RT-qPCR

full length of *SSN6* was amplified PCR using a primer SSN14 (5'- CCA GAT CTA TGT ATG CGA CAG CCC -3') and a SSN41 (5'- CTC GAG TTC ATC ATC ATA ATT TTC -3'). About 3.2 kb of the PCR product was ligated into pGEM T-Easy vector (Promega), yielding the pSSN-1441. *C. albicans URA3* gene was amplified with URA1-F; 5'- CCG TCG ACA ATA GGA ATT GAT TT -3' and URA1-R; 5'- GAG CTC AGG ACC ACC TTT GAT TGT AAA -3' by a PCR to replace the selection marker Sc*HIS3* to Ca*URA3* in a TAP cassette containing vector, pJS-HPM53H. About 1.4kb PCR product was digested with *Sac*I/*Sal*I and subsequently inserted into pJS-HPM53H yielding pHPM53U. Then, 2.1kb *Sac*I/*Xho*I fragment of pHPM53U was ligated into pSSN-1441 yielding pSSN-2. Targeted integration was performed through transformation with the plasmid which was digested at the unique *Afl*II site of *SSN6*.

3.1.2. Tagging CPP TAP tag

C. albicans URA3 gene was amplified with URA2-F; 5'- CTG GGC CCA ATA GGA ATT GAT TTG GAT GG -3' and URA2-R; 5'- TCA AGC TTG ACC ACC TTT GAT TGT AAA -3' by PCR to replace the selection marker *SkTRP1* of pBS1479 to *CaURA3* in the same manner of the HPM TAP. About 1.4kb PCR product was digested with *Apa*I/*Hind*III and subsequently inserted into pBS1479 yielding pJO-1. Resultant CPP TAP tag was integrated into c-terminus of *SSN6* using PCR products with 100-bp short flanking homologies to *SSN6*. This PCR was performed using primers of TAP-F; 5'- GAT GAA AAT CCA GAA CCT CCA ATG AGA AAG ATT GAA GAA GAT GAA AAT TAT GAT GAT GAA TCC ATG GAA AAG AGA AGA TG -3' and TAP-R; 5'- CCC GTC AAA TAT ATA TAT ATA TTC AAC TAT CAT ATA CAA TTT CAC CAC TTT TCT TTA TCT TAC GAC TCA CTA TAG GGC G -3' that contain flanking

regions of both the *SSN6* and the pJO-1.

3.2. TAP purification

3.2.1. Purification of HPM TAP tag

HPM TAP purification was performed according to a protocol (Graumann *et al*., 2004) with some modification. About 5 g (wet weight) of *C. albicans* cells were resuspended with TNET (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1 mM EDTA and 0.2% Triton X-100) supplemented with 1/100 volumes of Protease Inhibitor Cocktail and homogenized by bead beater (Biospec). To dissipate a heat, bead beater was turned on and off every 30 sec for 2 hours. The crude extract was incubated with 100 μl of α-myc conjugated protein A sepharose beads (Sigma, St. Louis) at 4 °C for 3 hours by rotating. After washing, the sample bound resin was eluted by adding PreScission Protease (Amersham, Piscataway). Then eluate was mixed with 50 μl of nickel nitrilotriacetic acid (Ni-NTA) agarose bead for 3 hours. After washing the bead, proteins were eluted by addition of 50 μl of 100 mM EDTA. The eluates were then precipitated by treatment of Trichloroacetic acid (TCA).

3.2.2. Purification of CPP TAP tag

CPP TAP-tagged Ssn6p was purified from *C albicans* according to a protocol (Gould *et al*., 2004; Puig *et al*., 2001) by some modifications. About 5 g (wet weight) of *C. albicans* cells were resuspended with Lysis buffer (20 mM Hepes, pH 7.6, 200 mM NaCl, 10 % glycerol, 0.3 % Tween 20, 10 mM EDTA and 100 μM zinc acetate) supplemented with 1/100 volumes of Protease Inhibitor Cocktail and 0.5 mM DTT then homogenized by bead beater (Biospec). To avoid heating, bead beater was turned on and off every 30 sec for

2 hours. The crude extract was incubated with 1 ml of IgG sepharose beads (GE healthcare) for 3 hours by rotating at 4 °C. After washing, the resin was eluted by treatment of TEV protease (Invitrogen). The eluant was mixed with 150 μl of calmodulin binding resin (GE healthcare) supplemented 6.25 μl/ml of 1M $CaCl_2$ for 3 hours. After washing the bead, proteins were eluted by calmodulin elution buffer (10 mM Tris-HCl, pH 8, 150 mM NaCl, 0.1 % NP-40, 10% glycerol, 100 μM zinc acetate, 1 mM magnesium acetate, 1 mM Imidazole, 2 mM EGTA and 10 mM β-mercaptoethanol). The eluates were then precipitated with 15% TCA of the sample volume.

4. MALDI-TOF mass spectrometry and database search

Sliced protein samples were destained by addition of 15 mM potassium ferricyanide and 50 mM sodium thiosulfate. After washing, the gel was dehydrated by acetonitrile. Dehydrated gel was then digested by 50 ng of trypsin for overnight at 37°C. Peptides were eluted by adding 5% trifluoroacetic acid in 50% acetonitrile solution. After elution and concentration, Delayed extractionTM (DE) MALDI time-of-flight (TOF) mass spectra of peptide samples were acquired by a Voyager-DETMSTR Biospectrometry Workstation (Applied Biosystems). Peptides were analyzed in the mass range 8000-25000 Da. Mass data were analyzed using the Mascot search algorithm. The mass tolerance of the monoisotopic masses was set to 50 ppm.

5. Gene disruption and overexpression in *C.*

albicans

5.1. Disruption of *RPD31* in *C. albicans*

Disruption of *RPD31* in *C. albicans* was carried out as described by Gola *et al*. (Gola *et al*., 2003). Each allele of *RPD31* gene was replaced by two distinct marker genes, *HIS1* and *ARG4*. First, 1.35 kb fragment of *HIS1* ORF containing 340 bp upstream from start and 240 bp downstream from stop region and 1.95 kb fragment of *ARG4* ORF containing 556 bp of upstream and 87 bp downstream regions from start and stop region were amplified by PCR. Each PCR product was then ligated into pGEM-T Easy vector (Promega), yielding p*HIS1* and p*ARG4* respectively. The cassettes for disrupt of *RPD31* genes were generated by PCR flanked at the 5'-end from a promoter region and at the 3'-end to the terminator region of *RPD31* to the *HIS1* and *ARG4* marker gene. In order to increase the recombination frequency, the flanking homology regions were comprised of a size of 100 bp. All Primers used in amplification of markers were as follows: 31HIS1- Forward; 5'- ACA ACT CAA CCA AAC TCG TCC ACC ATG TAT ACT GAA CTT CCA TTT GAT GAA TTG AAA GTT GAT CCA AAC CAA AAG AAA CGT TGG AGG ATG AGG AGA CAG AAG TTA GTA GTA -3', 31HIS1-Reverse; 5'- TGA ACT TTT ATT TAG TTC TTC AAT TTC TTT CAA TTC TTC TTC AGT CAA CTT TGC AGC ATC TTC CAT TGG TTG ATC CTC TGG TTT CAG TTC TGT GGT GGT CAA ACG AGA ATG CCT ATT GAC -3', 31ARG4-Forward; 5'- AAA GAA ACG TAT TGC TTA TTT CTA TGA TGC TGA TAT TGG GAA TTA TGC TTA TGG GGC TGG TCA TCC CAT GAA ACC TCA TCG CCC CTT TAG TTA GAT TTT TCA AGA GTA GTC -3', 31ARG4-Reverse ; 5'- GCA TCT TCC ATT GGT TGA TCC TCT GGT TTC AGT TCT GTG GTG GTC TCT GTA AGG

GTT TCT GCT GGT TTA TCT TCA TGT TTG TAT TGG AGT ACA AGG TAT CTC -3'. Transformants were verified by a diagnostic PCR and confirmed by a southern blot analysis.

5.2. Reintegration of *RPD31* in *C. albicans*

In order to reintegration, 2.72 kb fragment of *RPD31* ORF containing 540 bp upstream from start codon, 450 bp downstream region from stop codon was amplified by PCR and cloned into pGEM-T Easy, yielding pRPDREV1. Then 1.3 kb *Sac*I/*Sal*I fragment of p*URA3* was inserted into same site of it, yielding pRPDREV2. Targeted integration was performed by digestion of the plasmid pRPDREV2 at the unique *Bgl*II site within promoter region of *RPD31*. The transformants were confirmed by a diagnostic PCR and a southern blot analysis.

5.3. Disruption of *SSN6* in *rpd31* mutant in *C. albicans*

Disruption of *SSN6* in *rpd31* mutant was carried out as described by Fonzi and Irwin (1993) (Fonzi and Irwin, 1993). The *hph-URA3-hph* cassettes generated from the *Kpn*I/*Hind*III-digested pQF181 or pOF182 which had been inserted the *Kpn*I/*Hind*III-cut pSSN-1441, yielding pSSN-181 and pSSN-182. The 5.1-kb fragment of the *Apa*I/*Sac*I-digested pSSN-181 was integrated in place of as portion of *SSN6*. Spontaneous *Ura*$^{-}$ derivatives of the heterozygous disruptants were selected on minimal defined medium supplemented with 625 mg of 5-fluoroorotic acid (FOA) and 30 mg of Uridine per liter. The resulting *Ura*$^{-}$ cells were repeated by this procedure with the *Apa*I/*Sac*I-digested pSSN-182 to delete the other allele of *SSN6*.

5.4 Disruption of *RPD32* in *C. albicans*

Disruption of *RPD32* in *C. albicans* was the same as the method of disruption of *RPD31*. All Primers for *RPD32* used in amplification of markers were as following: 32HIS1- Forward; 5'- GAA TGG CAC ATT CAT TGA TTA TGA ATT ATG AAT TAT ATA AAA AAA TGG AAA TTT ATC GAG CTC AAC CAG CAA CAA ATT TGA TGG AGG ATG AGG AGA CAG AAG TTA GTA GT -3', 32HIS1-Reverse; 5'- GAA CTC TCC ATC AGC TTG AAC TAA TTT ATC TTT AGC AAA TTG TGA TCC ACC TTT TGT ATC TAT AGC ATC AGG CAT ATC TTC ATC CAA ACG AGA ATG CCT ATT GAC TTT -3', 32ARG4-Forward; 5'- AAT GGC ACA TTC ATT GAT TAT GAA TTA TGA ATT ATA TAA AAA AAT GGA AAT TTA TCG AGC TCA ACC AGC TCA ACC AGC AAC AAA TTT AGC CCC TTT AGT AAG ATT TTT CAA GAG TAG TC -3', 32ARG4-Reverse; 5'- TCA TAG AAC TCT CCA TCA GCT TGA ACT AAT TTA TCT TTA GCA AAT TGT GAT CCA CCT TTT GTA TCT ATA GCA TCA GGC ATA TCT TCA TCT ATT GGA GTA CAA GGT ATC TC -3'. Transformants were verified by a diagnostic PCR and confirmed by a southern blot analysis.

5.5 Disruption of *rpd31 rpd32* in *C. albicans*

Disruption of *RPD32* in *rpd31* mutant was carried out as following the method that mentioned above in 5.3. The 4.5-kb fragment of the *Hind*Ⅲ/*Sac*I-digested pR32-181 was integrated in place of a portion of *RPD32*. Spontaneous *Ura*$^{-}$ derivatives of the heterozygous disruptant were selected on minimal defined medium supplemented with 625 mg of 5-fluoroorotic acid (FOA) and 30 mg of Uridine per liter. The resulting *Ura*$^{-}$ cells were repeated by this

procedure with the *Hind*Ⅲ/*Sac*I-digested pR32 -182 to delete the other allele of *RPD32*.

5.6. Disruption of *SDS3* in *C. albicans*

Disruption of *SDS3* in *C. albicans* was applying the same as the method of disruption of *RPD31* and *RPD32*. All Primers for *SDS3* used in amplification of markers were as follows: SDS3HIS1- Forward; 5'- GAA TGG CAC ATT CAT TGA TTA TGA ATT ATG AAT TAT ATA AAA AAA TGG AAA TTT ATC GAG CTC AAC CAG CAA CAA ATT TGA TGG AGG ATG AGG AGA CAG AAG TTA GTA GT –3', SDS3HIS1-Reverse; 5'- GAA CTC TCC ATC AGC TTG AAC TAA TTT ATC TTT AGC AAA TTG TGA TCC ACC TTT TGT ATC TAT AGC ATC AGG CAT ATC TTC ATC CAA ACG AGA ATG CCT ATT GAC TTT -3', SDS3ARG4-Forward; 5'- AAT GGC ACA TTC ATT GAT TAT GAA TTA TGA ATT ATA TAA AAA AAT GGA AAT TTA TCG AGC TCA ACC AGC TCA ACC AGC AAC AAA TTT AGC CCC TTT AGT AAG ATT TTT CAA GAG TAG TC -3', SDS3ARG4-Reverse; 5'- TCA TAG AAC TCT CCA TCA GCT TGA ACT AAT TTA TCT TTA GCA AAT TGT GAT CCA CCT TTT GTA TCT ATA GCA TCA GGC ATA TCT TCA TCT ATT GGA GTA CAA GGT ATC TC -3'. Transformants were verified by a diagnostic PCR and confirmed by a southern blot analysis.

5.7. Overexpression of *RPD31* in *C. albicans*

To overexpress *RPD31* in *C. albicans*, YPB1-*ADH*pt vector containing the *C. albicans ADH1* promoter (Talibi *et al*., 1999) was used. PCR was performed by using primers contained *Bgl*II-for forward primer- , *Xho*I-for reverse primer-

sites of the ORF region of *RPD31* respectively. A PCR product of 1.73 kb fragment was inserted into pGEM-T Easy vector yielding pRPDOV1. A 1.73 kb *Bgl*II/*Xho*I fragment containing the entire coding sequence of *RPD31* and its 5' and 3' flanking regions was isolated from pRPDOV1 and ligated into the same sites of YPB1-*ADH*pt, which contain *URA3* from *C. albicans* as a selectable marker and autonomously replicating sequence from *C. albicans* for replication in *C. albicans*. The resulting plasmid pJO-2 was transformed into CAI4 strain and resulting Ura^+ transformants were selected from the minimal media.

5.8. Overexpression of *SSN6* in *C. albicans*

To overexpress *SSN6* in *C. albicans*, YPB1-*ADH*pt vector containing the *C. albicans ADH1* promoter (Talibi and Raymond, 1999) was used. PCR was performed by using primers contained *Bgl*II-for forward primer-, *Xho*I-for reverse primer- sites of the ORF region of *SSN6* respectively. A PCR product of 3.5 kb fragment was inserted into pGEM-T Easy vector yielding pSSN1441. A 3.5 kb *Bgl*II/*Xho*I fragment containing the entire coding sequence of *SSN6* and its 5' and 3' flanking regions was isolated from p*SSN6* and ligated into the same sites of YPB1-*ADH*pt, which contain *URA3* from *C. albicans* as a selectable marker and autonomously replicating sequence from *C. albicans* for replication in *C. albicans*. The resulting plasmid p*SSN6* was transformed into CAI4 strain and resulting Ura^+ transformants were selected from the minimal media.

6. Assays used in this study

6.1. Assay for the activity of histone deacetylase in *C. albicans*

ChIP assays were performed as described previously (Bhaumik and Green, 2003; Shukla et al., 2006), with minor modifications for *C. albicans.* Cells were grown until an A_{600} of 1.0 and then cross-linked with formaldehyde. 2.5M glycine was added to quench the formaldehyde and terminates the cross-linking reaction. For H4K5ac ChIP, chromatin solutions were pre-cleared with A/G agarose resin (Sigma) for 1 hour at 4 °C. Then, supernatants were incubated with A/G agarose resin (Sigma) and H4K5ac-antibody for 3 hours at 4 °C. Then, the purified DNA was analyzed using quantitative PCR.

6. 2. Virulence test

The *ura3*$^{-}$ strain showed attenuated virulence and proteome changes in *C. albicans* (Brand *et al.*, 2004). To avoid any interference by the *ura3*$^{-}$ strain, *Stu*I-digested p*RP10-URA3* vector was integrated at the *RPS10* locus of all strains (SC5314, JO103 and JO103R) for the virulence assay. The *Candida* strains on YPD plates for 48 hours at 28 °C, suspending the cells in phosphate-buffered saline (pH 7.5), and adjusting them to the desired concentration(10^6 cells) after measurement of cell density (A600 = 0.5). BALB/c (white, female) mice weighing 17-20 g were used to test the virulence of each strain.

6.3. Chromatin immunoprecipitation (ChIP)

ChIP assays were performed as described previously (Bhaumik and Green, 2003; Shukla *et al*., 2006), with minor modifications for *C. albicans*. For hyphal induction, cells were grown until an A_{600} of 0.9 and transferred to fresh 50 ml YPD supplemented with 10% FBS (Gibco). Cells were induced to form hyphae for 30 min at 37 °C and then cross-linked. For TAP tag ChIP, each sonicated chromatin extract after zymolyase treatment was pre-cleared with

Sepharose CL-4B (Sigma) for 1 h at 4 °C. Then, supernatants were incubated with IgG Sepharose 6 Fast Flow (GE Healthcare) for 3 h at 4 °C. Next, the purified DNA was analyzed using a quantitative PCR.

III. RESULTS

1. Identification of Ssn6-interacting proteins through TAP method in *C. albicans*

1.1. Construction a strain of bipartite-TAP tagged in the *SSN6* locus

To identify newly proteins associated with Ssn6 in *C. albicans*, the 3' end region of endogenous *SSN6* was replaced with sequences encoding for bipartite affinity tags (CBP TAP) through a PCR-based homologous recombination (Fig. III-1A). The cassette amplified from pJO-1, of which selection marker was replaced from *S. cerevisiae TRP1* to *CaURA3*, was transformed to *C. albicans* and correct insertion of cassette to genome were confirmed by a southern blot analysis (Fig. III-1B). The resulting Ssn6 was C-terminally tagged with two IgG binding domains of *Staphylococcus aureus* protein A (Protein A) and a calmodulin binding peptide (CBP), which are separated by a Tobacco etch viral protease cleavage (TEV) site (Fig. III-1A) (Puig *et al*., 2001).

1.2. Purification and identification of Ssn6 complex

Preliminary experiment revealed that the expression of C-terminally tagged Ssn6 was highly elevated in cells grown to ~2.5 of A_{600} in YPD media at 28 °C (data not shown). The cultured CBP TAP cells in that condition were lysed and resultant crude extract was purified through tandem affinity purification (TAP) (Puig *et al*., 2001). The purified Ssn6 and its associated components were resolved through SDS-PAGE, followed by trypsin in-gel digestion and MALDI-TOF mass spectroscopy (Fig. III-2A).

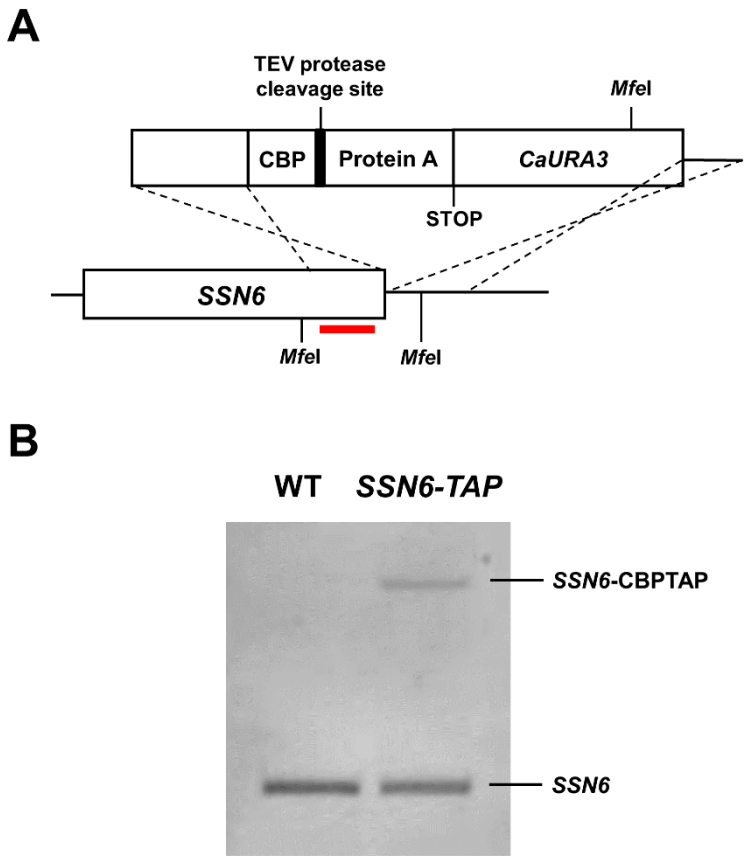
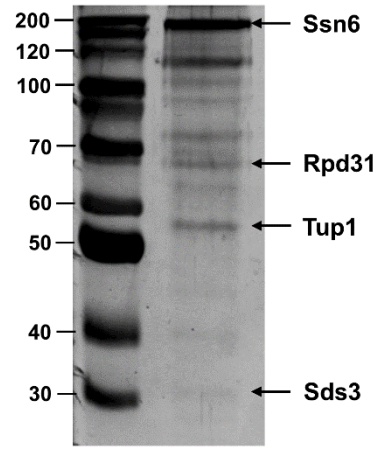


Fig. III-1. Strategy of TAP tagging of *SSN6* and confirmation by Southern blot analysis (unpublished data, Oh, 2008). (A) The Schematic view of CBP TAP and integration strategy of CBP TAP into 3'-end of *SSN6*. *Mfe*I-digested fragments were detected with DNA probe (red bar) by (B) southern blot analysis of wild-type and *SSN6*-TAP cells.

A



B

Protein name	MW (kDa)	Number of matched peptides	Sequence coverage (%)	Score	ORF
Ssn6	119.9	14	19%	77	orf19.6798
Tup1	58.3	8	27%	68	orf19.6109
Rpd31	65.3	6	24%	59	orf19.6801
Sds3	33.4	5	24%	61	orf19.1856

Fig. III-2. Identification of Ssn6 complex through Tandem affinity purification (TAP) method (unpublished data, Oh, 2008). (A) Purified Ssn6 complex was resolved on a 10% denaturing gel and stained with silver nitrate. Identified proteins from MALDI-TOF mass spectrometry shown in (B) are indicated by arrows and labeled. M represents the size marker for proteins and the indicated numbers correspond to size in kDa.

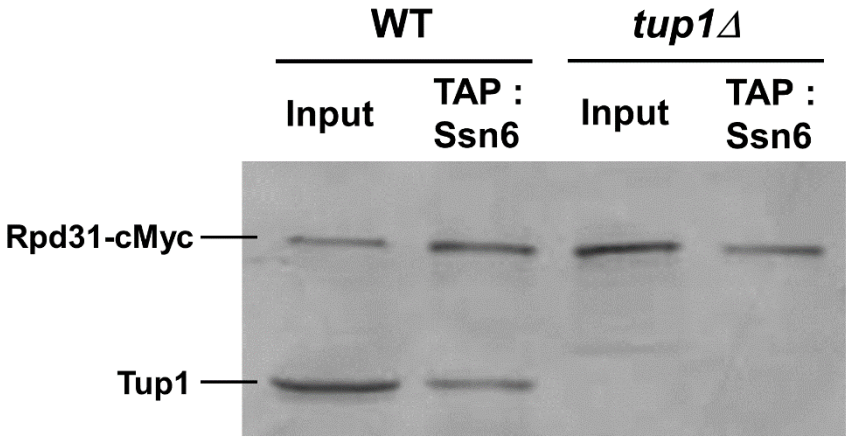


Fig. III-3. Interaction of Ssn6 and Rpd31 in Tup1-independent manner. Ssn6 and Rpd31 were C-terminally tagged with CBPTAP and HPMTAP respectively. C-terminally tagged Ssn6 was purified by using tandem affinity purification for CBP TAP-tagged protein. Input and purified Ssn6 from wild type and Tup1-absent cells were resolved on a 10% denaturing gel and immunoblotted against Tup1 and c-Myc.

As shown in Fig. III-2B, Tup1, Rpd31, and Sds3 were identified as Ssn6-associated proteins. The Tup1 is a general transcriptional repressor and has already been known to form a complex with Ssn6 in *S. cerevisiae* (Malave and Dent, 2006; Smith and Johnson, 2000; Tzamarias and Struhl, 1994, 1995; Varanasi *et al*., 1996). It has also been reported that Ssn6 and Tup1 exist as a complex in *C. albicans* (Kaneko *et al*., 2006). The Sds3 is homologous to Sds3 in *S. cerevisiae*. The *Sc*Sds3 is the integral component of Rpd3L histone deacetylase complex (Rpd3L HDAC complex) and is responsible for structural integrity and catalytic activity of Rpd3L HDAC complex in *S. cerevisiae* (Carrozza *et al*., 2005; Keogh *et al*., 2005; Lechner *et al*., 2000). Because histone deacetylase Rpd3 interacts with Ssn6 in *S. cerevisiae* (Davie *et al*., 2003), its homologous protein in *C. albicans*, Rpd31, was also identified as a component associated to Ssn6. Therefore, it indicates Sds3 might be co-purified with the Rpd31 rather than Ssn6.

Since it has already been demonstrated that *S. cerevisiae* Rpd3 can interact with Ssn6 in a Tup1-independent manner (Davie *et al*., 2003), Ssn6 and Rpd31 were C-terminally tagged with CBP TAP and HPM TAP (which contains nine histidines and nine c-Mycs) respectively in wild- type and Tup1-absent cells to examine the interaction in *C. albicans*. The immunoblotting against Rpd31 of Ssn6 complex purified from each cell revealed that Rpd31 could also interact with Ssn6 in a Tup1-independent manner (Fig. III-3). This result revealed that histone deacetylase Rpd31 could directly interact with Ssn6 in a Tup1-independent manner like interaction of Rpd3 with Ssn6 in *S. cerevisiae*.

2. Characterization of *RPD31* deletion mutant in

C. albicans

Previously, it has been reported that Ssn6 is a transcriptional regulator of filamentous growth in *C. albicans* (Hwang *et al*., 2003). To investigate the roles of Rpd31 in filamentous growth in *C. albicans*, both alleles of *RPD31* were disrupted by a PCR-based homologous recombination (Fig. III-4A) and the disruption of *RPD31* was confirmed by southern blot analysis (Fig. III-4B). In addition, a single copy of wild-type *RPD31* was integrated in a disrupted *RPD31* locus to verify the effects caused from the deletion of *RPD31* (Fig. III-4B), and it was also confirmed by southern blot analysis (Fig. III-4C).

When grown for 4 days on solid Spider media at 37 °C, the *rpd31Δ* mutant showed completely smooth colony morphology and failed to develop filaments (Fig. III-5A). This developmental defect was quite restored when one allele of *RPD31* was recovered (Fig. III-5A). The filament-specific genes *HWP1* and *ECE1* were also hardly expressed in the *rpd31Δ* mutant even though it was grown on solid Spider media at 37 °C, in contrast to those of the wild-type and *rpd31::RPD31* cells (Fig. III-5B). However, *INO1*, which encodes inositol-1-phosphate synthase, was induced in the *rpd31Δ* mutant (Fig. III5-B) as was its homologous gene in *S. cerevisiae* (Rundlett *et al*., 1998). The increased expression of *INO1* and the decreased expression of *HWP1* and *ECE1* in the *rpd31Δ* mutant suggest that Rpd31 could function as a repressor for metabolite genes but as an activator for filamentous genes.

To determine whether the failure of filament development in the *rpd31Δ* mutant is linked to virulence in *C. albicans*, wild-type, *rpd31Δ* and *rpd31::RPD31* strains were injected intravenously into immunocompetent mice. Although all of the mice injected with the wild-type and the *rpd31::RPD31* strain died within 10 days of infection, those injected with the *rpd31Δ* mutant

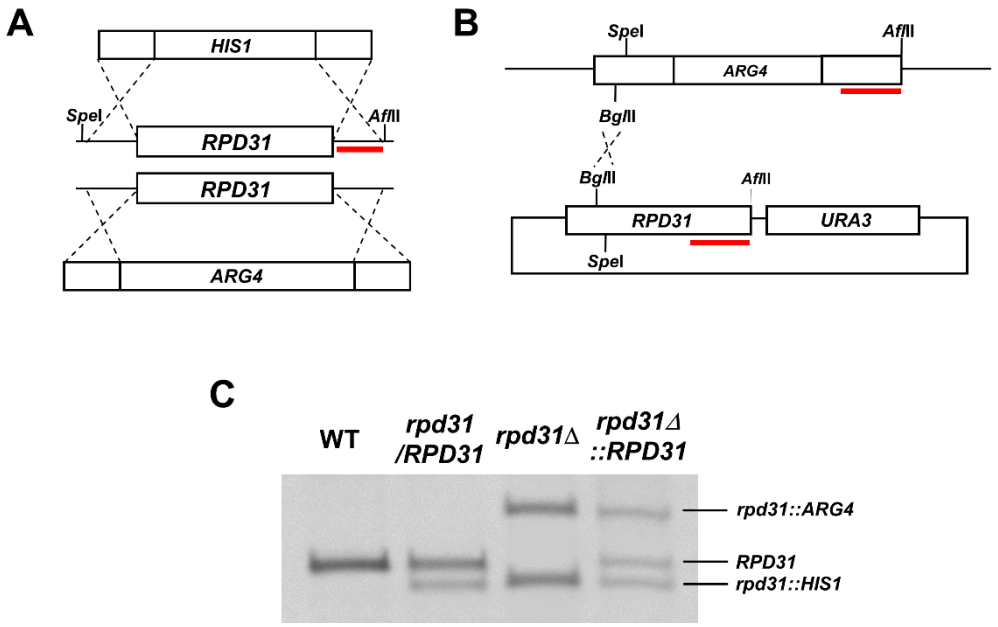


Fig. III-4. Disruption and reintegration of *RPD31* into *RPD31* locus (unpublished data, Oh, 2008). (A) Schematic representation of disruption for *RPD31*. Each allele of *RPD31* was replaced with selective marker *HIS1* and *ARG4*, respectively. (B) Schematic representation of reintegration for *RPD31*. One allele of *RPD31* and nutrient marker *URA3* was integrated into *RPD31* locus. (C) *Spe*I/*Afl*II-digested DNA fragments from each strain were detected by probe indicated as red bar in (A) and (B) through southern blot analysis.

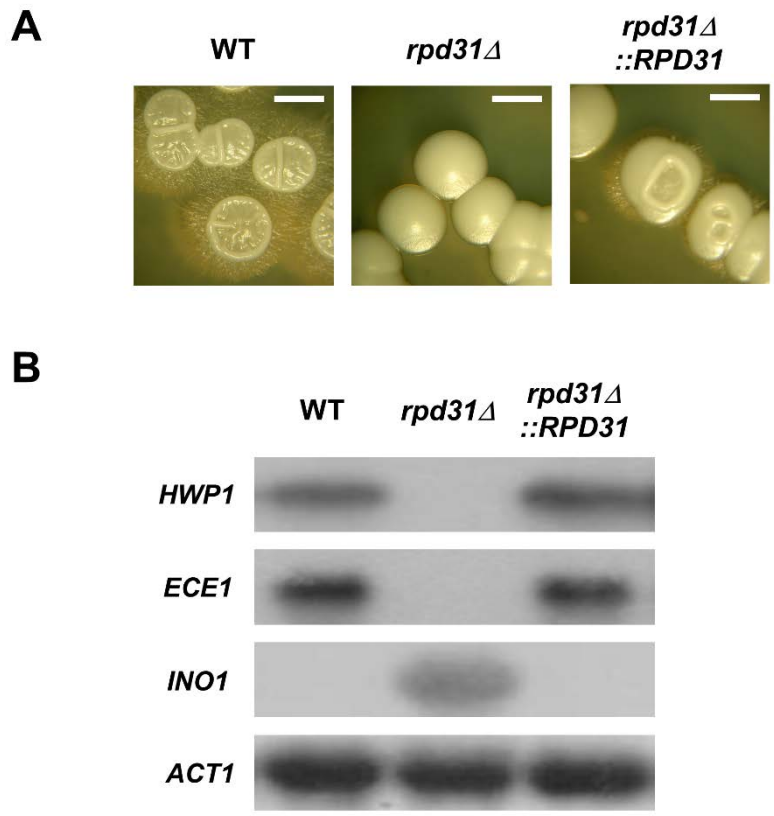


Fig. III-5. *RPD31*, as a critical activator of hyphal formation in *C. albicans* (unpublished data, Oh, 2008). (A) Colony morphology of the wild-type, *rpd31Δ* and its revertant *rpd31Δ::RPD31*. Each strain was grown for 4 days on Spider medium at 37 °C. Each scale bar represents 2 mm. (B) Expression of *HWP1*, *ECE1*, and *INO1* in wild-type, *rpd31Δ* and *rpd31Δ::RPD31* strains. Each strain was grown at 37 °C in Spider liquid medium for 6 hours. 40 μg of RNA was prepared from each strain and northern analysis was carried out with probes for the indicated genes. *ACT1* was probed as a control.

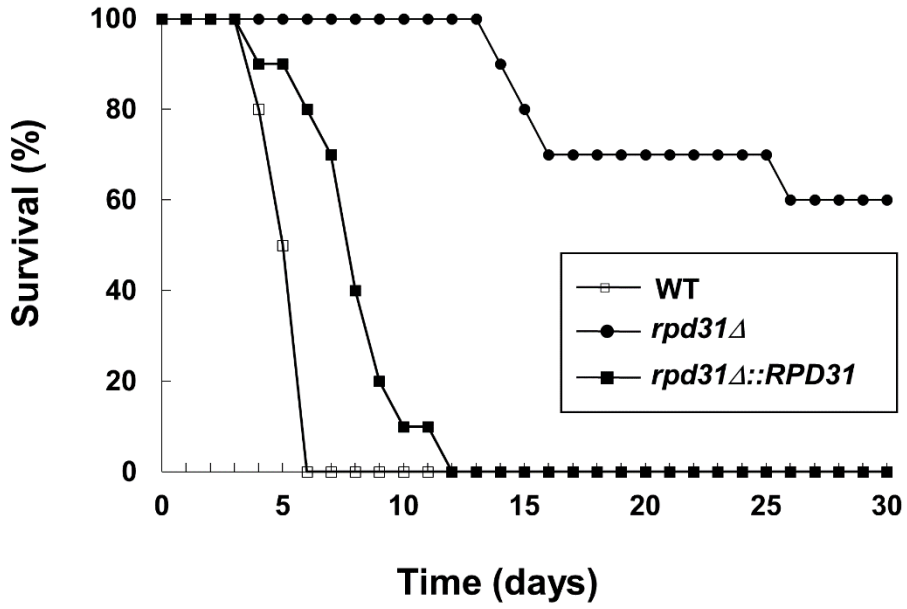


Fig. III-6. *RPD31* is an important factor for virulence in *C. albicans* (unpublished data, Oh, 2008). BALB/c mice were inoculated with 0.1 ml of 10^6/ml cells grown on YPD plates for 48 h at 28 °C. Each of following that wild-type (open square), *rpd31Δ* (closed circle), or *rpd31Δ::RPD31* (closed square).

survived by more than 60% up to 30 days (Fig. III-6). The defect in virulence of *rpd31Δ* mutant indicates that the failure in filamentous development of *rpd31Δ* mutant is directly linked with virulence. This result also supports that Rpd31 acts as an activator for filamentous development in *C. albicans*.

3. Characterization of *SSN6* and *RPD31* deletion mutant in *C. albicans*

3.1. *SSN6* and *RPD31* deletion mutant at elevated temperature

Since Rpd31 and Ssn6 assemble to the complex and the function of each of them are both connected to filamentous development and virulence in *C. albicans*, the functional interaction analysis was performed by further disrupting both alleles of *SSN6* through a PCR-based homologous recombination (Fig. III-7A) in *rpd31Δ* mutant. The disruption of *SSN6* in *rpd31Δ* mutant was confirmed by southern blot analysis (Fig. III-7B) .When grown for 3 days on solid YPD media at 28 °C, wild-type, *rpd31Δ* and *ssn6Δ* cells formed colonies with morphology of yeast-like smooth type, but *rpd31Δ ssn6Δ* mutant cells exhibited slightly wrinkled colony morphology (Fig. III-8). Because it has been previously reported that deletion of *SSN6* in *C. albicans* caused roughly wrinkled colony and stubby filament cell morphologies at an elevated temperature even under filament-inducing conditions (Hwang *et al*., 2003), each strain was also grown at 37 °C on a same media. Interestingly, temperature shift to 37 °C gave rise to distinct morphological differences among strains (Fig. III-8).

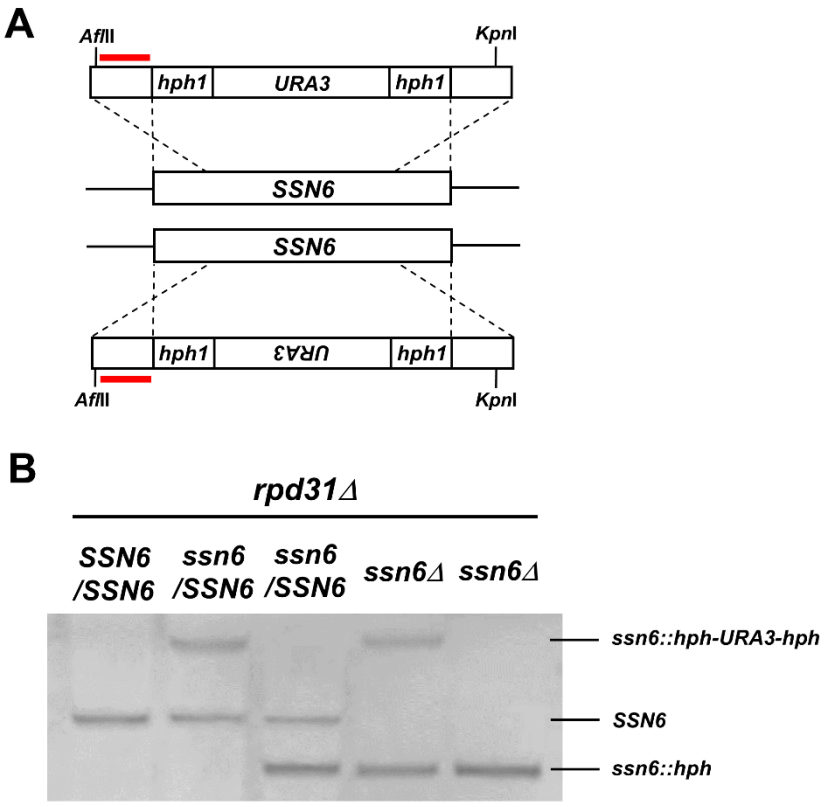


Fig. III-7. Disruption of *SSN6* in wild-type and *rpd31Δ* mutant. (A) Schematic representation of disruption for *SSN6*. Each allele of *SSN6* was replaced with *hph-URA3-hph* cassette. (B) *Kpn*I/*Afl*II-digested DNA fragments from each strain were detected by probe indicated as red bar in (A) through southern blot analysis.

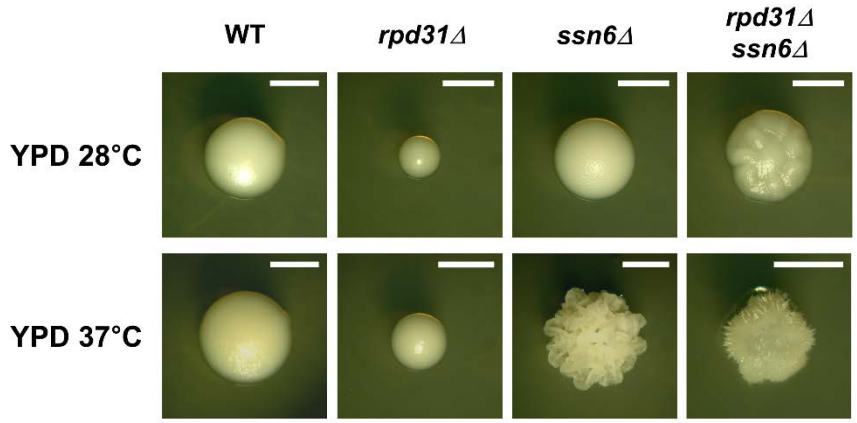


Fig. III-8. Colony morphology of wild-type, *rpd31Δ*, *ssn6Δ*, and *rpd31Δ ssn6Δ* strains on YPD media. Each strain was grown for 3 days on YPD medium at 28 °C or 37 °C. Each scale bar represents 2 mm.

When grown for 3 days, *ssn6Δ* mutant exhibited roughly wrinkled colony morphology as previously reported, and *rpd31Δ* mutant maintained colony morphology at 28 °C except the difference in colony sizes. However, *rpd31Δ ssn6Δ* showed unique type of colony morphology such as fluffy-surfaced and wrinkled (Fig. III-8).

The microscopic observation of those cells were scraped from each colony and stained by calcofluor-white revealed that that the *rpd31Δ ssn6Δ* mutant developed partially elongated filaments at 28 °C and fully elongated filaments at 37 °C while only the *ssn6Δ* mutant developed stubby filaments at 37 °C (Fig. III-9). The *rpd31Δ ssn6Δ* mutant cultured in liquid YPD media for 6 hours showed development of more elongated filaments at 28 °C than those on solid media, and also showed fully elongated filaments at 37 °C (Fig. III-10). Along with the cell morphology, expression of filament-specific genes was also induced in the *rpd31Δ ssn6Δ* mutant at 28 °C, and was more increased at 37 °C, while expression of the same genes in the *ssn6Δ* mutant was induced only at 37 °C (Fig. III-11). Interestingly, the expression of *ECE1*, which is related to filament elongation (Birse *et al*., 1993), was more elevated in the *rpd31Δ ssn6Δ* mutant than in the *ssn6Δ* mutant at 37 °C (Fig. III-11). This observation partially explains why the *rpd31Δ ssn6Δ* mutant developed more elongated filaments than *ssn6Δ* mutant. With given combined the results that Ssn6 could interact with Rpd31, these observations are connected to the possible role of Ssn6-Rpd31 complex as a repressor for filament-specific genes. However, since filament-specific genes, *HWP1* and *ECE1*, was neither induced in the *rpd31Δ* nor the *ssn6Δ* mutant but only induced in the *rpd31Δ ssn6Δ* mutant at 28 °C, it suggests that Ssn6 and Rpd31 might repress filament-specific genes independently from each other (Fig. III-11).

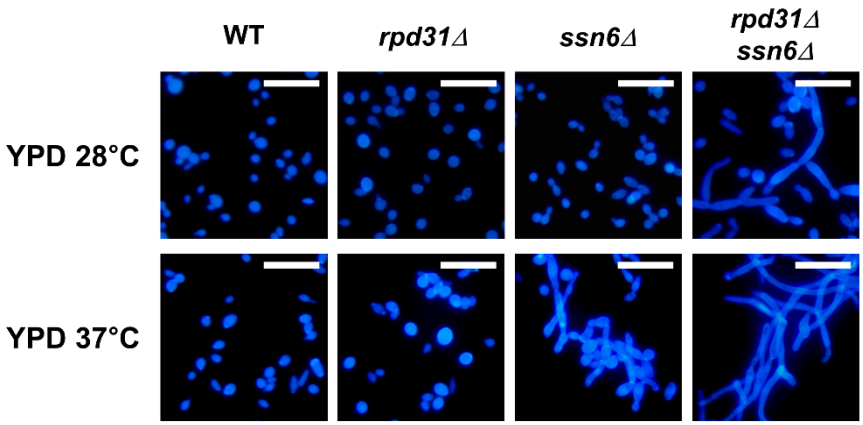


Fig. III-9. Cell morphology of wild-type, *rpd31Δ*, *ssn6Δ*, and *rpd31Δ ssn6Δ* strains. Each cell was scraped from a colony of Fig. III-8. Each cell was stained with calcofluor white and visualized by fluorescence microscopy. Each scale bar represents 10 μm.

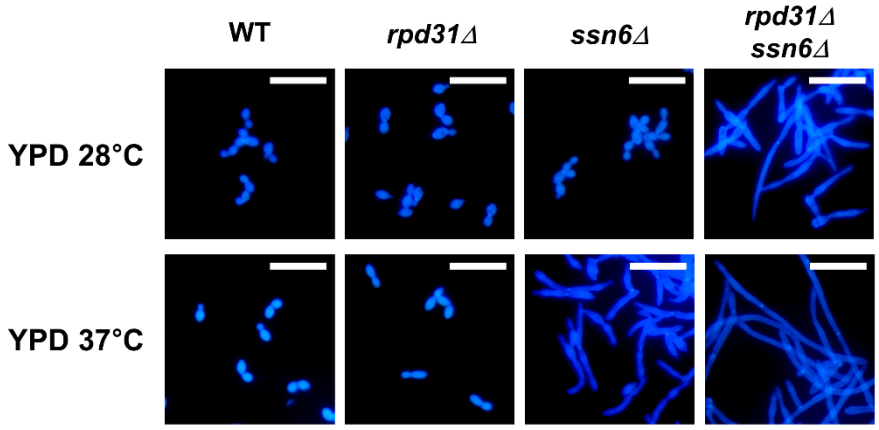


Fig. III-10. Ssn6 and Rpd31 are important for suppression of hyphal formation. Each cell was grown for 6 hours in liquid YPD media at indicated temperature. Each cell was stained with calcofluor white and visualized by fluorescence microscopy. Each scale bar represents 10 μm.

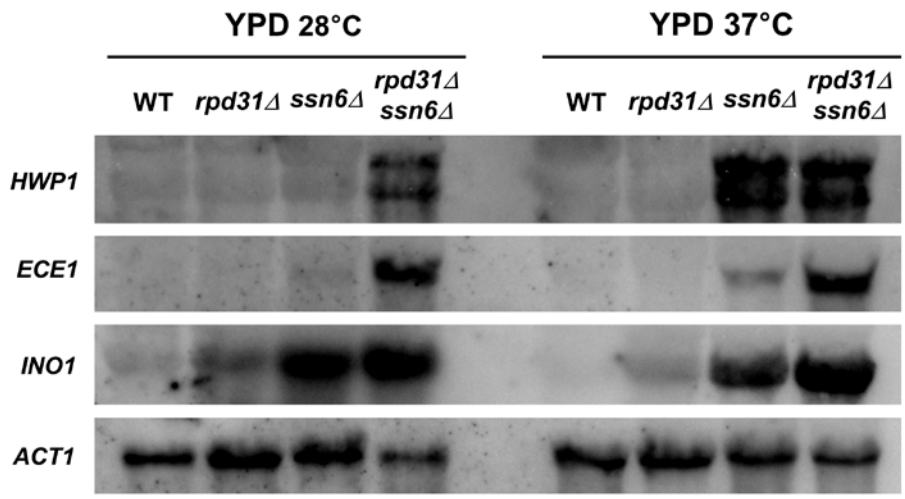


Fig. III-11. Expression of *HWP1*, *ECE1*, and *INO1* in wild-type, *rpd31Δ*, *ssn6Δ*, and *rpd31Δ ssn6Δ* strains. Each strain was grown at 28 °C or 37 °C in YPD liquid medium for 6 hours. 40 μg of RNA was prepared from each strain, and northern analysis was carried out with probes for the indicated genes. *ACT1* was used as the loading control.

The expression of *HWP1* and *ECE1* at 37 °C further suggests that Ssn6 might be more dominant for the repression of filament-specific genes than Rpd31 (Fig. III-11). The expression of *INO1* was also mostly dependent on Ssn6 rather than Rpd31 regardless of temperature (Fig. III-11). These results were contradictory to the inference that Ssn6 and Rpd31 might be functionally synchronized through interaction with each other.

3.2. *SSN6* and *RPD31* deletion mutant in filament-inducing conditions

To understand and solve this contradictory problem, based on that deletion of *RPD31* and *SSN6* induces development of elongated filaments, the *rpd31Δ ssn6Δ* mutant cells were observed under the filament-inducing conditions. When grown on solid Spider media at 28 °C or 37 °C for 4 days, all of the cells except *rpd31Δ* mutant showed slightly different colony morphology depending on temperature (Fig. III-12). After prolonged incubation up to 6 days, the colonies of wild-type and *ssn6Δ* mutant at 28 °C showed the morphology similar to those grown at 37 °C for 4 days (Fig. III-12). This indicates that the shift of temperature affects, at least in Spider media, the rate of the filaments development but not the program of filamentous growth. However, the *rpd31Δ ssn6Δ* mutant retained its own morphology in each temperature (Fig. III-12).

To investigate whether this difference is caused by temperature or phase of Spider media, each cell was incubated in liquid Spider media for 6 hours at 28 °C or 37 °C (Fig. III-13). The wild-type showed yeast-like morphology at 28 °C and extremely long filamentous morphology at 37 °C. Because the cells in liquid Spider media were incubated for shorter time than those on solid one, this result might be impacted due to different rate of filamentous growth

depending on temperature. Although the *ssn6Δ* mutant also showed similar pattern to the shift of temperature, at 28 °C, *ssn6Δ* mutant cells formed an aggregate of which post-mitotic cells could not segregates each other. Considering that the *ssn6Δ* mutant cells at 37 °C also showed aggregate-like forms similar to those at 28 °C except stubby elongated morphology, *ssn6Δ* mutant might have defects in the filament-developmental process which is not significantly affected by temperature. On the other hand, the *rpd31Δ ssn6Δ* mutant cells, surprisingly, exhibited more elongated filaments than those of *ssn6Δ* mutant cells regardless of temperature (Fig. III-13). This morphology of *rpd31Δ ssn6Δ* mutant cells coincided with those in liquid YPD media suggests that *rpd31Δ ssn6Δ* mutant cells could develop elongated filaments but could not response appropriately to filament-inducing condition. Interestingly, when grown at 37 °C and in filament-inducing liquid media such as YPD media containing 10% FBS and Spider, the *rpd31Δ ssn6Δ* mutant could not further elongate its filaments as did the *ssn6Δ* mutant, while the wild-type could develop extremely elongated filaments. In addition to shorter filament compartments than wild-type, the width of filaments was also irregular in the *rpd31Δ ssn6Δ* mutant as that in *ssn6Δ* mutant, while wild-type developed relatively narrow and constant-width filaments (Fig. III-14). However, expression of the filament-specific genes, *HWP1* and *ECE1*, and the positive hyphal regulator *EFG1* in the *rpd31Δ ssn6Δ* mutant was similar or more induced in Spider media at 37 °C compared to those of wild-type and *ssn6Δ* mutant (Fig. III-15). It is noteworthy that the *rpd31Δ ssn6Δ* mutant showed most elevated expression of *ECE1* among other strains despite its halted filament-extension. The expression of metabolic gene *INO1* showed nearly same patterns in Spider media at 37 °C and YPD at 37 °C (Fig. III-15), which

also supported that depletion of Ssn6 and Rpd31, resulted in similar effects caused by the release of the Ssn6 and Rpd31 from their target genes. These results suggest that Ssn6 and Rpd31 are both required for induction of filament-extension by activation of another genes even though filament-specific genes were induced following the release of Ssn6 and Rpd31 from their regulatory regions.

4. The requirement of Ssn6 and Rpd31 for the induction of filamentous extension in *C. albicans*

It has been previously reported that *UME6* is specifically important for filament extension and virulence (Banerjee *et al*., 2008; Carlisle *et al*., 2009; Zeidler *et al*., 2009). *UME6* was expressed transiently from 15 minutes to 3 hours after serum induction in YPD medium at 37 °C (Banerjee *et al*., 2008). As shown in Fig III-16, the mRNA level of *UME6* was most increased at 30 minutes after serum induction in same condition. To examine whether Ssn6 and Rpd31 is required for the activation of *UME6*, the mRNA level of *UME6* in wild-type and *rpd31Δ*, *ssn6Δ* and *rpd31Δ ssn6Δ* mutants was detected after 10% FBS induction for 30 minutes in YPD medium at 37 °C. Surprisingly, the mRNA level of *UME6* was distinctly decreased in the *rpd31Δ*, *ssn6Δ* and *rpd31Δ ssn6Δ* mutants (Fig. III-17). This result indicates that Ssn6 and Rpd31 are both required for the activation of *UME6* followed by the extension of developed filaments. It is also coinciding with the morphology of *rpd31Δ ssn6Δ* mutants which cannot develop extremely long filaments like those of wild-type (Fig. III-14).

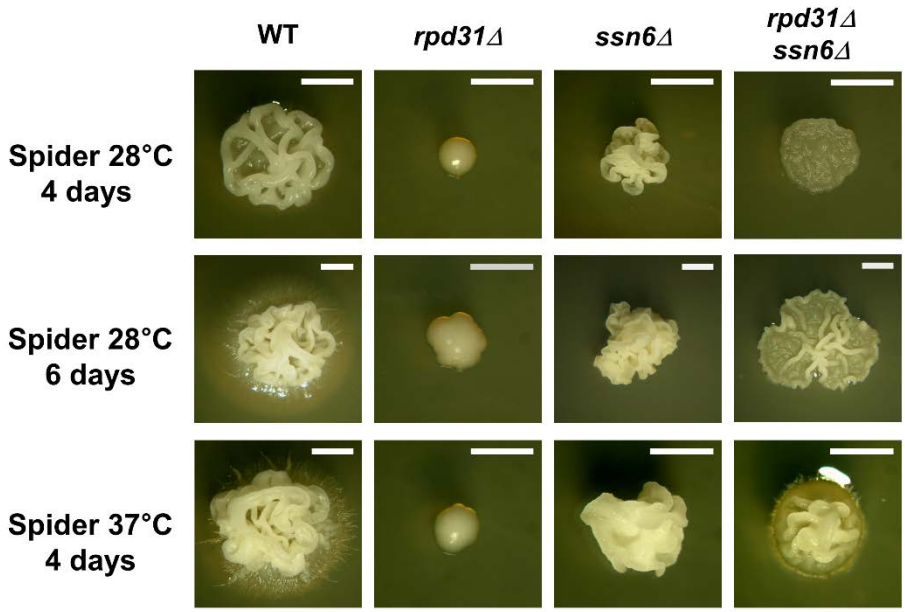


Fig. III-12. Colony morphology of wild-type, *rpd31Δ*, *ssn6Δ*, and *rpd31Δ ssn6Δ* strains on Spider media. Each strain was grown for 4 days or 6 days on Spider medium at indicated temperature. Each scale bar represents 2 mm.

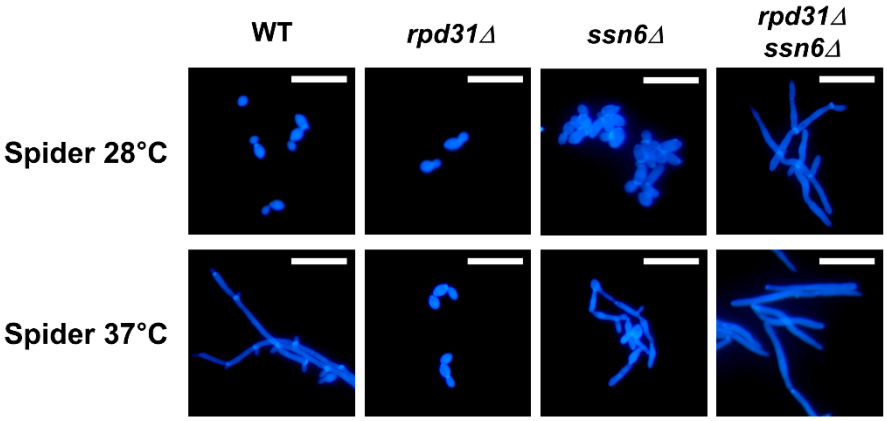


Fig. III-13. Ssn6 and Rpd31 together act like a repressor of hyphal formation under the hyphal inducing condition. Each cell was grown for 6 hours in liquid Spider media at indicated temperature. Each cell was stained with calcofluor white and visualized by fluorescence microscopy. Each scale bar represents 10 μm.

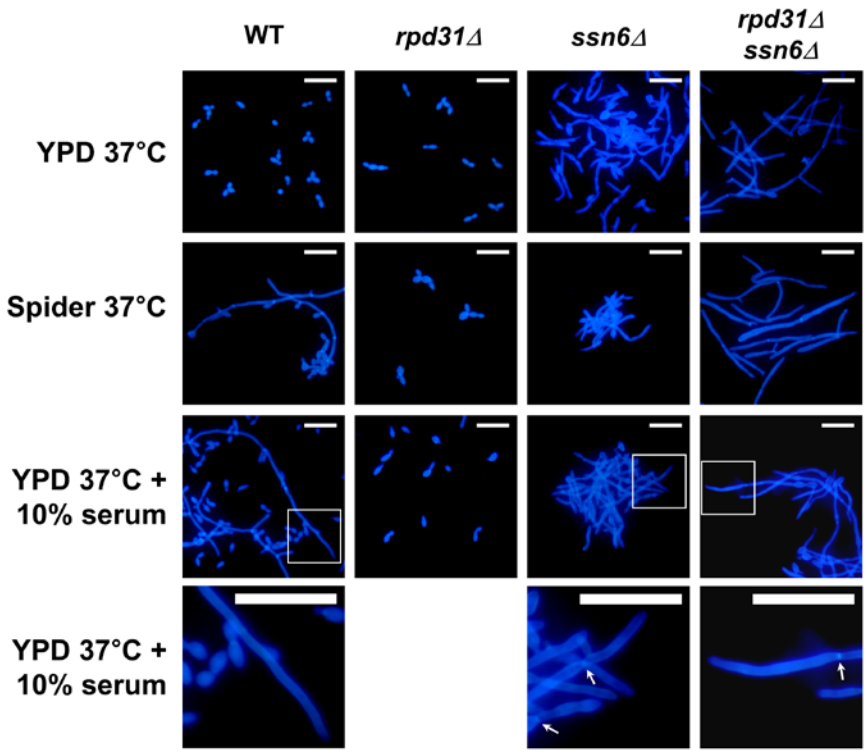


Fig. III-14. Cell morphology of wild-type, *rpd31Δ*, *ssn6Δ*, and *rpd31Δ ssn6Δ* strains in various conditions that induce a hyphal development. Each cell was grown for 6 hours in indicated media at 37 °C. Each cell was stained with calcofluor white and visualized by fluorescence microscopy. The cell morphology of wild-type, *ssn6Δ*, and *rpd31Δ ssn6Δ* strains in YPD+10% serum liquid media at 37°C (indicated by white box) are magnified in the last row. The septic junctions of each cell are indicated by white arrows. Each scale bar represents 10 μm.

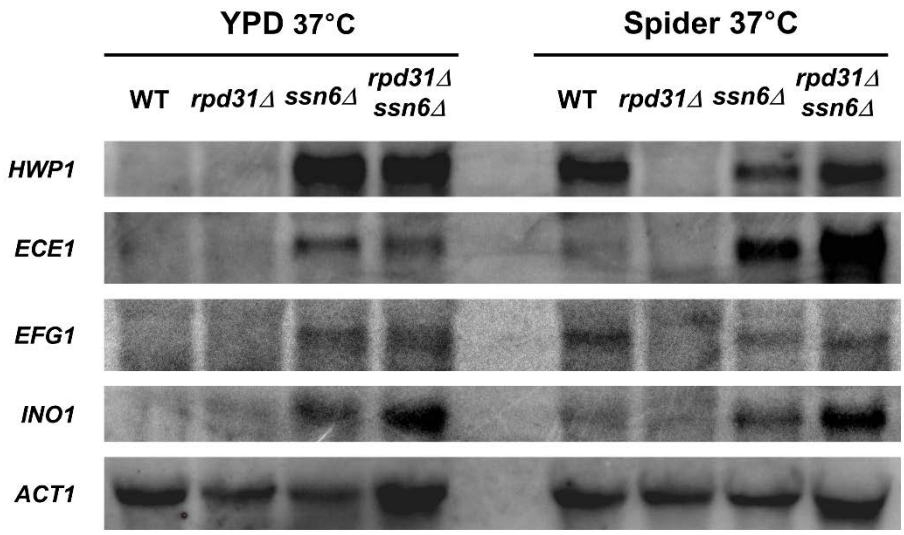


Fig. III-15. Expression of *HWP1*, *ECE1*, *EFG1*, and *INO1* in wild-type, *rpd31Δ*, *ssn6Δ*, and *rpd31Δ ssn6Δ* strains. Each strain was grown at 37 °C in YPD liquid media or Spider liquid media for 6 hours. 40 μg of RNA was prepared from each strain, and northern analysis was carried out with probes for the indicated genes. *ACT1* was used as the loading control.

To further understand how the roles of Ssn6 and Rpd31 are determined as a repressor or as an activator depending on target genes, the occupancies of Ssn6 and Rpd31 at the promoters of *INO1* and *UME6* were examined under the condition that induces the expression of *UME6*. At both promoters, Ssn6 was more enriched in the *rpd31Δ* mutant than in the wild-type (Fig. III-18), which indicates that interaction of Ssn6 with both genes is strengthened in the absence of Rpd31. This result also partly explained why the *RPD31* deletion caused the suppressive effects on the repression of filament-specific genes. This result also indicates that Ssn6 interacts with *INO1* and *UME6* in similar manner and, therefore Ssn6 is not the determinant for action of Ssn6-Rpd31 complex. Surprisingly, Rpd31 was more enriched at the promoter of *UME6* than that of *INO1*, and at the promoter of *UME6*, Rpd31 was less enriched in *ssn6Δ* mutant than in the wild-type (Fig. III-18). This finding indicates that in filament-inducing condition, Rpd31 interacts with *UME6* more than with *INO1* and this interaction of Rpd31 with *UME6* is mediated by Ssn6. Therefore, it suggests that the role of Ssn6-Rpd31 complex as a repressor for *INO1* (Fig. III-15) or as an activator for *UME6* (Fig. III-17) is determined by Rpd31 in Ssn6-dependent manner. That is, in Ssn6-Rpd31 complex, Ssn6 is mainly required for the repression of *INO1*, but both Ssn6 and Rpd31 are required for the activation of *UME6* in filament-inducing condition.

5. Functional relationship between Rpd31 and Rpd32 in *C. albicans*

5.1. Another regulator for filament-development corresponding with Rpd31

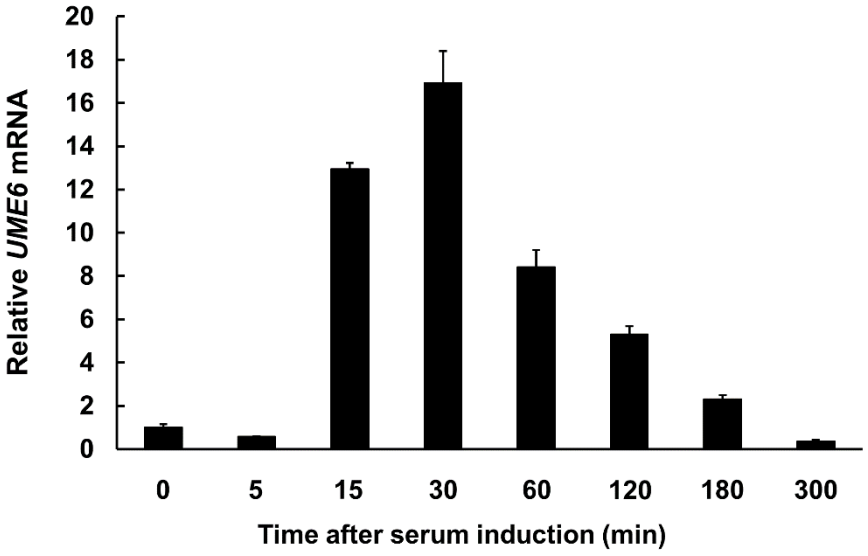


Fig. III-16. Expression of *UME6* in wild-type after serum induction. Wild-type cells were induced at 37 °C in YPD liquid medium with addition of 10% serum. 500 ng of RNA was prepared from cells of each time point, and applied to RT-qPCR analysis. Each measured *UME6* transcript level relative to *ACT1* was normalized to the wild-type. The results represent the average from three independent experiments, with bars representing SEM.

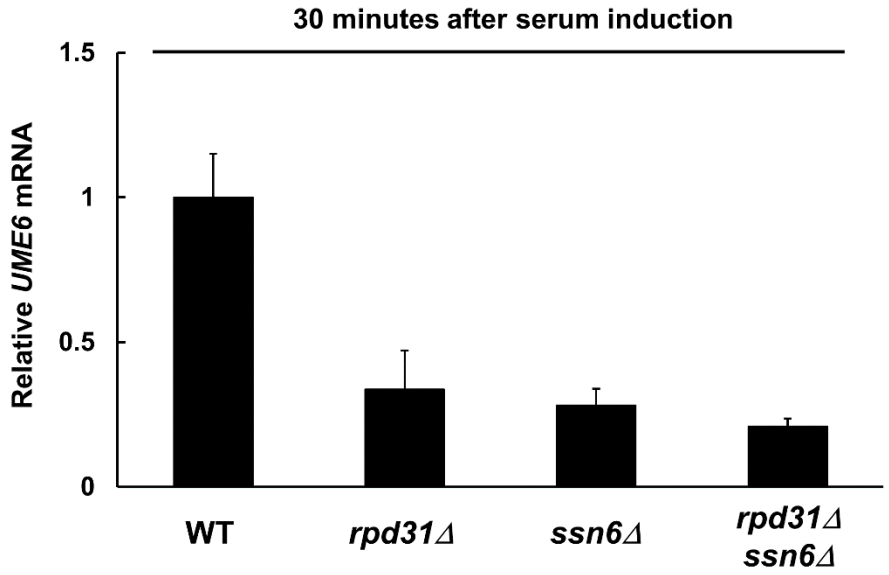


Fig. III-17. Expression of *UME6* in wild-type, *rpd31Δ*, *ssn6Δ* and *rpd31Δ ssn6Δ* strains under the filament-inducing conditions. Each cell was induced at 37 °C in YPD liquid medium for 30 minutes after addition of 10% serum. 500 ng of RNA was prepared from each strain, and applied to RT-qPCR analysis. Each measured *UME6* transcript level relative to *ACT1* was normalized to the wild-type. The results represent the average from three independent experiments, with bars representing SEM.

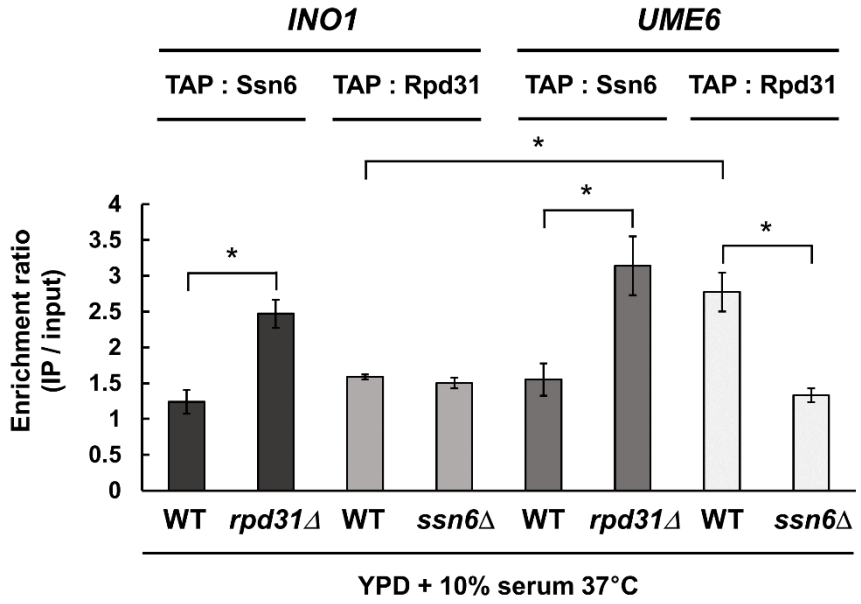


Fig. III-18. Occupancy of Ssn6 and Rpd31 at the promoter regions of *INO1* and *UME6* under the filament-inducing conditions. Under the same conditions as in Fig. III-17, Ssn6 ChIP analysis at each promoter region was carried out in the wild-type and *rpd31Δ* strain, and Rpd31 ChIP analysis at each promoter region was carried out in the wild-type and *ssn6Δ* strains. Each enrichment was normalized with 1% input. The results represent the average from three independent experiments, with bars representing SEM. The asterisk indicates a statistically significant difference between paired values as determined by the Student's t test ($P < 0.05$).

The requirement of Ssn6 and Rpd31 for the activation of filament-extension, as previously described, raised a question whether elevation in protein level of Ssn6 or Rpd31 could accelerate the filament-development and filament-extension. To investigate this possibility, the overexpression system of Ssn6 or Rpd31 depending on strong *ADH1* promoter was introduced into wild-type strain. After 30 minutes following serum induction, the overexpression of Ssn6 or Rpd31 could not accelerate the filament-development or the filament-extension when wild-type did not develop its filaments (Fig. III-19A). Under the same condition, the level of *UME6* expression was also not affected by the overexpression of Ssn6 or Rpd31 (Fig. III-19B). These results suggest that the process of filamentous growth in *C. albicans* is more complicatedly regulated and that is another regulator for filament-development corresponding to the action of Ssn6 and Rpd31.

Interestingly, in *C. albicans*, there are two homologues of Rpd3 in *S. cerevisiae* (Fig. III-20). These paralogues are Rpd31 (orf19.6801) and Rpd32 (orf19.2834), which showed identity of 84.06% and 75.29% over entire sequence with *S. cerevisiae* Rpd3 respectively. The high similarity between overall sequences of Rpd31 and Rpd32 suggests that two paralogues operate in similar molecular mechanism and show physiologically redundant function. However, in addition to Rpd31 only being identified through tandem affinity purification of Ssn6, the expression of *RPD32* was distinctive from those of Ssn6 and Rpd31. While *SSN6* and *RPD31* showed basal or slightly decreased level of mRNA along the time after serum induction, the mRNA level of *RPD32* increased gradually up to 5 hours after serum induction (Fig. III-21). This suggests that Rpd32 has same molecular function with Rpd31, but Rpd32 is not physiologically redundant protein of Rpd31.

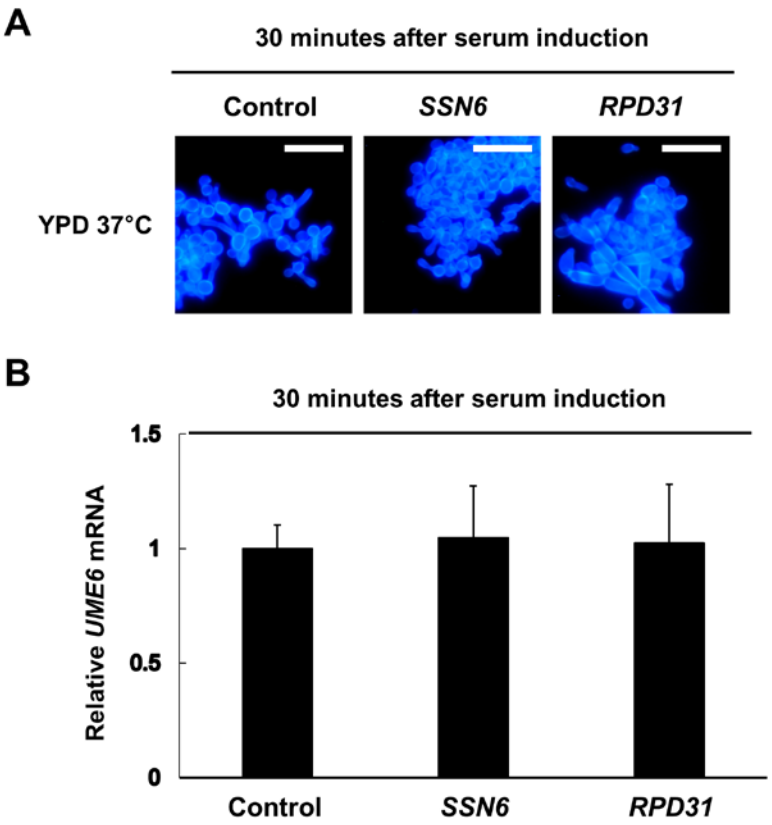


Fig. III-19. Neither of the overexpression of *SSN6* or *RPD31* can accelerate the expression of *UME6* compare to wild type. (A) Each cell was grown at 37 °C in YPD liquid media for 30 minutes after the addition of 10% serum. Each cell was stained with calcofluor white and visualized by fluorescence microscopy. Each scale bar represents 10 μm. (B) Each cell was induced at 37 °C in YPD liquid medium for 30 minutes after addition of 10% serum. 500 ng of RNA was prepared from each strain, and applied to RT-qPCR analysis. Each measured *UME6* transcript level relative to *ACT1* was normalized to the wild-type. The results represent the average from three independent experiments, with bars, representing SEM.

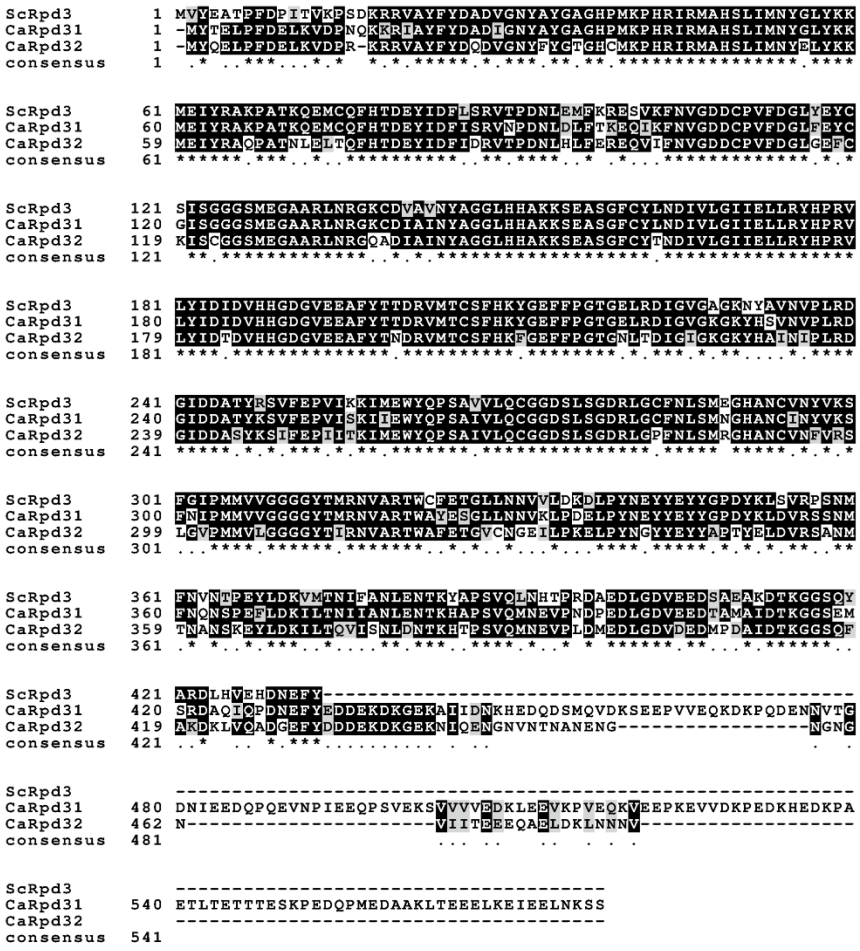


Fig. III-20. Comparison of the deduced amino acid sequence of Rpd31 and Rpd32 in *C. albicans* with those of Rpd3 in *S. cerevisiae*. Multiple sequence alignment among *Ca*Rpd31, *Ca*Rpd32, and *Sc*Rpd3 was produced using ClulstalW2. The asterisks in consensus row indicates highly conserver region among them.

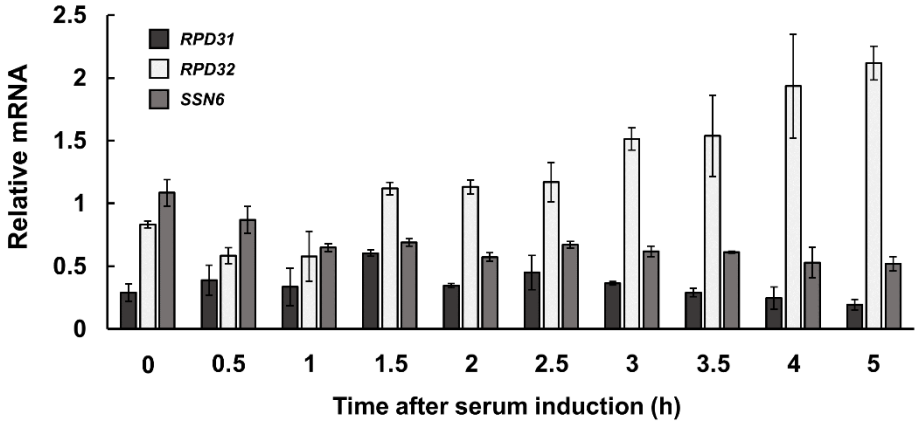


Fig. III-21. Expression patterns of *RPD31*, *RPD32* and *SSN6* in wild-type according to serum induction time. Wild-type cells were induced at 37 °C in YPD liquid medium with addition of 10% serum. 500 ng of RNA was prepared from cells of each time point, and applied to RT-qPCR analysis. Each measured transcript level was normalized to the transcript level of *ACT1* from each cell. The results represent the average from three independent experiments, with bars representing SEM.

5.2. Phenotypic aspects of *RPD31* and *RPD32* deletion mutants

To investigate functional differences between Rpd31 and Rpd32, both alleles of *RPD32* were disrupted in wild-type or *rpd31Δ* mutant by a PCR- based homologous recombination (Fig. III-22) and disruption of *RPD32* in both strains were confirmed by southern blot analysis (Fig. III-23). When grown for 3 days in solid YPD media at 28 °C, all of the strains showed smooth and round colony morphology. In Spider media at 37 °C after 4 days, only wild-type showed fully filamentous growth but the other strains showed yeast-like growth except center-collapsed colony morphology of *rpd32Δ* mutant (Fig. III-24). Although the cells of all strains showed yeast-like morphology in liquid YPD media at 28 °C, they showed distinct differences among them in liquid Spider media at 37 °C (Fig. III-25). The *rpd31Δ* mutant failed to develop its filaments as previously observed, but the *rpd32Δ* mutant exhibited extremely stubby filaments when wild-type normally developed its filamentous structure. Surprisingly, the *rpd31Δ rpd32Δ* mutant also showed stubby but relatively elongated filamentous structure compared to that of *rpd32Δ* mutant. In liquid Spider media at 37 °C, the expression of *HWP1*, which is related to the formation of hyphal cell wall, was not detected only in the *rpd31Δ* mutant and the mRNA level of *HWP1* was nearly same in wild-type, *rpd32Δ* mutant, and *rpd31Δ rpd32Δ* mutant. However, the expression of *ECE1* was most increased in the *rpd31Δ rpd32Δ* mutant, which indicated that Rpd32 was also related to the elongation of filaments as Rpd31 did (Fig. III-26).

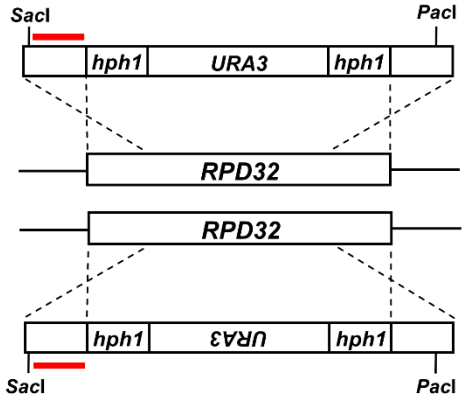


Fig. III-22. Schematic representation of disruption for *RPD32* in wild-type and *rpd31Δ* mutant. Strategy of disruption for *RPD32* in wild-type and *rpd31Δ* mutant. Each allele of *RPD32* was replaced with *hph-URA3-hph* cassette.

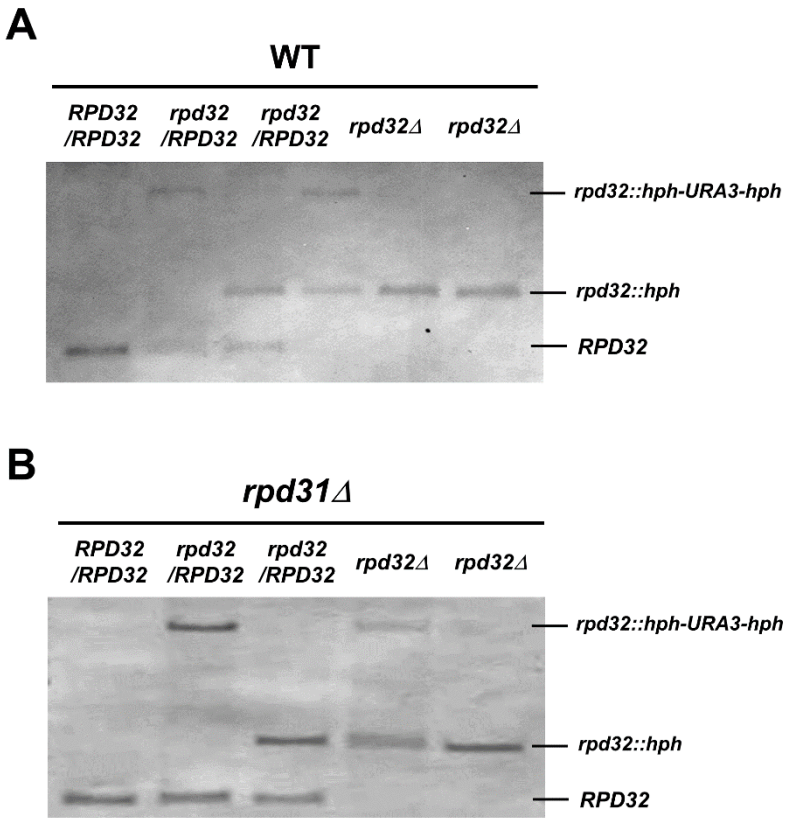


Fig. III-23. Disruption of *RPD32* in wild-type and *rpd31Δ* mutant. *Sac*I/*Pac*I-digested DNA fragments from each strain were detected by probe indicated as red bar in Fig. III-22 through southern blot analysis.

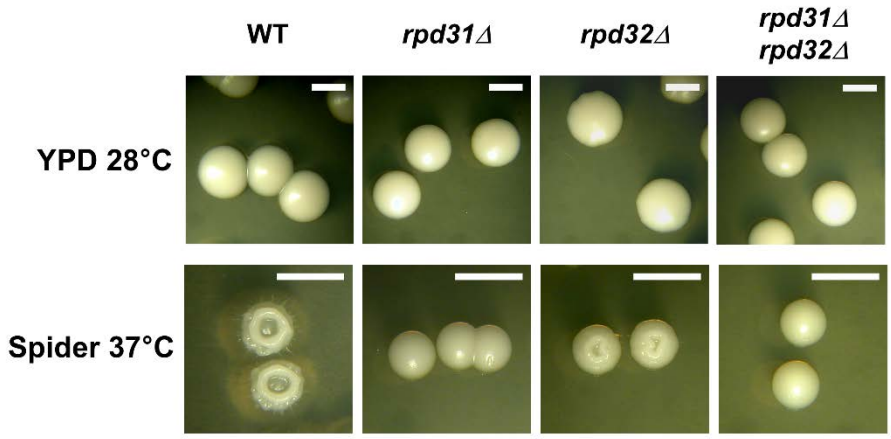


Fig. III-24. Colony morphology of wild-type, *rpd31Δ*, *rpd32Δ*, and *rpd31Δ rpd32Δ* strains. Each strain was grown for 3 days on YPD medium at 28 °C or for 4 days on Spider medium at 37 °C. Each scale bar represents 2 mm.

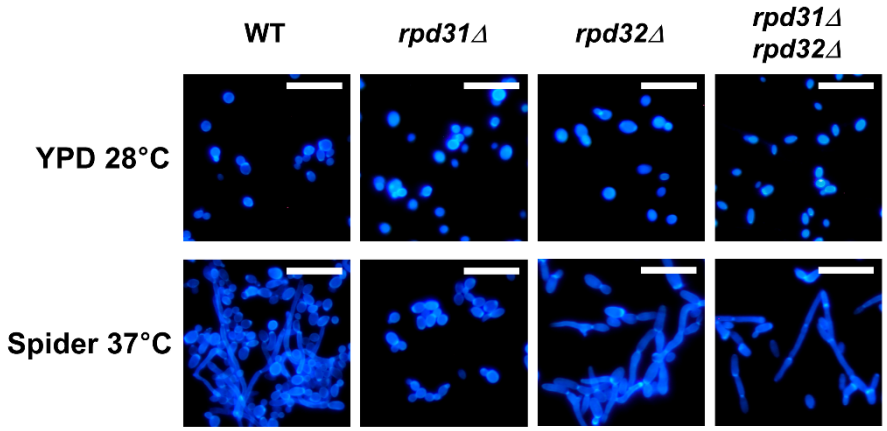


Fig. III-25. *RPD31* and *RPD32* regulate a hyphal development in a different manner. Each cell was grown for 6 hours in YPD liquid media at 28 °C or Spider media at 37 °C. Each cell was stained with calcofluor white and visualized by fluorescence microscopy. Each scale bar represents 10 μm.

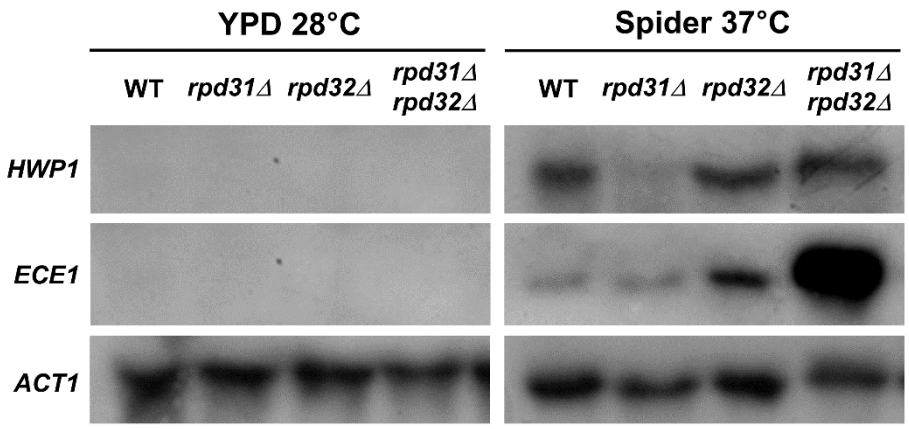


Fig. III-26. Hyphal specific genes levels in wild-type, *rpd31Δ*, *rpd32Δ* and *rpd31Δ rpd32Δ* strains. Each strain was grown in YPD liquid media at 28 °C or Spider liquid media at 37 °C for 6 hours. 40 μg of RNA was prepared from each strain, and northern analysis was carried out with probes for the indicated genes. *ACT1* was used as the loading control.

5.3. Identification of proteins interacting with Rpd32

To further investigate the roles of Rpd32 for filament-development in *C. albicans*, the proteins interacting with Rpd32 were examined through tandem affinity purification. Because Rpd32 has a highly sequence similarity to that of Rpd31 and is seemed to be related to filament-development, it is possible that Rpd32 acts as a component of Ssn6 complex and interacts with Ssn6 or Rpd31.

To verify this hypothesis, endogenous Ssn6 or Rpd31 was c-terminally tagged with CBP TAP and exogenous Rpd32 tagged with HPM TAP was introduced into the *RPS10* locus (Fig. III-27A). As shown in Fig. III-27B, Rpd32 neither interact with Ssn6 nor Rpd31, which indicated that Rpd32 functions in Ssn6- and Rpd3-independent manner. To identify the proteins assembled with Rpd32, similar to previous *SSN6*, the 3' end region of endogenous *RPD32* was replaced with sequences encoding for CBP TAP through a PCR-based homologous recombination (Fig. III-28A) and correct insertion of cassette to genome were confirmed by southern blot analysis (Fig. III-28B). As the mRNA level of Rpd32 was increased after serum induction (Fig. III-21), the cells expressing Rpd32-CBP TAP were cultured in liquid YPD media at 28 °C and in liquid YPD media including 10% FBS at 37 °C. The cultured Rpd32-CBP TAP cells were lysed and performed the TAP as the same of Ssn6.

The purified Rpd32 complex did not show significant differences between profiles of normal growth and filamentous growth (Fig. III-29A) and was revealed that Rpd32 associated with the proteins homologous to *S. cerevisiae* Sin3, Hat2, and Rxt2 by MALDI-TOF mass spectroscopy (Fig. III-29B). In *S. cerevisiae*, the Sin3 is the component of Rpd3S and Rpd3L histone deacetylase complex (Rpd3S and Rpd3L HDAC complex) and involved in regulation of

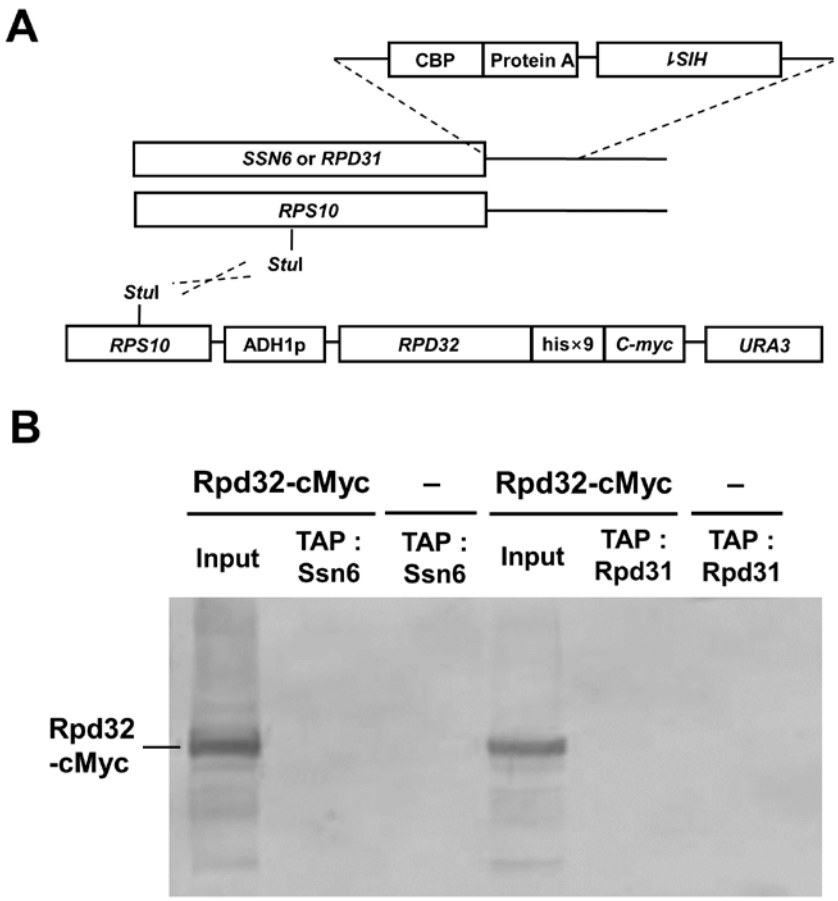


Fig. III-27. Rpd32 cannot interact with either Ssn6 or Rpd31. (A) Ssn6 and Rpd31 were C-terminally tagged with CBP TAP, respectively. The *RPD32* fused with coding sequence for HPM TAP was introduced into the *RPS10* locus. The expression of Rpd32-HPM TAP was dependent on the promoter of *ADH1*. (B) C-terminally tagged Ssn6 or Rpd31 was purified using tandem affinity purification for CBP TAP-tagged protein. Input and purified Ssn6 or Rpd31 from indicated cells were resolved on a 10% denaturing gel and immunoblotted against c-Myc.

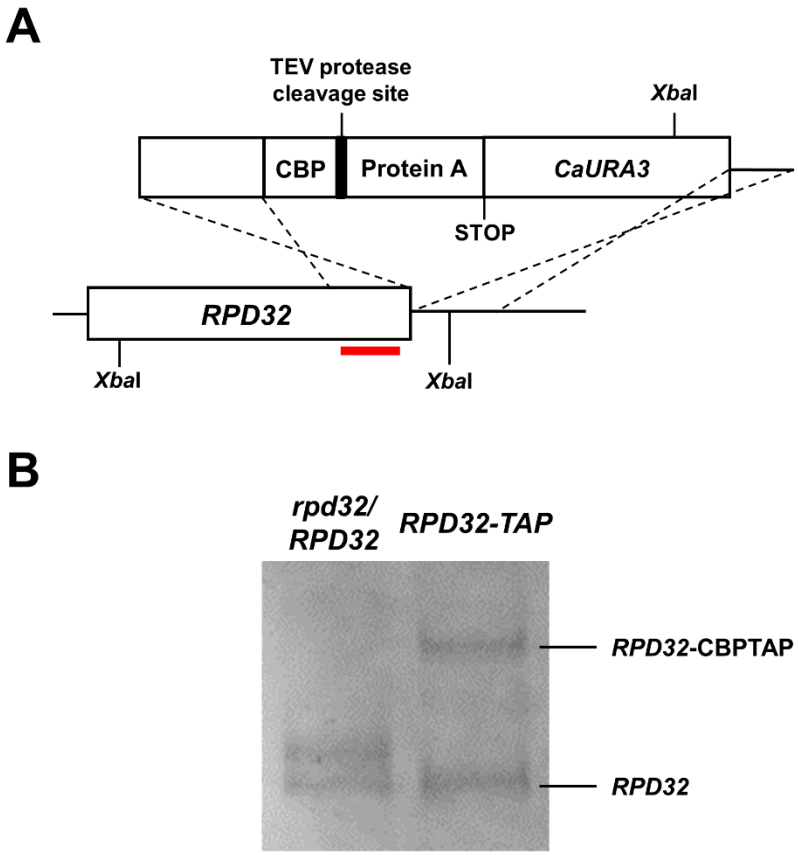
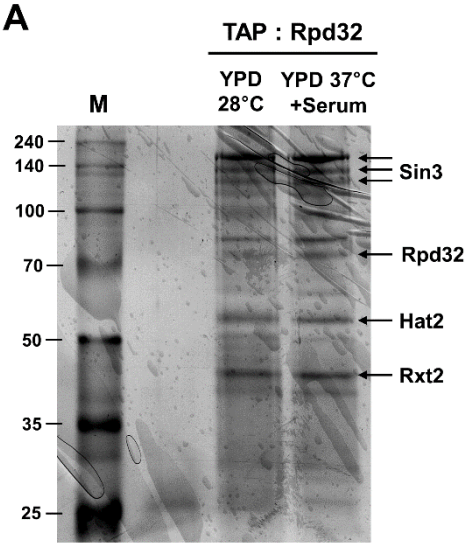


Fig. III-28. Strategy of TAP tagging of *RPD32* and confirmation through Southern blot analysis. (A) The Schematic view of CBP TAP and integration strategy of CBP TAP into 3'-end of *RPD32*. *Xba*I-digested fragments were detected with DNA probe (red bar) by (B) southern blot analysis of *rpd32/RPD32* cells and *RPD32*-TAP cells.

A



B

Protein Name	MW (kDa)	Sequence Coverage (%)	MOWSE score	ORF
Sin3	160	29.8	1.72E+14	orf19.6011
Sin3	160	27.8	8.89E+16	orf19.6011
Sin3	160	19	3.57E+08	orf19.6011
Rpd32	53.9	23.3	688371	orf19.2834
Hat2	55.3	28	2.29E+08	orf19.7185
Rxt2	37.4	17	970	orf19.12085

Fig. III-29. Tandem affinity purification and identification of Rpd32 complex. (A) Purified Rpd32 complex was resolved on a 10% denaturing gel and stained with silver nitrate. Identified proteins from MALDI-TOF mass spectrometry shown in (B) are indicated by arrows and labeled. M represents the size marker for proteins and the indicated numbers correspond to size in kDa.

diverse processes (Carrozza *et al*., 2005; Grzenda *et al*., 2009; Keogh *et al*., 2005). The Rxt2 in *S. cerevisiae* is also the component of Rpd3 HDAC complex but it is only assembled in Rpd3L HDAC complex (Carrozza *et al*., 2005; Colina and Young, 2005; Keogh *et al*., 2005). The *S. cerevisiae* Hat2 is the component of histone acetyltransferase (HAT) complex (Kelly *et al*., 2000; Parthun *et al*., 1996; Ruiz-Garcia *et al*., 1998) and is usually embedded in large multimolecular complex together with HDAC complex (Legube and Trouche,2003). Given that the proteins homologous to the components associated with Rpd3 in *S. cerevisiae* were identified as Rpd32-associated proteins, this result suggests that *C. albicans* Rpd32 is the histone deacetylase more functionally similar to *S. cerevisiae* Rpd3 than *C. albicans* Rpd31.

5.4. Characterization of *SDS3* deletion mutant in relation to Rpd31 and Rpd32

The purification of Ssn6 complex and Rpd32 complex through tandem affinity purification suggests that Sds3 is associated with Rpd31 but not with Rpd32. Because *S. cerevisiae* Sds3 is responsible for structural integrity and catalytic activity of Rpd3L HDAC complex (Carrozza *et al*., 2005; Keogh *et al*., 2005; Lechner *et al*., 2000), it might be also necessary for Rpd32-associated HDAC complex in *C. albicans* although it is not detected in the purifier Rpd32 complex. To examine the functional contribution of Sds3 in *C. albicans*, both alleles of *SDS3* were disrupted in wild-type by a PCR-based homologous recombination (Fig. III-30A) and correct disruption of *SDS3* were confirmed by southern blot analysis (Fig. III-30B). While the *sds3Δ* mutant was not distinguishable from wild-type and *rpd31Δ* mutant in liquid YPD media at

28 °C, the *sds3Δ* mutant showed nearly same morphology with the *rpd31Δ* mutant, which failed to develop filaments in liquid Spider media at 37 °C (Fig. III-31). This result indicates that Sds3 is functionally related to Rpd31 but not to Rpd32.

To investigate whether the genes regulated by Rpd31 is also affected by Sds3, the expression of *UME6* was also examined in the condition of *UME6* induction. The expression of *UME6* was distinctly decreased in the *sds3Δ* mutant as in the *rpd31Δ* mutant (Fig. III-32), which indicates that Rpds31 and Sds3 are physically and functionally synchronized. Surprisingly, the decreased expression of *UME6* caused by the depletion of Rpd31 is suppressed by depletion of Rpd32 despite *rpd32* deletion alone did not affect the expression of *UME6* (Fig. III-32). It suggests that there is more complicated regulatory correlation between Rpd31 and Rpd32, considering their cell morphology (Fig. III-25) and the increased expression of *UME6* in the *rpd32Δ* and *rpd31Δ rpd32Δ* mutants compared to those in the *rpd31Δ* mutants.

5.5. The regulation of metabolic gene *INO1* in relation to Rpd31 and Rpd32

The profiles of *UME6* mRNA level shown in Fig. III-32 suggests that Rpd31 and Rpd32 are not associated physically with each other but physiologically associated to regulate their target genes. To further understand the functional relation between Rpd31 and Rpd32, the expression of the metabolic gene *INO1* was examined in wild-type, *rpd31Δ*, *rpd32Δ*, *rpd31Δ rpd32Δ*, and *sds3Δ* strains. Interestingly, the expression of *INO1* was only increased in the *rpd31Δ rpd32Δ* or *sds3Δ* mutant (Fig. III-33). The *rpd31Δ* and

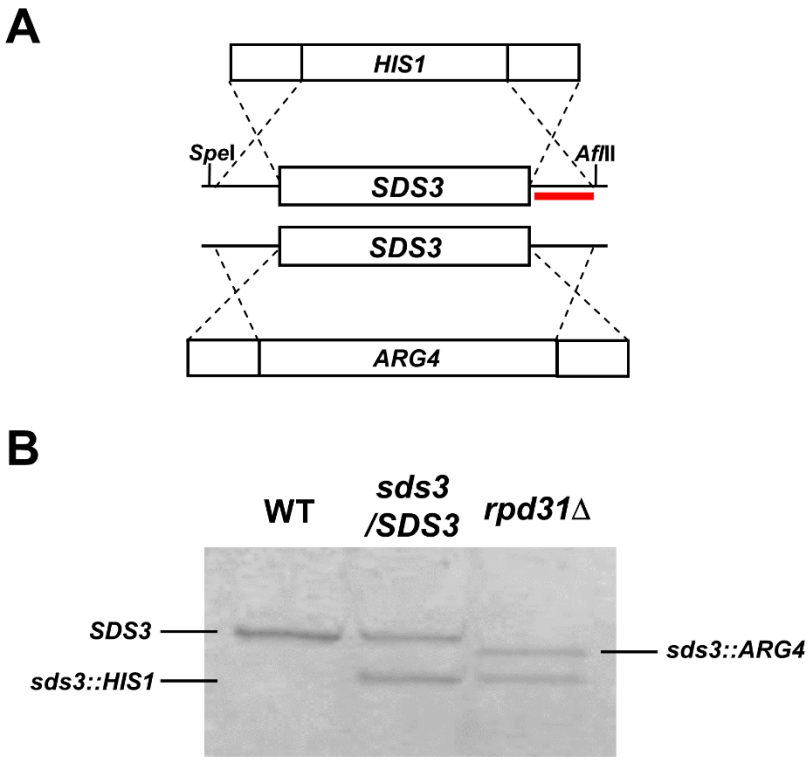


Fig. III-30. Disruption of *SDS3* in wild-type cells. (A) Schematic representation of disruption for *SDS3*. Each allele of *RPD31* was replaced with nutrient marker *HIS1* and *ARG4*, respectively. (B) *Spe*I/*Afl*II-digested DNA fragments from each strain were detected by probe indicated as red bar indicated in (A) through southern blot analysis.

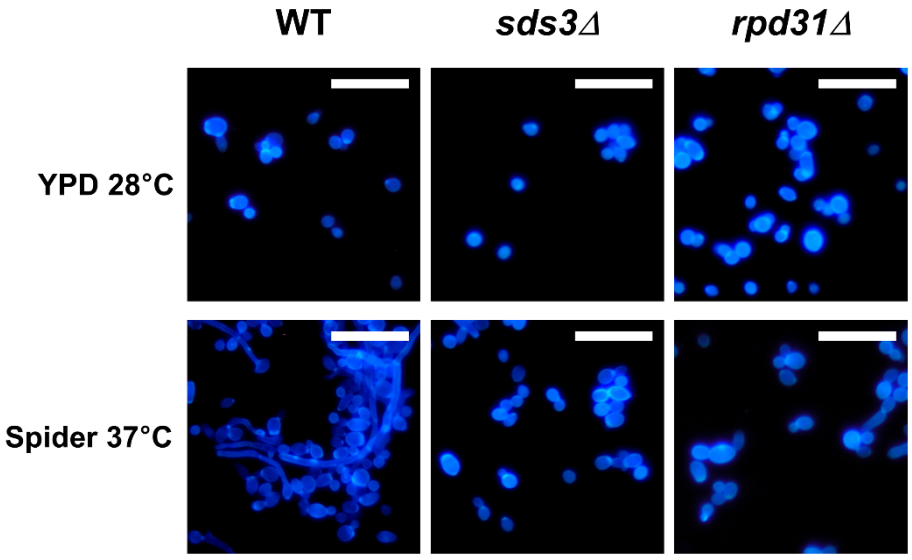


Fig. III-31. Cell morphology of wild-type, *sds3Δ*, and *rpd31Δ* strains. Each cell was grown for 6 hours in YPD liquid media at 28 °C or Spider media at 37 °C. Each cell was stained with calcofluor white and visualized by fluorescence microscopy. Each scale bar represents 10 μm.

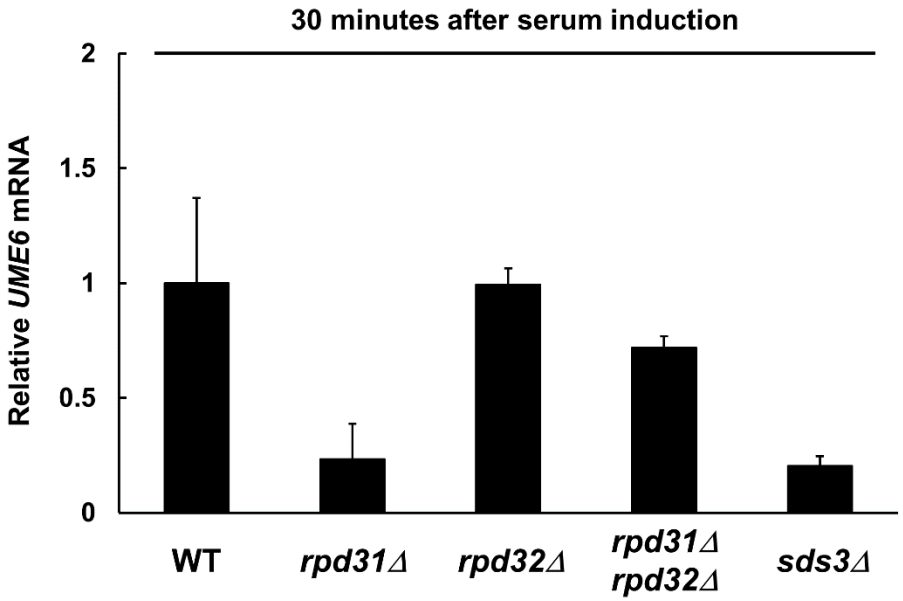


Fig. III-32. Expression of *UME6* in wild-type, *rpd31Δ*, *rpd32Δ*, *rpd31Δ rpd32Δ*, and *sds3Δ* strains under the filament-inducing conditions. Each cell was induced at 37 °C in YPD liquid medium for 30 minutes after addition of 10% serum. 500 ng of RNA was prepared from each strain, and applied to RT-qPCR analysis. Each measured *UME6* transcript level relative to *ACT1* was normalized to the wild-type. The results represent the average from three independent experiments, with bars representing SEM.

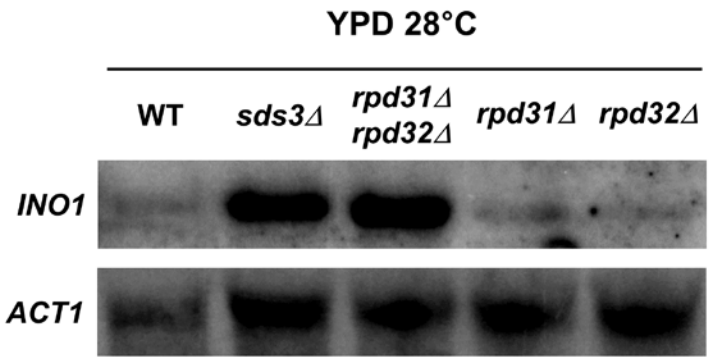


Fig. III-33. Expression of *INO1* in wild-type, *rpd31Δ*, *rpd32Δ*, *rpd31Δ rpd32Δ*, and *sds3Δ* strains. Each strain was grown at 28 °C in YPD liquid media for 6 hours. 40 μg of RNA was prepared from each strain, and northern analysis was carried out with probes for the indicated genes. *ACT1* was used as the loading control.

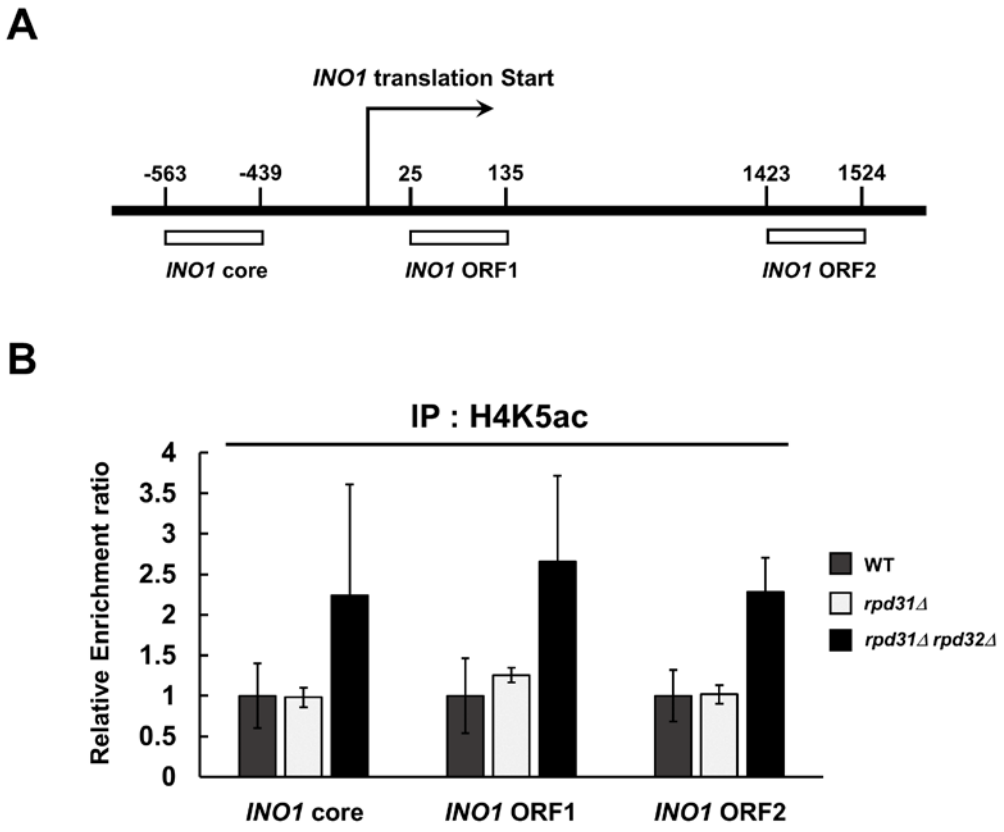


Fig. III-34. The acetylation state of H4-K5 along with *INO1* gene. Under the same conditions as in Fig. III-32, H4K5ac ChIP analysis at each region as shown in (A) was carried out in the wild-type, *rpd31Δ* and *rpd31Δ rpd32Δ* strains. Each relative enrichment to 1% input was normalized with the relative enrichment of wild-type strain. The results represent the average from three independent experiments, with bars representing SEM.

rpd32Δ mutants showed the mRNA level of INO1 similar to that in the wild-type which was not induced. This result indicated that both Rpd31 and Rpd32 repress the *INO1* in complementary manner and the Sds3 was required for this repression. In coinciding with this observation, the acetylation state of K5 residue in histone H4 along with *INO1* gene (Fig. III-34A) was also distinctly increased in the *rpd31Δ rpd32Δ* mutant compared to that in the wild-type while the *rpd31Δ* mutant showed similar level with that in the wild-type. That is, the deacetylation state of H4-K5 along with *INO1* was maintained by Rpd32 despite the absence of the Rpd31. These results suggests that the Rpd31 and Rpd32 exert their regulation in different manners depending on target genes and, according to the mode of Rpd31 or Rpd32, Sds3 is also differently associated with Rpd31 or Rpd32.

IV. DISCUSSION

In *S. cerevisiae*, Ssn6 is known as a general transcriptional repressor, which exists as a complex with the transcriptional repressor Tup1 through its TPR motifs (Keleher *et al*., 1992; Tzamarias and Struhl, 1994, 1995; Varanasi *et al*., 1996; Williams *et al*., 1991) and regulates the genes of many cellular processes including nutrient utilization, osmotic stress, meiosis, mating, and sporulation in *Saccharomyces cerevisiae* (Malave and Dent, 2006; Smith and Johnson, 2000). In *C. albicans*, Ssn6 also functions as a complex with Tup1 (Kaneko *et al*., 2006), which is a distinct repressor for morphogenesis (Argimon *et al*., 2007; Braun and Johnson, 1997; Goyard *et al*., 2008; Kadosh and Johnson, 2005; Sharkey *et al*., 1999), but previous data revealed that Ssn6 is a critical factor for filamentous growth and virulence independently of Tup1 (Hwang *et al*., 2003). Despite many studies focusing on the Ssn6-Tup1 complex in *S. cerevisiae* (Fleming *et al*., 2014; Hanlon *et al*., 2011; Jaschke *et al*., 2011) and Tup1 in *C. albicans* (Banerjee *et al*., 2008; Braun and Johnson, 1997; Goyard *et al*., 2008; Kaneko *et al*., 2006; Kebaara *et al*., 2008; Mao *et al*., 2008; Sharkey *et al*., 1999), how Ssn6 can regulate the morphological transition and virulence of *C. albicans* in Tup1-independent manner has not been well understood yet. Therefore, the interaction partner of Ssn6 was screened through tandem affinity purification (TAP) and histone deacetylase Rpd31 was identified as an interaction partner of Ssn6 (Fig. III-2). In addition, the interaction of Ssn6 and Rpd31 occurred in Tup1-independent manner (Fig. III-3) like Ssn6 and Rpd3 does in *S. cerevisiae* (Davie *et al*., 2003). This suggests that the function of Ssn6 and Rpd3 is synchronized for regulation of filament-development in *C. albicans*, independently of Tup1.

The filament-development and virulence of *C. albicans* was completely blocked in the *rpd31Δ* mutant and these defects were recovered by introduction

of one allele *RPD31* into the *rpd31Δ* mutant (Fig. III-5 and III-6). This indicates that Rpd31 is a crucial activator for filament-development in *C. albicans*. However, the further disruption of *SSN6* in the *rpd31Δ* mutant caused the development of elongated filaments at elevated temperature (Fig. III-9 and III-10). This morphology was not matched with the stubby filamentous morphology of *ssn6Δ* mutant and yeast-like morphology of *rpd31Δ* mutant. The expression level of *ECE1* indicated that the cell morphology of *rpd31Δ ssn6Δ* mutant is partly caused by induction of genes related to filament-elongation, but the increased mRNA level of *ECE1* in the *rpd31Δ ssn6Δ* mutant also indicated that Rpd31 is not only an activator but also a repressor for filament development in *C. albicans* (Fig. III-11). Interestingly, in filament-inducing condition, the *rpd31Δ ssn6Δ* mutant failed in development of extremely elongated filaments in contrary to wild-type (Fig. III-13 and III-14). The unique morphology of *rpd31Δ ssn6Δ* mutant which developed elongated but not extremely elongated filaments in filament-inducing condition was not caused by the failure in expression of filament-specific genes (Fig. III-15). The expression of *ECE1*, which is related to filament elongation of *C. albicans*, was most increased in the *rpd31Δ ssn6Δ* mutant than the other strains grown in Spider media at 37 °C.

Furthermore, the morphology and the expression of filament-specific genes in the deletion mutant of *RPD31* and *SSN6* showed that Ssn6 might function as a predominant repressor regardless of Rpd31 in *C. albicans* (Fig. III-11 and III-15). In the absence of Rpd31, Ssn6 is likely to exert more strengthened repression of target genes than in the presence of Rpd31, because the deletion of *RPD31* fails to develop filaments and express filament-specific genes (Fig. III-5). As the deletion of *RPD3* in *S. cerevisiae* often causes

repression or has little effect on the transcription of many genes (Kurdistani *et al*., 2002), similar effects caused by deletion of *RPD31* in *C. albicans* might be related to the magnitude of repression exerted by Ssn6. This suggests that the repression of target genes exerted by Ssn6-Rpd31 complex is achieved mainly by action of Ssn6 but the activation of genes related to filament-extension requires the associated action of Ssn6 and Rpd31.

Because Ume6 is not only a positive regulator involved in filament extension (Banerjee *et al*., 2008; Carlisle *et al*., 2009), but is also a crucial downstream target of other filamentation-related transcriptional regulators such as Nrg1-Tup1, Efg1, Cph1 (Zeidler *et al*., 2009), the requirement of Ssn6 and Rpd31 for filament-extension was supposed to be related to the expression of *UME6*. The expression of *UME6* was distinctly decreased in *rpd31Δ*, *ssn6Δ*, and *rpd31Δ ssn6Δ* mutant (Fig. III-17), which indicates that Ssn6-Rpd31 complex induces filament-extension through activation of *UME6*. The necessity of Ssn6 and Rpd31 for the induction of *UME6* illuminates another side of Ssn6-Rpd31 complex as an activator of filament development in *C. albicans*. In the side of as a repressor, Ssn6-Rpd31 complex suppresses filamentous growth of *C. albicans* until it encounters filament-inducing condition such as elevated temperature, serum, and nutrient sources. Although the release of Ssn6-Rpd31 complex from target genes induced the expression of filament-specific genes sequentially followed by development of filaments, it did not cross the threshold which induces the filament extension of *C. albicans.*

The occupancies of Ssn6 and Rpd31 at the promoters of *INO1* and *UME6* under the condition that induces the expression of *UME6* showed more specified mechanism of regulation mediated by Ssn6-Rpd31 complex (Fig. III-18). The similar changes in the enrichment of Ssn6 at both promoters depending

on the presence of Rpd31 indicate that Ssn6 interacts with target genes in similar manner regardless of the roles of Ssn6 as a repressor or as an activator. However, the Rpd31 showed clearly different enrichment depending on target genes, which was more enriched at the promoter of *UME6* than that of *INO1*. In addition, the interaction of Rpd31 with the promoter of *UME6* was dependent on the presence of Ssn6. These indicate that the role of Ssn6-Rpd31 complex is determined by Ssn6-mediated interaction of Rpd31 with target genes. That is, whether Rpd31 interacts with target genes or not, Ssn6-Rpd31 complex acts as an activator or as a repressor for target genes.

These present results suggest a model for how Ssn6-Rpd31 complex regulates filament-specific genes in a dual manner as a repressor and an activator (Fig. III-35). Under non-filament-inducing condition, the Ssn6 would mainly repress a set of filament-specific genes related to filament development. The Rpd31 would also participate in this repression with another repressor rather than Ssn6. Even though independent repression of same genes by Ssn6 and Rpd31 might be competitive, Ssn6 is a predominant repressor for target genes. Under filament-inducing condition, the release of Ssn6 complex and Rpd31 complex from filament-specific genes would induce the development of filaments. In this process of filamentation, another set of filament-specific genes also should be activated for extremely extension of filaments. The positive regulator Ume6 is involved in regulation of filament-extension and the activation of *UME6* is mediated by Ssn6-Rpd31 complex. In contrary to a mode of repressor, both Ssn6 and Rpd31 would be required as a complex for a mode of activator.

In *C. albicans*, the presence of Rpd31 and Rpd32, which both are homologues of Rpd3 in *S. cerevisiae* (Fig. III-20), raise another question

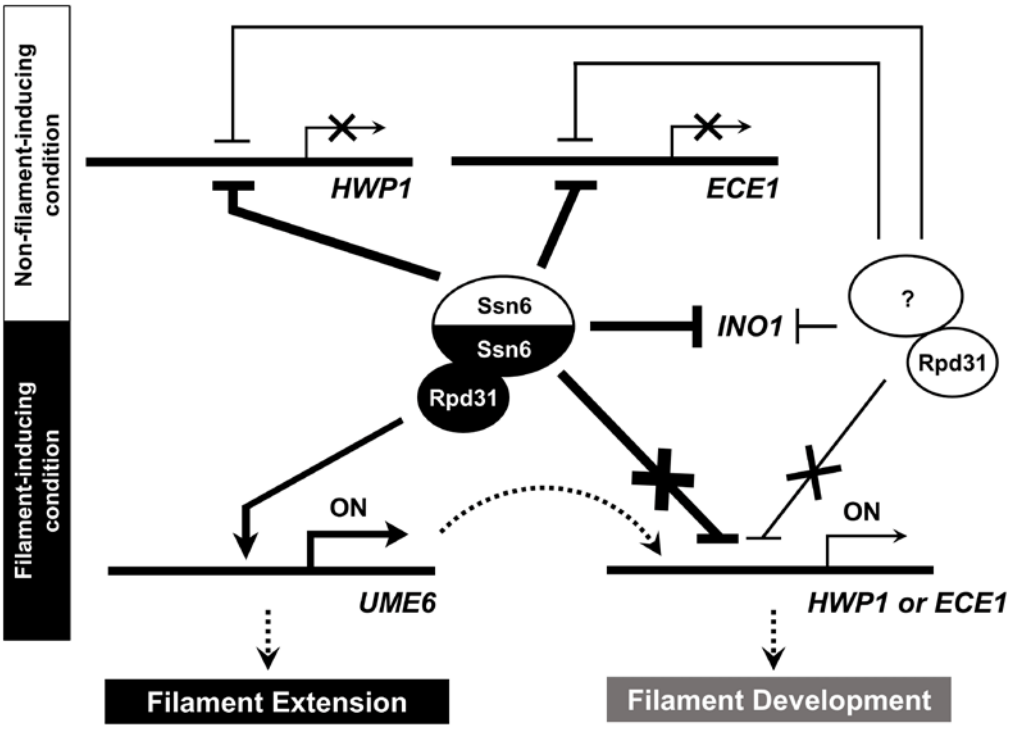


Fig. III-35. The model for regulation of genes by Ssn6 and Rpd31. Under non-filament-inducing condition, the Ssn6 mainly repress a set of filament-specific genes involved in filament development. The Rpd31 would also participate in this repression with another repressor rather than Ssn6. Despite competitive repression of same genes by Ssn6 and Rpd31, Ssn6 is a predominant repressor for target genes. Under filament-inducing condition, the release of Ssn6 complex and Rpd31 complex from filament-specific genes induces the development of filaments. Another set of filament-specific genes also is also induced for extremely extension of filaments. The positive regulator Ume6 is involved in the regulation of filament-extension and the activation of *UME6* is mediated both by Ssn6 and Rpd31 in Ssn6-Rpd31 complex.

whether the function of Rpd31 and Rpd32 is redundant or competitive for the regulation of filament-development. However, the increase of Ssn6 or Rpd31 in *C. albicans* could not affect the rate of filament-development and expression of *UME6* (Fig. III-19). This result also might be caused by stoichiometric difference between Ssn6 and Rpd31, but the increase of Ssn6 or Rpd31 also might cause the equilibrium shift to the Ssn6-Rpd31 complex. Therefore, it is reasonable that there is another regulatory system related to Ssn6-Rpd31 complex or Rpd31 alone. Interestingly, the expression level of *RPD32* was gradually increased up to 5 hours after serum induction, which indicated that Rpd32 exert the regulation for filament-development in different manner with that of Rpd31 (Fig. III-21).

The cell morphology of the *rpd32Δ* mutant showed stubby elongated filaments in contrary to yeast-like cell morphology of the *rpd31Δ* mutant, and the cell morphology of *rpd31Δ rpd32Δ* mutant exhibited more elongated filaments than that of the *rpd32Δ* mutant. The expression of filament-specific genes such as *HWP1* and *ECE1* was also induced in the *rpd32Δ* and *rpd31Δ rpd32Δ* mutants but not in the *rpd31Δ* mutant (Fig. III-26). These indicate that the deletion of *RPD32* suppresses the deletion of *RPD31* and suggest that Rpd32 has roles opposite to those of Rpd31. Although the development of elongated filaments in the *rpd31Δ rpd32Δ* mutant supports the opposite roles of Rpd31 and Rpd32, there is a remaining question why the deletion of *RPD32* could not induce filament-elongation more than wild-type.

To understand this question, the interaction of Rpd32 with Ssn6 or Rpd31 was examined and Rpd32 complex was purified through tandem affinity purification (Fig. III-27 and III-29). The Rpd32 formed a complex with Sin3, Rxt2, and Hat2 which are components of Rpd3 HDAC complex (Carrozza *et al.*,

2005; Keogh *et al*., 2005) and a HAT complex (Kelly *et al*., 2000; Parthun *et al*., 1996; Ruiz-Garcia *et al*., 1998) but not with Rpd31 or Ssn6 (Fig. III-27). The Sds3 required for structural integrity and catalytic activity of HDAC complex was not detected in Rpd32 complex but detected in Ssn6 complex through tandem affinity purification (Fig. III-2 and III-29). These results suggest that Rpd32 assembles in the different complex from the complex in which Rpd31 assembles. The similar morphology of the *sds3Δ* mutant to the *rpd31Δ* mutant also supports this suggestion (Fig. III-31).

The expression of *UME6* also showed that Rpd31 and Sds3 are physically and functionally synchronized (Fig. III-32). The depletion of Rpd32 did not affect the expression of *UME6* but it suppressed the decreased expression of *UME6* caused by the depletion of Rpd31. This result indicates that the decreased expression caused by depletion of Rpd31 is achieved by the repression of *UME6* by Rpd32. However, when the expression of *UME6* is induced in wild-type cells, the induction of *UME6* by Ssn6-Rpd31 is more dominant that repression of that by Rpd32 complex. This suggests that Rpd31 and Rpd32 might participate in the regulation of same target genes.

The regulation of same genes by Rpd31 and Rpd32 is supported and specified with the regulation of metabolite gene *INO1*. In contrary to the competitive regulation of *UME6* by Rpd31 and Rpd32, *INO1* was repressed cooperatively by Rpd31 and Rpd32. The expression of *INO1* was only induced in the *sds3Δ* or *rpd31Δ rpd32Δ* mutant and was not in the *rpd31Δ* or *rpd32Δ* mutant (Fig. III-33). Therefore, it might be suggested that Rpd31 and Rpd32 exert competitive or complementary regulation depending on target genes and that, depending on mode of Rpd31 or Rpd32, Sds3 associated differentially with Rpd31 or Rpd32. The acetylation state of K5 residue in histone H4 along

with *INO1* gene also supports that Rpd31 and Rpd32 regulate the expression of *INO1* in complementary manner through histone deacetylation of chromatin adjacent to regulatory region of *INO1* (Fig. III-34).

These results propose another model for regulation of genes by Rpd31 and Rpd32 (Fig. III-36). In the repression of genes such as *INO1*, both Rpd31 and Rpd32 are responsible for the repression of target genes in complementary manner. This complementarity might be caused by the association of Sds3 with each regulatory complex containing Rpd31 or Rpd32. However, in the regulation of genes such as *UME6*, Rpd31 and Rpd32 regulate target genes in competitive and opposite manner. That is, the Rpd31 associated with Ssn6 would positively regulate genes, but the Rpd32 associated with Sin3 would negatively regulate genes. In this opposite regulation, the Sds3 is only associated with the regulatory complex containing Rpd31.

In conclusion, transcriptional regulator Ssn6 interacts with histone deacetylase Rpd31 in Tup1-independent manner and Ssn6-Rpd31 complex regulates the filamentous growth of *C. albicans* in two different manners. Although Ssn6 mainly contributes for the repression of filament-specific genes in the Ssn6-Rpd31 complex, Ssn6 and Rpd31 are both required for the activation of filament-extension genes. This regulatory system is also involved with the action of Rpd32. Rather than that assembles in one complex, Rpd31 and Rpd32 are associated into the different complex of representing Ssn6 or Sin3. Two different complexes would regulate the same genes competitively or cooperatively depending on intracellular condition or their target genes. This complicated regulatory system might contribute the flexible pathogenicity of *C. albicans*.

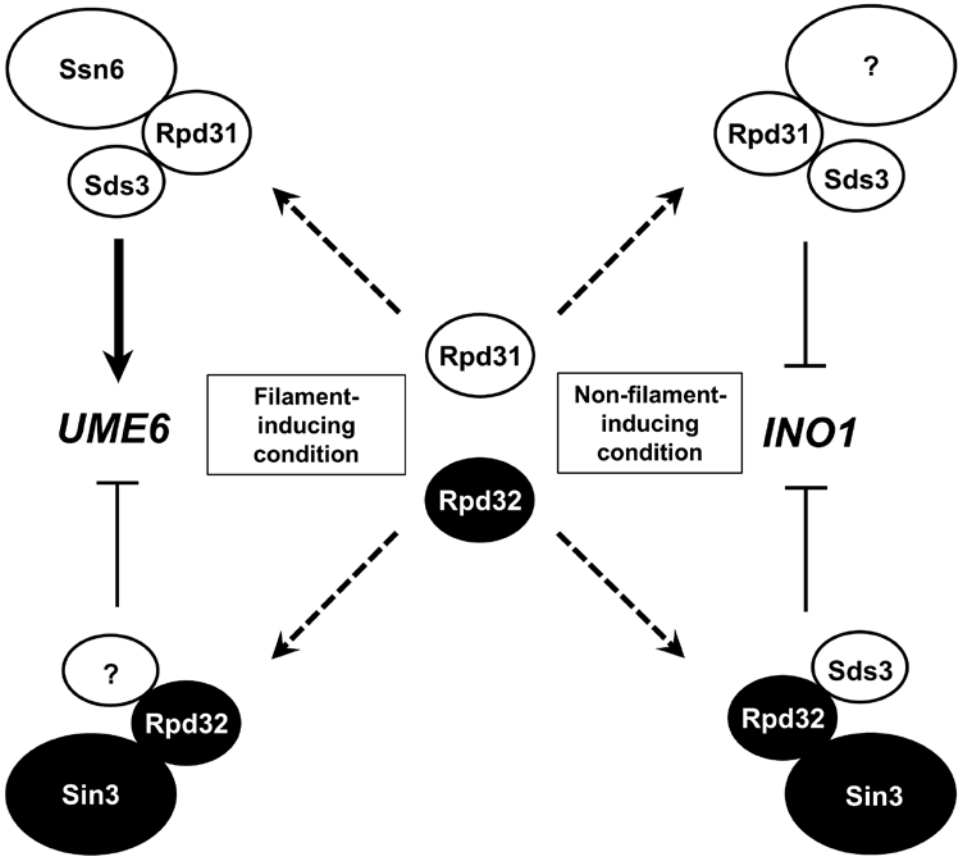


Fig. III-36. The model for regulation of genes by Rpd31 and Rpd32. In the repression of genes such as *INO1*, both Rpd31 and Rpd32 function as repressors in complementary manner. This complementarity might be depending on the association of Sds3 with Rpd31 or Rpd32. In the regulation of genes such as *UME6*, Rpd31 and Rpd32 regulate target genes in competitive and opposite manner. The Rpd31 associated with Ssn6 positively regulates genes, but the Rpd32 associated with Sin3 negatively regulate genes. In this regulation, the Sds3 is only associated with the regulatory complex containing Rpd31.

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국문초록

*Candida albicans*는 사람에게 빈번하게 칸디다증을 일으키는 병원성 곰팡이로, 이러한 *C. albicans*의 병원성은 둥근 효모 형태의 세포에서 균사를 형성하는 능력과 밀접한 연관이 있다. *C. albicans*의 이형성은 혈청, 프롤린, N-아세틸글루코사민 및 여러 탄소 영양원과 같은 다양한 형태로 이루어진 외부 환경에 대한 반응으로 일어난다. 외부 환경에 의한 신호는 단순하게 *C. albicans*의 형태 변화로 이어지는 것이 아니라, 다양하고 복잡한 신호 전달 경로에 의해 이루어진다.

Ssn6는 효모인 *Saccharomyce cerivisiae*에서 잘 알려진 전사 억제체로, Ssn6의 TPR 모티프를 통해 또 다른 전사 억제체인 Tup1과 결합체 형태로 존재하는 것으로 알려져 있다. 또한, Ssn6는 주로 Tup1과의 결합체 형태로 세포 내 영양 이용, 삼투압에 대한 저항, 감수 분열, 교미, 포자 형성 등 효모 내에서 다양한 세포 작용을 조절하는 것으로 알려져 있다. 이러한 Ssn6는 *C. albicans* 내에도 존재하며, 효모에서와 같이 Tup1과 결합체 형태로 존재하는 것으로 알려져 있다. 하지만, 본 연구진에서 이루어진 선행 연구들은 Ssn6가 Tup1과는 독립적으로 기능하여 균사 형성 능력 및 병원성을 조절함을 보여주었다.

대부분의 연구들이 *S. cerevisiae*에서의 Ssn6-Tup1 결합체의 기능과 *C. albicans*에서의 Tup1 자체의 기능에 초점이 맞추어져 있으나, *C. albicans* 내에서 Ssn6가 Tup1과 독립적으로 균사 형성과 병원성을 어떻게 조절하는가에 대한 연구는 거의 이루어지지 않고 있다. 따라서, 본 연구에서는 Tandem affinity purification 방법의 도입을 통해 *C. albicans* 내에서 Ssn6와 결합체로 존재하는 단백질을 분리하고자 하였고, 결과적으로 histone deacetylase인 Rpd31을 동정할 수 있었다.

본 연구를 통해 Rpd31은 Tup1이 없는 세포에서도 Sssn6와 결합할 수 있음을 밝혔고, 또한 Rpd31 자체만으로도 *C. albicans*의 균사 형성이나 병원성 조절에 상당히 중요한 역할을 하고 있음을 밝혔다. 또한, Ssn6와 Rpd31 모두가 존재하지 않는 균주를 제작하여, 그것을 이용한 실험 결과, 균사 형성과 초기 신장 과정은 Ssn6-Rpd31의 결합체 중 주로 Ssn6에 의해 억제되고 있으나, 균사의 확장적 신장을 활성화하기 위해서는 Ssn6와 Rpd31 모두가 필요함을 밝혀내었다. 이러한 조절 과정에 있어 *C. albicans*의 균사가 확장적으로 신장에 관여하는 Ume6가 관련되어 있는 것이 밝혀졌다. 크로마틴 항체 침전법을 이용해 Ssn6와 Rpd31가 균사의 확장적 신장과 연관된 *UME6* 유전자와 대사 관련 유전자인 *INO1* 유전자에서 서로 다른 유형으로 해당 유전자와 상호작용하는 것을 밝혔고, 특히 이러한 차이가 주로 Rpd31에 의한 것임을 밝혔다. 따라서, Ssn6는 전사 억제체나 전사 활성체 모두로 기능할 수 있으며, 이러한 기능을 결정하는 것이 Rpd31과 목적 유전자와의 상호작용에 의한 것임을

밝혀 내었다.

더 나아가, Rpd31과 상당히 유사한 아미노산 서열을 갖는 Rpd32에 대한 연구를 수행하였다. Rpd31은 Ssn6로 대표되는 결합체에, Rpd32는 Sin3로 대표되는 결합체에 결합하여, 서로 다른 결합체에서 기능하나 목적 유전자나 세포 내 환경에 따라 경쟁적으로 혹은 협력적으로 같은 유전자의 활성을 조절하는 기작을 밝혀 내었다. 또한, 이러한 조절 과정에서 Rpd31이나 Rpd32의 역할에 따라 Sds3가 기능적으로 상이하게 Rpd31, Rpd32와 결합되어 있음 역시 밝혀 내었다. *INO1* 유전자의 발현 억제와 같이 Rpd31, Rpd32가 모두 전사 억제체로 기능할 때, Sds3는 두 histone deacetylase 모두와 기능적으로 결합되어 있는 반면, *UME6* 유전자의 전사 조절과 같이 Rpd31이 전사 활성체로, Rpd32가 전사 억제체로 기능할 때는 Sds3는 Rpd31과만 기능적으로 결합되어 있음을 이번 연구를 통해 확인할 수 있었다. 이러한 Ssn6-Rpd31, 그리고 Sin3-Rpd32 결합체 형성과 그에 따른 경쟁적 혹은 협력적 유전자 조절은 *C. albicans*가 다양한 환경에 유연하게 적응하는 병원성 곰팡이로 존재하는데 중요한 역할을 하는 것으로 사료된다.

주요어: Tandem affinity purification, *SSN6*, *RPD31*, *RPD32*, *Candida albicans*