NHP6P AND MED3P REGULATE GENE EXPRESSION BY CONTROLLING THE LOCAL SUBUNIT COMPOSITION OF RNA POLYMERASE II

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SUMMARY

In this project, the Split-Ubiquitin system was used to isolate *S. cerevisiae* proteins that interacted with the non-histone chromosomal protein Nhp6p *in vivo*, and GST pull-down experiments confirmed eleven of these interactions *in vitro*. Most of the Nhp6p-interacting proteins were involved in transcription and DNA repair. The *ZDS1* gene, whose transcription was repressed by Nhp6p and its interacting partners Rpb4p and Med3p, was utilized to study their chromosomal co-localization. Nhp6p, Med3p and the essential RNA polymerase II (RNA Pol II) subunit Rpb2p were found at the entire *ZDS1* locus, while Rpb4p was found at the *ZDS1* promoter only, suggesting that the RNA Pol II that had transcribed *ZDS1* was lacking the dissociable Rpb4p subunit. The deletion of *NHP6* reduced binding of Rpb4p to the *ZDS1* promoter, while the deletion of *MED3* allowed Rpb4p to enter the *ZDS1* open reading frame. This indicates that Nhp6p loaded Rpb4p onto RNA Pol II at the *ZDS1* promoter, while Med3p prevented *ZDS1* promoter clearance of RNA Pol II that contained Rpb4p. Therefore, Nhp6p and Med3p repressed transcription of *ZDS1* by controlling the local subunit composition of RNA Pol II. On the other hand, Nhp6p generally supports transcription elongation, as suggested by the 6-AU phenotype of the *NHP6* deletion strain. The deletion of *RPB4* reduced growth on 6-AU plates and the over-expression of Rpb4p suppressed the 6-AU phenotype of the *NHP6* deletion strain, indicating that Nhp6p generally supported transcription elongation via Rpb4p.

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AD   activating domain

6-AU   6-azauracil

BD   binding domain

CAK   cyclin-activating kinase

ChIP   chromatin immunoprecipitation

CHL   Chloramphenicol

C_{ub} C-terminal half of ubiquitin

CTD   carboxy-terminal domain

dNTP   deoxyribonucleotide tri-phosphate

FOA   5-fluorooratic acid

FRET   fluorescence resonance-energy transfer

GEMs   gene expression machines

GST   glutathione S-transferase

GTFs   general transcription factors

HA   Haemagglutinin

HATs   histone acetyltransferases

HDACs   histone deacetyltransferases

HMG   high mobility group

HMTs   histone methyltransferases

LB   Luria Bertani

NBD	nucleosomal binding domain
NER	nucleotide excision repair
N_{ub}	N-terminal half of ubiquitin
ORF	open reading frame
PCR	polymerase chain reaction
PIC	preinitiation complex
PPI	protein-protein interaction
PTMs	post-translational modifications
RNA Pol II	RNA polymerase II
RUra3	orotidine-5'phosphate decarboxylase reporter modified to begin with an arginine residue
SAGA	Spt-Ada-Gcn5-acetyltransferase
SDS	sodium dodecyle sulfate
SPR	surface plasmon resonance
TAFs	TBP-associated factors
TBP	TATA-binding protein
TCR	transcription coupled repair
TEMED	N, N, N', N'-tetramethlethylene-diamine
TSS	transcription start site
UAS	upstream activating sequence
UBPs	ubiquitin-specific proteases
URS	upstream repression sequence

CHAPTER 1

INTRODUCTION

1. Introduction

1.1 Introduction

Expression of protein-coding genes in eukaryotic cells is tightly related to environmental stimulation, life cycle of the organisms and genetics of the species. Transcription of protein-coding genes is a complicated process that requires the concerted functions of multiple proteins and transcription factors. Protein-coding genes consist of a transcription start site, TATA box and sequences such as the upstream activating sequences (UAS), enhancer, upstream repression sequences (URS) and silencers, which can be bound by transcriptional regulators (Lee and Young, 2000).

Transcriptional activation often occurs upon the binding of an activator to an upstream activating sequence linked to a gene (Ptashne, 2005). Upon transcription initiation, the activator binds to proximal promoter elements or more distal regulatory sequences (i.e., enhancers). Promoter-bound activators then recruit chromatin modifying and remodelling complexes that switch the chromatin structure of the gene from an *off* state to an *on* state (Daniel and Grant, 2007). The activator also recruits and/or activates the transcription machinery, which include RNA polymerase II (RNA Pol II), the General Transcription Factors (GTFs) and the Mediator, a complex of about twenty proteins that is conserved from yeast to human (Kornberg, 2005). Transcriptional repression often occurs upon the binding of a repressor to a silencing region linked to a gene (Courey and Jia,

2001). Upon repression, the transcriptional repressor binds to promoter elements or repression regions (e.g., silencers) to block the RNA polymerase machinery and result in a decrease of transcription activity. The repressor also recruits chromatin modifying and chromatin remodeling complexes that switch the chromatin structure of a gene from the *on* state to the *off* state (Jacobson et al., 2004). The Mediator plays a key role in activation, bridging DNA-bound activators, the general transcriptional machinery, especially RNA polymerase II and proteins bound to the core promoter. The Mediator subunits are necessary for a variety of positive and negative regulatory processes and serve as the direct targets of activators themselves (Lewis and Reinberg, 2003). The Mediator components Med3p and Srb7p have been described as direct repressor targets (Papamichos-Chronakis et al., 2000; Gromöller and Lehming, 2000). Santangelo (2006) proposed a new model called "reverse recruitment" to explain the eukaryotic transcriptional activation and repression. This model states a link between transcription regulation and nuclear periphery. According to the reverse recruitment hypothesis, the proteins required for gene transcription are part of gene expression machines (GEMs) (Maniatis and Reed, 2002) in the nuclear periphery and uninduced genes are located in the centre of the nucleus. Upon gene induction, an activator recruits the gene to a GEM that is associated with a nuclear pore, the gene is transcribed, and the mRNA is exported out of the nucleus through the associated nuclear pore (Casolari et al., 2004). Upon gene repression, a repressor recruits the gene to a GEM that is not associated with a nuclear pore

and the gene is silenced by the SIR complex, which is associated with repressive

GEMs (Sarma et al., 2007).

High mobility group (HMG) proteins, present in all tissues of eukaryotes, are an

abundant class of chromosomal proteins facilitating assembly of higher order

strucutures (Aleporou-Marinou et al., 2003). HMG proteins act as architectural

factors in the nucleus, facilitating various DNA-dependent processes such as

transcription and recombination (Bustin et al., 1990). There are three HMG

protein families which have been classified due to their characteristic primary

structures: the HMGB protein family, the HMGN protein family and the HMGA

protein family. Each of these protein families contains distinct sequence motifs.

The HMGB family is the most abundant HMG family, which are distinguished by

the presence of one or two copies of the HMG-box which is responsible for DNA

binding (Lu et al., 1996). The general property of HMG proteins is to bend or

wrap DNA (Giese et al., 1992). The HMG proteins are required for efficient gene

activation due to their ability to promote assembly of preinitiation complexes.

They play a general role in controlling chromatin structure and a specific role in

controlling transcription and DNA replication. HMGB proteins are

non-sequence-specific DNA-binding proteins. They bend DNA strands to

facilitate the formation of higher order DNA-protein structures which are required

for transcription initiation (Tremethick and Molley, 1996; Tremethick and

Molley, 1998).

Nhp6p is an architectural transcription factor that is related to the high-mobility

group B family of non-histone chromosomal proteins that bend DNA sharply (Travers, 2003). In *Saccharomyces cerevisiae*, Nhp6p is encoded by two highly homologous genes, *NHP6A* and *NHP6B*. They are very similar and functionally redundant (Formosa et al., 2002). Nhp6p contains a single 70-residue HMG-box motif of the type found in the HMGB family, and it is homologous to the middle segment of the chromatin-associated high mobility group B protein from calf. Nhp6p shares certain biological functions with HMGB proteins. Nhp6p binds to the minor groove of double-stranded DNA in a non-sequence-specific manner (Masse et al., 2002) and contributes to stabilize bent DNA confirmations within the preinitiation complexes (Lopez et al., 2001). Loss of Nhp6p leads to increased genomic instability, hypersensitivity to DNA-damaging agents, and shortened yeast cell life span (Giavara et al., 2005). In addition, both Nhp6ap and Nhp6bp contain a highly basic amino acid region that precedes the HMG box, which confers Nhp6p a higher affinity to bend DNA more efficiently than mammalian HMGB proteins.

Transcriptional activation requires the recruitment of the transcription machinery, which consists of RNA polymerase II (RNA Pol II), the General Transcription Factors (GTFs) and the Mediator (Kornberg, 2005). The critical step in transcriptional activation by RNA polymerase II is the formation of the preinitiation complex, which contains the TBP-TFIIA-TFIIB-DNA complex. This complex recruits RNA polymerase II and other general transcription factors required for transcriptional initiation (Biswas et al., 2004; Biswas et al., 2006).

Nhp6p supports transcription initiation by facilitating the formation of the TFIIA-TBP-TATA complex. The binding of Tbp1p to DNA is a two-step process, starting with an unstable complex containing unbent DNA and then slowly isomerizing into a stable complex with bent DNA. Nhp6 proteins bend DNA and promote formation of the stable TBP-bent DNA complex. TFIIB also stimulates the formation of the stable TBP-DNA complex and its association with this complex plays an important role to maintain the bent DNA form. Nhp6p increases the affinity of TFIIB association to the TBP-TFIIA-DNA complex (Yu et al., 2003). Nhp6p regulates both the positive and negative transcription of a number of RNA polymerase II-transcribed genes in a variety of cellular processes. A genome-wide analysis of cells lacking *NHP6A/B* showed that 114 genes were up-regulated and 83 genes were down-regulated in an *nhp6a nhp6b* double mutant, indicating an important role for Nhp6p in chromatin-mediated gene regulation (Moreira and Holmberg, 2000). Nhp6p is involved in the transcriptional activation of *HO, FRE2, CUP1, CYC1*, *URA3, DDR2* and *DDR8* (Cosma et al., 1999; Fragiadakis et al., 2004; Paull, 1996). It also plays a role in the transcription repression of *GAL1*, *SUC2* (Laser et al., 2000) and *CHA1* (Moreira and Holmberg, 2000).

Nhp6p functions with the yeast FACT complex to promote transcriptional elongation (Formosa et al., 2001). Chromatin modulator FACT was identified to mediate the transcription of protein-coding genes by RNA polymerase II and works at the level of transcriptional elongation. The FACT complex acts as a

histone chaperone to promote H2A-H2B dimer dissociation from the nucleosome and allow RNA polymerase II move along the DNA template (Belotserkovskaya and Reinberg, 2004). The chromatin remodeller FACT has two main subunits. The larger one is Spt16p, and the smaller one is SSRP1(vertebrates) or Pob3p (yeast). Yeast Pob3p protein is structurally related to SSRP1 proteins but lacks the HMG-box domain at the C-terminus of SSRP1. The function of this domain for yeast FACT is supplied by the small yeast HMG-box protein Nhp6p (Wittmeyer and Formosa, 1997). Nhp6p is involved in a two-step nucleosome remodelling mechanism: multiple Nhp6p molecules bind to the nucleosome first and induce a change in nucleosome structure to convert it to a substrate for Spt16p-Pob3p or other chromatin-modifying factors; then these Nhp6p-nucleosomes recruit Spt16p-Pob3p to form SPN-nucleosomes (Ruone et al., 2003). The complex of Spt16p-Pob3p and Nhp6p (yFACT) with nucleosomes causes changes in the electrophoretic mobility and nuclease sensitivity of the nucleosomes (Formosa et al., 2002). In this way, Nhp6p promotes the formation of the yeast FACT complex to facilitate transcriptional elongation.

In addition, Nhp6p is reported as a transcriptional initiation fidelity factor for RNA polymerase III transcription *in vitro* and *in vivo* (Kassavetis and Steiner, 2006). Nhp6p participates in the activation of the RNA Pol III *SNR6* gene (Lopez et al., 2001) and the *nhp6a nhp6b* double mutant is temperature sensitive due to inefficient transcription of the essential *SNR6* gene by RNA Pol III (Kruppa et al., 2001). Nhp6p is important for transcription of a set of tRNA genes and

heterochromatin barrier function (Braglia et al., 2007). Nhp6p also plays a role in DNA repair and genome maintenance (Giavara et al., 2005).

RNA polymerase II catalyzes the transcription of DNA to synthesize the precursors of mRNA and small nuclear RNAs that take part in RNA splicing (Lewin, 2004). A wide range of transcription factors are required for RNA Pol II to bind to its promoters and begin transcription. Transcriptional activators recruit RNA polymerase II combined with specific transcription factors and other auxiliary proteins to form the preinitiation complex, which directs transcription from specific promoters (Hahn, 2004). RNA Pol II of *S. cerevisiae* is composed of twelve subunits designated Rpb1p–12p, ranged in size from approximately 6 kd to 200 kd (Young, 1991; Levine and Tjian, 2003). Rpb1p, Rpb2p, Rpb3p and Rpb11p are responsible for the basic catalytic activity; Rpb5p, Rpb6p, Rpb8p, Rpb10p and Rpb12p constitute the bulk of RNA Pol II structure and maintain structural integrity (Choder, 2004; Sampath and Sadhale, 2005); Rpb9p influences start site selection (Hampsey, 1998). These ten subunits form the core of RNA Pol II. Rpb4p and Rpb7p form a conserved complex and perform multiple functions in transcription, mRNA transport and DNA repair (Edwards et al., 1991). The Rpb4p/Rpb7p subcomplex of RNA Pol II interacts with transcriptional activators and the general transcription factors TFIIB and TFIIF to promote the assembly of the initiation complex in the promoter region (Choder, 2004). Rpb4p is a non-essential subunit of the RNA Pol II. It is not essential for cell viability, but cells lacking *RPB4* exhibit slow growth at moderate temperature, poor recovery

from stationary phase and are sensitivity to extreme temperatures (Woychik and Young, 1989; Choder and Young, 1993; Rosenheck and Choder, 1998). Rpb4p has some unique features distinguishing it from other subunits. The stoichiometry of Rpb4p is dependent upon growth conditions. In optimally growing cells, the fraction of RNA Pol II containing Rpb4p is approximately 20%, and it gradually increases following the shift to post-logarithmic phases (Kolodziej et al., 1990; Choder and Young, 1993).

The Split-Ubiquitin system was originally developed by Johnsson and Varshavsky (1994). It is an alternative yeast two-hybrid assay that is based on a conditional proteolysis design (Lehming, 2002; Reichel and Johnsson, 2005). The Split-Ubiquitin system is based on conditional proteolysis that occurs upon the re-association of the N- and C-terminal halves of ubiquitin designated N_{ub} and C_{ub}, respectively. Each half of ubiquitin is fused to either protein of interest. In our split-ubiquitin screen, the bait protein Nhp6ap or Nhp6bp was fused to C_{ub} immediately followed by the reporter protein RUra3p. The first residue of Ura3p had been changed to arginine to cause the degradation of the free RUra3p reporter by the enzymes of the N-end rule. The *URA3* gene encodes orotidine-5'-phosphate decarboxylase. This enzyme is required for biosynthesis of uracil. It also converts non-toxic 5-fluoroorotic acid (FOA) to 5-fluorouracil. The new product is highly toxic and causes cell death. A N_{ub} fusion library had been constructed by fusing genomic *S. cerevisiae* Sau3A-partially digested DNA fragments in all three reading frames 3' to the N_{ub} moiety. The N_{ub} fusion library was transformed into a

S. cerevisiae strain that expressed Nhp6a-C_{ub}-RUra3p or Nhp6b-C_{ub}-RUra3p as bait. When the N_{ub} fusion protein interacted with the C_{ub} fusion protein inside the cell, the two halves of ubiquitin were brought together to form a native-like ubiquitin. Ubiquitin-specific proteases (UBPs) recognized the reconstituted ubiquitin and cleaved off RUra3p. The enzymes of the N-end rule degraded the released RUra3p rapidly. Thus the interaction between N_{ub} and C_{ub} fusion proteins within the cells could be indicated by the FOA resistance.

1.2 Aim of the study

Our research is focused on the isolation and identification of new interacting partners of the non-histone chromosomal protein Nhp6p. The purpose of this study is to investigate how these cooperator proteins function together with Nhp6p to regulate specific gene transcription in yeast. We have isolated Nhp6p-interacting proteins using the Split-Ubiquitin system. We have used the *ZDS1* gene, which is repressed by Nhp6p and its interacting partners to study chromosomal co-localization of Nhp6p and its interacting partners in wild-type and deletion strains. Our study will provide further understanding of how non-histone chromosomal proteins are involved in the regulation of transcription and how they cooperate with their associated proteins to regulate gene expression.

CHAPTER 2

SURVEY OF LITERATURE

2. Survey of literature

2.1 Eukaryotic Transcription

2.1.1 Transcription of protein-coding genes

Cells express protein-coding genes according to requirement. The regulation of protein-coding genes can be achieved through activation and repression via regulator proteins that lead to the chromatin modification at the genes. In general, protein-coding genes consist of a transcription start site (TSS), TATA box and sequences that can be bound by transcriptional regulators such as the upstream activating sequences (UAS), enhancer, upstream repression sequences (URS) and silencers. The core promoter element of the protein-coding gene is approximately 100 bp and contains the transcription site (Lee and Young, 2000).

Transcription of eukaryotic protein-coding genes is a complicated process that requires the concerted functions of multiple proteins and transcription factors. During transcription initiation, sequence-specific DNA-binding transcriptional regulators, such as activators, bind to proximal promoter elements or more distal regulatory sequences (i.e., enhancers) to modulate the rate of transcription of specific target genes in response to physiological or environmental stimuli. Then co-activators are recruited by promoter-bound activators to remodel the chromatin structure to stimulate the recruitment or activity of the basal and general transcription factors, which include RNA polymerase II (RNA Pol II) and a set of accessory general transcription initiation factors (GTFs) that bind to core promoter

DNA elements (e.g., TATA box, initiator) and allow the specific recruitment of RNA Pol II to the core promoter (Martinez, 2002; Ptashne, 2005; Daniel and Grant, 2007). Transcriptional repression often occurs upon the binding of a repressor to a silencing region linked to a gene (Courey and Jia, 2001). In this case, the sequence-specific DNA-binding regulator is the transcriptional repressor. Transcriptional repressors binding to repressing regions (e.g., silencers) can block the RNA polymerase machinery and result in a decrease of transcription (Keaveney and Struhl, 1998). They recruit chromatin-modifying and chromatin-remodeling complexes that switch the chromatin structure of a gene from the *on* state to the *off* state (Jacobson et al., 2004). The Mediator, a complex of twenty proteins that is conserved from yeast to human, is also involved in transcriptional regulation (Kornberg, 2005). The Mediator components Med3p and Srb7p have been described as direct repressor targets (Papamichos-Chronakis et al., 2000; Gromoller and Lehming, 2000). Santangelo (2006) proposed a new model for eukaryotic gene regulation, called "reverse recruitment". The reverse recruitment model states that a link exists between the nuclear periphery and transcriptional activation. According to this hypothesis, upon transcriptional activation, a transcriptional activator recruits a specific gene to a GEM that is associated with a nuclear pore, the gene is transcribed and the mRNA is exported out of the nucleus through the associated nuclear pore (Casolari et al., 2004); upon transcriptional repression, a transcriptional repressor recruits the gene to a GEM that is not associated with a nuclear pore and the gene is silenced by the SIR

complex, which is associated with repressive GEMs (Sarma et al., 2007).

2.1.2 RNA polymerase II

Transcription in eukaryotic cells is divided into three classes. Each class is transcribed by a different RNA polymerase. RNA polymerase I, functions in the transcription of precursor ribosomal RNA (rRNA), which is processed into 28S, 5.8S and 18S rRNA. RNA polymerase II catalyzes the transcription of DNA to synthesize the precursors of mRNA and four of the five small nuclear RNAs that take part in RNA splicing. RNA polymerase III is needed for the synthesis of transfer RNA (tRNA) and other small nuclear RNAs (including the small 5S rRNA) (Lewin, 2004).

RNA polymerase II (also called RNA Pol II) is the most studied type of RNA polymerase. A wide range of transcription factors are required for it to bind to promoters and begin transcription. Transcriptional activators recruit RNA polymerase II together with the transcription initiation apparatus to the promoters of protein-coding genes. The assembled initiation apparatus consists of specific transcription factors and other auxiliary proteins to direct transcription from specific promoters (Hahn, 2004). These auxiliary proteins comprising of GTFs, coactivators and mediators along with RNA polymerase II make up the holoenzyme. RNA polymerase II holoenzyme exists in most eukaryotic organisms, even though the holoenzyme composition shows some species specificities. Yeast RNA polymerase holoenzyme contains five major components: the core RNA polymerase II, the GTFs, the core Srb-mediator complex, the Srb10

cyclin-dependent kinase (CDK) complex and the SWI-SNF complex (Myer and Young, 1998).

The core RNA polymerase is highly conserved among eukaryotes. Yeast core RNA polymerase is composed of 12 subunits, Rpb1p-Rpb12p, ranged in size from approximately 6 kd to 200 kd (Young, 1991; Levine and Tjian, 2003). Of the 12 core subunits of RNA Pol II, Rpb1p, Rpb2p, Rpb3p and Rpb11p are responsible for the basic catalytic activity; Rpb5p, Rpb6p, Rpb8p, Rpb10p and Rpb12p are shared between the three RNA polymerases and constitute the bulk of RNA Pol II structure maintaining structural integrity (Sampath and Sadhale, 2005; Choder and Young, 2004); Rpb9p influences start site selection (Hampsey, 1998); these ten subunits form the core of RNA Pol II. Rpb4p and Rpb7p form a conserved complex in all three RNA polymerases and perform multiple functions. The Rpb4p/Rpb7p sub-complex originally characterized in *S. cerevisiae* was identified as a dissociable sub-complex of yeast RNA Pol II (Ruet et al., 1980; Edwards et al., 1991). This heterodimer plays a role in transcription, mRNA transport and DNA repair. Rpb4p/Rpb7p interacts with both transcriptional activators and general transcription factors such as RNA Pol II, TFIIB and TFIIF to promote the assembly of the initiation complex in the promoter region (Choder, 2004). Rpb4p/Rpb7p is recruited to the RNA Pol II complex to prevent conformational changes during long-term starvation (Choder, 1993). The interaction between RNA Pol II and Rpb4p/Rpb7p is promoter specific, the Rpb4p/Rpb7p complex is recruited in only 20% of the initiation events in optimally proliferating cells

(Khazak et al., 1995; Petermann et al., 1998; Na et al., 2003). However, the recruitment of the Rpb4p/Rpb7p heterodimer to initiation sites does not occur often. Rpb4p/Rpb7p is not required for stable recruitment of polymerase to pre-initiation complexes. The interaction of heterodimer and RNA Pol II only occurs during some specific stages of the transcription cycle (Choder, 2004).

Apart from its role in transcription, Rpb4p is also involved in the appropriate response of a cell to various stressful conditions. Rpb4p is a non-essential subunit of the RNA Pol II; the deletion of *RPB4* causes slow-growth at moderate temperature, poor recovery from stationary phase and sensitivity to extreme temperatures (Woychik and Young, 1989; Choder and Young, 1993; Rosenheck and Choder, 1998). Furthermore, yeast cells lacking *RPB4* sporulate poorly under severe starvation and are defective for mating, cell wall integrity and display Na^+/Li^+ ion sensitivity (Bourbonnais et al., 2001). Rpb4p has been associated with post-transcriptional processes like Transcription Coupled Repair (TCR) and mRNA export under stress (Li and Smerdon, 2002; Farago et al., 2003). Rpb4p is also involved in cell-cycle regulation. Cells lacking *RPB4* display a cell-cycle arrest as large unbudded cells in G1 phase (Sampath and Sadhale, 2005).

2.1.3 General Transcription Factors

General transcription factors (GTFs) are protein transcription factors which are involved in the transcription of class II genes to mRNA templates. Most of GTFs are involved in the formation of the preinitiation complex together with RNA

polymerase II for transcription initiation. Some of them are also required for facilitation of RNA Pol II movement on gene-coding regions to promote transcriptional elongation. The most common general transcription factors are TFIIA, TFIIB, TFIID, TFIIE, TFIIF and TFIIH.

TFIIA is one of the general transcription factors required for transcription. These factors are responsible for promoter recognition and the formation of a transcription preinitiation complex (PIC) capable of initiating RNA synthesis from a DNA template. TFIIA is involved in RNA polymerase II-dependent transcription of DNA and it is essential for viability. TFIIA interacts with the Tbp1p subunit of TFIID and aids in the binding of Tbp1p to TATA-box containing promoters. Although TFIIA does not recognize DNA itself, its interactions with TFIID allow it to stabilize and facilitate the formation of the PIC. TFIIA also acts as a coactivator for some transcriptional activators, assisting their ability to increase or activate transcription (Gill, 2001; Martinez, 2002).

TFIIB is an essential part of the multi-protein transcription initiator complex that assembles on RNA polymerase II-dependent promoters. It contains a zinc finger domain at the N-terminus and a direct repeat in the C-terminal domain. TFIIB interacts with both the C-terminal stirrup of Tbp1p and with the deformed DNA backbone on either side of the TATA box. The Tbp1p-TFIIB complex serves as a platform to recruit RNA polymerase II and the rest of the transcription machinery (Bartlett, 2005; Deng and Robert, 2007).

TFIID is a multi-component transcription factor that recognizes and binds

promoters. TFIID consists of a DNA-binding subunit that recognizes the TATA element and is therefore designated TATA-binding protein (Tbp1p), as well as several TBP-associated factors (TAFs). TFIID binding is thought to be the first step in transcription initiation. TFIID acts to nucleate the transcription complex, recruiting the rest of the factors through a direct interaction with TFIIB. The Tbp1p subunit of TFIID is sufficient binding to the TATA element and for interaction with TFIIB to support basal transcription. When Tbp1p binds to the TATA box in the promoter region, it distorts the DNA to create a 90 degree bend. The bend of DNA increases the DNA-protein interaction and recruits other factors required for RNA Pol II to begin transcription (Lerner et al., 2006; Thomas and Chiang, 2006). Tbp1p is also a necessary component of RNA polymerase I and RNA polymerase III. Several *in vivo* studies have shown that Tbp1p plays a specific role in the activation of a subset of cellular genes controlling the cell-cycle (Davidson et al., 2004). Some of the TAFs also bind to initiator elements. The TAFs of TFIID are necessary to increase the rate of transcription when bound by activators (Green, 2000).

TFIIE (Transcription factor II E) is composed of two subunits of 56 kd and 34 kd, and it is a tetramer consisting two molecules of each subunit. The general transcription factor TFIIE recruits TFIIH at a late stage of transcription initiation complex formation and markedly stimulates TFIIH-dependent phosphorylation of the carboxy-terminal domain (CTD) of RNA polymerase II. TFIIE modulates the helicase and kinase activities of TFIIH and the two factors show species-specific

interactions. Published results suggest that TFIIE, via its effect on TFIIH, may act as a checkpoint for the formation of the preinitiation complex (Ohkuma et al., 1995; Thomas and Chiang, 2006).

TFIIF (transcription factor II F) binds directly to RNA polymerase II and it is necessary for RNA polymerase II to stably associate with the TFIIA-TFIIB-promoter complex. TFIIF is a component of the yeast holoenzyme and mediator complexes. It interacts with TFIIB and the dissociable Rpb4p/Rpb7p polymerase subunit to recruit RNA polymerase II to the initiation complex. It remains associated with the elongating polymerase to promote transcription elongation (Thomas and Chiang, 2006).

TFIIH (Transcription factor II H) is a large multi-subunit complex which comprises two sub-complexes – the core complex and the cyclin-activating kinase complex (CAK). It is involved in three of the most important functions of cells: DNA repair, cell-cycle control and transcription. TFIIH participates in nucleotide excision repair (NER) in DNA repair. It eliminates large adducts in DNA and repairs oxidative DNA damage (Le Page et al., 2005). TFIIH is a component of the basal RNA Pol II transcription machinery and plays important roles in both transcription initiation and elongation. During transcription initiation, the ATP-dependent helicase activities of TFIIH are essential for the formation of the open complex. Cdk7p phosphorylates the fifth serine of the heptapeptide repeat in the C-terminal domain of the RNA Pol II large subunit after the open complex is established. This phosphorylation enables RNA Pol II to release most of the basal

transcription factors, initiating elongation of the mRNA. TFIIH plays a regulatory role in transcription by interacting with diverse transcription factors (Zurita and Merino, 2003). TFIIH associates with the TIF-IB-SL1 factor (Reese, 2003), which is a complex of the RNA Pol I holoenzyme. The TIF-IB-SL1 complex interacts with the high mobility group protein (HMG) family factor to facilitate the recruitment of RNA Pol I and auxiliary factors to the ribosomal gene cluster promoter (Grummt, 1999).

2.1.4 Chromatin

Chromatin is the complex of DNA and protein found inside the nuclei of eukaryotic cells. The nucleic acids and major proteins involved in chromatin are double-stranded DNA and histone proteins. The basic chromatin structure unit is the nucleosome, which contains a 146 bp piece of DNA wrapped around a histone octamer. In nucleosome, histone H3 and H4 form a dimer, association of two H3-H4 dimers constructs a $(H3-H4)_2$ tetramer. DNA wraps around this tetramer, forming a tetrameric particle. Histones H2A and H2B heterodimerize and the heterodimers associate on each side of the tetrameric particle to form a nucleosome (Arents and Moudrianakis, 1993; Rando and Ahmad, 2007). Nucleosomes in chromatin are connected by 10-60 bp of linker DNA, resulting in an extended bead-on-a-string structure. Nucleosomes are an invariant component of euchromatin and heterochromatin in the interphase nucleus and in the mitotic chromosomes. The second level of organization is the coiling of the series of

nucleosomes into a helical array to constitute the fiber of 30 nm diameter. This structure is found in both interphase chromatin and mitotic chromosomes. The third level of organization is the packaging of the 30 nm fiber itself. Such 30 nm fibers are then further condensed *in vivo* to form 100-400 nm thick interphase fibers or the more highly compacted metaphase chromosome structures (Sivolob and Prunell, 2004). Chromatin structure imposes significant obstacles on all aspects of transcription that are mediated by RNA polymerase II. The dynamics of the chromatin structure is tightly regulated through multiple mechanisms including histone modification, chromatin remodeling, histone variant incorporation, and histone eviction.

The general process of inducing changes in chromatin structure is called chromatin remodeling. The most common use of chromatin remodeling is to change the organization of nucleosomes at the gene promoter. To achieve such chromatin structural changes, two major mechanisms have been proposed: the action of ATP-dependent chromatin-remodeling complexes and the post-transcriptional modification of histones. The latter will be discussed in the next section. Yeast has two major types of complexes depending on the type of ATPase subunit present in the complex: SWI/SNF and ISWI. The ATPase activity of the SWI/SNF complex is preferentially stimulated by naked DNA. The SWI/SNF complex remodels chromatin *in vitro* without overall loss of histones and only partially dissociates DNA from nucleosomes. The ATPase activity of the ISWI complex is stimulated by nucleosomes. The ISWI complex induces a

nucleosome sliding on the DNA (Hamiche et al., 1999; Langst et al., 1999). It was shown for the yeast cell-cycle regulated *HO* promoter, that the DNA binding factor Swi5p can recruit the SWI/SNF complex, followed by the recruitment of the SAGA complex, which acetylates the histone and facilitate transcription. However, this mechanism is far from being general and the mechanism for other ATP-dependent chromatin-remodeling complexes are not well understood (Morales et al., 2001).

2.1.5 Histones

Histones are the major protein components of chromatin. They play a role in gene regulation by remodeling chromatin structure. Six major histone classes have been identified: H1, H2A, H2B, H3, H4 and archaeal histone. Histone H2A, H2B, H3 and H4 are called the core histones. Two of each of the four core histones assembly to form one octameric nucleosome core particle with 146 bp DNA wrapped around. The linker histone H1 binds to the nucleosome and the entry and exit sites of the DNA to lock the DNA into place, allowing the formation of higher order structures.

The four core histones are relatively similar in structure and highly conserved due to constraints to maintain the overall structure of the nucleosomal octameric core. All of the core histones are characterized by the presence of a histone fold domain, a 'helix-turn-helix-turn-helix' motif. They also share the feature of N-terminal tails of variable length that are the subject of extensive post-translational

modifications (PTMs), which have been implicated in transcriptional activation, silencing, chromatin assembly and DNA replication (Peterson and Laniel, 2004). PTMs are components of the epigenome that induce changes to DNA and its connected proteins. These epigenetic modifications are switches for the regulation of gene expression and they are chemical modifications of the DNA and histones that do not result in changes to the DNA sequence (Marino-Ramriez et al., 2005).

2.2 Function of histone in eukaryotic transcription

2.2.1 Post-translational modification of histone

Besides acting as tools for DNA packing, histones are subjected to an enormous number of post-translational modifications. Post-translation modification is the chemical modification of a protein after its translation. The spectrum of modifications ranges from the addition of relatively small groups such as methyl, acetyl and phosphoryl groups to the attachment of larger sugar moieties or the generation of isopeptidic bonds between the molecule of interest and the small peotides ubiquitin or SUMO (Walsh et al., 2005). There are at least eight distinct types of modifications found on histone as listed in the following table.

Chromatin Modifications	Residues Modified		Functions Regulated
Acetylation	**K**-ac		Transcription, Repair, Replication, Condensation
Methylation (lysines)	**K**-me1 **K**-me3	**K**-me2	Transcription, Repair
Methylation (arginines)	**R**-me1 **R**-me2s	**R**-me2a	Transcription
Phosphorylation	**S**-ph **T**-ph		Transcription, Repair, Condensation
Ubiquitylation	**K**-ub		Transcription, Repair
Sumoylation	**K**-su		Transcription
ADP ribosylation	**E**-ar		Transcription
Deimination	**R > Cit**		Transcription
Proline Isomerization	**P-cis > P-trans**		Transcription

Table 2.2.1 Different classes of modifications identified for histones

Over 60 different residues on histones can be modified. The majority of these post-translational marks occurs at the amino-terminal and carboxy-terminal histone tail domains. Two commonly found modifications in eukaryotic histones to regulate protein function are acetylation and methylation at lysine residues. Extra complexity comes partly from the fact that methylation at lysines or arginines may be one of three differnet forms: mono-, di-, or trimethyl for lysines and mono- or di- for arginines. This vast array of modifications gives enormous potential for functional responses (Kouzarides, 2007). Recent studies have shown that site-specific combinations of histone modifications correlate well with particular biological functions to active or inactive genes. Acetylation of H4K8, H3K14 combined with phosphorylation of H3S10 is often found at active genes. Conversely, tri-methylation of H3K9 and the lack of H3 and H4 acetylation correlate with transcription repression in higher eukaryotes (Peterson and Laniel,

2004). Modifications that are localized to inactive genes or regions, such as H3K9me and H3K27me, are often termed heterochromatin modifications. Most modifications are distributed in distinct localized patterns within the upstream region, the core promoter, the 5' end of the open reading frame (ORF) and the 3' end of the ORF. Indeed, the location of a modification is tightly regulated and is crucial for its effect on transcription (Li et al., 2007).

2.2.2 The role of histone modifications in the remodelling of chromatin structure

The general process of inducing changes in the structure of chromatin is called chromatin-remodeling (Studitsky et al., 2004). The change of chromatin organization via covalent modification provides access to the genes for the transcription apparatus (Ito, 2007). Changes in chromatin structure are initiated by modifying the N-terminal tails of the histones, especially H3 and H4. Histone modification may directly affect nucleosome structure or create binding sites for the attachment of non-histone proteins that change the properties of chromatin (Lewin, 2004). Histone modifications, including lysine acetylation and methylation, serine phosphorylation and arginine methylation, play major regulatory roles in many genetic events such as transcriptional activation and elongation, silencing and epigenetic cellular memory (Strahl and Allis, 2000; Berger, 2002; Turner, 2002).

Recent studies have shown that reversible and rapid changes in histone acetylation play important roles in chromatin modification, induce genome-wide and specific

changes in gene expression, and affect a variety of biological processes (Chen and Tian, 2007). The balance between these modifications is achieved through the action of enzymes called histone acetyltransferases (HATs) and histone deacetyltransferases (HDACs). These specific enzymes catalyze the transfer of an acetyl group from acetyl-CoA molecules to the lysine ε-amino groups on the N-terminal tails of histones (Yang, 2004). Acetylation of lysines neutralizes the charge on histones, therefore increasing chromatin accessibility. Acetylation regulates nucleosome structure in both replication and transcription. At replication, acetylation of histones allows them to be incorporated into new cores more easily. At transcription, a similar effect allows the histone core to be displaced from DNA. Furthermore, acetylation is an important signal for the binding of activation factors. In yeast, GCN5, the catalytic subunits of the HAT SAGA complex, acts as an adaptor complex that is necessary for the interaction between certain enhancers and their target promoters. The SAGA complex is recruited to acetylated histones via its Bromo-domain, manifesting the acetylated state. Histone deacetylases are associated with repression of gene activity. Yeast cells contain a group of related HDACs that include Rpd3p, Hda1p, Hos1p, Hos2p and Hos3p. Mutations in *RPD3* derepress a variety of genes (Verdone et al., 2006).

Methylation of histones in *S. cerevisiae* is carried out by histone methyltransferases, which are capable of covalently modifying specific lysine residues (K4, K9, K36 and K79) in the tail of H3 and arginine residue (R3) in the tail of H4 with methyl groups (Peterson and Laniel, 2004). The histone

methyltransferase (HMT) Set1p is responsible for histone H3K4 methylation (Briggs et al., 2001; Bernstein et al., 2002; Krogan et al., 2003). Peaks of histone H3K4 trimethylation occur at the beginning of actively transcribed genes. Methylation of H3K4 is a mark for gene activation (Santos-Rosa et al., 2002). Methylation of H3K9 by HMT SUV39H1 is a feature of condensed regions of chromatin, leading to gene silencing (Pokholok et al., 2005). Trimethylation of H3K36, which is catalyzed by HMT Set2 (Strahl and Allis, 2002), is enriched throughout the coding region, peaking near the 3′ ends of transcription units. H3K36 trimethylation correlates with transcriptional activity (Pokholok et al., 2005). The HMT Dot1 modifies histone H3 lysine 79 (H3K79), which occurs within the core domain of histone H3 (Feng et al., 2002; Ng et al., 2002). Methylation of this residue is associated with telomeric silencing control in yeast (Ng et al., 2003; Van Leeuwen et al., 2002). Methylation of H4R3 is catalyzed by histone methytransferase PRMT1 and this methylation facilitates transcriptional activation (Wang et al., 2001).

Histones are phosphorylated in two circumstances: cyclically during the cell-cycle and in association with chromatin remodeling. Histone phosphorylation affects chromatin structure. Loss of phosphorylation of H3S10 has devastating effects on chromatin structure. H3 phosphoraltion is required to generate the more extended chromosome structure of euchromatic regions. Phosphorylation of different histones has opposite effects on chromatin structure (Lewin, 2004).

2.3 High Mobility Group (HMG) family proteins

High mobility group (HMG) proteins are an abundant class of chromosomal proteins that facilitate assembly of higher order strucutures (Aleporou-Marinou et al., 2003). The animal HMG proteins have been classified in three families due to their characteristic primary structures: HMGB (previously HMG1/2) family proteins, HMGN (previously HMG14/17) family proteins and HMGA (previously HMGI/Y) family proteins. Each of these families of proteins contains distinct sequence motifs. HMG proteins are present in all tissues of eukaryotes. They act as architectural factors in the nucleus, facilitating various DNA-dependent processes such as transcription and recombination (Bustin et al., 1990).

The HMGB family is the most abundant HMG family. Members of the HMGB family proteins are distinguished by the presence of one or two copies of an 80-amino acid domain termed HMG-box, which is responsible for DNA-binding activity (Lu et al., 1996). The HMGB class can be further divided into two subfamilies based upon the number of HMG domains, the DNA sequence-binding specificity, and their evolutionary relationship: transcription factors and chromatin-associated proteins. Members of the transcription factor class usually contain a single box and recognize dsDNA with good sequence specificity (Jantzen et al., 1990). The HMG box present in a number of human sequence-specific transcription factors such as UBF, SRY, LEF-1 and TCF (Jantzen et al., 1990; Sinclair et al., 1990; Travis et al., 1991; Van de Wetering et

al., 1991). Members of the chromatin-associated class recognize DNA with little or no sequence specificity and recognize altered DNA conformations. They contain two HMG boxes and a highly acidic C-terminus (Lu et al., 1996). A general property of both classes of HMGs is the ability to bend or wrap DNA (Javaherian et al., 1979; Giese et al., 1992). They may play a general role in controlling chromatin structure and a specific role in controlling transcription and DNA replication (Tremethick and Molly, 1986; Tremethick and Molly,1988). Furthermore, efficient activated transcription of certain genes requires HMG proteins due to their ability to promote assembly of preinitiation complexes. HMGB proteins can stimulate the formation of transcription initiation complexes of RNA polymerase II and III by facilitating the binding of transcription factors to template DNA to form the initiation complexes (Tremethick and Molly, 1998). The DNA in the assemblies may be tightly bent, and the role of the HMG proteins is to overcome the axial rigidity of the DNA. The formation of higher order protein-DNA structures often requires bending of DNA strands between specific sites. Non-specific DNA-binding HMGB proteins which serve as assembly factors facilitate this process. A model for this activity is the formation of the invertasome, an intermediate structure created in the Hin-mediated site-specific DNA in inversion reaction. In mammals, HMGB proteins efficiently stimulate invertasome formation in the Hin DNA inversion reaction (Paull and Johnson, 1995).

The HMGN protein family contains the only non-histones chromosomal proteins known to have a higher affinity for nucleosomes than for DNA (Bustin and

Reeves, 1996). They form complexes with nucleosomes on actively transcribed genes where their presence is associated with sensitivity to DNase I. HMGN proteins bind to the inner side of the nucleosomal DNA potentially affecting histone-DNA interaction. The HMGN family is composed of the HMGN1 (previously HMG14) and HMGN2 (previously HMG17) proteins. The functional motif of the HMGN family is the nucleosomal binding domain (NBD). HMGN1 and HMGN2 are similar to each other in size and structure and share greater than 90% amino acid sequence identity (Aleporou-Marinou et al., 2002). Several experiments suggested that HMGN proteins may modulate the chromatin structure of transcriptionally active genes. The binding of HMGN may induce specific changes in the formation of the nucleosome core particle (Postnikov et al., 1995) and thus change the architechture of the higher order chromatin structure.

The third family of HMG proteins is composed of the HMGA proteins. There are three members in this family, HMGA1a (previously HMGI), HMGA1b (previously HMGY), and HMG2 (previously HMGI-C). The characteristic features of HMGA proteins are the presence of a sequence motif called AT-hook (Gupta et al., 1997). The AT-hook motif is a short positively charged sequence containing the invariant peptide core motif Arg-Gly-Arg-Pro (GRP), which is usually flanked on either side by conserved positively charged arginine and proline residues (Bustin and Reeves, 1996). The AT-hook mediates preferential binding of HMGA proteins to DNA substrates with altered structures such as synthetic four-way junctions (Hill and Reeves, 1997). HMGA proteins recruit the

transcription factors NF-κB and ATF-2/c-Jun to enhancers and promote enhanceosome assembly. Both Brm/SNF2α and BRG-1/SNF2ß have an AT-hook-like DNA-binding motif in their C-terminal regions. Experiments have demonstrated that when this region was deleted from the Brm/SNF2α protein, the SWI/SNF complex lost its *in vivo* functional activity and was no longer tethered to chromatin. This supported a role for the AT-hook-like proteins in the process of structural alterations of chromatin in *vivo*. In addition, HMGA proteins assist the formation of higher-order transcription factor complexes, regulating gene expression by orchestrating multiple protein-protein and protein-DNA interactions (Merika and Thanos, 2001; Reeves and Beckerbauer, 2001). Besides their roles in gene transcription, HMGA proteins are also involved in the regulation of chromatin structure and actively participate in pathologic processes. HMGA proteins also perform important functions as host-supplied factors involved in viral gene regulation and retroviral integration events (Grasser, 2003).

2.4 The non-histone chromosomal protein Nhp6p

2.4.1 Structure of Nhp6p

The non-histone chromosomal protein Nhp6p is an architectural transcription factor that belongs to the high-mobility group B family of proteins that bend DNA sharply and regulate gene expression (Travers, 2003). In *Saccharomyces cerevisiae*, Nhp6p is encoded by two highly homologous genes, *NHP6A* and *NHP6B*. Nhp6ap and Nhp6bp are 92- and 98-residue proteins representing single

HMGB motif. They are highly identical and functionally redundant (Formosa et al., 2001). Nucleotide sequence analysis revealed that *NHP6A* and *NHP6B* match at 87% of their sequences besides that *NHP6B* has six extra amino acids at its amino terminus. Although Nhp6a and Nhp6b proteins encoded by duplicated genes are functionally equivalent, the levels of protein synthesized from each gene of a duplicated set is not equal. In wild type cells, Nhp6ap is present at three times the level of Nhp6bp. Deletion of Nhp6ap led to a three-fold increase in Nhp6bp synthesis while an extra copy of Nhp6ap reduced Nhp6bp expression two-fold (Kolodrubet et al., 2001). Nhp6p appears to interact with or through another protein in regulating transcription from the *NHP6* gene since purified Nhp6ap does not bind specifically to the *NHP6B* promoter region (Kolodrubet et al., 2001).

Nhp6p contains a single 70-residue HMG-box motif of the type found in the HMGB family. It is the most related HMGB family protein in *S. cerevisiaes* (Ruone et al., 2003). Nhp6ap and Nhp6bp are homologous to the middle segment of the chromatin-associated high mobility group B protein from calf. HMGB protein contains three domains: the amino-terminal 80-amino acid (domain A), the middle 70-amino acid (domain B), and the highly acidic carboxyl-terminal 50-amino acid domain (domain C). Yeast Nhp6ap and Nhp6bp share the most identities with the middle domain and are missing the amino-third and the carboxyl-third of the other HMGB proteins (Kolodrubetz and Burgum, 1990).

The significant identity of amino acid sequence between Nhp6p and HMGB

indicates that Nhp6p may share certain biological functions with HMGB proteins.

HMGB proteins function as architectural factors through their ability to strongly distort DNA structure. They promote assembly of specialized recombination complexes (Paull et al., 1993; Segall et al., 1994; Lavoie and Chaconas, 1994) and facilitate nucleosome assembly and disassembly *in vitro* (Bonne-andrea et al., 1986; Ura et al., 1996). Moreover, efficient activated transcription of certain genes requires HMG proteins to promote assembly of preinitiation complexes (Shykind et al., 1995). Nhp6p binds to the minor groove of double-stranded DNA in a non-sequence-specific manner and thereby influence chromatin structure (Masse et al., 2002). Nhp6p stabilizes bent DNA confirmations within the preinitiation complexes (Lopez et al., 2001). It may alter the DNA structure to promote appropriate promoter architecture and enable co-complex formation or recruitment (Paull et al., 1996; Formosa et al., 2001). Loss of Nhp6p leads to increased genomic instability, hypersensitivity to DNA-damaging agents, and shortened yeast cell life span (Gravara et al., 2005).

In addition, Nhp6ap and Nhp6bp both contain a highly basic amino acid region that precedes the HMG box. This structure confers Nhp6p a higher affinity to bend DNA more efficiently than mammalian HMGB protein. Yen and group (1998) demostrated that the N-termial segment of Nhp6a is critical for its DNA binding activities and functional properties. The basic N-terminal arm of Nhp6a presumably associates with DNA to anchor the complex upon Nhp6a-DNA interaction.

2.4.2 Function of Nhp6p in transcription initiation

Transcriptional activation often occurs upon the binding of an activator to an upsteam activating sequence linked to a gene (Ptashne, 2005). The activator recruits the transcription machinery that consists of RNA polymerase II (RNA Pol II), the General Transcription Factors (GTFs) and the Mediator (Kornberg, 2005). One critical step in transcriptional activation by RNA polymerase II is the formation of the preinitiation complex: the general transcription factors TFIIA, TFIIB and TFIID are recruited onto TATA sequence-containing promoter DNA in a sequential and cooperative manner to form the TFIID-TFIIA-TFIIB-DNA complex. This complex recruits RNA polymerase II and other general transcription factors required for transcriptional initiation (Biswas et al., 2004; Biswas et al., 2006). Nhp6p is responsible for modulating chromatin structure at target promoters, allowing additional regulatory factors to be recruited (Moreira and Holmberg, 2000).

Zhao and Herr (2002) demostrated that TFIID binding to DNA is a two-step process, starting with an unstable complex containing unbent DNA and then slowly isomerizing into a stable complex with bent DNA. HMG proteins bend DNA and promote formation of the stable TFIID-bent DNA complex. TFIIB stimulates the formation of a stable TFIID-DNA complex and its association with this complex plays an important role to maintain the stable bent DNA form. *In vitro* binding experiments showed that TFIIB has a higher affinity for a

TFIID-TFIIA-DNA complex than for the complex lacking Nhp6p. These results suggested that Nhp6p, by bending DNA, promotes the formation of the multiprotein complex of TFIID, TFIIA, and TFIIB with DNA. Since the formation of the TFIID-TFIIA-TFIIB-DNA complex is critical for transcriptional initiation, Nhp6p stimulates transcription by promoting the formation of this preinitiation complex (Yu et al., 2003).

Nhp6p affects the expression of a number of RNA polymerase II-transcribed genes, positively as well as negatively, involved in a variety of cellular processes. A genome-wide analysis of cells lacking *NHP6A/B* found that 114 genes were up-regulated and 83 genes were down-regulated in an *nhp6a nhp6b* double mutant, indicating an important role for Nhp6p in chromatin-mediated gene regulation (Moreira and Holmberg, 2000).

One example of Nhp6p regulating gene transcription by affecting the recruitment of transcription factors is the expression of *S. cerevisiae HO* gene. The *HO* gene encodes an endonuclease that is responsible for initiating mating type switching in yeast. Activation of *HO* transcription involves ordered recruitment of transcription factors (Cosma et al., 1999). Swi5p enters the nucleus and binds to the promoter first, and then recruits SWI/SNF. SWI/SNF, in turn, recruits SAGA. SWI/SNF and SAGA are both required for SBF binding. SBF is directly responsible for *HO* activation. Yu and colleagues (2000) found that *HO* expression was reduced in either an *nhp6a nhp6b* double mutant or a *gcn5* mutant, suggesting Nhp6p and Gcn5p, which is the catalytic subunit of SAGA complex, are required for the

expression of *HO*. The *nhp6a nhp6b* double mutant showed the same suppression pattern as the *gcn5* mutant, indicating Nhp6p functions together with the Gcn5p HAT. Nhp6p might assist in the recruitment of SAGA to the *HO* promoter, by stabilizing the binding by SAGA; or it might facilitate DNA binding of SBF. The *gcn5 nhp6a nhp6b* triple mutant was extremely sick, suggesting that the SAGA complex and the Nhp6p architectural factor function in two distinct pathways to activate *HO* transcription. Nhp6p also plays an important role in regulating *FRE2* transcription. *S. cerevisiae FRE2* encoding a plasma membrane ferric reductase that is induced by the trancriptional activator Aft1p. Ssn6p is a major transcriptional coregulator in yeast. It was identified as the corepressor in a complex with Tup1p for the transcriptional inhibition of various genes. Genetic and biochemical analysis have revealed that the Ssn6p-Tup1p complex can also act as a transcriptional coactivator (Conlan et al., 1999) and a number of natural genes are targets of Ssn6p-Tup1p positive action (Proft and Struhl, 2002). Nhp6p acts at the *FRE2* UAS regulating predominantly Aft1p-DNA binding. Formation of an Aft1p-Nhp6p-DNA complex on the *FRE2* promoter allows Ssn6p recruitment, which is a crucial step for activation. Thus, Nhp6p facilitates Aft1p binding and Ssn6p recruitment to the *FRE2* promoter for this gene activation (Fragiadakis et al., 2004). Furthermore, the activated expression of *CUP1*, *CYC1*, *URA3*, *DDR2* and *DDR8* were decreased more than 50% in cells lacking *NHP6*. This effect might have been due to the lack of formation of a TFIID-TFIIA-NHP6 complex at the TATA box (Paull et al., 1996).

Nhp6p also plays a role in silencing. The *S. cerevisiae GAL1* promoter is a well-studied example of transcriptional regulation by nutrients. When cells were grown in galactose as the sole carbon source, *GAL1* is activated by Gal4p, which binds specifically to *GAL1* promoter. Gal4p interacts with the holoenzyme component Srb4p to recruit the transcription apparatus to the *GAL1* promoter. Tup1p is the repressor thought to directly influence the transcription machinery. Laser and group (2000) found that Nhp6p, as a binding partner of Gal4p and Tup1p, influences the glucose repression of the *GAL1* gene through its interaction with Tup1p. Transcription of the *SUC2* gene is also controlled by glucose repression and deprepresion mechanisms in *S. cerevisiae*. In the presence of high amounts of glucose in the growth media, Mig1p-Ssn6p-Tup1p and nucleosomes form a repressed chromatin structure on the *SUC2* promoter region. Nhp6p is required for the modulation of the *SUC2* promoter region in response to glucose repression or derepression signals. Nhp6a and Nhp6b might be involved in the rapid formation of the repressed chromatin structure by interacting with Mig1p-Tup1p complex and HDAC (Turkel, 2004). An increase in *CHA1* basal expression in an *nhp6a nhp6b* double mutant suggested again that Nhp6p somehow was involved in gene repression. A likely function for Nhp6a/b proteins at the *CHA1* promoter is the assembly of a repressive nucleoprotein complex (e.g. nucleosome) or as auxiliary co-repressors, facilitating the recruitment/binding of repressive factors working through chromatin (e.g. histone deacetylase activity) (Moreira and Holmberg, 2000).

Taken together, the above results suggest that Nhp6p functions *in vivo* as a co-regulatory factor, directly involved in recruiting or in stabilizing interactions of trans-acting factors with cognate sequences. Nhp6p could be responsible for modulating chromatin structure at target promoters, allowing additional regulatory factors to be recruited.

2.4.3 Function of Nhp6p in transcription elongation

The chromatin configuration of DNA inhibits access of enzymes and other proteins that facilitate gene transcription, DNA replication, and DNA repair. RNA Pol II-dependent transcription is inhibited at both initiation and elongation steps. Binding of an activator to an upstream activating sequence linked to a gene recruits the transcription machinery that consists of RNA Pol II, the general transcription factors and the mediator to induce transcription initiation (Ptashne, 2005). However, elongating RNA Pol II still has to contend with the intrinsically inhibitory nucleosomal configuration of the DNA template (Hartzog, 2003). A chromatin modulator called FACT (facilitates chromatin transcription) was identified to mediate the transcription of protein-coding genes by RNA polymerase II and works at the level of transcriptional elongation. Nhp6p, which is a functional domain of the yeast FACT complex, facilitates the nucleosome reconfiguration to allow RNA Pol II transit along DNA templates, thus facilitating transcription elongation (Rhoades et al., 2004).

In human and yeast cells, FACT is present in large amounts, with a relative

abundance approaching that of the nucleosome that make up euchromatin (Ghaemmaghami et al., 2003). The chromatin remodulator FACT has two main subunits. The larger one is Spt16p, and the smaller one is termed SSRP1(vertebrates) or Pob3p (yeast). The name SSRP1 is short for structure-specific recognition protein, which describes its initial identification (Bruhn et al., 1992). Pob3p is the small subunit of yeast FACT. It was originally identified through its ability to bind to DNA polymerase α, which is involved in the initiation of DNA replication. Yeast Pob3p is structurally related to SSRP1 proteins from various sources. However, the HMG-box domain at the C-terminus of SSRP1 is absent from Pob3p and the function of this domain for yeast FACT is supplied by the small yeast HMG-box protein Nhp6p (Wittmeyer and Formosa, 1997). Spt16p (also known as Cdc68p) and Pob3p form a stable heterodimer called SP or CP (Wittmeyer et al., 1999). Nhp6p displays strong genetic interactions with Spt16p-Pob3p and it is required to recruit Spt16p-Pob3p to form yeast FACT complexes (Ruone et al., 2003). Ruone et al.. (2003) claimed that Nhp6p is involved in a two-step nucleosome-remodelling mechanism: multiple Nhp6 molecules bind to a nucleosome first and induce a change in nucleosome structure to convert it into a substrate for Spt16p-Pob3p or other chromatin-modifying factors; then these Nhp6p-nucleosomes can recruit Spt16p-Pob3p to form SPN-nucleosomes. The complex of Spt16p-Pob3p and Nhp6p (SPN or yFACT) with nucleosomes causes changes in the electrophoretic mobility and nuclease sensitivity (Formosa et al., 2001). These results suggest that

Spt16p-Pob3p and Nhp6p coorperate to form yFACT to function as a novel nucleosome-reorganizing factor.

FACT stimulates transcription elongation by facilitating the passage of RNA polymerase II along a nucleosomal template. The barrier to transcriptional elongation that a nucleosome presents can be alleviated *in vitro* by conditions that allow the displacement of an H2A-H2B dimer (Kireeva et al., 2002). Several studies have indicated that transcription correlates with the generation of a nucleosome depleted for one H2A-H2B dimer. RNA Pol II has been demonstrated to selectively bind to the chromatin fraction in which nucleosomes are deficient in one H2A-H2B dimer (Baer and Rhodes, 1983) and quantitative removal of a dimer from the nucleosomes results in enhanced transcription *in vitro* (Gonzalez and Palacian, 1989). These results suggest that transcription elongation by RNA Pol II requires depletion of at least one H2A-H2B dimer and possibly nucleosome sliding. The role of FACT in transcription elongation was revealed by the Beltserkovskaya and Reinberg (2004). They suggested that FACT acts as a histone chaperone to promote H2A-H2B dimer dissociation from the nucleosome and allow RNA Pol II transcription on chromatin: first, FACT interacts physically with nucleosomes and with the histone H2A-H2B dimers; second, near equimolar amounts of FACT, relative to nucleosomes, are needed for optimal transcription efficiency; and third, FACT activity requires disruption of the histone octamer. Thus, Nhp6p cooperates with Spt16p-Pob3p to facilitate the formation of yFACT and promote transcription elongation by dissociating H2A-H2B dimer from

nucleosome (Singer and Johnston, 2004). yFACT is also indicated to facilitate interaction of TBP with TFIIA on nucleosomal DNA and plays a direct role in transcription initiation (Biswas et al., 2005).

In addition to its function in RNA Pol II gene transcription, Nhp6p is reported as a transcriptional initiation fidelity factor for RNA polymerase III transcription *in vitro* and *in vivo* (Kassavetis and Steiner, 2006). Nhp6p participates in the activation of the RNA Pol III *SNR6* gene (Lopez et al., 2001). The *nhp6a nhp6b* double mutant displays temperature-sensitivity due to inefficient transcription of the essential *SNR6* gene by RNA Pol III (Kruppa et al., 2001). Nhp6p is also important for the transcription of a set of tRNA genes and Nhp6p has heterochromatin barrier function (Braglia et al., 2007).

2.5 Protein-protein interaction systems

2.5.1 Significance of protein-protein interactions

Protein-protein interactions are of central importance for every cellular process in living cells. Most proteins function through interaction with other proteins. Enzymes interact with their substrates, inhibitors interact with enzymes, transport proteins interact with structural proteins, hormones interact with receptors – and that's just a few of the interactions that occur in a cell. Some proteins are made up of more than one polypeptide chain, and the interactions between the different peptides are necessary for the whole protein to function. In yeast, 85% of the proteins associate with other proteins, and promiscuous proteins have more than

95 associates. Protein interactions can be characterized as stable or transient. Stable interactions are those associated with proteins that are purified as multi-subunit complexes. Hemoglobin and core RNA polymerase are two examples of stable multi-subunit complex interactions. Stable interactions are best studied by co-immunoprecipitation, pull-down or far-Western methods. Transient interactions are expected to control the majority of cellular processes. Transient interactions are on/off or temporary in nature and typically require a set of conditions that promote the interaction. Transient interactions can be strong or weak, fast or slow. While in contact with their binding partners, transiently interacting proteins are expected to be involved in the whole range of cellular processes including protein modification, transport, folding, signaling, cell-cycling, etc. Transient interactions can be captured by cross-linking or label transfer methods (Phizichk and Fields, 1995). There are four forces involved in protein-protein interaction (PPI): hydrophobic interaction, hydrogen bonds, ionic interaction, Van Der Waals force. The interactions between proteins are important to many biologic processes. First, protein interactions can alter the kinetic properties of enzyme, resulting in subtle changes at the level of substrate binding or at the level of an allosteric effect. Second, protein-protein interactions allow substrate channeling by moving a substrate between or among subunits, resulting ultimately in an intended end product. Third, PPIs create new binding sites for small effector molecules. Fourth, proteins can be activated or destroyed through interaction with other proteins. Fifth, PPIs are involved in changing the specificity

of a protein for its substrate through interaction with different binding partners; e.g., demonstrate a new function that neither protein can exhibit alone. Finally, PPIs serve regulatory roles in upstream or downstream actions (Espelin et al., 1997).

The study of protein interactions has been vital to the understanding of how proteins function within the cell. Implications about protein functions can be made via protein-protein interaction studies based on the premise that the function of unknown proteins may be discovered if captured through their interaction with a known protein target of known function. There are a multitude of methods to detect PPI. Several innovative technologies which are currently used in laboratories are introduced below.

2.5.2 GST pull-down assay

GST (Glutathione S-transferase) pull-down assays are useful to confirm a suspected interaction between two proteins. GST pull-down assays can also be used for an initial screen to identify novel interactions between a probe protein and unknown targets. The minimal requirement for a pull-down assay is the availability of a purified and tagged protein (probe) which will be used to capture and pull-down a protein-binding partner (target). In the GST pull-down system, the probe protein is a GST fusion, which is commonly expressed in bacteria (e.g., *Escherichia coli*) and purified by affinity chromatography on glutathione beads. Target proteins are usually lysates of cells, which may be radio-active labeled or

epitope-tagged. The cell lysate and the GST fusion protein probe, which are immobilized on glutathione-agarose beads, are incubated together and then collected and separated by SDS-PAGE. Protein-protein interactions can be visualized by associated detection methods as Coomassie or silver staining, Western blotting and [35S] radioisotopic detection (Sambrook and Russell, 2001). The GST pull-down assay is a relative sensitive method and it has many advantages. First, the GST pull-down assay allows the researcher to work with a single purified no coupling protein and eliminates confusing results, which could arise from interaction of the bait protein with other interaction proteins present in the endogenous system that are not under study. It can indicate a direct physical interaction between two proteins if both proteins had been purified prior to the interaction assay. Second, the bait protein can be expressed in different environments. Although the GST-tagged bait protein is originally expressed in bacteria, which abolishes protein modifications, the GST tag could be cloned in a proper vector or integrated into the chromosome to express the GST fusion bait protein in its native environment, contributing a better understanding for the protein modification function to their interactions. Third, the 26 kd GST affinity tag enhances the solubility of many eukaryotic proteins expressed in bacteria. However, artifacts have been seen using this method and proper control must be included.

2.5.3 Yeast two-hybrid system

The yeast two-hybrid system, which was proposed by Fields and Song in 1989, is probably the most widely used assay to study protein-protein interactions. It was originally designed to detect the protein-protein interaction between Gal4p and Gal80p (Ma et al., 1988). The yeast two-hybrid system uses the transcription process to make predictions about protein interaction, which is based on the ability of an interacting protein pair to bring together the DNA-binding domain and the activation domain of a transcription factor *in vivo* to produce a functional activator of transcription. The interaction can be detected by expression of linked reporter genes. The premise behind the test is the activation of downstream reporter genes by the binding of a transcription factor onto the respective upstream activating sequences (UAS). For the purposes of two-hybrid screening, the transcription factor is split into two separate fragments, called the binding domain (BD) and activating domain (AD). The BD is the domain responsible for binding to the UAS and the AD is the domain responsible for activation of transcription (Fields and Song, 1989; Young, 1998).

Yeast two-hybrid systems utilize genetically engineered strains of yeast in which the biosynthesis of certain nutrients (usually amino acids or nucleic acids) is lacking. When grown in media that lacks these nutrients, the yeast fails to grow. This mutant yeast strain can be made to incorporate foreign DNA in the form of plasmids. In the yeast two-hybrid screening, separate bait and prey plasmids are

simultaneously introduced into the mutant yeast strain. Plasmids are engineered to produce a protein product in which the DNA-binding domain (BD) fragment is fused onto a protein while another plasmid is engineered to produce a protein product in which the activation domain (AD) fragment is fused onto another protein. The protein fused to the BD may be referred to as the bait protein and is typically a known protein that the investigator is using to identify new binding partners. The protein fused to the AD may be referred to as the prey protein and can be either a single known protein or a library of known or unknown proteins. In this context, a library may consist of a collection of protein-encoding sequences that represent all the proteins expressed in a particular organism or tissue or may be generated by synthesising random DNA sequences. If the bait and prey proteins interact (i.e. bind), then the AD and BD of the transcription factor are indirectly connected, bringing the AD in proximity to the transcription start site and transcription of reporter gene can occur. If the two proteins do not interact, there is no transcription of the reporter gene. In this way, an interaction between the fused proteins loads to a change in the cell phenotype (Gietz et al., 1997; Joung et al., 2000).

Yeast two-hybrid screens are now routinely performed in many labs. They can provide an important first hint for the identification of interaction partners. The yeast two-hybrid assay is an *in vivo* method. The interaction between bait and prey proteins can be observed in living cells, and no purification is needed. It is highly sensitive and widely applicable, and many commercial kits are available. The

main criticism applied to the yeast two-hybrid screen of protein-protein interactions is the possibility of a high number of false positive (and false negative) identifications. The reason for this high error rate lies in the principle of the screen: The assay investigates the interaction between (i) overexpressed (ii) fusion proteins in the (iii) yeast (iv) nucleus. Each of these points (i-iv) alone can give rise to false results. For example, protein overexpression can result in non-specific interactions. Moreover, a mammalian protein is sometimes not correctly modified in yeast (e.g. missing phosphorylation), which can also lead to false negative results. Finally, some proteins might specifically interact when they are co-expressed in the yeast, although in reality they are never present in the same cell at the same time. This means that all interactions have to be confirmed by other assays like co-immunoprecipitation of the endogenous proteins (Deane et al., 2002).

2.5.4 The Split-Ubiquitin system

2.5.4.1 The principle of the Split-Ubiquitin system

The Split-Ubiquitin system was originally developed by Johnsson and Varshavsky (1994). It is the most commonly used alternative to the standard yeast two-hybrid assay. The split-ubiquitin system is based on the conditional proteolysis that occurs upon the re-association of the N- and C-terminal halves of ubiquitin, designated N_{ub} and C_{ub}, respectively. Each half of ubiquitin is fused to either protein of interest. If the two proteins interact, the two halves of ubiquitin are

brought into close proximity. This reconstituted native-like ubiquitin is recognized by ubiquitin specific proteases (UBPs), and a reporter attached to C_{ub} is then released. As a result, the reduction in size of the reporter protein can easily be detected through Western blot (Dunnwald et al., 1999). The split-ubiquitin system used in this project is shown in Figure 2.5.4.1.

To decrease spontaneous interactions between N_{ub} and C_{ub}, some N_{ub} mutants were constructed (Johnsson and Varshavsky, 1994). This resulted in more sensitive assays, as it allowed to adjust for the background level. Wild type N_{ub} carries an isoleucine in position 13, and two mutations were created such that N_{ub} now carries an alanine ($N_{ub}A$) or glycine ($N_{ub}G$) at that position. The strength of interaction with C_{ub} as compared to wild type N_{ub} was reduced in $N_{ub}A$, and for $N_{ub}G$, it was further decreased. This allowed limited quantitative analysis of the strength of protein-protein interactions.

This assay enables the detection of interactions between two proteins, but has a disadvantage in that it is not possible for the selection of new interacting partners from random fusion library. Two variants of the original assay have been reported to date: one is based on conditional compartmentalization (Stagljar et al., 1998), while the basis of the other strategy is conditional protein degradation (Wittke et al., 1999).
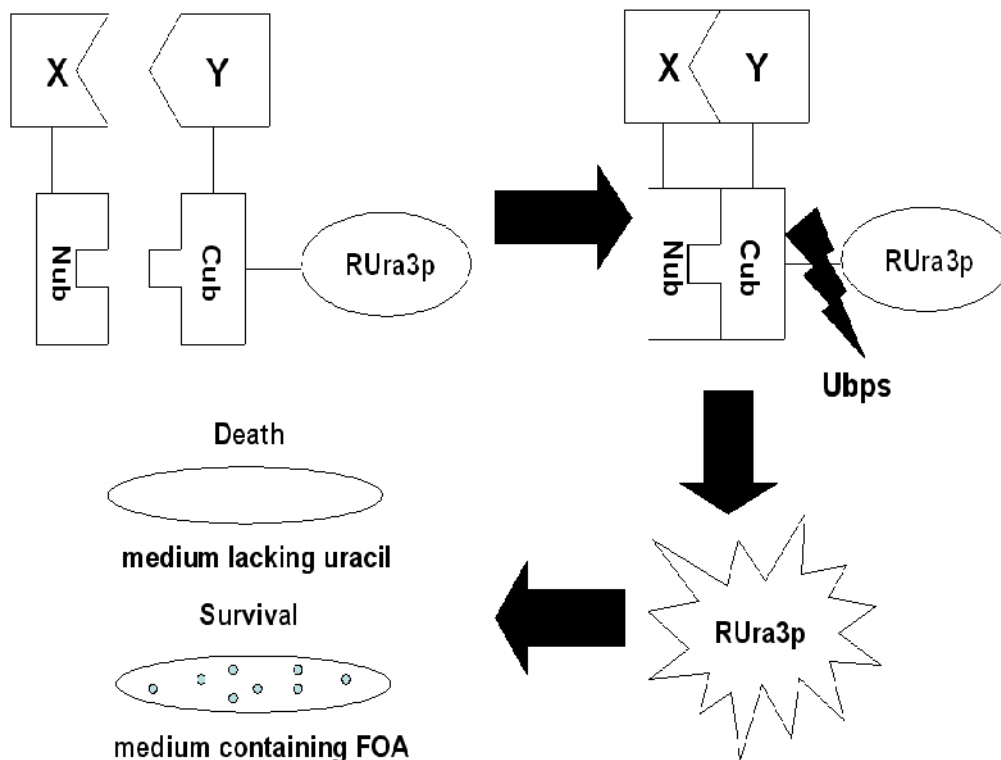
Figure 2.5.4.1 Identification of protein-protein interaction with the split-ubiquitin system.
Split-Ubiquitin system is an alternative yeast two-hybrid assay that is based on a conditional proteolysis design. Yeast cells expressing a fusion of a protein Y to the C-terminal half of ubiquitin that is extended by Ura3p whose first amino acid had been replaced by arginine (Y-C_{ub}-RUra3p) are uracil prototroph and sensitive to 5-fluoroorotic acid (FOA), as RUra3p converts FOA into toxic 5-fluorouracil. Yeast cells co-expressing a fusion of the N-terminal half of ubiquitin to a protein X that interacts with Y (N_{ub}-X) are uracil auxotroph and resistant to FOA. Upon interaction of X and Y, the two halves of ubiquitin form an ubiquitin-like moiety, which is recognized by the ubiquitin-specific proteases. These cleave off RUra3p, which is degraded so rapidly by the enzymes of the N-end rule that the yeast cells are unable to grow on plates lacking uracil and they are resistant to FOA.

2.5.4.2 Advantages of the Split-Ubiquitin system

The Split-Ubiquitin system is a protein fragment complementation assay, which is based on conditional proteolysis instead of the traditional transcription readout. Therefore, it can be utilized to explore transcription factors (Wellhausen and Lehming, 1999). In addition, since UBPs are present in the cytosol as well as in the nucleus (Varshavsky, 1997; Byrd et al., 1998), this assay can be used to probe protein-protein interactions that do not occur in the nucleus. This overcomes a limitation of the standard yeast two-hybrid assay where protein-protein interactions are required to occur in the nucleus. Thus the Split-Ubiquitin assay has been used to investigate membrane proteins in their native environment (Dunnwald et al., 1999). The Split-Ubiquitin system is suitable to investigate membrane proteins. Two membrane-based split-ubiquitin systems have been described: Ura3p-based split-ubiquitin system and the trans-activator-based split-ubiquitin system. A further advantage of the split-ubiquitin system is that the signal for an interaction can be changed by changing the nature of the reporter protein (eg. transcription factor or enzyme activated by ubiquitin cleavage). Furthermore, the small size of the ubiquitin fragments in the hybrid proteins may also be advantageous because it minimizes the possibility of steric hindrance.

2.5.4.3 Applications of the Split-Ubiquitin system

The modified split-ubiquitin system has been widely used to study membrane proteins involved in signaling and transporters in yeast, plants and mammals, and

it was also used to investigate interacting partners of membrane proteins with enzymatic functions like oligosaccharyl transferase in yeast (Pandey and Assmann, 2004; Pasch et al., 2005; Yan et al., 2005). This modified split-ubiquitin system has also been used to study viral membrane proteins like the Hepatitis B surface antigen in understanding viral morphogenesis (Toh et al., 2005). Wang and group, Miller and group (2004), respectively, was reportedly the first group to use the yeast split-ubiquitin system based on the compartmentalization approach to screen a cDNA library. They used the endoplasmic reticulum membrane protein BAP31p as bait to screen a cDNA library for interacting-proteins, and a novel human member of the protein tyrosine phosphatase-like B family was isolated. A library screen with the conditional protolysis method had been described in 2000 (Laser et al., 2000).

In addition, the large-scaled analysis of the integral membrane proteins of *Saccharomyces cerevisiae* was recently carried out by Miller et al. (2005) using the modified split-ubiquitin system. A total of 705 membrane proteins were short-listed for protein-protein interaction analysis, but only 365 proteins were found to be competent for use in the screening. This study was successful in isolating previously undiscovered interactions. As such, the coupling of computational tools together with experimental data in large-scale protein-protein interaction studies is useful in weeding out the false-positive and false-negative interactors. However, like for most protein-protein interaction screens, one must perform other experimental strategies to confirm the results.

In this project, Nhp6p-interacting partners were isolated using the split-ubiquitin system. *ZDS1* gene, whose transcription is repressed by Nhp6p and its interacting proteins, was selected to study how Nhp6 protein functions with its interacting partners in gene regulation. Chromatin immunoprecipitation was used to study the chromosomal localization of Nhp6p and its interacting partners on *ZDS1* gene in wild-type and deletion strains. Our study will provide further understanding of how HMG proteins are involved in transcription regulation and how they cooperate with their association proteins to regulate gene transcription.

CHAPTER 3

MATERIALS AND METHODS

3. Materials and Methods

3.1 Materials

3.1.1 Plasmids

1. P_{cup1}-C_{ub}-RUra314 (*TRP1* marker, ampicillin marker) (Sikorski and Hieter, 1989)

   NHP6A-C_{ub}-RUra314

   NHP6B-C_{ub}-RUra314

The fusion vector for the C-terminal portion of ubiquitin (C_{ub}) was the P_{cup1}-C_{ub}-RUra314 single-copy plasmid as described by Johnsson and Varshavsky (1994), which expressed the Nhp6a-C_{ub}-RUra3p or Nhp6b-C_{ub}-RUra3p under the control of a copper-inducible *CUP1* promoter.

2. PACNX-N_{ub}IBC and PADNX-N_{ub}IBC (*LEU2* marker, chloramphenicol marker) (Colicelli et al., 1989; Laser et al., 2000)

PACNX is a single-copy yeast vector while PADNX is a multi-copy yeast vector. These two vectors express the N_{ub} fusion library under the control of the constitutive *ADH1* promoter.

3. YEplac181 (*LEU2* marker, ampicillin marker, multi-copy vector)

   YEplac181-RPB4

YEplac181 is a multi-copy yeast vector. YEplac181-RPB4 over-expresses Rpb4p

from its own promoter.

4. pGEX-5X-1 (ampicillin marker)

    pGEX-5X-1

    pGEX-NHP6B

GEX-5X-1 is a bacterial GST expression vector. GEX-NHP6B expresses

GST-Nhp6bp in *E. coli*.

5. RS314-MYC9

    NHP6B-MYC9-314

    RPB4-MYC9-314

RS314 is a single-copy yeast vector with the *TRP1* marker (Sikorshi and Hieter,

1989). RS314-MYC9 carries nine myc tags. NHP6B-MYC9-314 and

RPB4-MYC9-314 express Nhp6b-myc9p and Rpb4-myc9p from their own

respective promoters.

6. RS304-MYC9

    RTT107C-MYC9-304

    MED3C-MYC9-304

    TFB1C-MYC9-304

    TFB4C-MYC9-304

    RPB2C-MYC9-304

RS304 is an integrative plasmid with the *TRP1* marker (Sikorshi and Hieter, 1989).

RS304-MYC9 carries nine myc tags. The "C" indicates that a C-terminal fragment

of the respective gene had been cloned into the vector. The plasmids were
linearized in the C-terminal gene fragments and integrated into the respective
chromosomal loci, replacing the endogenous full-length proteins with the myc
tagged derivatives.

3.1.2   Yeast strains

1. *JD52 (mata ura3-52 leu2-3 his3Δ200 lys 2-801 trp1Δ63)* (Dohmen et al.,
   1995)

   JD52::RTT107-MYC9-304

   JD52::MED3-MYC9-304

   JD52::RPB2-MYC9-304

2. *JD52ΔNHP6 (mata ura3-52 leu2-3 his3Δ200 lys 2-801 trp1Δ63 nph6a::hisG*

   nhp6b::hisG）

   JD52ΔNHP6::MED3-MYC9-304

   JD52ΔNHP6::RPB2-MYC9-304

The *NHP6* deletion strains were generated by successive deletion of the entire
NHP6A and *NHP6B* ORFs with the help of a *HisG* knockout construct.

3. *JD52ΔRPB4 (mata ura3-52 leu2-3 his3Δ200 lys 2-801 trp1Δ63 rpb4::HIS3)*

   JD52ΔRPB4::MED3-MYC9-304

   JD52ΔRPB4::RPB2-MYC9-304

The endogenous *RPB4* gene in the deletion strain has been replaced by the *HIS3*

gene flanked by approximately 500 bp of the wild-type *RPB4* promoter and terminator with the help of homologous recombination.

4. *JD52ΔRTT107 (mata ura3-52 leu2-3 his3Δ200 lys 2-801 trp1Δ63 rtt107::his3)*

   JD52ΔRTT107::MED3-MYC9-304

   JD52ΔRTT107::RPB2-MYC9-304

The endogenous *RTT107* gene in the deletion strain has been replaced by the *HIS3* gene flanked by approximately 500 bp of the wild-type *RTT107* promoter and terminator with the help of homologous recombination.

5. *JD52ΔMED3 (mata ura3-52 leu2-3 his3Δ200 lys 2-801 trp1Δ63 med3::his3)*

   JD52ΔMED3::MED3-MYC9-304

   JD52ΔMED3::RPB2-MYC9-304

The endogenous *MED3* gene in the deletion strain has been replaced by the *HIS3* gene flanked by approximately 500 bp of the wild-type *MED3* promoter and terminator with the help of homologous recombination.

6. *JD55 (mata trp1-Δ63 ura3-52 his3-Δ200 leu2-3,112 lys2-801, ubr1::HIS3)*

   (Dohmen et al., 1995)

3.1.3   Bacterial Strains

1. *DH10B (F⁻ mcrA Δ(mrr⁻ hsdRMS⁻ mcrBC) Φ80dlacZΔM15 ΔlacX74 deoR recA1*

      endA1 araD139Δ(ara, leu) 7497 galU galKλ⁻rpsL nupG)

DH10B is an *Escherichia coli* strain that was used for plasmid electroporation.

2. *DH5α* (F$^-$*Φ80dlacZΔM15 Δ(lacZYA-argF)U169 deoR recA1 endA1 hasR17(r$_k^-$m$_k^+$) supE44λ$^-$ thi-1 gyrA96 relA1)*

DH5α is an *Escherichia coli* strain that was used for heat transformation of plasmids.

3. *BL21 LysS* (F$^-$ *ompT gal dcm Ion hsdS$_B$(r$_B^-$m$_B^-$) λ (DE3) plysS(cm^R))*

BL21LysS is an *Escherichia coli* strain that was used for protein expression.

3.1.4   Primers

The primers used in this thesis were listed in Table 3.1.4

Primer Name	primer sequence 5' to 3'
Library sequencing	5'N$_{ub}$: GCCAAGCTTATGCAGATTTTCGTCAAG 3'1995: CTACCAACGATTTGACCCTT
RPB4 promoter	5'P-831-SacI:GCGGAGCTCATTCCAAGGGTTAACAA 3'P-1-SmaI:CGCGCCCGGGAAACATTCATTTTTCTATATTC
RPB4 terminator	3'T+848-SalI:GCTAGTCGACCGATTCAGGGCTAGTTG
RTT107 promoter	5'P-579-SacI:GCGGAGCTCCAATAAGATATCCCAATA 3'P-1-SmaI:CGACCCGGGATCGTATATTTACCAGTATG
RTT107 terminator	3'T+894-SalI:CGCGCGTCGACGTAAATCTATGGATGTAAC
MED3 promoter	5'P-EcoRI: GCCGAATTCTGTAAAATATGCGATGAC 3'P-SmaI: GCCGCCCCGGGAAAATCCTCAGGAAAGTGT
MED3 terminator	5'T-NsiI: GCCGCCATGCATCTATGTATCCAAGAT 3'T-SalI: GCCGCCGTCGACCATTTCTAGAGTTTGAG
ZDS1 Promoter	5'P'zds1-207: ACGGACTCAATAACAAGTCG 3'Pzds1-8: GACAGCTAAATTCTCCTTTG
ZDS1 ORF	5'Ozds1+1274: TGAGTTCGCCTGGGTCAATT 3'Ozds1+1458: TGTTGTCATCCGTTGCGCTT

Table 3.1.4: List of primers used in gene cloning, PCR and ChIP assays

3.1.5 Buffers

1. Yeast breaking buffer

Reagents	Volume (ml)
Triton X-100	10
10% SDS	50
5 M NaCl	10
1 M Tris-HCl (pH8)	5
0.5 M EDTA (pH8)	1
Distilled water	424
Total	**500**

2. Miniprepatation solution buffers

   A. Miniprep Solution I buffer

Reagents	Amount
1 M Tris-HCl (pH7.5)	25 ml
0.5 M EDTA (pH8.0)	10 ml
Distilled water	Top up to 500
RNase A (added after autoclave)	25 mg
Total	**500 ml**

   B. Miniprep Solution II buffer (Do not autoclave)

Reagents	Volume (ml)
Sterile water	430
5 N NaOH	20
10% SDS	50
Total	**500**

   C. Miniprep Solution III buffer

Reagents	Amount
Potassium Acetate	65 g
Distilled water	Top up to 200 ml

Note: 1. Adjust to pH4.8 using 100% acetic acid. 2. Top up to 500 ml with distilled water. 3. Autoclave.

3. Yeast lysis buffer

Reagents	Volume (ml)
1M Tris-HCl (pH7.4)	50
1 M KCl	25
0.5 M EDTA	1
50% NP40	1
Sterile water	423
Total	**500**

4. 5× Transfer buffer

Reagents	Amount
100% Tris	29 g
100% Glucine	145 g
100% SDS	5 g
Sterile water	Top up to 1000 ml
Total	1000 ml

5. 1 ×Transfer buffer

Reagents	Volume (ml)
5 × Transfer buffer	10
100% Methanol	10
Sterile water	30
Total	**50**

Note: For 1 ×Transfer buffer, it should be made at the time of use.

6. ChIP washing buffer

Reagents	Volume (ml)
1 M Tris-HCl (pH7.5)	5
1 M LiCl	125
50% Nonidet P-40	5
50% Sodium deoxycholate	5
Sterile water	360
Total	**500**

7. ChIP elusion buffer

Reagents	Volume (ml)
1 M Tris-HCl (pH7.5)	25
0.5 M EDTA	10
10% SDS	50
Sterile water	415
Total	**500**

8. Pronase working solution

Reagents	Volume (ml)
1 M Tris-HCl (pH7.5)	5 ml
10% SDS	2.5 ml
Sterile water	42.5 ml
Total	**50 ml**

3.2 Methods

3.2.1 Library Screening

3.2.1.1 The Split-Ubiquitin screen

The Split-Ubiquitin system is based on a conditional proteolysis design. In the split-ubiquitin system, Ubiquitin is split into two halves, the C-terminal half (C_{ub}) and the N-terminal half (N_{ub}). Each half is fused to proteins under study for interaction (such as X and Y). The fusion proteins are not recognized by the ubiquitin-specific protease (Ubps) in the separate states. However, if X and Y interact, they bring together the ubiquitin halves, which reconstitute into a native-like ubiquitin. Ubps recognizes the reconstituted ubiquitin and cleave it at the C-terminal half that had been attached to a reporter protein. In our Split-Ubiquitin system, ubiquitin is fused to an Ura3 reporter protein whose first amino acid had been substituted by an arginine (RUra3p). When the cleavage was done by the Ubps, the arginine of the RUra3p moiety was exposed. According to the N-end rule pathway of protein degradation, the half-life of a protein in a cell is based on its N-terminal amino acid. Since arginine is the most destabilizing

residue, the exposure leads to the rapid degradation of RUra3p. This conditional proteolysis allows for negative and positive selection. Cells lacking the *URA3* gene would not be able to grow in medium without uracil, but they are resistant to the drug 5-fluoorotic acid (FOA), since Ura3p converts FOA into toxic 5-fluorouracil, which causes cell death. If the split-ubiquitin screen were done in a uracil-deficient medium, then interaction would be indicated by the absence of growth, as RUra3p would be degraded if the ubiquitin halves were brought together. On the other hand, in FOA containing media, an interaction would be indicated by growth as the RUra3p was degraded upon bringing the two ubiquitin halves together.

3.2.1.2 Preparation of competent yeast cells

Competent cells were prepared by using the lithium acetate method (Ausubel et al., 2006). Cells were inoculated into 50 ml YPDA media and incubated at 28℃ until the culture density had reached OD_{600nm} of 0.8-1.0. Cells were transferred to 50 ml centrifuge tube and centrifuged at 4,000 rpm for 5 min at room temperature. The supernatant was discarded. The cell pellet was resuspended in 1 ml sterile distilled water and then transferred to a 1.5 ml microtube. After centrifugation at 7,000 rpm for 1 min, the supernatant was removed and the cell pellet was resuspended in 500 μl 0.1 M lithium acetate (LiAc)/TE (pH7.0). The cells were then incubated at 28℃ for 1 h followed by transformation or they were stored at 4℃ for a maximum of two weeks.

3.2.1.3 Transformation of plasmids into competent cells

No.	Reagents	Volume (µl)
1	Plasmid library PACNX-N_{ub}IBC-Bank13 Fish sperm DNA Competent *JD52* cells containing Nhp6a-C_{ub}-RUra3p 40% PEG	50 250 300 3000
2	Plasmid library PADNX-N_{ub}IBC-Bank13 Fish sperm DNA Competent *JD52* cells containing Nhp6a-C_{ub}-RUra3p 40% PEG	50 250 300 3000
3	Plasmid library PACNX-N_{ub}IBC-Bank13 Fish sperm DNA Competent *JD52* cells containing Nhp6b-C_{ub}-RUra3p 40% PEG	50 250 300 3000
4	Plasmid library PADNX-N_{ub}IBC-Bank13 Fish sperm DNA Competent *JD52* cells containing Nhp6b-C_{ub}-RUra3p 40% PEG	50 250 300 3000

Table 3.2.1.3 Components of transformation reaction

Individual reagents were added and mixed into four tubes as indicated in Table 3.2.1.3. Samples were incubated at 28℃ for 1 h, followed by heat shock incubation for 15 min in a 42℃ water bath. The cells were centrifuged at 7,000 rpm for 1 min. The supernatants were removed by pipette and the cell pellets were resuspended in 1.8 ml sterile distilled water and plated on six selective plates.

3.2.1.4 Plasmid isolation from *S. cerevisiae*

Individual colonies from each transformation plate were inoculated into 10 ml of leucine-deficient medium. Cells were grown at 28℃ until the OD_{600nm} reached 1.0. Cells were transferred into 15 ml centrifuge tubes and centrifuged at 13,000

rpm for 5 min. The supernatants were removed and the cell pellets were resuspended with 1 ml sterile distilled water and transferred into fresh eppendorf tubes. After centrifuged at 7,000 rpm for 1 min, the supernatants were removed and the cell pellets were stored at -20℃, before they were broken with glass beads.

Cell pellets were resuspended in 400 μl of yeast breaking buffer. One scoop of glass beads and 400 μl 5:1 (pH4.7) phenol/chloroform were added to each tube. The mixtures were subjected to vortex for 2 min at highest speed to shear the yeast cells. After centrifugation at 13,000 rpm for 5 min, the mixtures were separated into four different phases: top aqueous DNA, cell debris, phenol and glass beads. 400 μl of the top aqueous layer from each sample were carefully pipetted into fresh microtubes and centrifuged at 13,000 rpm for 5 min. The top aqueous layer was carefully extracted using a pipette and transferred into a new 1.5 ml microtube. 1 ml of 100% ethanol was added into each tube. The tubes were inverted several times to mix the contents. After centrifugation at 13,000 rpm for 10 min, the supernatant was removed. 400 μl 0.3 M sodium acetate (NaAc) and 1 ml of 100% ethanol were added to each tube. The tubes were inverted to mix the contents and then centrifuged at 13,000 rpm for 10 min. The supernatants were removed. Cell pellets were washed with 800 μl 70% ethanol and centrifuged at 13,000 rpm for 15 min. The supernatants were discarded and the cell pellets were dried in a speedvac for 20 min. The dried DNA pellets were dissolved in 50 μl of sterile distilled water each. The DNA samples were stored at -20℃.

3.2.1.5 Electroporation

Electroporation cuvettes were prepared by treatment with denatured ethanol followed by drying in a laminar flow hood. The treated cuvettes were placed under UV light for 10 min and chilled on ice for 10 min prior to usage. 40 µl of competent *E. coli DH10B* cells were mixed with 4 µl of N_{ub}-fusion plasmid in a 1.5 ml microtube. The mixture was transferred carefully into the pre-chilled cuvette to avoid bubble formation. The samples were electroporated at 1.8 KV. 400 µl Luria Bertani (LB) broth was added immediately and mixed vigorously using a pipette. All the contents was pipetted back to the original 1.5 ml microtube and incubated at 37℃ for 1 h. Individual samples were plated onto LB plates containing chloramphenicol and incubated at 37℃ overnight.

3.2.1.6 Plasmid Minipreparation from *E. coli*

Colonies formed on the LB selection plates were picked and inoculated into 2 ml LB broth containing chloramphenicol in culture tubes. The culture tubes were incubated on a shaker at 37℃ overnight. The broth from each tube was then transferred to 1.5 ml microtube and centrifuged at 13,000 rpm for 1 min. The supernatant was discarded. The cell pellet was resuspended in 200 µl Minipreparation Solution I buffer using vortex. After addition of 200 µl Minipreparation Solution II buffer, the tube was inverted 5 times to mix the contents. Upon addition of 200 µl Minipreparation Solution III buffer, the tube was then inverted 5 times to mix all the contents. The mixture was centrifuged at

13,000 rpm for 15 min, and the supernatant was poured to a fresh 1.5 ml microtube. 300 μl of isopropanol was added into each tube and the content was mixed with vortex. After centrifugation at 13,000 rpm for 15 min, the supernatant was discarded and 1 ml 70% ethanol was added to each tube. The tube was centrifuged at 13,000 rpm for 5 min and the supernatant was removed. The plasmid DNA pellet was dried in a speedvac for 20 min, and then resuspended in 50 μl of sterile distilled water. The minipreparation plasmid DNA samples were stored at -20℃ for further usage.

3.2.1.7 Restriction endonuclease digestion

The restriction endonuclease reaction was carried out at 37℃ for 2 h in a total volume of 20 μl, and consisted of 10 μl plasmid DNA, and 0.25 μl of each enzyme used for the digestion. The components for each reaction are listed in Table 3.2.1.7.

Reagents	Volume (μl)
Sterile distilled water	7.5
10×RE buffer	2
*Hind*III	0.25
*Sal*I	0.25
Plasmid DNA (from minipreparation)	10
Total volume per reaction	**20**

Table 3.2.1.7 Components of restriction endonuclease reaction

3.2.1.8 Agarose gel electrophoresis

100 ml 1×TBE buffer was poured into an Ethidium Bromide (EtBr)-specific conical flask. 1 gram agarose powder was added to make a 1% agarose gel. The

mixture was heated in a microwave oven for 2 min, till the solution was clear. After making up the lost buffer, the solution was cooled till safe for handing. 2.5 μl EtBr was added and swirled evenly to mix. The gel casting flatform was set up and the gel solution was poured slowly and evenly in to the center of the flatform. The combs were placed in their slots. After the gel solidified, the combs were removed and the flatform was placed into an electrophoresis tank. 1×TBE buffer was poured in to cover the gel about 1mm about its surface. 3 μl of 6×loading dye was added to each sample tube. The mixture was tapped to mix and loaded into each well. 3 μl of 1 kb DNA marker was loaded to the first well of each lane. The electrophoresis was carried out at 100 V for 40 min. After the electrophoresis, the gel was removed and viewed on an illuminator to check the sizes of the insert DNA fragments.

3.2.1.9 Amplification of N_{ub} fusion vectors

1 μl of N_{ub} fusion vector and 10 μl of competent *DH5α* cells were mixed in an eppendorf tube. The samples were incubated on ice for 20 min and then subjected to heat shock for 90 sec at 42℃ in a water bath. 100 μl of LB broth was added to each sample and the tubes were incubated at 37℃ for 1 h. After the incubation, the samples were plated onto LB plates with selective agent and incubated at 37℃ overnight. Amplified N_{ub} fusion vectors were obtained by plasmid minipreparation.

3.2.1.10 Cycle sequencing of N_{ub} fusion candidates

Cycle sequencing was carried out using the 5'N_{ub} forward primer and 3'1995 reverse primer listed in Table 3.1.4. Only one primer was used in each reaction. The components of each sequencing reaction are listed in Table 3.2.1.11.

Reagents	Volume (µl)
Terminater Ready Reaction Mix (big dye)	4
Template, double-stranded DNA	0.5 (100-250 ng)
Primer (final concentration: 2 pmol)	0.5
Deionized water	5
Total volume per reaction	10

Table 3.2.1.10 Contents in cycling sequencing reaction

The following cycling parameters were used: denaturation at 96℃ for 30 sec, annealing at 50℃ for 15 sec and extension at 60℃ for 4 min. The reaction was held at 4℃. Upon completion of the reaction, the purification of the extension products was carried out. The contents were each transferred into a 1.5 ml microtube containing 80 µl of ethanol/sodium acetate solution (consisting of 3 µl 3 M sodium acetate, pH 4.6, 62.5 µl of non-denatured 95% ethanol and 14.5 µl of deionized water). The microtubes were then vortexed briefly and left at room temperature for 15 min to precipitate the extension products. After centrifugation at 13,000 rpm for 10 min, the supernatants were removed carefully by pipetting and discarded. 1 ml 75% ethanol was added to rinse the pellet and the microtube was centrifuged at 13,000 rpm for 5 min. The supernatant was discarded and the pellets were dried in a speedvac concentrator for 15 min. Subsequently, the dried samples were handed to the Department of Microbiology Sequencing Facility for

separation on a DNA sequence.

3.2.1.11 Droplet Assay

With the samples ten-fold serial dilutions from 10^0 to 10^{-5} were performed in a 96-well plate, 90 μl of sterile distilled water was pipetted into 6 wells horizontally. Cells were picked from culture plates using a pipette tip and resuspended into the first well. Then 10 μl mixture from the first well was drawn and pipetted into the second well, followed by mixing. The process was repeated until the sixth well was reached, whereby cells were diluted 10^{-5} fold. 5 μl of each dilution was dropped onto selective plates. The plates were incubated under various conditions and the cells were observed for growth phenotypes.

3.2.1.12 Construction of YEplac181-RPB4 and YEplac181-RTT107

YEplac181-RPB4 was constructed by cloning the *Sac*I-*Sal*I RPB4 ORF fragment and 500 bp of promoter and terminator into YEplac181. The oligonucleotides used to amplify the RPB4 gene were GCGGAGCTCATTCCAAGGGTTAACAA and GCTAGTCGACCGATTCAGGGCTAGTTG. YEplac181-RTT107 was generated using the same strategy. The oligonucleotides used here to amplify the RTT107 gene were GCGGAGCTCCAATAAGATATCCCAATA and CGCGCGTCGACGTAAATCTATGGATGTAAC.

The purified PCR products were cut with specific enzymes. After incubated at 37 ℃ for 2 h, 200 μl Binding Buffer was added and mixed with the RE digestion

mixture. The total content was transferred to a clean spin column. After centrifuged at 3,000 rpm for 1 min, the flow-through was discarded and 500 μl 70% ethanol was added. The column was centrifuged at 3,000 rpm for 1 min. The flow-through was discarded and 100 μl 70% ethanol was added. After centrifugation at 3,000 rpm for 1 min, the flow-through was removed. The spin column was placed into a 1.5 ml microtube and 100 μl sterile distilled water was added directly to the column membrane. The column was left to stand at room temperature for 10 min. After centrifugation at 13,000 rpm for 1 min, the eluted DNA samples were placed in a speedvac for 15 min to get rid of the excess ethanol. The purified DNA samples could be used for ligation. In a 1.5 ml microtube, 2.4 μl steril water, 0.5 μl ligase buffer, 0.1 μl T4 DNA ligase, 1 μl of cut YEplac181 vector and 3 μl of cut DNA fragment were added and mixed. The mixture was incubated at 4℃ overnight. Upon addition of 40 μl *DH5α* cells, the mixture was placed on ice for 15 min, following by heat shock at 42℃ water bath for 1 min. 200 μl LB was added immediately and mixed using a pipette. The mixture was incubated at 37℃ for 1 h, then plated on a LB + ampicillin plate and incubated at 37℃ overnight. Colonies were picked from the plates and cultured in LB+Amp broth. Reconstructed plasmid was obtained by plasmid minipreparation. RE digestion was carried out to check the insert fragment sizes. PCR and gene complementation assay were further performed to confirm the successful construction of the plasmids.

3.2.2 GST pull-down assay

3.2.2.1 Preparation of GSTp and GST-Nhp6bp

The GST-NHP6B fusion was made by cloning the entire *NHP6B* open reading frame into GEX-5X-1 (Amersham Pharmacia). GEX-5X-1 and GEX-NHP6B were transformed into *E. coli BL21* and plated on selective plates. The obtained colonies were grown in 2 ml of LB broth containing ampicillin selective reagent on a shaker at 37℃ overnight. The pre-cultured 2 ml broth was transferred into 50 ml of LB broth containing ampicillin and cultured on a shaker at 37℃ to reach OD_{600nm} of 0.8-1.0. 50 μl of 0.1 M IPTG (final concentration was 0.1 mM) was added and the cultures were continued to incubate at 37℃ to induce protein expression. After incubation for 2-4 h, cells were harvested and cell pellets were washed and then resuspended in 5 ml PBS buffer. The cells underwent three cycles of freeze-thaw to obtain cell extracts. 2.5 μl of DNase and 25 μl of $MgCl_2$ (25 μl from 1 M stock, final concentration was 5 mM) to the *E. coli* extracts to digest the DNA. After incubation at room temperature for 30 min, the sample tubes were centrifuged at 4℃ at the speed of 8,000 rpm for 10 min. The supernatants were transferred into new tubes and stored at -80℃ until needed. Western blot was carried out with anti-GST antibody to check the correct expression of GSTp and GST-Nhp6bp.

3.2.2.2 GST pull-down assay

20 μl of glutathione sepharose beads were added to 5 μl of GSTp or 100 μl of GST-Nhp6bp supernatants in order to start with equal amounts of GSTp and GST-Nhp6bp. Samples were topped up to 200 μl with yeast lysis buffer. PMSF and DTT (final concentration: 1 mM) were added to inhibit protein degradation. Sample tubes were attached to an end-over-end rotator. After incubation at 4℃ for 1 h, the beads were centrifuged at 2,000 rpm for 1 min. The supernatants were discarded. The beads were washed with PBS buffer for three times, and then washed with yeast lysis buffer for another three times.

JD52 cells containing HA-tagged N_{ub}-fusion candidates were grown in leucine-deficient broth at 28℃ to reach OD_{600nm} of 1.0. The cell pellets were washed once, and then resuspended in 1 ml yeast lysis buffer. Yeast extracts expressing HA-tagged proteins were prepared using a bead beater. Samples were agitated for 1 min three times, with 1 min rest on ice between every two agitations.

20 μl of GSTp- or GST-Nhp6bp-coupled beads were added to 200 μl of yeast extracts containing HA-tagged N_{ub}-fusion proteins. Samples were topped up to 500 μl with yeast lysis buffer with the addition of PMSF and DTT. Sample tubes were attached to an end-over-end rotator and incubated at 4℃. After incubation 2 h in a cold room at 4℃, the tubes were centrifuged at 2,000 rpm for 1 min. The supernatants were removed and the beads were washed six times with yeast lysis buffer (1 ml per washing). The washed beads were then resuspended with 20 μl of

yeast extract buffer. 8 µl 6×loading buffer was added to each tube. The content of each tube were distributed into two equal parts and heated at 95℃ for 5 min.

3.2.2.3 SDS-PAGE and Western blot

8%-12% separating gels and 4% stacking gel were used in this study. Formulations of SDS-polyacrylamide separating and stacking gels were listed in Table 3.2.2.3. Recipes are sufficient for the preparation of one gel and the components were mixed in the order shown. Polymerization would begin as soon as N,N,N',N'-tetramethlethylene-diamine (TEMED) had been added. 4 µl of the molecular marker standard-Kaleidoscope Pre-stained Standards (Bio-Rad laboratories) was heated for 1 min at 95℃ and then loaded onto the first well. 24 µl of each sample was separately loaded into the sample wells. Electrophoresis was carried out at 100 V at room temperature. The voltage was increased to 150 V when the samples had entered the separating gel and monitored till the dye reached the end of the separating gel.

The separated proteins were then transferred onto a nitrocellulose membrane using a Semi-Dry Electrophoretic Transfer Cell (Bio-Rad laboratories). Eight pieces of 5.5×8.5 cm filter paper and one piece of nitrocellulose membrane were cut and soaked in freshly prepared transfer buffer. The separating gel was removed from the gel tank and the plates were gently pried apart. A sandwiched was assembled by placing 4 pieces of filter papers onto the platinum anode, followed by the nitrocellulose membrane, the gel and 4 more pieces of filter papers. Appropriate

marking was made to indicate the relative positions between the gel and the membrane. Air bubbles were carefully removed from each layer. After the cathode and safe cover were placed onto the stack, the electrophoretic transfer was performed at 0.2 A for 60 min.

The blotted membrane was then soaked in blocking buffer that contained 5% non-fat milk in 100 ml distilled water with 10 mM Tris-HCl (pH7.0). The incubation was performed at 4℃ in the cold room for 1 h on an orbital shaker. This was to block non-specific binding sites. The membrane was then incubated with the primary anti-HA antibody (BabCO, Freiburg, Germany) at 4℃ in the cold room. The dilution was 1:10,000 in 5% non-fat milk in TBST (20 mM Tris, 150 mM NaCl, 0.1%Tween-20). The incubation was performed overnight, with the tray placed on an orbital shaker. After three 15-minute washes with 1% non-fat milk to remove the unbound primary antibody, the membrane was incubated with a horseradish peroxidase-coupled anti-mouse secondary antibody (Bio-Rad, München, Germany). This incubation was performed at 4℃ in the cold room for 2 h on an orbital shaker. The dilution of the secondary antibody was 1: 10,000 in 5% non-fat milk in TBST. Following this, the membrane was washed with TBST for three times, each time lasted 15 min. The nitrocellulose membrane was then placed protein side-up on a clean box.

ECL Plus (Amersham Pharmacia Biotech) system was used to detect the protein. The detection reagents were prepared by mixing solution A and B in the ratio of 40:1. The mixed detection reagent was then pipetted onto the entire surface of the

membrane and incubated for 1 min. Excess detection reagent was removed. The

membrane was gently wrapped up with Saran Wrap. The wrapped membrane was

placed protein side-up onto an X-ray film cassette. The film was exposed and

developed in a dark room with red safe light. A sheet of autoradiography film was

placed on top of the membrane, and the cassette was closed for various exposure

times. The film was then developed at the dark room at the Clinical Research

Center.

Separating gel reagent	8%	10%	12%
Sterile water	4.6 ml	4 ml	3.3 ml
30% Bisacrylamide	2.7 ml	3.3 ml	4 ml
pH8.8 Tris-HCl	2.5 ml	2.5 ml	2.5 ml
10% SDS	100 μl	100 μl	100 μl
10% APS	100 μl	100 μl	100 μl
TEMED	4 μl	4 μl	4 μl
Total Volume	10 ml	10 ml	10 ml

Stacking gel reagent	4%
Sterile water	3 ml
30% Bisacrylamide	0.65 ml
pH8.8 Tris-HCl	1.25 ml
10% SDS	50 μl
10% APS	50 μl
TEMED	5 μl
Total Volume	5 ml

Table 3.2.2.3: Contents of separating and stacking gels for SDS-PAGE

3.2.3 Analysis of phenotype

3.2.3.1 Construction of *NHP6*, *RPB4*, *RTT107* and *MED3* deletion strains

JD52ΔNHP6 strain was generated by successive deletion of the entire *NHP6A* and

NHP6B ORFs with *hisG* knockout constructs as described (Laser et al., 2000)**.**

The deletion of *RPB4, RTT107* and *MED3* was performed in the yeast strain *JD52*

by homologous recombination using *HIS3*-based knockout vectors containing

approximately 500 bp of the respective promoters and terminators. The

mechanism of homologous recombination is shown in Figure 3.2.3.1. Genomic

DNA was isolated from all deletion strains and successful deletions of the

respective genes were confirmed by PCR using primers specific to the target

genes.
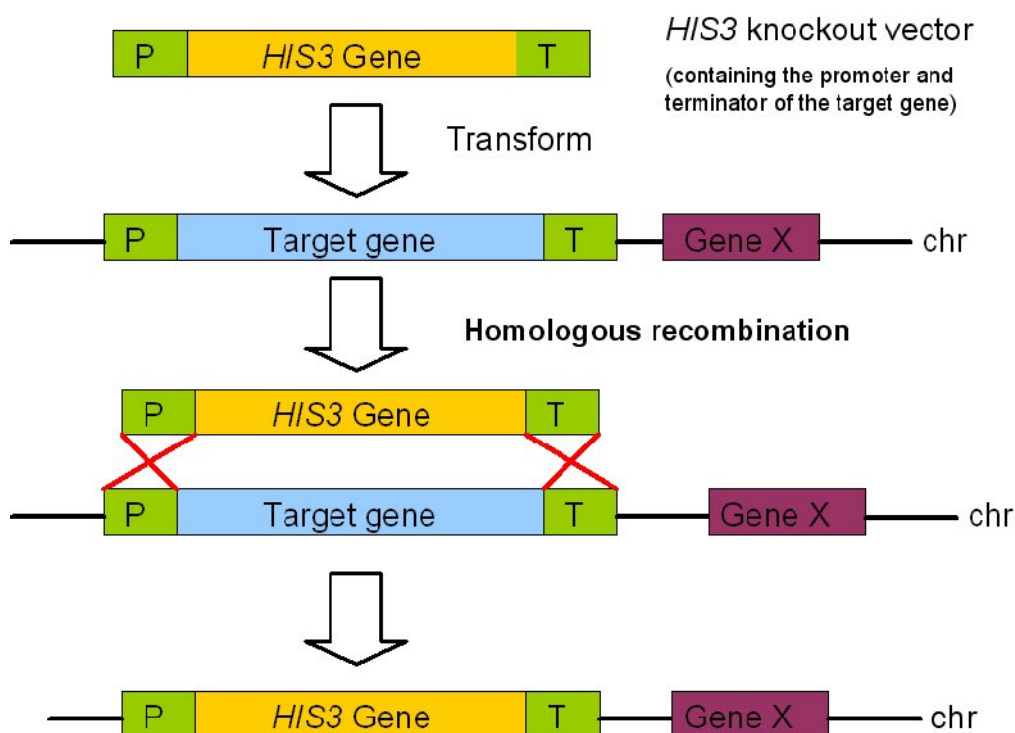
Figure 3.2.3.1: Mechanism of homologous recombination. Homologous
recombination is a process of physical rearrangement occurring between two
strands of DNA. It involves the alignment of similar sequences, a crossover
between the aligned DNA strands, and breaking/repair of the DNA to produce an
exchange of material between the strands. Homologous recombination is utilized
as a molecular biology technique for introducing genetic changes into organism.

3.2.3.2 Analysis of 6-AU phenotype

S. cerevisiae JD52 wild-type and isogenic deletion strains *JD52ΔNHP6*, *JD52ΔRPB4*, *JD52ΔRTT107* and *JD52ΔMED3* were diluted ten-fold and titrated onto plates lacking or containing 6-azauracil (6-AU). *S. cerevisiae JD52* cells lacking *NHP6* transformed with the plasmid expressing Nhp6b-C$_{ub}$-Rura3p or the plasmid YEplac181-RPB4 were ten-fold diluted and titrated onto plates lacking or containing 6-AU. Plates were incubated at 28℃ for three days to observe the growth phenotype.

3.2.4 Real-time PCR analysis

3.2.4.1 Construction of myc-tagged proteins

1. Cloning of *NHP6B* into RS314-MYC9 (generated by Dr. Lehming)

2. Cloning of *RPB4* into RS314-MYC9

RPB4-MYC9-314 was constructed by cloning a genomic *Pst*I–*Sal*I RPB4go PCR fragment lacking the stop-codon into RS314-MYC9. The constructed RPB4-MYC9-314 expresses myc-tagged Rpb4p from its own promoter.

3. Construction of RTT107C-MYC9-304

RTT107C-MYC9-304 was constructed by cloning a C-terminal *Cla*I-*Sal*I RTT107cgo PCR fragment lacking the stop-codon into RS304-MYC9. The RTT107C-MYC9-304 construct was linearized with *Afl*II and transformed into

wild-type *JD52* for chromosomal integration. The transformants were selected on a tryptophan-lacking plate.

4. Construction of MED3C-MYC9-304

MED3C-MYC9-304 was constructed by cloning a C-terminal *EcoR*I-*Xho*I MED3cgo PCR fragment lacking the stop-codon into RS304-MYC9 cut with *EcoR*I-*Sal*I. The MED3C-MYC9-304 was linearized with *Sal*I and transformed into wild-type *JD52* for chromosomal integration. The transformants were selected on a tryptophan-lacking plate.

5. Construction of TFB1C-MYC9-304

TFB1C-MYC9-304 was made by cloning a C-terminal *EcoR*I-*Mlu*I TFB1cgo fragment lacking a stop-codon into RS304-MYC9. TFB1C-MYC9-304 was linearized with *Cla*I and transformed into wild-type *JD52* for chromosomal integration. The transformants were selected on a tryptophan-lacking plate.

6. Construction of TFB4C-MYC9-304

TFB4C-MYC9-304 was constructed by cloning a C-terminal *Nhe*I-*Sal*I TFB4cgo fragment lacking the stop-codon into RS304-MYC9 cut with *Spe*I-*Sal*I. TFB4C-MYC9-304 was linearized with *Nco*I and transformed into wild-type *JD52* for chromosomal integration. The transformants were selected on a tryptophan-lacking plate. Figure 3.2.4 shows the mechanism of the myc-tagged protein replacement.
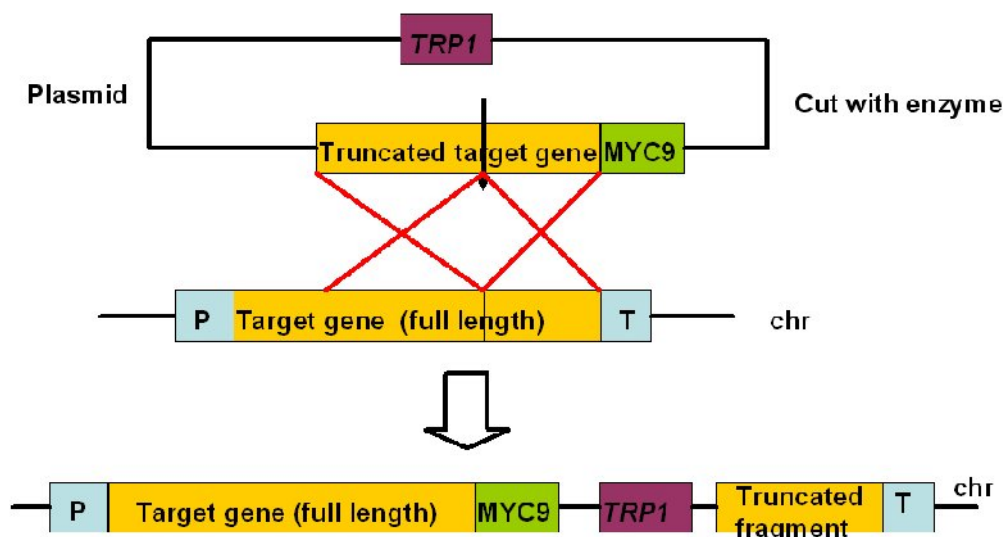
Figure 3.2.4.1: Mechanism of myc-tagged protein replacement. A truncated C-terminal target gene fragment lacking a stop-codon was cloned into RS304-MYC9. The construction was linearized and transformed into wild-type *JD52* for chromosomal integration.

3.2.4.2 Isolation of total RNA

The transcription levels of the *ZDS1* gene in wild-type *JD52* and in isogenic deletion strains were studied. Cells were grown in 50 ml of complete glucose medium or in glucose medium lacking tryptophan at 28℃ until the culture density had reached OD_{600nm} of 0.8-1.0. Cell pellets were washed with sterile water and then transferred to 1.5 ml centrifuge tubes. Total RNA was isolated using the RNeasy Mini kit (Qiagen) according to the protocol for isolation of total RNA from yeast from the manufacturer's manual. The RNA was eluted with 50 μl of 0.1% DEPC water.

Next, the absorbances of the samples were taken, and the concentration of the RNA was determined. A 200× dilution was performed by mixing 4 μl of total RNA sample with 800 μl of 0.1% DEPC water. RNA cuvettes were washed with

0.1% DEPC water and RNase-free water and then dried. The concentration of the total RNA was determined by measuring absorbance at OD_{260nm}. The concentration of total RNA could be calculated by using the following formula (A_{260nm} reading of 1 = 40 μg/ml RNA): concentration of total RNA = 40 μg/ml × OD_{260nm} × 200 (dilution factor). The ratio of the readings at 260 nm and 280 nm (A_{260nm}/A_{280nm}) provides an estimate of the purity of RNA with respect to contaminants that absorb the UV-light. Pure RNA has an A_{260}/A_{280} ration of 1.9-2.1. 6 μl of each isolated RNA sample was subjected to denaturing agarose gel electrophoresis and ethidium bromide staining to verify the integrity and size distribution. 4 μl RNA ladder (MBI Fermentas) was included. This was performed according to manufacturer's protocol (Qiagen). The 18S and 28S bands should appear as sharp bands under the UV detector.

After the concentration of the total RNA was measured and calculated, dilution was performed to obtain 100-200 μg/ml total RNA in a volume of 30 μl. Diluted total RNA sample was then treated with DNaseI to remove the genomic DNA contamination. In order to make sure that the treated RNA sample did not contain any genomic DNA contaminant, a gene-specific PCR reaction was performed using treated RNA sample as template. The contents for each reaction were listed in Table 3.2.4.2. The PCR products were analyzed by 2% agarose gel electrophoresis to ensure that there was no DNA contamination in the treated RNA samples.

Reagents in PCR	Volume (μl)
10× Taqman buffer	2.5
25 mM MgCl$_2$	1.5
2.5 mM dNTPs	0.5
Taq polymerase	0.25
Nuclease free water	14.25
10 μM Forward primer	0.5
10 μM Reverse primer	0.5
DNase treated total RNA sample	5
Total Volume	**25**

Table 3.2.4.2 Contents in gene-specific PCR

3.2.4.3 Reverse-transcription polymerase chain reaction

The reagents of the reverse transcription reactions are listed in Table 3.2.4.3. The cycling parameters were performed as 25℃ for 10 min, 37℃ for 60 min, 95℃ for 5 min. After the reverse-transcription PCR, the cDNA samples were removed and stored at -80℃ for use.

Reagents in reverse transcription	Volume (μl)
10× Taqman buffer	3
25 mM MgCl$_2$	6.6
2.5 mM dNTPs	6
20 μg/ L RNase inhibitor	0.6
50 μg/L Reverse Transcriptase	0.75
Nuclease free water	5.55
100 ng/μl RNA	6
50 μM Random Hexamer	1.5
Total Volume	**30**

Table 3.2.4.3: Contents in reverse transcription PCR

In order to determine the quality of cDNA, a gene-specific PCR reaction was performed before the cDNA sample was used for the real-time PCR reaction. The

cDNA product was used as template to amplify the specific gene which would be analyzed by real-time PCR. The PCR products were analyzed by electrophoresis. The defined sharp band with the correct presumed size indicated good quality of the cDNA sample.

3.2.4.4 Quantitative Real-time PCR

In quantitative real-time PCR, two sets of duplicates were prepared for each sample. One set used the gene-specific ORF primer pair and the other set used an *ACT I* primer pair as control. The contents for each real-time reaction tube were listed in Table 3.2.4.4. Real-time PCR was performed using ABI Prism® 7700 (Applied Biosystems). The cycling parameters used for the real-time PCR were as follows: denaturation at 50℃ for 2 min, 95℃ for 10 min, and subsequently 40 amplification cycles at 95℃ for 15 sec and 60℃ for 60 sec. A dissociation curve was carried out after each run to ensure that no unspecific products had been formed. The relative mRNA levels of *ZDS1* in wild-type *JD52* and in isogenic deletion strains were determined using the comparative threshold (Ct) method. The fold changes in *ZDS1* expression between wild-type *JD52* and isogenic deletion strains were calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Real-time PCR reagents	Volume (µl)
2× SYBR Green	12.5
10 µM Forward primer	0.3
10 µM Reverse primer	0.3
Nuclease-free water	6.9
cDNA	5
Total Volume	**25 µl / tube**

Table 3.2.4.4: Contents in quantitative real-time PCR

3.2.5 Chromatin Immunoprecipitation (ChIP)

3.2.5.1 Cross-linking of protein-DNA complexes *in vivo*

For each sample, 50 ml of yeast cells were grown in tryptophan-deficient broth.

1.5 ml 37% formaldehyde (1% final concentration) was added into the culturing

broth when the cells reached OD_{600nm} of 0.8-1.0. Samples were cross-linked

15 min at room temperature by shaking slowly on a platform. The cross-linked

samples were harvested by centrifuged at 4,000 rpm for 5 min. Supernatants were

discarded into a chemical waster container. Cell pellets were washed with 50 ml

ice-cold water twice and then transferred into fresh microtubes. After

centrifugation on a benchtop centrifuge at 7,000 rpm for 1 min at room

temperature, supernatants were discarded and cell pellets were stored in -80℃.

3.2.5.2 Preparation of chromatin solution

Cell pellets were resuspended in 1 ml ice-cold yeast lysis buffer containing 20 µl

PMSF (50 pM final concentration) and 10 µl DTT (50 pM final concentration) and

transferred into screw-cap microcentrifuge tubes. The microcentifuge tubes were

filled up to three quarters with glass beads. The caps were screwed tightly to avoid leakage. Cells were broken by 3 min agitation with a mini bead beater at maximum speed. Samples were removed and incubated for 3 min on ice. Five times breakings were carried out to make a total breakage time of 18 min. A 5 ml syringe was cut below the flared opening with a razor. The smaller portion was inserted into a 15 ml disposable conical tube so that the flared portion of the truncated syringe rested on top of the conical tube opening, forming a microcentrifuge-tube holder. Sample tubes were inverted and a hole was punched in the bottom with a 27-G needle. Sample tubes were placed into the syringe/conical tube and a hole was punched in the top cover with the same needle. The assemblies were centrifuged at 1,000 rpm for 1 min at room temperature. The conical tube was placed on ice and the 2 ml centrifuge tube containing the dry beads was discarded. The eluted samples were transferred to a fresh 1.5 ml microcentrifuge tube and centrifuged at 13,000 rpm at 4℃ for 20 min. Supernatants were discarded and 600 ml ice-cold yeast lysis buffer containing PMSF and DTT was added to resuspend the pellets. Sample tubes were sonicated for 1 min in an ice-water bath using a continuous pulse at a power output of 5%. Samples were then cooled in an ice-water bath for 1 min. The same sonication cycle was repeated once. Sample tubes were centrifuged at 13,000 rpm for 30 min at 4℃. The supernatants were transferred to a fresh microtube. 100 μl of chromatin solution was removed for DNA fragment testing and the remaining chromatin solution was frozen in -80℃ for the IP assay.

3.2.5.3 Determination of chromatin-fragment size

100 µl of chromatin aliquot, 10 µl of 20 mg/ml Pronase and 100 µl of Pronase working buffer were added into PCR tube. Sample tubes were placed into PCR machine, first incubated at 42℃ for 2 h and then switched to 65℃ for 4 h. DNA fragments were phenol extracted and ethanol precipitated, and then dried in a speedvac for 20 min. DNA fragments were resuspended in 30 µl DNase-free water. 10 µl of DNA was mixed with 1 µl of 10×loading buffer and electrophoretically separated on a 2% agarose gel. The fragments should be between 100 to 1000 bp, with an average length of 400 to 500 bp.

3.2.5.4 Immunoprecipitation

200 µl of chromatin solution was incubated with 4 µl of anti-myc-antibody-coupled Protein A-Sepharose beads (Sigma) and topped up to 500 µl with yeast lysis buffer. Sample tubes were bound on an end-over-end rotator and incubated at 4℃ for 2 h. Beads were collected by centrifuged at 3,000 rpm for 2 min and supernatants were discarded. The beads were then washed with yeast lysis buffer, yeast lysis buffer containing NaCl (0.5M final concentration), ChIP washing buffer and finally TE buffer. With each type of buffer, beads were washed for three times. The washed beads were then resuspended in 100 µl of ChIP elution buffer and gently pipetted up and down two or three times to dislodge the beads from the tube wall. Sample tubes were incubated at 65℃ for 10 min. After incubation, tubes were centrifuged at

3,000 rpm for 2 min at room temperature. The beads were discarded and the

elution was transferred to 0.5 ml PCR tubes labeled as IP.

3.2.5.5 Reversion of cross-link

100 μl of Pronase working solution and 20 μl of Pronase were added into the PCR

tube that contained 200 μl eluted IP sample. 20 μl of chromatin solution labeled as

input was combined with 100 μl of Pronase working solution and 20 μl of Pronase

in the PCR tube. The PCR tubes were first incubated at 42℃ for 2 h, and then

switched to 65℃ for 4 h. The decross-linked samples were stored at -80℃ until

use.

3.2.5.6 Gene specific quantitative PCR

The primers used here for the analyzed genomic regions were *ZDS1* ORF primers

and *ZDS1* promoter primers listed in Table 3.1.4. To ensure that the PCR results

were in the linear range, a PCR standard curve was generated from a dilution

series of the "input" sample. The dilution factors used here were 1-fold, 10-fold

and 100-fold, respectively. Each dilution sample was amplified by PCR under 25

cycles, 30 cycles and 35 cycles, respectively. PCR products were analyzed on a

2% agarose gel. The fluorescence intensity was monitored and quantified by an

UV illuminator with specific software. The PCR was optimized and performed

with the following parameters: 95℃ for 10 min, 95℃ for 10 sec, 50℃ for 1 min,

42℃ for 1 min (30cycles) and 72℃ for 10 min. The contents for the ChIP

quantitative PCR are listed in Table 3.2.5.6.

PCR reagent	Volume (µl)
5×PCR buffer	5
25 mM MgCl$_2$	1.5
2.5 mM dNTPs	0.5
10 µM Forward primer	0.5
10 µM Reverse primer	0.5
Taq polymerase	0.2
DNase-free water	16.8
Total Volume	**25**

Table 3.2.5.6: Contents in ChIP quantitative PCR

3.2.5.7 Quantitative analysis of ChIP PCR products

The PCR products were electrophoretically analyzed on a 2% agarose gel. The relative intensity of PCR products were quantified using proper software with the accompanying instrument. The relative protein occupancy at the *ZDS1* locus was normalized to 10% input sample. Three separate PCRs were carried out to calculate the standard division.

CHAPTER 4

RESULTS

4. Results

4.1 Nhp6p-interacting proteins isolated with Split-Ubiquitin screens

4.1.1 Screening for Nhp6p-interacting partners with the Split-Ubiquitin system

The Split-Ubiquitin system is a fragment complementation assay that is based on a conditional proteolysis design. It allows for the study of protein interactions between transcription factors within living cells (Lehming, 2002). The HMG protein Nhp6p plays an important role in transcriptional regulation and genome stability. Identification of the interaction partners of Nhp6p would help to understand how HMG proteins regulate gene expression. In this project, a N_{ub} *S. cerevisiae* genomic fusion library and a collection of N_{ub} fusions to more than 100 *S. cerevisiae* transcription factors were directly tested for their interactions with C_{ub} fused to Nhp6ap and Nhp6bp. The main steps, which were taken to isolate and identify the Nhp6p-interacting partners are described in the flow chart shown in Figure 4.1.1.

NHP6A-C$_{ub}$-RUra314
NHP6B-C$_{ub}$-RUra314

PACNX-N$_{ub}$IBC-library
PADNX-N$_{ub}$IBC-library

Co-transformed into the *S. cerevisiae* strain *JD52*,
plated onto FOA plates and incubated at 28℃

Colonies from the FOA plates were restreaked and inoculated in 10 ml L$^-$ broth. N$_{ub}$ fusion vectors were isolated from the *S. cerevisiae* cells.

The isolated N$_{ub}$ fusion vectors were transformed into *E. coli DH10B* by electroporation. Cells were amplified in 2 ml LB medium. DNA minipreparation was carried out to purify the N$_{ub}$ fusion vectors.

N$_{ub}$ fusion vectors were analyzed by digestion with *Hind*III and *Sal*I, followed by agarose gel electrophoresis to determine the sizes of inserts. The amplified N$_{ub}$ fusion vectors were transformed into *JD52* expressing Nhp6-C$_{ub}$-RUra3p to test for plasmid-linkage.

N$_{ub}$ fusion vectors isolated from the library screens were cycle sequenced.

The obtained sequences were used to conduct internet BLAST searches in order to determine the sequence identity.

The existence of the interaction was confirmed by testing the isolated N$_{ub}$ fusion candidates with Nhp6-C$_{ub}$-RUra3p in *JD55*.

The specificity of the interaction was determined by testing the isolated N$_{ub}$ fusion candidates

Figure 4.1.1: Steps taken to screen and identify N$_{ub}$ fusion candidates which interacted with Nhp6-C$_{ub}$-RUra3p in library screening.

An expression library containing yeast genomic DNA fragments fused to N_{ub} was transformed into yeast *JD52* cells expressing the bait protein Nhp6a-C_{ub}-RUra314p or Nhp6b-C_{ub}-RUra314p. Approximately 400,000 primary transformants had been plated onto FOA plates. Proteins interacting with Nhp6ap or Nhp6bp were isolated by their ability to confer growth on the FOA plates. After 10 days incubation at 28℃, a total of 94 colonies were obtained from FWL plates. The N_{ub} fusion vectors obtained from the 94 colonies were transformed into the *E. coli* strain *DH10B* in order to amplify the vectors and test for plasmid-linkage. Four single colonies were picked from each of the 94 plates. Amplified N_{ub} fusion vectors from the 376 candidates were obtained by DNA mini-preparation.

4.1.2 Restriction endonuclease digestion to check the size of the insert DNA

The 376 N_{ub} fusion vectors were cut with the restriction enzymes *Hind*III and *Sal*I in order to determine the size of the inserts. The cut N_{ub} fusion vectors were then separated on a 1% agarose gel together with a DNA marker. The vector without insert, PACNX-N_{ub}-IBC1 or PADNX-N_{ub}-IBC1, was included as a negative control. The candidates were denoted as A1.1-A43.4 and B1.1-B51.4. "A" or "B" indicates that the potential candidates were isolated from the library screens with Nhp6ap or Nhp6bp as bait, while the number represents the candidate number of the yeast clone from which the N_{ub} fusion vector was originally obtained. Restriction endonuclease digestion and agarose gel electrophoresis analysis indicated that 243 out of 376 samples had different insert sizes. Figure 4.1.2 is a

gel electrophoresis photo, showing the inserts of some of the N_{ub} candidates. Lane 1 shows the pGEM3 DNA ladder, with the size of each DNA band indicated on the left. From lane 2 to lane 11, the N_{ub} fusion plasmids had been subjected to restriction digestion with *Hind*III and *Sal*I to give two bands (plasmid back-bone and insert). The multiple bands in the bottom part in lanes 4, 5 and 6 indicated that there were *Hind*III and/or *Sal*I sites present within the insert. In lane 11, N_{ub} empty vector without insert was included as a control. Some samples gave bands of the same size (compared the samples in the upper part in lanes 5, 7), indicting that they were duplicates from the same colonies.
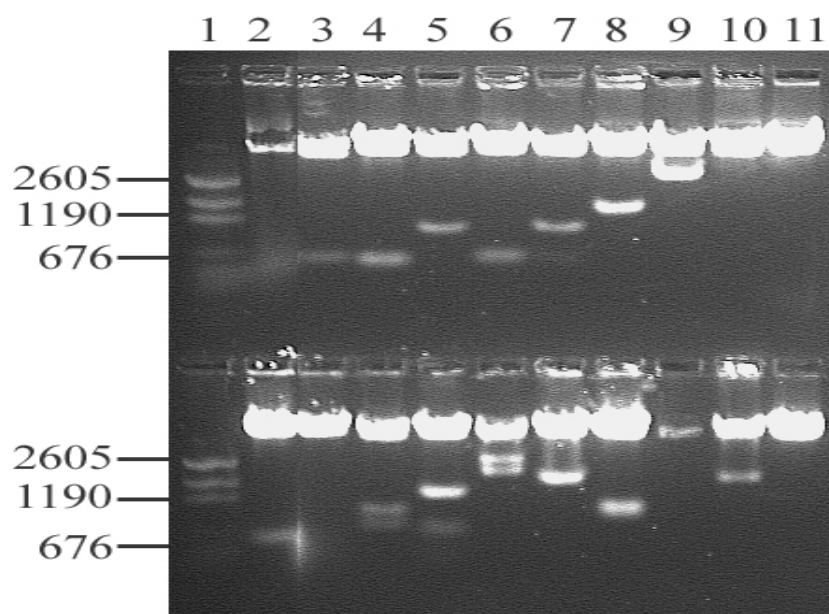
Figure 4.1.2: Gel electrophoresis photo showing N_{ub} insert sizes in 2% agarose gel. The N_{ub} fusion vectors have been digested with *Hind*III and *Sal*I. Lane 1 is the pGEM3 DNA marker, with the size of each DNA band indicated on the left. Lane 11 is the N_{ub} empty vector which is included as a control. From lanes 2 to lane 11, the N_{ub} fusion plasmids had been subjected to restriction digestion with *Hind*III and *Sal*I.

4.1.3 Testing for Plasmid Linkage

The 243 N_{ub} fusion vectors, which showed different insert sizes in the restriction digestion were then transformed back into yeast *JD52* cells containing either the NHP6A-C_{ub}-RUra314 or the NHP6B-C_{ub}-RUra314 vector in order to test for plasmid linkage. Equal amounts of transformed cells were plated on WL plates and FWL plates. Since the C_{ub} vector containing the *TRP1* gene confers tryptophan prototrophy to transformed yeast cells, and the N_{ub} vector containing the *LEU2* gene confers leucine prototrophy to the transformed cells, cells co-expressing both C_{ub} and N_{ub} fusion vectors were selected for on WL plates. The FWL plates were used to test for the specific protein interaction. Of the 243 plasmid-linkage tests carried out, 34 vectors were confirmed to cause plasmid-linked FOA resistance. Table 4.1.3.1 shows the 34 N_{ub} fusion candidates.

No.	N_{ub} library	No. of colonies		Plasmid-linkage
		WL	FWL	
1	A1.1	++	4	Y
2	A2.1	++	+	Y
3	A3.1	++	++	Y
4	A4.1	++	9	Y
5	A5.1	++	++	Y
6	A6.2	++	51	Y
7	A9.1	++	3	Y
8	A10.1	++	65	Y
9	A11.1	++	5	Y
10	A12.1	++	++	Y
11	A14.1	++	+	Y
12	A14.2	++	++	Y
13	A15.1	++	++	Y
14	A16.1	++	7	Y
15	A16.2	++	61	Y
16	A17.4	++	++	Y
17	A19.2	++	++	Y
18	A20.1	++	++	Y
19	A26.3	++	41	Y
20	A38.1	++	28	Y
21	B1.1	++	++	Y
22	B3.1	++	5	Y
23	B4.1	++	11	Y
24	B4.4	++	++	Y
25	B6.1	++	14	Y
26	B6.2	++	64	Y
27	B8.1	++	++	Y
28	B15.1	++	29	Y
29	B23.2	++	21	Y
30	B29.1	++	3	Y
31	B30.2	++	9	Y
32	B32.1	++	4	Y
33	B32.2	++	17	Y
34	B47.1	++	++	Y

Table 4.1.3.1: The 34 N_{ub} fusion candidates isolated from the *S. cerevisiae* genomic library showed plasmid-linkage with Nhp6-C_{ub}-Rura3p in *JD52*.
The relative amount of cell growth is recorded as abundant (++) or moderate (+).

The 34 N_{ub} fusion candidates that showed plasmid-linkage in *JD52* cells were then transformed into *JD55* cells to test for their interaction with Nhp6a-C_{ub}-RUra3p and Nhp6b-C_{ub}-RUra3p. The yeast strain *JD55* is an *UBR1* deletion derivative of the *JD52* strain. This means that the *UBR1* gene, which encodes the E3 ubiquitin ligase that ubiquitinates substrates of the N-end rule pathway of protein degradation, had been disrupted with the *HIS3* gene. In *JD55* cells, RUra3p is stable due to the loss of the E3 ubiquitin ligase. If interaction between the N_{ub} fusion protein and Nhp6-C_{ub}-RUra3p occurred in *JD55* cells, they still can form the ubiquitin-like moiety and the UBPs still can cleave RUra3p off the C_{ub} fusion. RUra3p, however, is perfectly stable and enzymatically active as the responsible E3 ubiquitin ligase is missing. The cells will remain uracil prototrophic and FOA sensitive regardless if the N_{ub} fusion protein interacts with the C_{ub} fusion protein or not. The *JD55* strain serves as a negative control compared to *JD52*. Since over-expression of some proteins, such as certain membrane transporters which can pump FOA out of the cells, result in FOA resistance, it is good to test the isolated N_{ub} fusion candidates in *JD55* to confirm that the FOA resistance in *JD52* was really due to the interaction between the N_{ub} fusion protein and the C_{ub} fusion protein and not due to the over-expression of an FOA resistance protein. The growth of the *JD55* cells containing Nhp6-C_{ub}-RUra3p and 34 N_{ub} fusion proteins on the WL and FWL plates is shown in Table 4.1.3.2. Seven of the 34 N_{ub} fusion candidates grew on the FWL plates, indicating that these seven N_{ub} fusion proteins caused FOA resistance. The seven candidates were sequenced and

compared with known genes in a yeast genomic database. The sequence results indicated that four of the seven candidates (A2.1, A5.1, B1.1, B4.4) were determined to express N_{ub}-Ydr007Wp and the other three candidates (A3.1, B8.1, B47.1) expressed N_{ub}-Ydr008Cp. *YDR007W* is the systematic name of the *TRP1* gene, which encodes phosphoribosylanthranilate isomerase that catalyzes the third step in tryptophan biosynthesis, and it confers tryptophan prototrophy to transformed cells. *YDR008C* is a dubious open reading frame unlikely to encode a protein. However, next to this gene, and also contained on the genomic DNA fragment, was the *TRP1* gene. The most likely explanation for the FOA resistance caused by these clones is that they combine both the *LEU2* and the *TRP1* markers on a single plasmid. Cells transformed with these clones were able to loose the Nhp6-C_{ub}-RUra3p-expressing *TRP1*-marked plasmid and grow on the FWL plate thus.

No.	N_{ub} library	No. of colonies		FOA resistance
		WL	FWL	
1	A1.1	++		
2	A2.1	+	+	Y
3	A3.1	++	++	Y
4	A4.1	+		
5	A5.1	++	++	Y
6	A6.2	++		
7	A9.1	++		
8	A10.1	+		
9	A11.1	+		
10	A12.1	++		
11	A14.1	+		
12	A14.2	+		
13	A15.1	+		
14	A16.1	+		
15	A16.2	++		
16	A17.4	++		
17	A19.2	++		
18	A20.1	++		
19	A26.3	++		
20	A38.1	+		
21	B1.1	++	+	Y
22	B3.1	++		
23	B4.1	++		
24	B4.4	++	++	Y
25	B6.1	++		
26	B6.2	++		
27	B8.1	++	++	Y
28	B15.1	+		
29	B23.2	++		
30	B29.1	++		
31	B30.2	++		
32	B32.1	+		
33	B32.2	+		
34	B47.1	++	+	Y

Table 4.1.3.2: Seven of the 34 N_{ub} fusion candidates isolated from the *S. cerevisiae* genomic library showed FOA resistance in *JD55*.
The relative amount of cell growth is recorded as abundant (++) or moderate (+).

4.1.4 DNA sequencing of the isolated Nhp6p-interacting candidates

Cycle sequencing was performed with the DNA mini-preparation samples of the 34 candidates, which had shown plasmid linkage in *JD52*. The obtained sequences were then used to conduct BLAST searches in the *S. cerevisiae* database (SGD: genome-www.stanford.edu/Saccharomyces) to check for sequence identity with known genes. A summary of the identified genes is given in Table 4.1.4.1 and Table 4.1.4.2. The sequencing results showed that most of the plasmids contained gene sequences which could be expressed as N_{ub}-fusion proteins in the correct orientation and reading frame. Two of the 34 candidates contained non-coding sequences. One is A9.1, whose sequence is located on the 2 μm plasmid from coordinates 1091 to 1308. The other one is A38.1, whose sequence spanned from 736553 to 736722 on chromosome XV. Seven of the 34 N_{ub} fusion candidates (A2.1, A3.1, A5.1, B1.1, B4.4, B8.1 and B47.1) either expressed the Trp1p protein as a N_{ub} fusion or they contained the entire *TRP1* gene on the genomic DNA fragment. Since the N_{ub} vector itself contains the *LEU2* gene, cells transformed with the above seven candidates combined both the *LEU2* and *TRP1* markers on one single plasmid. They grew on the FWL plates as they did not have to keep the Nhp6-C_{ub}-RUra3p-expressing *TRP1*-marked plasmid. Such candidates were termed as false positives in the split-ubiquitin assay since their FOA resistance was not due to the interaction between the N_{ub} and C_{ub} fusion proteins. These results were consistent with the plasmid linkage results in *JD55* and explained why the seven candidates displayed FOA resistance in *JD55*. Some of the

Nhp6-interacting candidates like Nsf1p, Yml108Wp, Ddc1p and H4 had been

isolated several times, indicating that the screens had nearly exhausted the library.

In total, 18 different N_{ub} proteins had been isolated as potential interactors of

Nhp6p.

No.	Gene name	Clone coordinates	FL chromatin location	Matched sequence	Insert length (AA-AA)
A1.1	NFS1	Chr III: 92014-93112	Chr III: 94270-92777	93112-92777	386-497 (497) (**B4.1, B32.1**)
A2.1	YDR007W(TRP1)	Chr IV: 461705-462592	Chr IV: 461839-462513	461839-462513	1-224 (224) (**A5.1, B1.1, B4.4**)
A3.1	YDR008C	Chr IV: 462343-462608	Chr IV: 462599-462249	462599-462343	1-85 (117) (**B8.1, B47.1**)
A4.1	YML108W	Chr XIII: 54716-55480	Chr XIII: 54793-55110	54793-55110	1-105 (105) (**B29.1, B30.2**)
A6.2	RPB4	Chr X: 151255-151838	Chr X:150958-151623	151255-151623	99-221 (221)
A10.1	SRP14	Chr IV: 293140-294654	Chr IV: 292781-293221	293140-293221	119-146 (146) (**A26.3**)
A11.1	YOL022C	Chr XV: 280123-280425	Chr XV: 281499-280273	280425-280273	358-408 (408)
A12.1	ARC15	Chr IX: 243624-244420	Chr IX: 244459-243995	244420-243995	13-154 (154)
A14.1	MLP1	Chr XI: 624703-625014	Chr XI: 619447-625074	624703-625014	1752-1855 (1875)
A14.2	SGF11	Chr XVI: 466088-466882	Chr XVI: 465959-466258	466088-466258	43-99 (99)
A15.1	CBF1	Chr X: 541576-549280	Chr X: 548752-549807	548752-549280	1-176 (351)
A16.1	DDC1	Chr XVI: 180822-183391	Chr XVI: 179276-181114	180822-181114	515-612 (612) (**A16.2**)
A17.4	HHF2	Chr XIV: 576728-577039	Chr XIV: 576728-577039	576728-577039	1-103 (103) (**B6.2**)
A19.2	ZRG8	Chr V: 219333-219699	Chr V: 221286-218056	219699-219333	529-651 (1076)
A20.1	RTT107	Chr VIII: 405138-408399	Chr VIII: 402969-406181	405138-406181	724-1070 (1070)
B3.1	YLR301W	Chr XII: 731238-731621	Chr XII: 730827-731561	731238-731561	137-244 (244)
B6.1	ACP1	Chr XI: 80089-92582	Chr XI: 80542-80165	80542-80165	1-125 (125)
B15.1	TUF1	Chr XV: 684565-685251	Chr XV: 684031-685344	684565-685251	178-406 (437)
B23.2	MSS4	Chr IV: 870489-871202	Chr IV: 868221-870560	870489-870560	756-779 (779)
B32.2	YDR186C	Chr IV: 833772-834292	Chr IV: 835489-832856	834292-833772	399-572 (877)

Table 4.1.4.1: Sequencing results of the N$_{ub}$ candidates isolated from the *S. cerevisiae* genomic library screens using Nhp6a-C$_{ub}$-Rura3p or Nhp6b-C$_{ub}$-Rura3p as bait.

Gene name	Systematic deletion	Product description
YCL017C (NFS1)	inviable	Cysteine desulfurase involved in iron-sulfur cluster (Fe/S) biogenesis; required for the post-transcriptional thio-modification of mitochondrial and cytoplasmic tRNAs; essential protein located predominantly in mitochondria (Kispal et al., 1999; Nakai et al., 2004; Muhlenhoff et al., 2004).
YDR007W (TRP1)	viable	Phosphoribosylanthranilate isomerase that catalyzes the third step in tryptophan biosynthesis (Braus, 1991).
YDR008C	viable	Dubious open reading frame unlikely to encode a protein (Fisk et al., 2006).
YML108W	viable	Putative protein of unknown function whose structure defines a new subfamily of the split beta-alpha-beta sandwiches; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm and nucleus (Giaever et al., 2002; Pineda-Lucena et al., 2003; Huh et al., 2003).
RPB4	viable	RNA polymerase II subunit B32; forms two subunit dissociable complex with Rpb7p; dispensable under some environmental conditions; involved in export of mRNA to cytoplasm under stress conditions; involved in telomere maintenance (Archambault and Friesen, 1993; Choder, 2004; Askree et al., 2004).
SRP14	inviable	Signal recognition particle (SRP) subunit, interacts with the RNA component of SRP to form the Alu domain, which is the region of SRP responsible for arrest of nascent chain elongation during membrane targeting; homolog of mammalian SRP1 (Brown et al., 1994; Strub et al., 1999; Mason et al., 2000).
YOL022C	inviable	Cytoplasmic protein of unknown function; essential gene in S288C (Rad et al., 1997; Huh et al., 2003).
ARC15	inviable	Subunit of the ARP2/3 complex, required for the motility and integrity of cortical actin patches (Winter et al., 1997).
MLP1	viable	Myosin-like protein associated with the nuclear envelope, connects the nuclear pore complex with the nuclear interior; involved with Tel1p in telomere length control; involved with Pml1p and Pml39p in nuclear retention of unspliced mRNAs (Strambio-de-Castillia et al., 1999; Palancade et al., 2005).

SGF11	viable	Integral subunit of SAGA histone acetyltransferase complex, regulates transcription of a subset of SAGA-regulated genes, required for the Ubp8p association with SAGA and for H2B deubiquitylation (Powell et al., 2004; Ingvarsdottir et al., 2005).
CBF1	viable	Helix-loop-helix protein that binds the motif CACRTG, which is present at several sites including MET gene promoters and centromere DNA element I, required for nucleosome positioning at this motif, targets Isw1p to DNA (Wieland et al., 2001; Moreau et al., 2003; Kent et al., 2004).
DDC1	viable	DNA damage checkpoint protein, part of a PCNA-like complex required for DNA damage response, required for pachytene checkpoint to inhibit cell cycle in response to unrepaired recombination intermediates; potential Cdc28p substrate (Hong and Roeder, 2002; Giannattasio et al., 2003).
HHF2	viable	One of two identical histone H4 proteins; core histone required for chromatin assembly and chromosome function; contributes to telomeric silencing; N-terminal domain involved in maintaining genomic integrity (Smith et al., 1996; Wyrick et al., 1999; Altheim and Schultz, 1999)
ZRG8	viable	Protein of unknown function; authentic, non-tagged protein is detected in highly purified mitochondria in high-throughput studies; GFP-fusion protein is localized to the cytoplasm; transcription induced under conditions of zinc deficiency (Yuan, 2000; Reinders et al., 2006).
RTT107	viable	Protein implicated in Mms22-dependent DNA repair during S phase, DNA damage induces phosphorylation by Mec1p at one or more SQ/TQ motifs; interacts with Mms22p and Slx4p; has four BRCT domains; has a role in regulation of Ty1 transposition (Rouse 2004; Baldwin et al., 2005; Roberts et al., 2006).
YLR301W	N/A	Protein of unknown function that interacts with Sec72p (Willer et al., 2003).
ACP1	inviable	Mitochondrial matrix acyl carrier protein, involved in biosynthesis of octanoate, which is a precursor to lipoic acid; activated by phosphopantetheinylation catalyzed by Ppt2p (Brody et al.,1997; Stuible et al., 1998).
TUF1	viable	Mitochondrial translation elongation factor Tu; comprises both GTPase and guanine nucleotide exchange factor activities, while these activities are found in separate proteins in S. pombe and humans (Nagata et al., 1983; Chiron et al., 2005).
MSS4	inviable	Phosphatidylinositol-4-phosphate 5-kinase, involved in actin cytoskeleton organization and cell morphogenesis; multicopy suppressor of stt4 mutation (Homma et al., 1998; Desrivieres et al., 1998).
YDR186C	viable	Putative protein of unknown function; may interact with ribosomes, based on co-purification experiments; green fluorescent protein (GFP)-fusion protein localizes to the cytoplasm (Huh et al., 2003; Fleischer et al., 2006).

Table 4.1.4.2: Summary of the N$_{ub}$ candidates isolated from the *S. cerevisiae* genomic library screens using Nhp6-C$_{ub}$-RUra3p as bait.

No.	Gene name	Systematic deletion	Product description
1	HHO1(H1)	viable	Histone H1, a linker histone required for nucleosome packaging at restricted sites; suppresses DNA repair involving homologous recombination; not required for telomeric silencing, basal transcriptional repression, or efficient sporulation (Escher and Schaffner 1997; Hellauer et al., 2001; Downs, 2003).
2	HTA1(H2A)	HTA1/2 double deletion inviable	One of two nearly identical (see also HTA2) histone H2A subtypes; core histone required for chromatin assembly and chromosome function; DNA damage-dependent phosphorylation by Mec1p facilitates DNA repair; acetylated by Nat4p (Norris et al., 1988; Downs et al., 2000; Song et al., 2003).
3	HTB1(H2B)	HTB1/2 double deletion inviable	One of two nearly identical (see HTB2) histone H2B subtypes required for chromatin assembly and chromosome function; Rad6p-Bre1p-Lge1p mediated ubiquitination regulates transcriptional activation, meiotic DSB formation and H3 methylation (Robzyk et al., 2000; Briggs et al., 2002; Kao et al., 2004).
4	HHT1(H3)	HHT1/2 double deletion inviable	One of two identical histone H3 proteins (see also HHT2); core histone required for chromatin assembly, involved in heterochromatin-mediated telomeric and HM silencing; regulated by acetylation, methylation, and mitotic phosphorylation (Zhang et al., 1998; van Leeuwen et al., 2002; Krogan et al., 2003; Boa et al., 2003).
5	MED3(PGD1)	viable	Subunit of the Mediator global transcriptional cofactor complex, which is part of the RNA polymerase II holoenzyme and plays an essential role in basal and activated transcription; direct target of the Cyc8p-Tup1p transcriptional corepressor (Papamichos-Chronakis et al., 2000; Reeves and Hahn, 2003; Guglielmi et al., 2004).
6	RAP1	inviable	DNA-binding protein involved in either activation or repression of transcription, depending on binding site context; also binds telomere sequences and plays a role in telomeric position effect (silencing) and telomere structure (Pina et al., 2003).
7	RPB6	inviable	RNA polymerase subunit ABC23, common to RNA polymerases I, II, and III; part of central core; similar to bacterial omega subunit (Cramer et al., 2000; Geiduschek and Kassavetis, 2001; Cramer, 2002).

8	SRB5	viable	Subunit of the RNA polymerase II mediator complex; associates with core polymerase subunits to form the RNA polymerase II holoenzyme; essential for transcriptional regulation; involved in telomere maintenance (Kim et al., 1994; Askree et al., 2004; Kornberg, 2005).
9	SWI3	viable	Subunit of the SWI/SNF chromatin remodeling complex, which regulates transcription by remodeling chromosomes; required for transcription of many genes, including ADH1, ADH2, GAL1, HO, INO1 and SUC2 (Peterson and Herskowitz, 1992; Peterson et al., 1998).
10	TAF1	inviable	TFIID subunit (145 kDa), involved in RNA polymerase II transcription initiation, has histone acetyltransferase activity, involved in promoter binding and G1/S progression (Walker et al., 1997; Lee and Young, 2000; Tora, 2002).
11	TFA1	inviable	TFIIE large subunit, involved in recruitment of RNA polymerase II to the promoter, activation of TFIIH, and promoter opening (Orphanides et al., 1996; Lee and Young, 2000).
12	TFA2	inviable	TFIIE small subunit, involved in RNA polymerase II transcription initiation (Feaver et al., 1994; Lee and Young, 2000).
13	TFB1	inviable	Subunit of TFIIH and nucleotide excision repair factor 3 complexes, required for nucleotide excision repair, target for transcriptional activators (Prakash and Prakash, 2000; Lee and Young, 2000).
14	TFB4	inviable	Subunit of TFIIH complex, involved in transcription initiation, similar to 34 kDa subunit of human TFIIH; interacts with Ssl1p (Feaver et al., 2000; Lee and Young, 2000).
15	TFG2	inviable	TFIIF (Transcription Factor II) middle subunit; involved in both transcription initiation and elongation of RNA polymerase II; homologous to human RAP30 (Hampsey, 1998).

Table 4.1.4.3: Description of the N_{ub} fusions isolated from a collection of N_{ub}-fused transcription factors using Nhp6a-C_{ub}-RUra3p as bait.

4.1.5 Comparing the interaction strength between the N_{ub} fusion proteins and

    C_{ub}-fusions to Nhp6ap/Nhp6bp and Tpi1p

The isolated N_{ub} fusion candidates were further tested for their interaction with

Nhp6p and triose phosphate isomerase (Tpi1p) in the *JD52* strain. Droplet assays

were carried out to deduce the strength of the protein-protein interactions. Either

NHP6A-C_{ub}-RUra314 or NHP6B-C_{ub}-RUra314 was transformed into competent

yeast *JD52* cells. The cells containing the C_{ub} fusion vector were then transformed

with the N_{ub} fusion vectors to test for protein-protein interactions with Nhp6p. The

N_{ub} expression vector PACNX-N_{ub}IBC1 or PADNX-N_{ub}IBC1 was used to indicate

the background FOA resistance not due to the interaction between C_{ub} and N_{ub}

fusion proteins. These co-transformants were ten-fold serial diluted and titrated

onto WL, UWL and FWL plates to test for protein interactions. The WL plates

were used to select cells co-expressing both C_{ub} and N_{ub} fusion vectors.

Uracil-depleted WL plates (UWL plates) and FWL plates were used to select for

yeast cells with N_{ub} and C_{ub} fusion protein interactions in the split-ubiquitin

system.

Figure 4.1.5.1 shows the growth of the *JD52* cells containing Nhp6a-C_{ub}-RUra3p

and the 18 plasmid-linked N_{ub} fusion proteins on various plates. The cells showed

good growth on the FOA-containing plates, indicating interactions between the

respective N_{ub} fusion proteins and Nhp6a-C_{ub}-RUra3p. However, 10 of the 18

tested candidates, Nfs1p, Yml108Wp, Yol022Cp, Mlp1p, Sgf11p, Cbf1p,

Ylr301Wp, Acp1p, Mss4p and Ydr186Cp, still displayed good growth on UWL

plates, indicating their interactions with Nhp6ap were not as strong as those who

showed poor growth on UWL plates. Since our main interest focused on how

Nhp6p and its interacting partners regulate gene expression, we put our emphasis

on proteins that are relevant to the regulation of gene expression and DNA repair.

Based on their interaction strength with Nhp6p and the information obtained from

SGD, nine candidates were selected for future study. The nine candidates were

Rpb4p, Srp14p, Mlp1p, Cbf1p, Ddc1p, H4, Rtt107p, Tuf1p and Mss4p. Besides

the N_{ub} fusion candidates isolated from the *S. cerevisiae* genomic library screen, a

collection of N_{ub} fusions to more than 100 *S. cerevisiae* transcription factors was

also tested for interaction with Nhp6ap and Nhp6bp. Figure 4.1.5.2 shows that 15

of these transcription factors interacted with Nhp6p (only positive results are

shown). The functions of the 15 transcription factors are listed in Table 4.1.4.3.
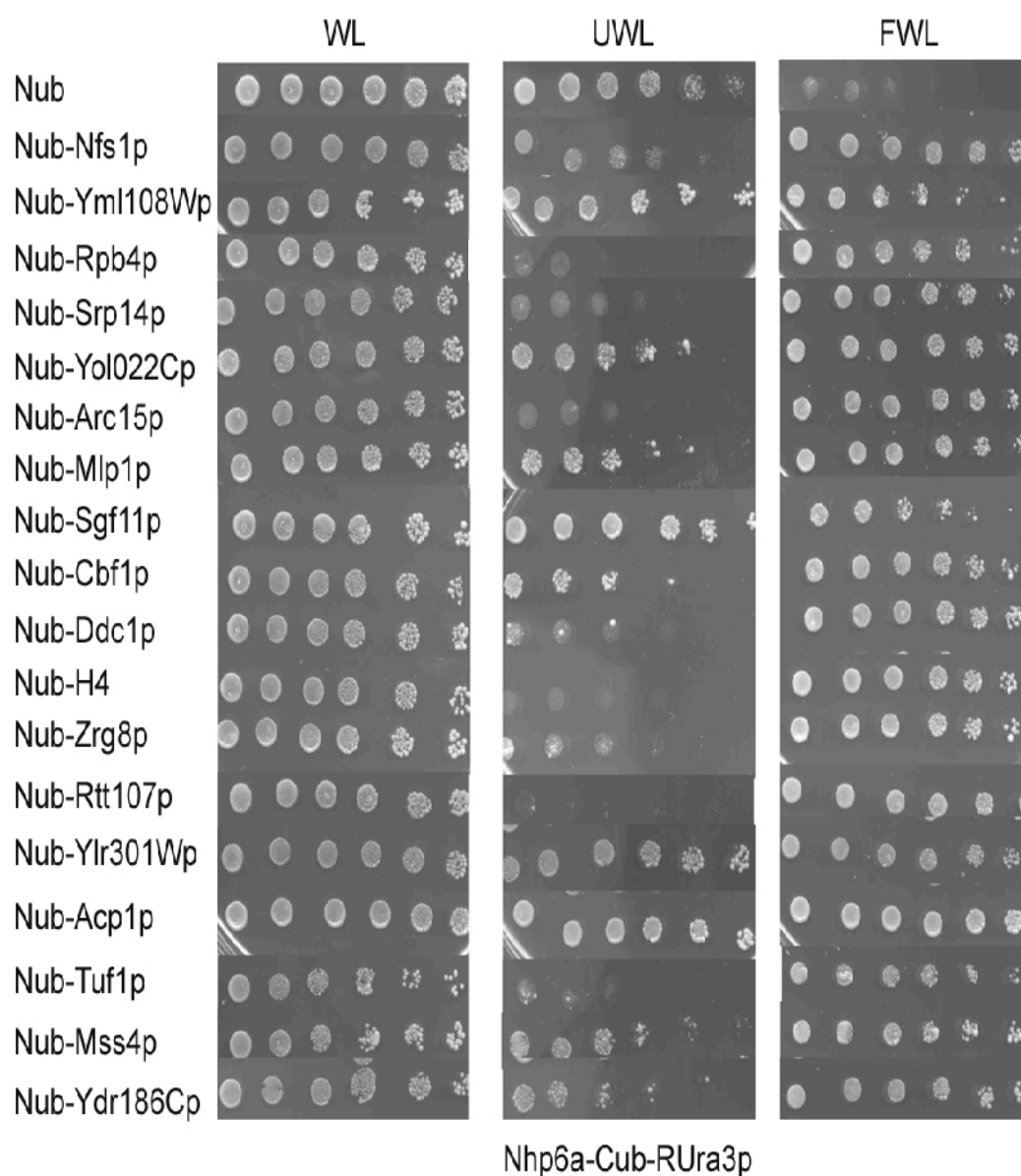
Figure 4.1.5.1: Droplet assays to determine the interaction between Nhp6a-C$_{ub}$-RUra3p and the N$_{ub}$ fusion candidates isolated from the *S. cerevisiae* **genomic library in** *JD52.* Ten-fold serial dilutions of cells co-expressing the depicted fusions were titrated onto the indicated plates to determine the strength of the protein interactions. Serial dilutions are in decreasing order from left (10^{-0}) to right (10^{-5}). Lack of growth on uracil-depleted plates and growth on FOA plates revealed protein-protein interaction in the split-ubiquitin system.
WL: plate lacking tryptophan and leucine. UWL: plate lacking uracil, tryptophan and leucine. FWL: FOA plate lacking tryptophan and leucine.
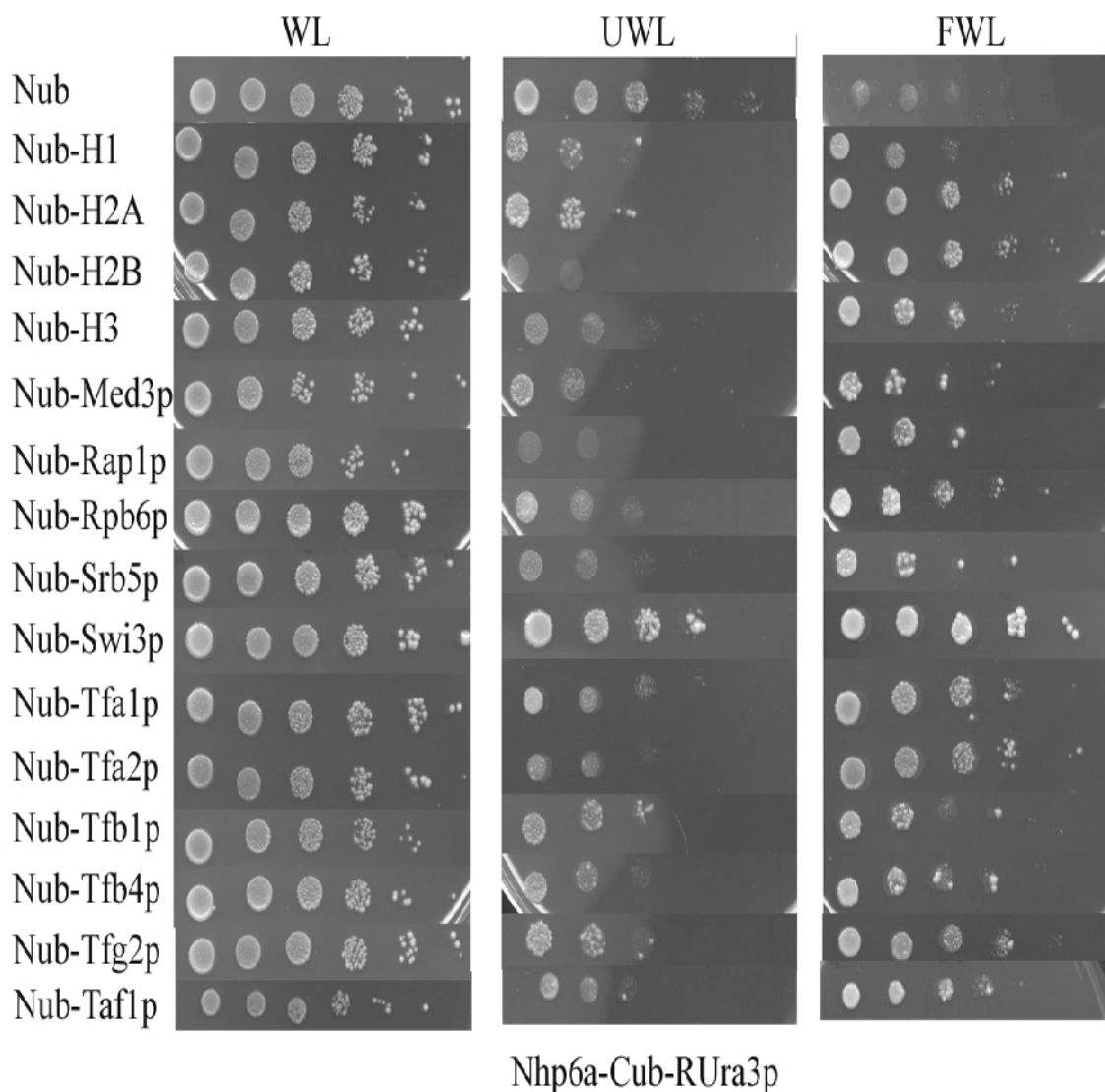
Figure 4.1.5.2: Droplet assays to determine the interaction between Nhp6a-C_{ub}-RUra3p and the N_{ub} fusion transcription factors in *JD52*. Ten-fold serial dilutions of cells coexpressing the depicted fusions were spotted onto the indicated plates to determine the interaction strength of the N_{ub} fusion proteins and C_{ub}-fused Nhp6a protein. Serial dilutions are in decreasing order from left (10^{-0}) to right (10^{-5}). Lack of growth on the uracil-deficient plates and growth on the FOA plates revealed protein-protein interaction in the split-ubiquitin system. Fifteen N_{ub}-fused transcription factors were confirmed to interact with Nhp6ap.

WL: plate lacking tryptophan and leucine. UWL: plate lacking uracil, tryptophan and leucine. FWL: plate with FOA and lacking tryptophan and leucine.

Although the interactions between Nhp6p and the N_{ub} fusion proteins can be seen from the growth on the plates, there is a need to compare the relative growth of the cells with a negative control in order to eliminate any background signals. Tpi1-C_{ub}-RUra3p was used as the negative control as triose phosphate isomerase is localized to the cytoplasm and was not expected to interact with any of the N_{ub} fusion candidates. The nine N_{ub} fusion candidates isolated from library screens and the 15 N_{ub} fusion transcription factors, which interacted with Nhp6p in *JD52* were tested for their interaction with Tpi1p. The interaction of the N_{ub}-fused proteins with Tpi1-C_{ub}-RUra3p is shown in Figure 4.1.5.3. The observation of cell growth on the UWL plates and lack of growth on the FWL plates indicated that the N_{ub} fusion proteins did not interact with Tpi1-C_{ub}-RUra3p. This allowed us to see if the interaction observed between the N_{ub} fusion proteins and Nhp6a-C_{ub}-RUra3p was specifically due to Nhp6a not due to the C_{ub} portion of the fusion. The N_{ub} fusion proteins were also tested for their interactions with Nhp6bp (data shown in appendix). All of the Nhp6a-interacting proteins interacted with Nhp6bp as well as with Nhp6ap.
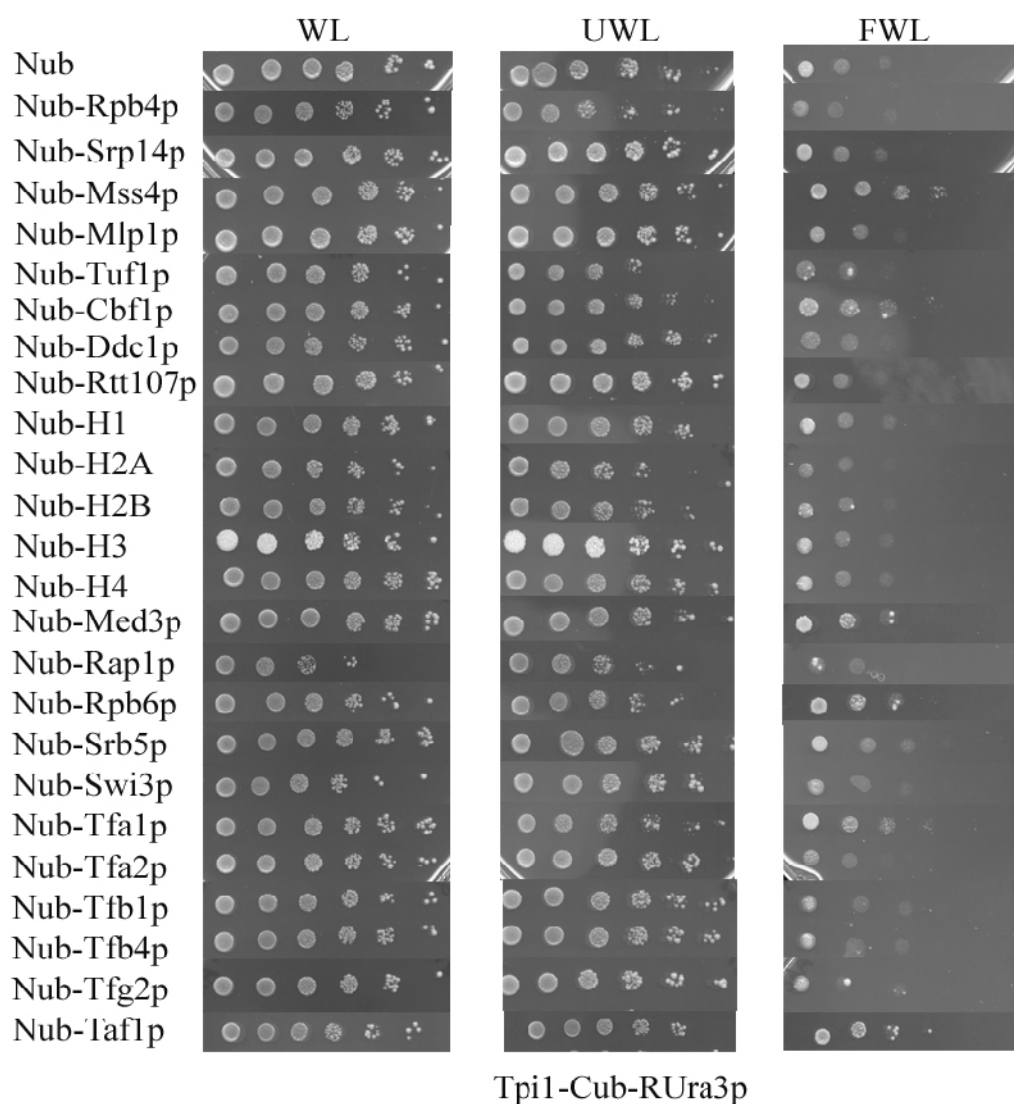
Figure 4.1.5.3: Droplet assays to determine the interaction between Tpi1p-C$_{ub}$-RUra3p and the N$_{ub}$ fusion candidates in *JD52*. Ten-fold serial dilutions of cells co-expressing the depicted fusions were spotted onto the indicated plates to determine the specificity of the protein interactions. Cell growth on uracil-depleted plates and lack of growth on FOA plates indicated the absence of protein-protein interactions between the N$_{ub}$ fusion candidates and Tpi1p.

WL: plate lacking tryptophan and leucine. UWL: plate lacking uracil, tryptophan and leucine. FWL: FOA plate lacking tryptophan and leucine.

The Split-Ubiquitin results for the 24 N_{ub}-fusion proteins with Nhp6a-C_{ub}-RUra3p, Nhp6b-C_{ub}-RUra3p and Tpi1-C_{ub}-RUra3p were quantified to allow comparisons with the negative controls and eliminate the background readings. Each double transformant was scored from zero to six according to the degree of growth on each plate by counting the number of serial dilutions for which there was substantial growth. For example, a score of six was given when there was growth of more than three colonies for all the six serial dilutions. When no growth occurred for all serial dilutions, it was scored as zero. These numbers were termed the raw scores for each of the double transformants. Formulae were then used for the conversion of the raw scores into relative scores, taking into account the background growth of the negative controls. To calculate the interaction strength on the plates lacking uracil, the following formula was used:

[(Score of double transformants on WL)-(Score of double transformants on UWL)] - [(Score of N_{ub} on WL)-(Score of N_{ub} on UWL)]

As shown in Figure 4.1.5.1 and Table 4.1.5.3, using the Nhp6a-C_{ub}-RUra3p/N_{ub}-Rpb4p co-transformant as example, the relative interaction score on UWL plate was: (6-0) - (6-6) = 6

For the FWL plates, the formula is stated below:

[(Score of double transformants on FWL)-(Score of double transformants on WL)] - [(Score of N_{ub} on FWL)-(Score of N_{ub} on WL)]

For example, as shown in Figure 4.1.5.1 and Table 4.1.5.3, the relative score of the Nhp6a-C_{ub}-RUra3p/N_{ub}-Rpb4p co-transformant on the FWL plate was:

(6-6) - (0-6) = 6

From the relative scores, an average score for the various N_{ub} fusion proteins interacting with Nhp6a-C_{ub}-RUra3p was calculated. This score was obtained by taking the average of the relative scores of the two plates:

Average score = (Relative score of UWL+ Relative score of FWL)/2

For example, as indicated in Table 4.1.5.3, the average score of the Nhp6a-C_{ub}-RUra3p/N_{ub}-Rpb4p co-transformant was: (6+6)/2 = 6

In order to eliminate the non-specific background growth with unrelated proteins, the average relative score for each double transformants was obtained by subtracting the average score of Tpi1-C_{ub}-RUra3p with the corresponding N_{ub} fusion protein. Using the Nhp6a-C_{ub}-RUra3p and N_{ub}-Rpb4p co-transformant as example, the average relative score was obtained by the following formula:

Final Score of Nhp6a-C_{ub}-RUra3p/N_{ub}-Rpb4p = (Average score of Nhp6a-C_{ub}-RUra3p/N_{ub}-Rpb4p) - (Average score of Tpi1-C_{ub}-RUra3p/N_{ub}-Rpb4p)

As indicated in Table 4.1.5.5, the final score for the Nhp6a-C_{ub}-RUra3p/ N_{ub}-Rpb4p = 6-0 = 6

The average interaction scores for the 24 N_{ub} fusion candidates with Nhp6a-C_{ub}-RUra3p and Tpi1-C_{ub}-RUra3p are listed in Table 4.1.5.3 and Table

4.1.5.4, respectively. The final scores for the 24 N_{ub} fusion proteins with Nhp6a-C_{ub}-RUra3p are shown in Table 4.1.5.5.

Using the Split-Ubiquitin system, I had isolated 24 N_{ub} fusion candidates which were potential interacting partners of Nhp6p.

JD52	Nhp6a-C_{ub}-RUra3p Raw scores			Nhp6a-C_{ub}-RUra3p Relative scores		
Library screens	WL	UWL	FWL	UWL	FWL	average
A6.2 Rpb4	6	0	6	6	6	6
A10.1 Srp14	6	0	6	6	6	6
A14.1 Mlp1	6	0	6	6	6	6
A15.1 Cbf1	6	3	6	3	6	4.5
A16.1 Ddc1	6	1	5	5	5	5
A17.4 H4	6	0	6	6	6	6
A20.1 Rtt107	6	0	6	6	6	6
B15.1 Tuf1	6	1	6	5	6	5.5
B23.2 Mss4	6	4	6	2	6	4
N_{ub}	6	6	0	0	0	0
Transcription factor	WL	UWL	FWL	UWL	FWL	average
H1 (HHO1)	5	2	3	1	4	2.5
H2A(HTA1)	5	2	4	1	5	3
H2B(HTB1)	5	0	4	3	5	4
H3(HHT1)	5	0	4	3	5	4
Med3	4	0	3	2	5	3.5
Rap1	5	0	3	3	4	3.5
Rpb6	5	1	4	2	5	3.5
Srb5	5	0	2	3	3	3
Swi3	6	4	5	0	5	2.5
Taf1	5	1	4	2	5	3.5
Tfa1	5	0	4	3	5	4
Tfa2	5	1	4	2	5	3.5
Tfb1	5	2	2	1	3	2
Tfb4	5	1	4	2	6	4
Tfg2	6	2	4	2	4	3
N_{ub}	6	4	0	0	0	0

Table 4.1.5.3: Average scores between the interactions of the C_{ub} fusion Nhp6ap and the 24 N_{ub} fusions

JD52	Tpi1-C_{ub}-RUra3p Raw scores				Tpi1-C_{ub}-RUra3p Relative scores		
Library screens	WL	UWL	FWL		UWL	FWL	average
A6.2 Rpb4	5	5	0		-1	1	0
A10.1 Srp14	6	6	1		-1	1	0
A14.1 Mlp1	5	5	1		-1	2	0.5
A15.1 Cbf1	5	5	1		-1	2	0.5
A16.1 Ddc1	5	5	1		-1	0	-0.5
A17.4 H4	6	6	1		0	0	0
A20.1 Rtt107	5	6	1		-2	2	0
B15.1 Tuf1	5	4	1		0	1	0.5
B23.2 Mss4	5	5	2		-1	3	1
N_{ub}	6	5	1		0	0	0
Transcription factor	WL	UWL	FWL		UWL	FWL	average
H1 (HHO1)	5	5	1		-1	1	0
H2A(HTA1)	5	4	0		0	0	0
H2B(HTB1)	5	4	1		0	1	0.5
H3(HHT1)	5	6	1		-2	1	-0.5
Med3	6	5	2		0	1	0.5
Rap1	4	3	0		0	1	0.5
Rpb6	5	5	2		-1	2	0.5
Srb5	6	5	1		0	0	0
Swi3	4	5	1		-2	2	0
Taf1	6	5	2		0	1	0.5
Tfa1	6	4	2		1	1	1
Tfa2	6	5	0		0	-1	-0.5
Tfb1	6	6	1		-1	0	-0.5
Tfb4	6	6	1		-1	0	-0.5
Tfg2	5	6	1		-2	1	-0.5
N_{ub}	6	5	1		0	0	0

Table 4.1.5.4: Average scores between the interactions of the C_{ub} fusion Tpi1p and the 24 N_{ub} fusions

Library screen candidates	Tpi1p-C$_{ub}$-Rura3p	Nhp6a-C$_{ub}$-Rura3p	NHP6a-Tpi1p
A6.2 Rpb4	0	6	6
A10.1 Srp14	0	6	6
A14.1 Mlp1	0.5	6	5.5
A15.1 Cbf1	0.5	4.5	4
A16.1 Ddc1	-0.5	5	5.5
A17.4 H4 (HHF2)	0	6	6
A20.1 Rtt107	0	6	6
B15.1 Tuf1	0.5	5.5	5
B23.2 Mss4	1	4	3
N$_{ub}$ fusion transcription factors	Tpi1p-C$_{ub}$-Rura3p	Nhp6a-C$_{ub}$-Rura3p	NHP6a-Tpi1p
H1 (HHO1)	0	2.5	2.5
H2A(HTA1)	0	3	3
H2B(HTB1)	0.5	4	3.5
H3(HHT1)	-0.5	4	4.5
Med3	0.5	3.5	3
Rap1	0.5	3.5	3
Rpb6	0.5	3.5	3
Srb5	0	3	3
Swi3	0	2.5	2.5
Taf1	0.5	3.5	3
Tfa1	1	4	3
Tfa2	-0.5	3.5	4
Tfb1	-0.5	2	2.5
Tfb4	-0.5	4	4.5
Tfg2	-0.5	3	3.5

Table 4.1.5.5: The final scores for Nhp6a-C$_{ub}$-RUra3p and 24 N$_{ub}$ fusions

4.2 GST pull-down assays confirmed the interactions with Nhp6bp

The Split-Ubiquitin system indicates a close proximity of two proteins in the cell but not necessarily a direct protein-protein interaction (Lehming, 2002). Therefore, the newly identified proteins had to be tested for their interactions with Nhp6p *in vitro*. In our split-ubiquitin screens, nine N_{ub} fusion candidates were identified from the *S. cerevisiae* genomic library and 15 candidates were isolated from the collection of N_{ub}-fusions to yeast transcription factors as novel interacting partners of Nhp6p. These 24 Nhp6p-interacing candidates were further tested for their interactions with Nhp6p in the GST pull-down assay.

4.2.1 N_{ub} fusion proteins were expressed in yeast *JD52* cells

Before the GST pull-down analysis was performed, all the 24 N_{ub} fusion candidates had been transformed into *JD52* to test for their expression in yeast cells. *JD52* cells containing the N_{ub} fusion vectors were grown in 10 ml leucine-deficient broth to logarithmic stage. Cell pellets were used directly for SDS polyacrylamide gel electrophoresis. Western blot was performed using an antibody against the haemagglutinin (HA) tag present in the N_{ub} fusions. The Western blot results indicated that 17 of the 24 N_{ub} fusion proteins showed detectable expression in yeast cells. Seven of the 24 N_{ub} fusion candidates (Ddc1p, Mlp1p, Mss4p, Tuf1p, Rpb6p, Srb5p, Tfa2p), however, were not detected in *JD52* cells. Figure 4.2.1 shows the expression of the 11 N_{ub} fusion proteins that were precipitated by GST- Nhp6bp in subsequent experiments.
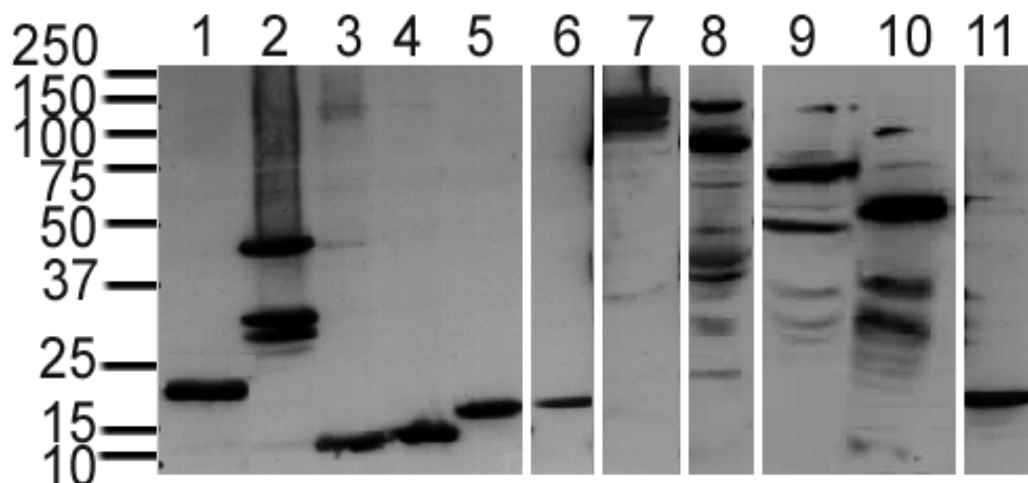
Figure 4.2.1: Expression of 11 N$_{ub}$ fusion proteins in *JD52*. *JD52* cells were transformed with the various N$_{ub}$ plasmids isolated in split-ubiquitin screens. Cells were cultured in leucine-deficient media. Cell pellets were resuspended with loading buffer and separated by SDS-PAGE. Western blot assay was performed using haemagglutinin primary antibody. Protein sizes were indicated as left. Lane 1: N$_{ub}$-Rpb4p (99-221AA), lane 2: N$_{ub}$-Rtt107p (724-1070AA), lane 3: N$_{ub}$-Srp14p (119-146AA), lane 4: N$_{ub}$-H4 (1-103AA), lane 5: N$_{ub}$-H2A (FL), lane 6: N$_{ub}$-H2B (FL), lane 7: N$_{ub}$-Tfb1-DsRed1p (FL), lane 8: N$_{ub}$-Med3-DsRed1p (FL), lane 9: N$_{ub}$-Tfb4-DsRed1p (FL), lane 10: N$_{ub}$-Tfg2 (FL), lane 11: N$_{ub}$-H3 (FL).

4.2.2 GST pull-down assays confirmed the interaction between bacterial expressed GST-Nhp6bp and yeast expressed candidate proteins

The 17 N$_{ub}$ fusion proteins, which showed detectable expression in *JD52* cells were tested for their interaction with GST-Nhp6bp in GST pull-down assays. Full length *NHP6B* was cloned into the pGEX-5X-1 vector. GST and GST-Nhp6bp were expressed in *E. coli BL21* and purified with the help of glutathione beads.

Yeast extracts expressing the HA-tagged N_{ub} fusion proteins were incubated with the glutathione beads on which GSTp or GST-Nhp6bp had been immobilized. After precipitation, the beads were washed and the bound proteins separated by SDS-PAGE. The precipitates were detected by Western blot using a monoclonal anti-HA antibody. Figure 4.2.2, panels (A) to (D), showed the 11 of the 17 N_{ub} fusion proteins were precipitated by GST-Nhp6bp, but not GSTp alone, confirming the results from the split-ubiquitin assays. The remaining six N_{ub} fusion proteins that had been isolated in the split-ubiquitin screens and displayed detectable expression in yeast cells (Cbf1p, H1, Rap1p, Swi3p, Taf1p and Tfa1p) were not precipitated with GST-Nhp6bp.

The 11 proteins which were confirmed to interact with Nhp6bp *in vitro* are all involved in gene regulation. Rpb4p is the fourth largest subunit of RNA polymerase II and it plays a role in transcription-coupled DNA repair and mRNA export under stress (Li and Smerdon, 2002; Farago et al., 2003). Rtt107p can establish silent chromatin and has also been implicated in DNA repair (Zappulla et al., 2006). Med3p is a component of the global transcriptional Mediator, which plays an essential role in basal and activated transcription (Kornberg, 2005). Srp14p is a component of the Signal Recognition Particle. It targets proteins to membranes and regulates RNA Pol III transcription (Grosshans et al., 2001). Tfb1p and Tfb4p are two components of the general transcription factor TFIIH. They are also involved in DNA repair (Bardwell et al., 1994). Tfg2p is an essential subunit of the general transcription factor TFIIF, which plays a role in the

initiation and elongation of transcription (Chung et al., 2003). The remaining four

proteins are yeast core histones H2A, H2B, H3 and H4, which are important for

transcription, DNA replication and DNA repair (Matsubara et al., 2007).

In addition to further confirmed 11 of the N_{ub} fusion candidates interacted with

Nhp6bp *in vitro*, the GST pull-down assays provided additional information about

the interactions between Nhp6p and its interacting partners. In the case of

N_{ub}-Rtt107 (724-1070)p, a C-terminally truncated degradation product of

approximately 30 kd was observed in the input but not in the pull down (Figure

4.2.2A, compare lane 4 with lane 8). This indicated that the C-terminal BRCT

domain, which is located between residue 840 and residue 910, could be required

for the interaction of Rtt107p with Nhp6bp. For N_{ub}-Med3-DsRed1p (DsRed1p

indicates that a red fluorescent protein of approximately 30 kd was immediately

followed Med3p), a degradation product of approximately 47 kd, which

corresponded in size to N_{ub}-Med3p, was precipitated with GST-Nhp6bp. However,

the shorter degradation products were not precipitated (Figure 4.2.2A, compare

lanes 6 with lane 9), indicating that the C-terminus of Med3p was required for the

interaction with Nhp6bp. The input of N_{ub}-Srp14 (119-146)p showed a

degradation product missing approximately 2 kd, which was not precipitated by

GST-Nhp6bp (Figure4.2.2B, compare lane 1 with lane 7), indicating that the

C-terminus of Srp14p was required for the interaction with Nhp6bp. The input of

N_{ub}-Tfb4-DsRed1p showed a degradation product of approximately 30 kd, which

was precipitated with GST-Nhp6bp (Figure 4.2.2B, compare lane 6 with lane 9),

indicating that the C-terminus of Tfb4p was not required for the interaction with GST-Nhp6bp. The input of N_{ub}-H2B showed a degradation product missing approximately 1 kd, which was not precipitated by GST-Nhp6bp (Figure 4.2.2C, compare lanes 4 with lane 8). This indicates that the C-terminus of H2B might be required for the interaction with Nhp6bp. A summary of the GST pull-down results is shown in Table 4.2.2. Y indicates positive GST pulldown results, N indicates negative pulldown results. Blank row means that the GST pulldown assay did not carry out since the HA tagged candidate proteins did not show detectable expression in yeast.
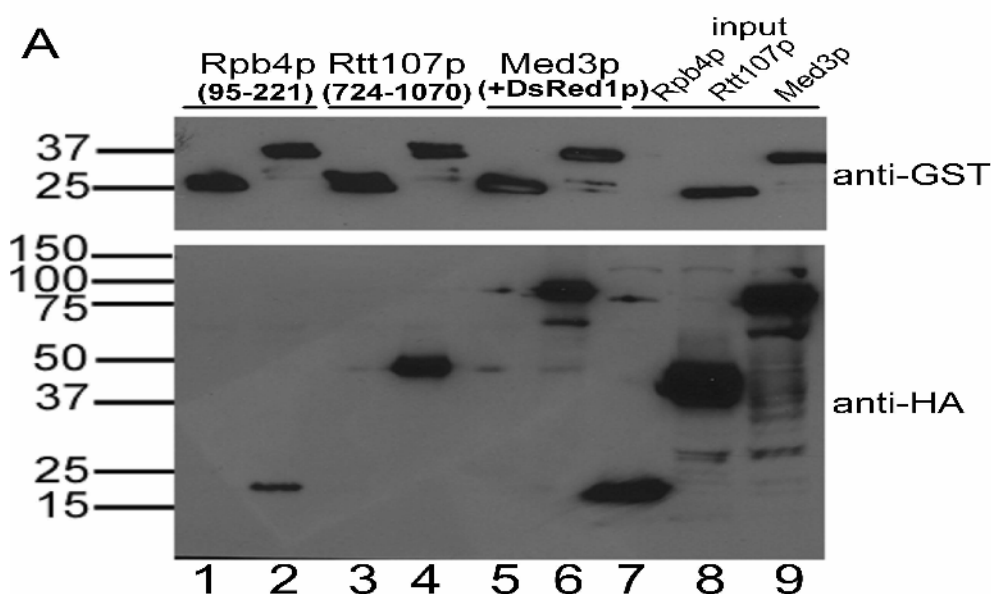
Figure 4.2.2 (A): GSTp was incubated with N_{ub}-Rpb4 (95-221)p (lane 1), N_{ub}-Rtt107 (724-1070)p (lane 3) and N_{ub}-Med3-DsRed1p (lane 5), respectively. GST-Nhp6bp was incubated with N_{ub}-Rpb4 (95-221)p (lane 2), N_{ub}-Rtt107 (724-1070)p (lane 4) and N_{ub}-Med3-DsRed1p(lane 6), respectively. 10% of the input of N_{ub}-Rpb4 (95-221)p, N_{ub}-Rtt107 (724-1070)p and N_{ub}-Med3-DsRed1p (lower panel, lane 7-9), 10% of the input of GSTp and GST-Nhp6bp (upper panel, lane 8-9), was directly loaded onto the gel.
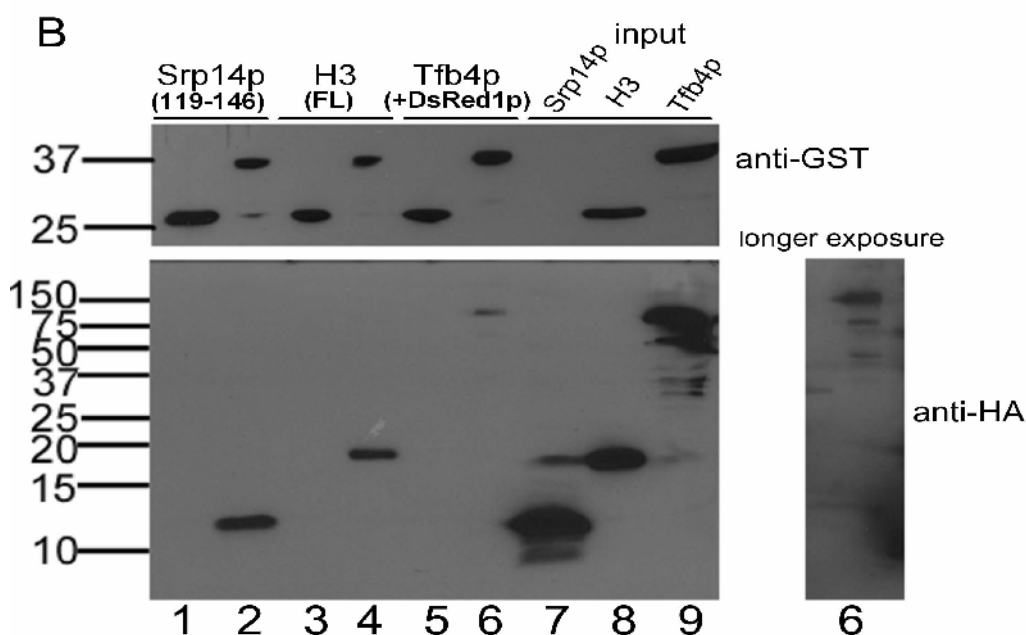
Figure 4.2.2(B): GSTp was incubated with N_{ub}-Srp14(119-146)p (lane 1), N_{ub}-H3 (lane 3) and N_{ub}-Tfb4-DsRed1p (lane 5), respectively. GST-Nhp6bp was incubated with N_{ub}-Srp14 (119-146)p (lane 2), N_{ub}-H3 (lane 4) and N_{ub}-Tfb4-DsRed1p (lane 6), respectively. 10% of the input of N_{ub}-Srp14(119-146)p, N_{ub}-H3 and N_{ub}-Tfb4-DsRed1p (lower pane, lane 7-9), 10% of the input of GSTp and GST-Nhp6bp (upper panel, lane 8-9), was directly loaded onto the gel.
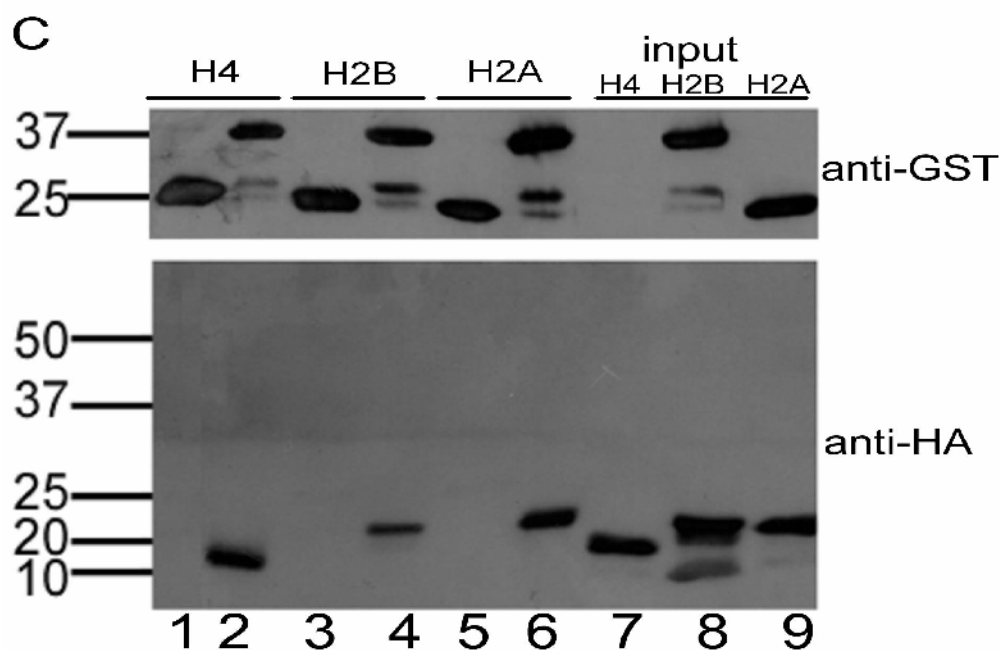
Figure 4.2.2 (C) GSTp was incubated with N_{ub}-H4 (lane 1), N_{ub}-H2B (lane 3) and N_{ub}-H2A (lane 5), respectively. GST-Nhp6bp was incubated with N_{ub}-H4 (lane 2), N_{ub}-H2B (lane 4) and N_{ub}-H2A (lane 6), respectively. 10% of the input of N_{ub}-H4, N_{ub}-H2B and N_{ub}-H2A (lower panel, lane 7-9), 10% of the input of GSTp and GST-Nhp6bp (upper panel, lane 8-9), was directly loaded onto the gel.
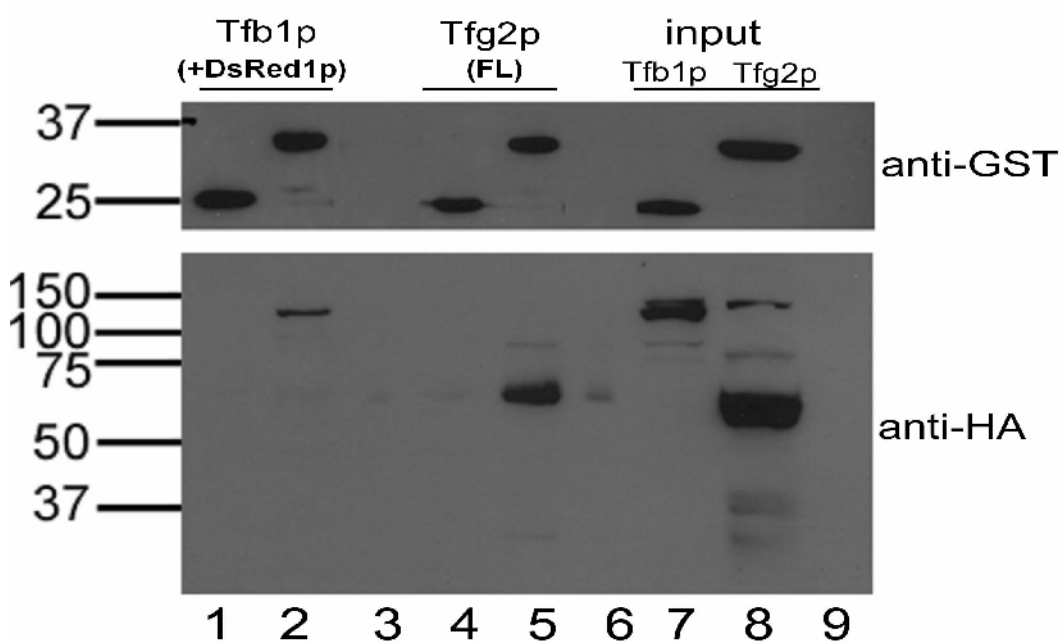
Figure 4.2.2 (D) GSTp was incubated with N_{ub}-Tfb1-DsRed1p (lane 1) and N_{ub}-Tfg2p (lane 4), respectively. GST-Nhp6bp was incubated with N_{ub}-Tfb1-DsRed1p (lane 2) and N_{ub}-Tfg2p (lane 5), respectively. 10% of the input of N_{ub}-Tfb1-DsRed1p and N_{ub}-Tfg2p (lower panel, lane 7-8), 10% of the input of GSTp and GST-Nhp6bp (upper panel, lane 7-8), was directly loaded onto the gel. Lane 3, 6 and lane 9 are empty.

| Gene Name	FL size (AA)	Fragments (AA)	System Deletion	Sizes in WB (+HA: 6KD): KD	Expression in yeast	Pull down by Nhp6Bp
RPB4	221	99-221	Viable	21	Y	Y
RTT107	1070	721-1070	Viable	48	Y	Y
SRP14	146	119-146	Inviable	9	Y	Y
CBF1	351	1-176	Viable	27	Y	N
DDC1	612	515-612	Viable	18	N	
MLP1	1875	1752-1855	Viable	18	N	
HHF2 (H4)	103	1-103	Inviable	18	Y	Y
MSS4	779	739-779	Inviable	11	N	
TUF1	437	174-437	Viable	38	N	
HHO1 (H1)	258	FL	Inviable	34	Y	N
HTA1 (H2A)	132	FL	Inviable	20	Y	Y
HTB1 (H2B)	132	FL	Inviable	20	Y	Y
HHT1 (H3)	136	FL	Inviable	21	Y	Y
MED3 (PGD1)	397	FL + DsRed (30K)	Viable	79	Y	Y
RAP1	827	FL	Inviable	99	Y	N
RPB6	155	FL	Inviable	26	N	
SRB5	307	FL	Viable	40	N	
SWI3	825	FL	Viable	99	Y	N
TAF1(YGR274C)	1066	FL	Inviable	127	Y	N
TFA1	482	FL	Inviable	61	Y	N
TFA2	328	FL	Inviable	43	N	
TFB1	642	FL + DsRed (30K)	Inviable	78	Y	Y
TFB4	338	FL + DsRed (30K)	Inviable	43	Y	Y
TFG2	400	FL	Inviable	53	Y	Y

Table 4.2.2: Summary of the Western blot and GST pull-down assays with the isolated N$_{ub}$ fusion proteins.

4.3 Phenotypes of strains lacking the genes for the non-essential Nhp6p-interacting proteins

To explore the function of Nhp6p and its interacting partners, the non-essential candidate genes were deleted. Nhp6p is encoded by two redundant, highly homologous genes in *Saccharomyces cerevisiae*, *NHP6A* and *NHP6B*. Only the deletion of both genes causes detectable phenotypes. The *JD52ΔNHP6* strain was generated by the successive deletion of the entire *NHP6A* and *NHP6B* ORFs with *hisG* knockout constructs as described (Laser et al., 2000). Since mutagenesis of essential genes is more time-consuming and not commercially available, we focused on the non-essential candidates. *RPB4*, *RTT107* and *MED3* are three non-essential genes and their deletions do not affect yeast growth under normal conditions. The deletion of *RPB4*, *RTT107* or *MED3* was performed in the yeast strain *JD52* by homologous recombination using *HIS3*-based knockout vectors containing approximately 500bp of the respective promoters and terminators.

Previous studies indicated that cells lacking *NHP6* are sensitive to elevated temperature and to 6-azauracil (Eriksson et al., 2004). 6-azauracil (6-AU) acts as an inhibitor for transcriptional elongation. Treatment of yeast cells with 6-AU leads to the depletion of intracellular uracil. The reduction in uracil is not itself lethal, but it can block yeast growth when combined with mutations that affect transcriptional elongation. 6-AU sensitivity can be used as an indicator to test for mutations that affect transcriptional elongation thus. Cells lacking *NHP6*, *RPB4*, *RTT107* or *MED3* were titrated on complete medium- or 6-AU containing plates to test for 6-AU sensitivity. The plates were incubated at 28℃ for 3 days.

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As shown in Figure 4.3, yeast cells lacking *NHP6* were sensitive to 6-AU (compare line 1 and line 2), indicating that Nhp6p supported the elongation of transcription. *S. cerevisiae* cells lacking *RPB4* also showed reduced growth on 6-AU plates (Figure 4.3, compare lines 1 and 3). This suggested that RNA polymerase II lacking Rpb4p was defective for transcription elongation. Figure 4.3 further shows that *S. cerevisiae* cells lacking *RTT107* and *MED3* were not sensitive to 6-AU (compare lines 1, 4, 5), indicating that Rtt107p and Med3p were not required for efficient transcription elongation. Next, we tested whether the expression of Nhp6b-C$_{ub}$-RUra3p and the over-expression of Rpb4p in the *NHP6* deletion strain were able to rescue the 6-AU sensitivity. *NHP6* deletion strains were transformed with NHP6B-C$_{ub}$-RUra3 or YEplac181-RPB4 and plated on complete medium- or 6-AU containing plates. *NHP6* deletion strains that expressed Nhp6b-C$_{ub}$-Rura3p were able to grow in the presence of 6-AU (Figure 4.3, line 6), indicating that Nhp6b-C$_{ub}$-RUra3p was functional and able to complement the *NHP6* deletion. The overexpression of Rpb4p suppressed the 6-AU sensitivity of *S. cerevisiae* cells lacking *NHP6* (Figure 4.3, compare line 2 with line 7), indicating that Nhp6p might have exerted its positive role in transcription elongation through Rpb4p.

The remaining eight Nhp6p-interacting proteins whose interactions had been confirmed in the GST pull-down assay were essential and gene deletions could not be tested for 6-AU sensitivity thus. Two of the eight Nhp6p-interacting proteins, Tfb1p and Tfb4p, are essential components of the general transcription factor

TFIIH. TFIIH phosphorylates RNA polymerase II (Hirose and Ohkuma, 2007).

Tfg2p is the middle subunit of the general transcription factor TFIIF. TFIIF and

histones (H2A, H2B, H3, H4) are known for their supporting role in transcription

elongation (Conaway et al., 2000; Li et al., 2007).
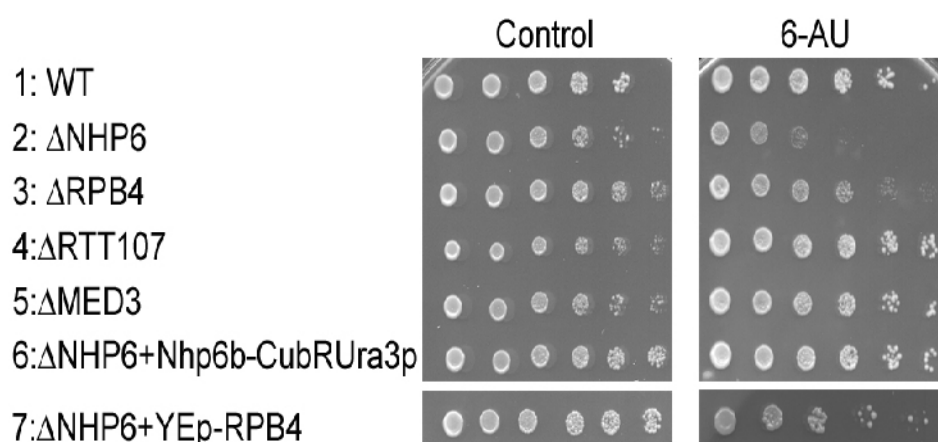
Figure 4.3: Nhp6bp and Rpb4p support transcription elongation.
Deletion of *NHP6* and *RPB4* caused sensitivity to 6-AU. *S. cerevisiae JD52*
wild-type (WT) and the indicated isogenic deletion strains were ten-fold serial
diluted and titrated onto plates lacking or containing 6-AU (lines 1 to 5).
Nhp6b-C_{ub}-RUra3p complemented the *NHP6* deletion strain. *S. cerevisiae JD52*
cells lacking *NHP6* transformed with the plasmid expressing Nhp6b-C_{ub}-RUra3p
were ten-fold serial diluted and titrated onto plates lacking or containing 6-AU
(line 6). The *NHP6* deletion strain transformed with a multi-copy vector
over-expressing Rpb4p was ten-fold serial diluted and titrated onto plates lacking
or containing 6-AU (line 7). The plates were incubated at 28°C for 3 days.

4.4 Nhp6p and Med3p repressed *ZDS1* transcription by controlling the local

    subunit composition of RNA Pol II

4.4.1 Nhp6p and its interacting proteins repressed expression of *ZDS1*

Nhp6p has multiple roles in the expression of a set of RNA polymerase II-transcribed genes in *S. cerevisiae*, such as *CUP1*, *CYC1*, *DDR2*, *GAL1*, *CHA*, *HO* and *FRE2* (Paull et al., 1996; Laser et al., 2000; Moreira and Holmberg, 2000; Fragiadakis et al., 2004). Previous studies suggested that *ZDS1* transcription was down-regulated by Nhp6p (Kerkmann, 2000). In order to see if Nhp6p-interacting proteins affected gene-specific transcriptional effects of Nhp6p, we determined the expression of the *ZDS1* gene in *S. cerevisiae* cells lacking *NHP6*, *RPB4*, *RTT107* and *MED3*.

The transcription of *ZDS1* in the wild type strain and in the isogenic deletion strains is shown in Figure 4.4.1. The top schematic presentation of *ZDS1* indicates the two fragments in the *ZDS1* promoter and ORF region which are amplified in ChIP analysis described in next section or in the real-time PCR assays, respectively. An approximately 200 bp fragment, which spans from position +1274 to +1458 at the *ZDS1* coding region, was amplified in the real-time PCR assays. The bottom bar graph in Figure 4.4.1 shows the transcription levels of the *ZDS1* gene in the wild type strain and in the isogenic deletion strains. mRNA quantification by real-time PCR showed that, consistent with previous reports (Kerkmann, 2000; Bourbonnais et al., 2001), the deletion of *NHP6* and *RPB4*

increased *ZDS1* mRNA transcription approximately four-fold (Figure 4.4.1,

bottom). This indicated that although Nhp6p and Rpb4p had generally positive

effects on transcription elongation, they acted as repressors of the *ZDS1* gene.

Transcription of the *ZDS1* gene was also increased approximately four-fold by the

deletion of *RTT107* and *MED3* (Figure 4.4.1), indicating that Rtt107p and Med3p

repressed transcription of the *ZDS1* gene approximately four-fold as well.
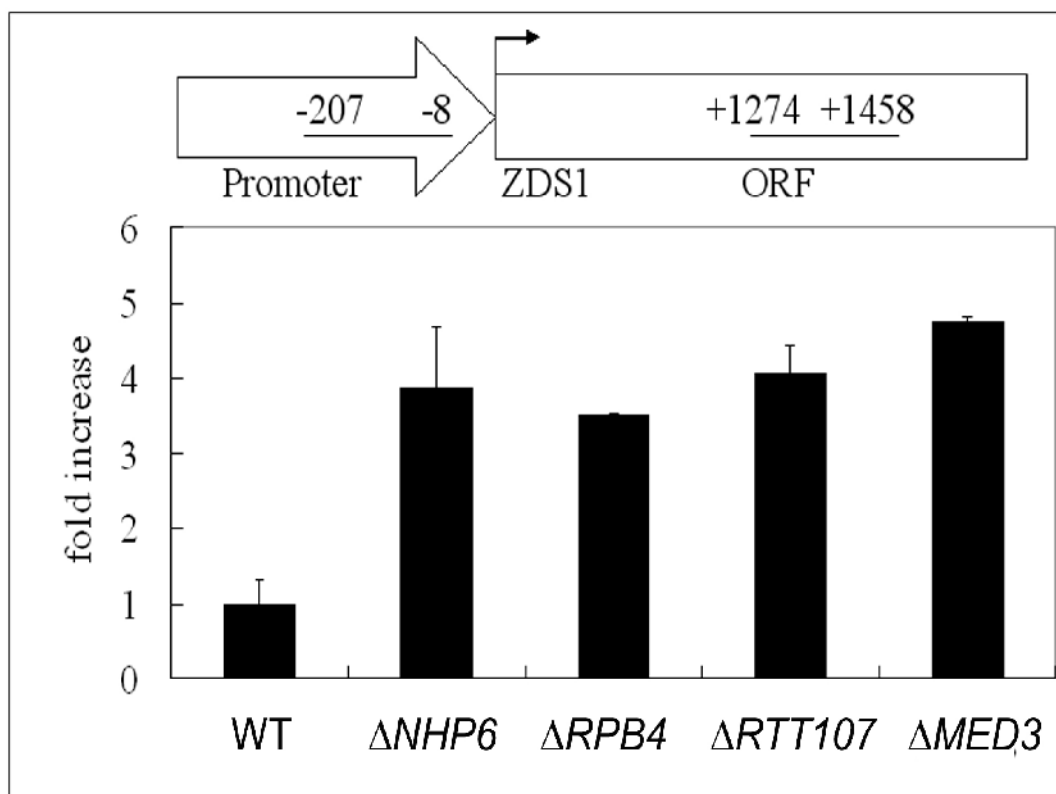
Figure 4.4.1: Nhp6p, Rpb4p, Rtt107p and Med3p repressed transcription of the *ZDS1* gene.

The wild-type *S. cerevisiae* strain *JD52* and the isogenic deletion strains *JD52ΔNHP6, JD52ΔRPB4, JD52ΔRTT107* and *JD52ΔMED3* were grown in complete glucose media to $OD_{600nm} = 1.0$. Total RNA was isolated and the mRNA levels of *ZDS1* were quantified by real-time PCR using primers specific to the *ZDS1* ORF region. The upper schematic presentation of *ZDS1* indicates the two fragments in the promoter and ORF regions amplified for the ChIP experiments and for the real-time PCR experiments, respectively. The bottom bar graph shows the relative *ZDS1* mRNA levels in wild-type and isogenic deletion strains.

4.4.2 Myc-tagged fusions of Nhp6bp and its interacting partners were functional

In order to study chromosomal localization by chromatin immunoprecipitation (ChIP), Nhp6bp, Rpb4p, Med3p, Rtt107p, Tfb1p, Tfb4p and Rpb2p were fused to nine myc tags and expressed under the control of their own respective promoters. Complementation experiments were performed to test whether the myc-tagged fusions were functional.

We first determined whether Nhp6b-myc9p and Rpb4-myc9p rescued the temperature-sensitivity of their respective deletion strains. Cells lacking *NHP6* and cells lacking *RPB4* both showed temperature sensitivity at 38℃ (Figure 4.4.2A, compare lines 1, 2 and 5), consistent with previous reports (Eriksson et al., 2004; Choder 2004). Cells transformed with the RS314 vector, which contained the *TRP1* gene conferring tryptophan prototrophy to the transformed cells, were used as control. Cells transformed with NHP6B-C_{ub}-RUra314 were included as positive control since this construction had been confirmed to be functional (Figure 4.4.2). The expression of Nhp6b-myc9p from a single-copy vector under the control of its own promoter complemented the temperature sensitivity of an *NHP6* deletion strain (Figure 4.4.2A, compare lines 1-3), indicating that Nhp6b-myc9p was functional. Figure 4.4.2A further shows that Rpb4-myc9p was able to complement the temperature sensitivity of an *RPB4* deletion strain as well (compare lines 1, 5 and 6), indicating that Rpb4-myc9p was also functional.

Since cells lacking *RTT107* or *MED3* displayed no growth phenotypes, we

expressed Rtt107-myc9p and Med3-myc9p from their chromosomal loci to see

whether the increased *ZDS1* transcription in the deletion strains was reduced by

the presence of Rtt107-myc9p and Med3-myc9p. Figure 4.4.2B shows that

Med3-myc9p repressed *ZDS1* transcription in a strain lacking *MED3*, while

Rtt107-myc9p repressed *ZDS1* transcription in a strain lacking *RTT107*, indicating

that both fusions were functional and able to complement the respective gene

deletion strains. Rpb2p, Tfb1p and Tfb4p were chromosomally myc-tagged as

well. As all three proteins are essential, Rbp2-myc9p, Tfb1-myc9p and

Tfb4-myc9p were apparently able to complement the essential functions of Rpb2p,

Tfb1p and Tfb4p.
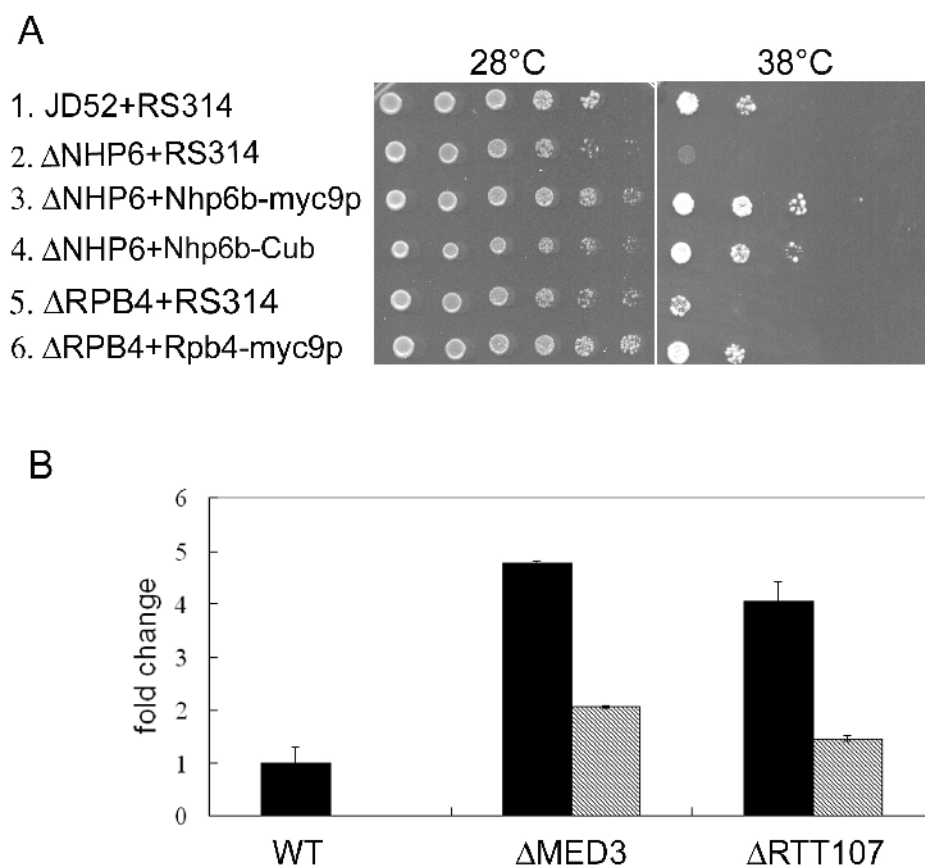
Figure 4.4.2: Function of myc-tagged Nhp6bp, Rpb4p, Med3p and Rtt107p.
(A) Expression of Nhp6b-myc9p and Rpb4-myc9p from single-copy vectors under the control of their own promoters in their respective gene deletion strains suppressed temperature sensitivity. Wild-type *JD52* cells transformed with the empty vector RS314 and the indicated deletion strains transformed with RS314 and RS314 expressing Nhp6b-myc9p, Nhp6b-C$_{ub}$-RUra3p and Rpb4-myc9p, respectively, were ten-fold serial diluted, plated and incubated at 28°C and 38°C for 3 days. (B) Expression of Med3-myc9p and Rtt107-myc9p under the control of their own promoters complemented repression of *ZDS1* transcription in the respective gene deletion strains. Total RNA was isolated from *JD52* containing RS314 (WT), *JD52ΔMED3* containing RS314, *JD52* expressing Med3-myc9p from the *MED3* locus in place of endogenous Med3p, *JD52ΔRTT107* containing RS314 and *JD52* expressing Rtt107-myc9p from the *RTT107* locus in place of endogenous Rtt107p. Black bars represent cells containing RS314 and striped bars represent cells expressing myc9 fusions in place of the respective endogenous protein. Total RNA was analyzed by quantitative real-time PCR using primers specific to *ZDS1* ORF.

4.4.3 The deletion of *RPB4*, *RTT107* and *MED3* did not affect the expression

    of Nhp6bp

In order to determine whether the expression of Nhp6bp or any of its interacting partners was affected by any of the deletions, myc-tagged Nhp6b, Rpb4p, Med3p, Rpb2p and Rtt107p were expressed in wild-type *JD52* and in the isogenic deletion strains. Equal amounts of cells were analyzed by Western blot using an anti-myc antibody. Figure 4.4.3A shows that expression of Nhp6b-myc9p was not reduced by the deletion of *RPB4*, *RTT107* or *MED3* (compare lane 1 with lanes 2-4), indicating that the deletion of the genes encoding the Nhp6p-interacting proteins did not exert their transcriptional effects by reducing the expression of Nhp6p. Figure 4.4.3A also shows that the expression of Rpb4-myc9p was not affected by the deletion of *RTT107* or *MED3* (compare lane 6 with lanes 8 and 9), indicating that the repressed transcription of *ZDS1* by Rtt107p and Med3p was not due to decreased amounts of Rpb4p. An increased expression of Rpb4-myc9p was observed in the *NHP6* deletion strain (Figure 4.4.3A, compare lane 6 with lane7). However, since the real-time PCR results had indicated that the deletion of *RPB4* increased *ZDS1* transcription (Figure 4.4.1), it is unlikely that Nhp6p repressed *ZDS1* transcription by reducing the expression of Rpb4p. The expression of Med3-myc9p was not affected by the deletion of *NHP6*, *RPB4* and *RTT107* (Figure 4.4.3B, compare lanes 2-9), and the expression of Rpb2-myc9p was not affected by the deletion of *NHP6*, *RTT107* and *MED3* (Figure 4.4.3C, compare lane 1 with lanes 2, 4 and 5). There was a reduction of Rpb2-myc9p expression in

cells lacking *RPB4* (Figure 4.4.3C, compare lane 1 and lane 3), however, since Rpb2 is an essential subunit of RNA polymerase II and actually required for gene expression, it is unlikely that Rpb4p repressed *ZDS1* transcription by increasing the amount of Rpb2p. Rtt107-myc9p was detected by Western blot (Figure 4.4.3D, lane 1). As Rtt107-myc9p was not detected at the *ZDS1* chromosomal locus by ChIP (Figure 4.4.4A), its expression was tested in the wild-type strain background only. For all Western blots, asterisks indicate the non-specific bands that were detected in the untagged wild-type strain and that served as loading controls.
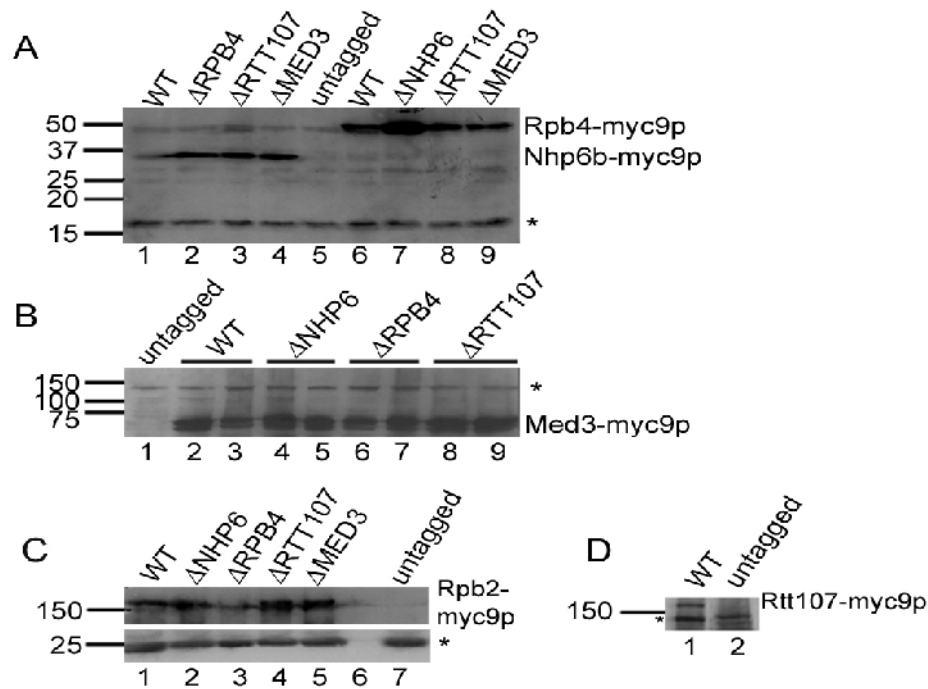
Figure 4.4.3 Expression of myc-tagged Nhp6bp, Rpb4p, Med3p, Rpb2p and Rtt107p. Myc-tagged Nhp6b, Rpb4p, Med3p, Rpb2p and Rtt107p were expressed in wild-type *JD52* and in the isogenic deletion strains. Equal amounts of cells were analyzed by Western blot using an anti-myc antibody. The asterisks indicate cross-reacting bands, which served as loading controls.
(A) Expression of Nhp6bp was not affected by the deletion of *RPB4*, *RTT107* and *MED3*, and expression of Rpb4p was not affected by the deletion of *RTT107* and *MED3*, while there was an increase in Rpb4p expression in the *NHP6* deletion strain. Nhp6b-myc9p (lanes 1-4) and Rpb4-myc9p (lanes 6-9) were expressed from single-copy plasmids under the control of their own respective promoters in wild-type *JD52* (lanes 1 and 6), *JD52ΔRPB4* (lane 2), *JD52ΔNHP6* (lane 7), *JD52ΔRTT107* (lanes 3 and 8) and *JD52ΔMED3* (lanes 4 and 9). Lane 5 contains untagged *JD52*. (B) Med3p was not affected by the deletion of *NHP6, RPB4* and *RTT107*. Med3-myc9p (lanes 2-9) was expressed from the *MED3* locus in the place of endogenous Med3p in *JD52* (lanes 2 and 3), *JD52ΔNHP6* (lanes 4 and 5), *JD52ΔRPB4* (lanes 6 and 7) and *JD52ΔRTT107* (lanes 8 and 9). Lane 1 contains untagged *JD52*. (C) Rpb2p was not affected by the deletion of *NHP6, RTT107* and *MED3*, while there was a decrease in Rpb2p expression in the strain deleted for *RPB4*. Rpb2-myc9p was expressed from the *RPB2* locus in place of endogenous Rpb2p in wild-type *JD52* (lane 1), *JD52ΔNHP6* (lane 2), *JD52ΔRPB4* (lane 3), *JD52ΔRTT107* (lane 4) and *JD52ΔMED3* (lane 5). Lane 6 contains untagged *JD52*. (D) Rtt107-myc9p was expressed from the *RTT107* locus in place of endogenous Rtt107p *in JD52* (lane 1). Lane 2 contains untagged *JD52*.

4.4.4 ChIP analysis determined Nhp6p and its interacting partners Rpb4p

and Med3p at the *ZDS1* chromosomal locus

In order to determine the occupancy of Nhp6bp and its interacting partners at the

ZDS1 chromosomal locus, ChIP analysis was performed with an anti-myc

antibody coupled to Protein A-Sepharose beads. Figure 4.4.4A shows that Nhp6bp

was found at the *ZDS1* promoter and ORF. This indicated that the observed

repression of *ZDS1* transcription by Nhp6bp had been a direct effect (compare

lane 1 and 2). Rpb4p was also found at the *ZDS1* promoter, however, it was not

detected at the *ZDS1* ORF (Figure 4.4.4A, compare lane 1 with lane 3). The

former suggested that the observed repression of *ZDS1* transcription by Rpb4p

had been a direct effect, while the latter indicated that the RNA Pol II transcribing

the *ZDS1* gene might not have contained the Rpb4p subunit. As shown in Figure

4.4.4A lane 4, Rtt107p was not detected at the *ZDS1* locus, indicating that Rtt107p

played its repression role in *ZDS1* transcription through an indirect way. The

presence of Med3p at the *ZDS1* promoter and ORF indicated that the repression of

ZDS1 transcription by Med3p had been a direct effect (Figure 4.4.4A, compare

lane 1 with lane 5). Med3p is one subunit of the Mediator of transcription. The

presence of Mediator and Med3p in promoters as well as in ORFs has been noted

previously (Andrau et al., 2006). Figure 4.4.4A further shows that two of

Nhp6p-interacting partners, Tfb1p and Tfb4p were also detected at the *ZDS1*

promoter and ORF. This is in consistent with the role of TFIIH in transcription

regulation (Figure 4.4.4A, compare lane 1 with lanes 6, 7).

A
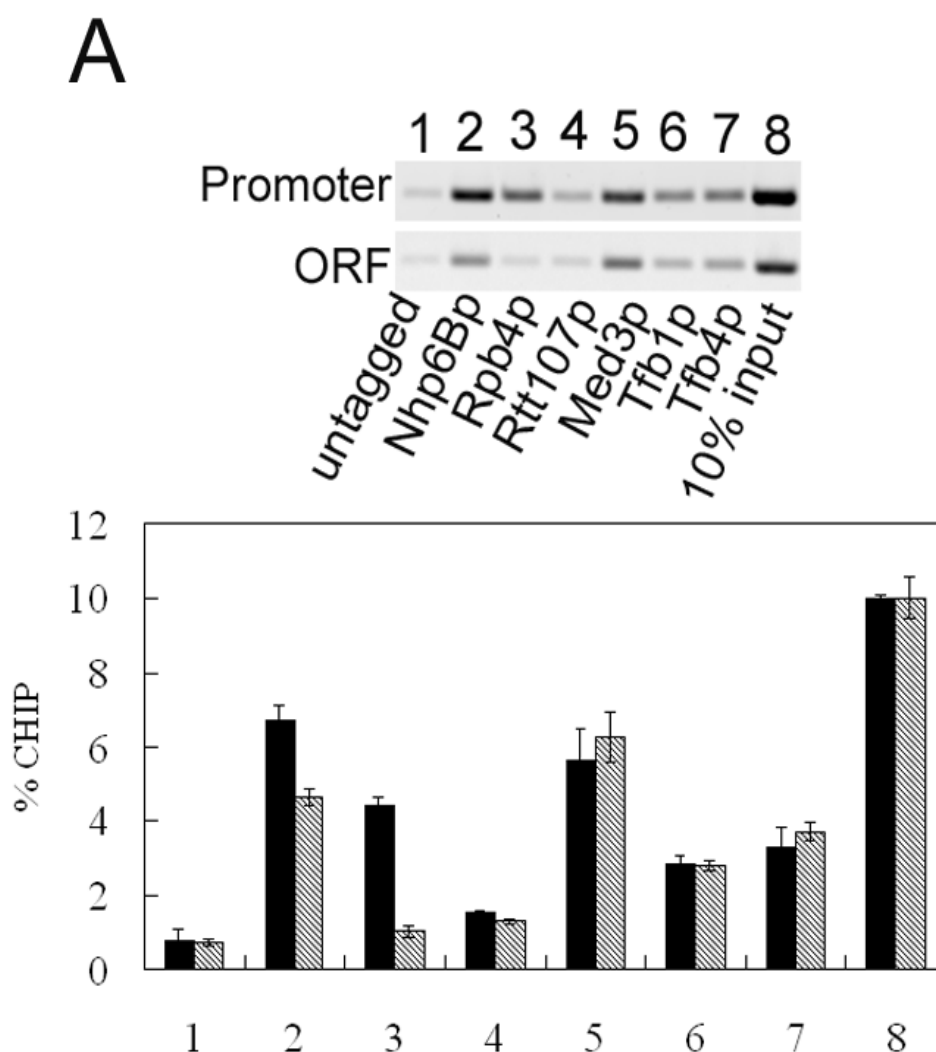
Figure 4.4.4 Association of myc-tagged proteins to the *ZDS1* locus in wild type and deletion strains. ChIP was performed with untagged and myc-tagged proteins in wild type and isogenic deletion strains. The cross-linked extracts were immunoprecipitated with anti-myc antibody-coupled beads. Precipitated DNA was quantified by PCR. Relative binding is shown, after normalization to 10% input control. Solid bars represent % ChIP of *ZDS1* promoter and striped bars represent % ChIP of *ZDS1* ORF. Error bars reflect variations between three replicate experiments.

(A): Nhp6b-myc9p, Med3-myc9p, Tfb1-myc9p and Tfb4-myc9p were detected at the *ZDS1* promoter and ORF in wild type cells. Rpb4-myc9p was detected at the *ZDS1* promoter but not at the *ZDS1* ORF in wild type cells. Rtt107p-myc9p was not present at the *ZDS1* locus.

We then investigated whether the deletion of *RPB4*, *RTT107* and *MED3* affected the association of Nhp6bp to the *ZDS1* locus. Figure 4.4.4B shows that the binding of Nhp6bp to the *ZDS1* locus was not affected by the deletion of *RPB4*, *RTT107* and *MED3* (compare lane 2 with lanes 3, 4 and 5). This suggested that Rpb4p, Rtt107p and Med3p had not been required for the association of Nhp6p to the *ZDS1* locus. As shown in Figure 4.4.4C, the binding of Med3-myc9p to the *ZDS1* locus was tested in cells lacking *NHP6*, *RPB4* and *RTT107*, respectively. The deletion of *NHP6*, *RPB4*, and *RTT107* did not affect the binding of Med3p to the *ZDS1* locus (compare lanes 2-5). This indicated that Nhp6p, Rpb4p and Rtt107p had not been necessary for the binding of Med3p to the *ZDS1* locus.

Figure 4.4.4D shows that the deletion of *RTT107* and *MED3* had no effect on the recruitment of Rpb4p to the *ZDS1* promoter (upper panel, compare lane 2 with lanes 4, 5), indicating that Rtt107p and Med3p had not been required for the binding of Rpb4p to the *ZDS1* promoter. However, the deletion of *NHP6* reduced the amount of Rpb4p detected at the *ZDS1* promoter significantly (Figure 4.4.4D, compare lane 2 with lane 3), even though the expression of Rpb4p had actually been increased in cells lacking *NHP6* (Figure 4.4.3A). This indicated that Nhp6bp might have been responsible for the recruitment of Rbp4p to the *ZDS1* promoter. Surprisingly, Rpb4p was found at the *ZDS1* ORF in the absence of *RTT107* and *MED3* (lower panel, compare lanes 2, 4 and 5). This indicated that Med3p might have exerted its repressing role in transcription elongation of *ZDS1* by removing Rpb4p from RNA polymerase II during the process of promoter clearance.

Since the transcription of *ZDS1* had been increased with the deletion of *NHP6*, *RPB4*, *RTT107* and *MED3*, we hypothesized that the increased transcription should coincide with an increase in the association of RNA polymerase II to the *ZDS1* chromosomal locus. Figure 4.4.4E shows that RNA Pol II was present at the entire *ZDS1* locus, as its essential subunit Rpb2p was detected at the *ZDS1* promoter and ORF. This indicated that RNA Pol II including Rpb2p and Rpb4p was bound to the *ZDS1* promoter in wild-type cells, but only RNA Pol II lacking the Rpb4 subunit had been able to enter the *ZDS1* ORF. The binding of Rpb2p was not affected by the deletion of *NHP6*, *RPB4*, and *MED3* (Figure 4.4.4E, compare lanes 2-5). A comparison of Figures 4.4.4D and 4.4.4E reveals that the binding of Rpb4p, but not Rpb2p, to the *ZDS1* promoter was decreased in *ΔNHP6* cells (lanes 3), suggesting that Nhp6bp had recruited Rpb4p to the *ZDS1* promoter and loaded it onto RNA Pol II. The comparison further shows that the binding of Rpb4p, but not Rpb2p, to the *ZDS1* ORF was increased in *ΔMED3* cells (lanes 5). This indicated that Med3p had dispelled Rpb4p from RNA Pol II during the process of promoter clearance. The real-time PCR results indicated that the amount of *ZDS1* mRNA was increased approximately four-fold upon the deletion of *NHP6*, *RPB4* and *MED3* (Figure 4.4.1), however, the amount of RNA Pol II at the *ZDS1* locus did not increase in the deletion strains (Figure 4.4.4E). One possible explanation is that RNA Pol II transcribed *ZDS1* four-times more efficiently in the absence of Nhp6p, Rpb4p and Med3p.
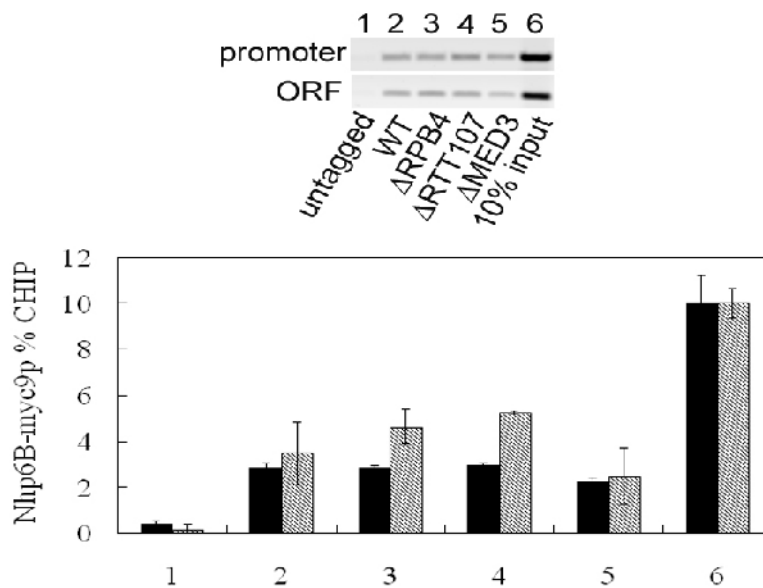
(B): Nhp6bp was detected at the *ZDS1* promoter (solid bars) and ORF (striped bars) in wild-type cells (lane 2). The occupancy of Nhp6bp at the *ZDS1* locus did not change in the indicated deletion strains (lane 3-5).
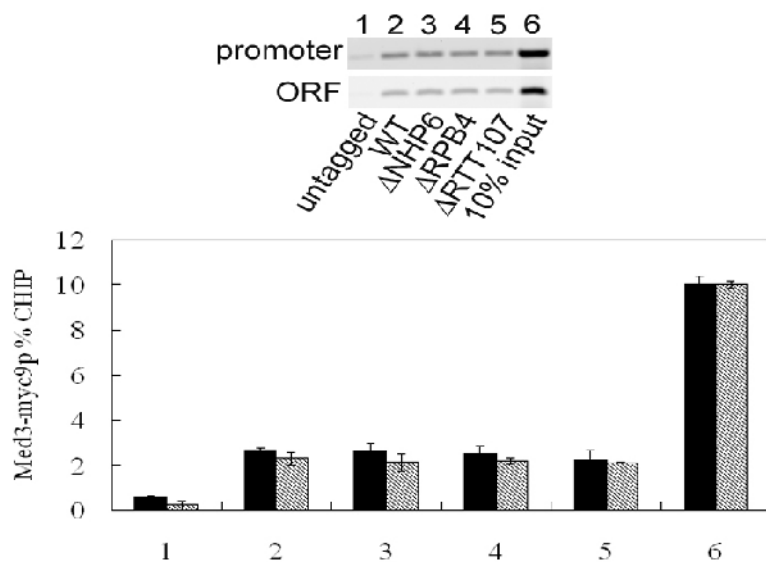
(C): Med3p was detected at the *ZDS1* promoter (solid bars) and ORF (striped bars) in wild-type cells (lane 2). The occupancy of Med3p at the *ZDS1* locus did not change in the indicated deletion strains (lane 3-5).
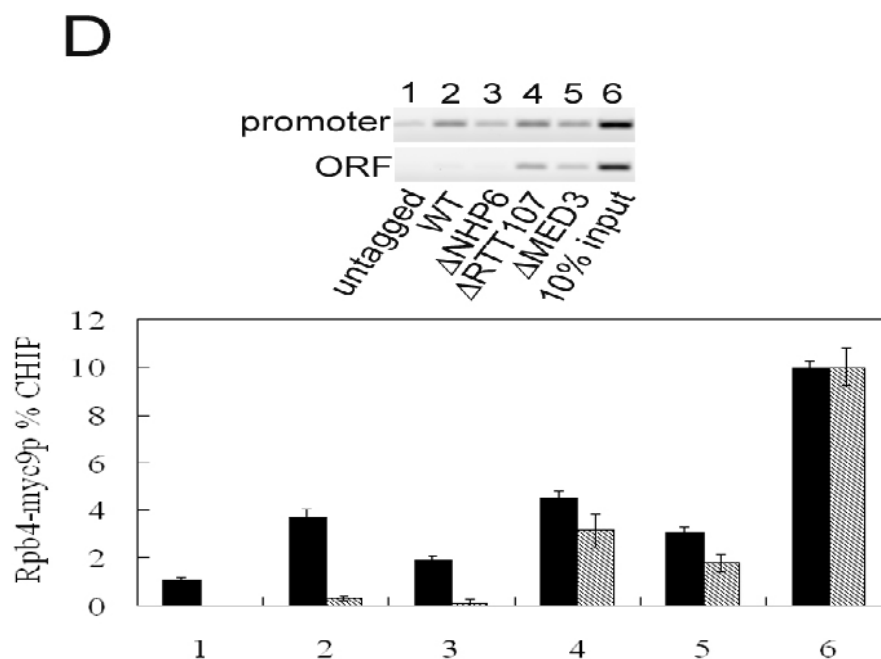
(D): Rpb4p was detected at the *ZDS1* promoter (solid bars) but not at the ORF (striped bars) in wild type cells (lane 2, compare upper and lower panel). The occupancy of Rpb4p at the *ZDS1* promoter was reduced in the *NHP6* deletion strain (lane 3, upper panel), while Rpb4p occupancy at the *ZDS1* ORF was increased in the *RTT107* and *MED3* deletion strains (lane 4 and lane 5, lower panel).
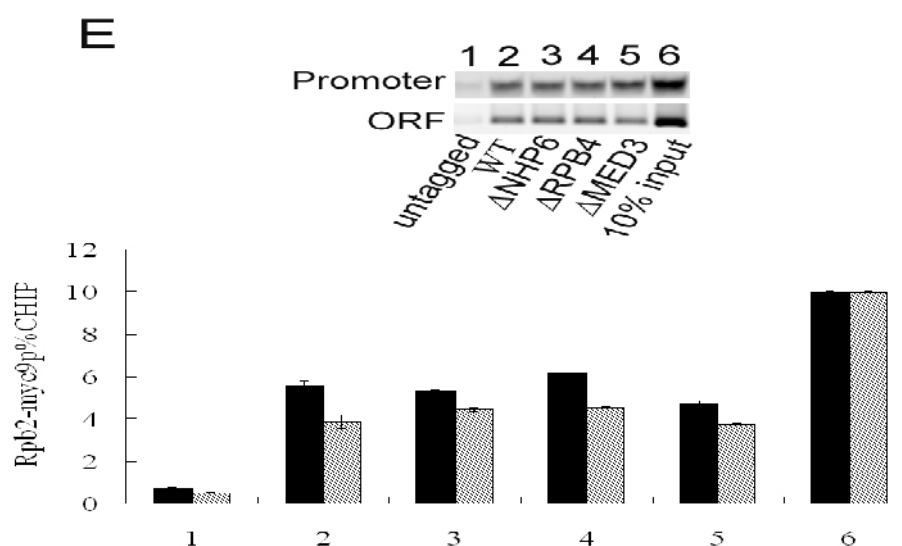
(E): Rpb2p was detected at the *ZDS1* promoter (solid bars) and ORF (striped bars) in wild type cells (lane 2). Its occupancy at the *ZDS1* locus did not change in the deletion strains (lane 3-5).

CHAPTER 5

DISCUSSION

5. Discussion

Saccharomyces cerevisiae Nhp6p is related to the high-mobility group B family of small, abundant non-histone chromosomal proteins that bend DNA sharply and modulate gene expression (Biswas et al, 2004). In this project, we used the Split-Ubiquitin system to isolate Nhp6p-interacting proteins *in vivo*. GST pull-down experiments confirmed eleven of these interactions *in vitro*. The *ZDS1* gene, whose transcription was repressed by Nhp6p and its interacting partners, was utilized to study their chromosomal co-localization.

5.1 Novel Nhp6p-interacting proteins were isolated with the help of the Split-Ubiquitin system

Nhp6p was used as a bait to screen an *S. cerevisiae* genomic library and a collection of N_{ub}-fused transcription factors for novel Nhp6p-interacting partners using the Split-Ubiquitin system. Of the 243 plasmid-linkage tests carried out, only 18 different N_{ub} fusion candidates were confirmed to be plasmid-linked. The reason for the high false positive rate lies in the principle of the screen. The Split-Ubiquitin system investigates the interaction between C_{ub} and N_{ub} fusion proteins. In our Split-Ubiquitin screens, the bait protein Nhp6p was fused to C_{ub} followed by the reporter protein RUra3p. The C_{ub} vector contains the *TRP1* marker and the N_{ub} vector contains the *LEU2* marker. Thus, cells co-expressing both C_{ub} and N_{ub} fusion vectors can be selected in WL plates and the specific

protein interaction between the N_{ub} fusion and C_{ub} fusion is indicated by uracil auxotrophy and FOA resistance. If the N_{ub} fusion contains the *TRP1* gene, for example, the single N_{ub} vector will combine both the *TRP1* and *LEU2* markers. Cells transformed with such a N_{ub} vector are able to loose the Nhp6-C_{ub}-RUra3p-expressing *TRP1*-marked plasmid and grow on the FWL plate. Such candidates are termed as false positives since their FOA resistance is not due to the interaction between the N_{ub} and C_{ub} fusion proteins. For example, as shown in Table 4.1.3.2 and Table 4.1.4.1, seven of the 34 plasmid-linked N_{ub} fusion candidates were confirmed to contain *TRP1* gene and displayed FOA resistance in *JD55*. Moreover, the N_{ub} vector contains the strong *ADH1* promoter. Expression of N_{ub} fusion proteins under the control of the *ADH1* promoter might be high as compared to the expression of the respective protein under its own endogenous promoter. Over-expression of some proteins can result in false positive results as well. For example, over-expression of certain membrane transporters can pump FOA out of the cells, resulting in FOA resistance. Finally, the Split-Ubiquitin assay indicates close proximity between two proteins inside living cells, but not necessarily a direct protein-protein interaction. This means that all the interactions detected with the Split-Ubiquitin system should be further confirmed by other high confidence assays, such as GST pull-down assay or co-immunoprecipitation assay.

GST pull-down assays were performed to further confirm the interactions using bacterially expressed GSTp, GST-Nhp6bp and yeast expressed HA-tagged N_{ub}

fusions. Of the 24 N_{ub} fusion proteins isolated from the yeast genomic library and collection of N_{ub} fused transcription factors by the split-ubiquitin screens, eleven N_{ub} fusions were able to be precipitated by GST-Nhp6bp, but not by GST alone (Figure 4.2.2 A-D). The other 13 proteins were either not expressed in the yeast cells (Ddc1p, Mlp1p, Mss4p, Tuf1p, Rpb6p, Srb5p and Tfa2p) or they could not be precipitated with GST-Nhp6bp (Cbf1p, H1, Rap1p, Swi3p, Taf1p and Tfa1p). For the six candidates who showed negative signals in the GST pull-down assay, it does not mean that they did not interact with Nhp6bp. There might be several reasons. One possible explanation is that some modifications of Nhp6p, such as acetylation or methylation, may be required for the interaction. The candidates may interact with properly modified Nhp6bp, but not with the non-modified Nhp6bp expressed in *E. coli*. To clarify this possibility, GST fused Nhp6p and HA tagged Nhp6p putative interacting partners should be expressed in yeast under their endogenous promoters. The co-immunoprecipitation should be performed using yeast extracts where the proteins of interest are present at native concentrations. These Co-IP results would provide more physiological clues about the interactions between Nhp6p and the various putative interactors. Another possibility which could explain the negative GST pulldown results is that the GST tag might have blocked the interacting surfaces of Nhp6bp with the candidates. Or the candidates might simply not interact with Nhp6bp. The GST pull-down assays we performed serve to detect the *in vitro* interactions between Nhp6p and its interacting partners isolated from split-ubiquitin screens. To confirm the direct

physical interactions between Nhp6p and the various putative interactors, the N_{ub} fusion candidates should be expressed and purified in bacteria. Since both Nhp6p and its putative interactors were expressed in bacteria, all relative modifications and third bridge proteins involved in the interactions would be avoided. The co-immunoprecipiation results will indicate the direct physical interaction between Nhp6p and its interacting partners. To delineate the exact domains for the interactions between Nhp6p and its interacting partners, truncated versions of the interactors could be used to directly test for their interaction regions. Since our main interesting focused on the functional analysis of protein interactions, we did not distinguish the exact regions required for the interactions.

The isolated N_{ub} candidates were sequenced and checked for sequence identity in the *Saccharomyces cerevisiae* genome database (SGD). The sequencing results indicated that most of our isolated Nhp6-interacting proteins are involved in transcription and DNA repair. Some of the Nhp6-interacting candidates like Nsf1p, Yml108wp, Ddc1p and H4 had been isolated several times, indicating that the expected screen were carried out to near saturation. (H3) had previously been shown to interact with Nhp6p (Formosa, 2002), which is in line with the interactions observed here. However, most of the interactions which were reported by others were not seen in our list. One example would be Spt16p interaction with Nhp6p. Spt16p associates with Pob3p to form the yeast chromatin regulator FACT, which mediates transcription elongation (Rhoades et al, 2004). Spt16p was shown not only to physically interaction with Nhp6p (Brewster et al, 2001), but also a

genetic interaction with Nhp6p has been demonstrated (Formosa et al, 2001).

However, we did not isolate Spt16p from the *S. cerevisiae* genomic library by the

Split-Ubiquitin system. Actually, few isolated proteins in our screens overlapped

with the reported Nhp6p-interacting partners. This discrepancy could be due to the

different methods used for isolating the Nhp6p-interacting proteins. In the

Split-Ubiquitin system, bait and prey proteins were expressed as C_{ub} and N_{ub}

fusions. The C_{ub} fusion could hinder the proper folding of the bait protein at the

C-terminus. Conversely, the N_{ub} fusion could pose steric hindrance at the

N-terminus of the prey proteins. The fusion proteins may fail to fold into the

intended shape and may not be able to interact in the correct orientation, giving

the impression that they do not interact. Another reason for false-negative results

could be that the interaction between the two proteins is not a direct one, and the

interaction between the two proteins might occurred in the presence of a third

bridging proteins only. The Split-Ubiquitin system measures the local

concentration of the N_{ub} and C_{ub} fusion proteins. If two proteins interact through

another linker protein, the distance between C_{ub} and N_{ub} might not be close

enough to reconstitute ubiquitin. Thus the interaction could not be detected in the

split-ubiquitin system. Moreover, interactions between two proteins might be

induced upon a given condition. For example, Nhp6p was reported to act together

with Tup1p specifically on *GAL1* and *SUC2* promoters in glucose-containing

medium (Laser et al, 2000). A comparison of the N_{ub} fusions isolated in the

umbiased library screen with the direct tests with N_{ub} fusions to transcription

factors reveals, however, that the library screen had missed many proteins that did interact with Nhp6bp in the Split-Ubiquitin assay. The most likely reason for this is the construction of the library. *Sau3A*I-partially digested genomic DNA had been fused to N_{ub} in all three reading frames. Some genes encoding Nhp6bp-interacting proteins, however, might had a *Sau3A*I site between the last in-frame stop codon before the ATG and the Nhp6p-interacting domain. Those proteins could not have been isolated by the library screen. One possible strategy to overcome this problem is to fuse all 6,000 yeast proteins to N_{ub} and to test them systematically with Nhp6-C_{ub}-Rura3p in the Split-Ubiquitin assay.

5.2 Nhp6p and its interacting proteins regulate gene transcription

Nhp6p plays an important role in transcription. Previous studies suggested that *ZDS1* transcription was down-regulated by Nhp6p and Med3p (Kerkmann, 2000; Bourbonnais et al, 2001), thus we chose the *ZDS1* gene to study how Nhp6p and its interacting partners affect specific gene transcription. *ZDS1* encodes a protein of 915 amino acids in yeast. It has a homolog, *ZDS2*, whose predicted product of 942 amino acids is 38% identical in sequence to Zds1p (Yu et al, 1996). Zds1p interacts with silencing proteins, such as Sir2p, Sir3p, Sir4p and Rap1p at the telomere, implementing Zds1p in transcriptional silencing (Roy and Runge, 1999). Zds1p plays a role in the localization of Bcy1p, a regulatory subunit of protein kinase A (Griffioen et al, 2001). Zds1p was also reported to associate with the essential transport complex formed by Dbp5p, Gfd1p and nucleoporins at the

nuclear pore complex, which is required for mRNA export (Estruch et al, 2005).

Most of the Nhp6p-interacting proteins isolated with the Split-Ubiquitin system are involved in transcription and DNA repair. Based on their interaction strength with Nhp6p and the information obtained from SGD, the three non-essential genes *RPB4*, *RTT107* and *MED3* were selected for the study of *ZDS1* gene transcription. Rpb4p, whose interaction with Nhp6p was confirmed *in vitro*, is a dissociable subunit of RNA Pol II, and in exponentially growing cells, only 20% of RNA Pol II contain Rpb4p, even though Rpb4p is present in excess over the other RNA Pol II subunits (Kolodziej et al, 1990; Choder, 2004). Rpb4p plays an important role for transcription-coupled DNA repair, where a stalled RNA Pol II signals DNA damage (Li and Smerdon, 2002). Rpb4p is also involved in mRNA export and shuttle between the nucleus and the cytoplasm (Selitrennik et al, 2006). Both Nhp6p and its interacting partner Rpb4p play a known role in DNA repair. Here, we have shown that the deletion of *RPB4*, like the deletion of *NHP6*, increased sensitivity to 6-AU (Figure 4.3), indicating that RNA Pol II generally elongated transcription more efficiently if it contained Rpb4p. Furthermore, over-expression of Rpb4p suppressed the 6-AU sensitivity of the *NHP6* deletion strain (Figure 4.3), suggesting that Nhp6p might have exerted its positive effect on transcription elongation via Rpb4p. Nhp6p changes the structure of chromatin by bending DNA, which can have a positive or negative effect on gene expression depending upon the architecture of the gene (Moreira and Holmberg, 2000). Nhp6p and Rpb4p directly repressed *ZDS1* transcription, as – consistent with previous reports

(Kerkmann, 2000; Bourbonnais et al., 2001) – the deletion of both genes increased *ZDS1* mRNA levels approximately four-fold and we have found both proteins at the *ZDS1* promoter. The expression of Nhp6p was not decreased by the deletion of *RPB4* (Figure 4.4.4B) and the expression of Rpb4p was not decreased by the deletion of *NHP6* (Figure 4.4.4D), indicating that both repressors did not exert their effects by preventing the expression of each other. While the binding of Nhp6p to the *ZDS1* promoter was not affected by the deletion of *RPB4*, the binding of Rpb4p to the *ZDS1* promoter was reduced two-fold in the *NHP6* deletion strain (Figure 4.4.4D, compare lane 2 and 3), indicating that Nhp6p was responsible for the recruitment of Rpb4p to the *ZDS1* promoter. The binding of Rpb2p to the *ZDS1* promoter, on the other hand, was not affected by the deletion of *NHP6* (Figure 4.4.4E, compare lane 2 and 3), indicating that Nhp6p recruited Rpb4p and not RNA Pol II. Nhp6p might have supported the preferential recruitment of RNA Pol II that contained Rpb4p, or Nhp6p might have directly recruited Rpb4p and loaded it onto RNA Pol II lacking Rpb4p. Our results are consistent with a dual role for Nhp6p in the gene expression of *ZDS1*. While Nhp6p inhibited transcription initiation, it stimulated transcription elongation. Rpb4p could have mediated both effects, as also Rpb4p appeared to inhibit *ZDS1* transcription initiation while generally stimulating transcription elongation. Furthermore, Rpb4p dosage-compensated the 6-AU phenotype of an *NHP6* deletion, supporting the hypothesis that Nhp6p generally activated transcription elongation via Rpb4p. The deletion of *NHP6* resulted in a net increase of *ZDS1*

mRNA, suggesting that the repressive effect on *ZDS1* initiation was stronger than the activating effect on elongation.

Med3p, whose interaction with Nhp6p was confirmed *in vitro*, is a component of the mediator. Med3p plays an important role in basal and activated transcription (Bjorklund and Gustafsson, 2005). Med3p is also a direct target for transcriptional repressors (Papamichos-Chronakis et al, 2000), and it has been found in promoters as well as in open reading frames (Andrau et al, 2006). Med3p is not known to play a role in DNA repair, however, it reduces the frequency of deletions in a *rad52-1* mutant (Santos-Rosa and Aguilera, 1995). We have found that the deletion of *MED3* increased expression of *ZDS1* approximately four-fold. We have detected Med3p at the *ZDS1* locus, which indicated that Med3p repressed *ZDS1* transcription directly. Med3p was found at the promoter as well as in the open reading frame of *ZDS1*, consistent with previous reports that had shown Mediator components including Med3p at promoters and at open reading frames (Andrau et al, 2006). The occupancy of Med3p at the *ZDS1* locus did not change upon the deletion of *NHP6*, *RPB4* and *RTT107*, indicating that none of these proteins was responsible for the recruitment of Med3p to the *ZDS1* locus. Surprisingly, Rpb4p, which was not found in the *ZDS1* open reading frame in wild-type or *NHP6*-deleted cells, was detected in the *ZDS1* open reading frame upon the deletion of *MED3* (Figure 4.4.4D, lane 5). *ZDS1* is transcribed in *NHP6*-deleted cells at the same level as in *MED3*-deleted cells, but Rpb4p was detected at the *ZDS1* ORF in *MED3*-deleted cells only. This indicated that the

form of RNA Pol II transcribing *ZDS1* in *NHP6*-deleted (and wild-type) cells was lacking the Rpb4p subunit. The presence of RNA Pol II at the *ZDS1* ORF was confirmed with the help of the essential subunit Rpb2p, which – contrary to Rpb4p – was detected at the *ZDS1* ORF even in wild-type cells (Figure 4.4.4E). Rpb4p, on the other hand, was detected at the *ZDS1* promoter together with Rpb2p in wild-type cells. This indicated that Med3p prevented Rpb4p-containing RNA Pol II from entering the *ZDS1* ORF, either by allowing only Rpb4p-lacking RNA Pol II to clear the promoter, or by actively removing the Rpb4p subunit from RNA Pol II during promoter clearance.

The deletion of *NHP6* and *MED3* increased transcription approximately four-fold, while the *ZDS1* local concentration of RNA Pol II, represented by the essential subunit Rpb2p, did not change upon the deletion of *NHP6* and *MED3*. This was surprising, as the local concentration of RNA Pol II correlated well with gene expression in wild-type *S. cerevisiae* cells in a genome-wide study (Steinmetz et al, 2006). However, the ChIP assay precipitates actively transcribing RNA Pol II as well as inactive, stalled RNA Pol II. The lack of increase in RNA Pol II *ZDS1* occupancy upon the deletion of *NHP6*, *RPB4* and *MED3* can be explained with the assumption that RNA Pol II did not transcribe *ZDS1* efficiently in the presence of Nhp6p, Rpb4p and Med3p. The deletion of the three repressors could have increased transcription by increasing the speed with which RNA Pol II transcribed *ZDS1*. This would have increased the amount of transcript without affecting the amount of precipitated RNA Pol II. The deletion of *NHP6* and *RPB4* might have

increased the speed with which RNA Pol II cleared the promoter, while the deletion of *MED3* might have increased the speed with which RNA Pol II elongated transcription.

Rtt107p, whose interaction with Nhp6p was confirmed *in vitro*, is required for DNA repair (Baldwin et al, 2005). It also binds to the silencing proteins Sir3p and Sir4p and establishes heterochromatin (Zapulla et al, 2006). *RTT107* is synthetically lethal with *GAL11*, a component of the Mediator, indicating a role for Rtt107p in transcription (Tong et al, 2004). The deletion of *RTT107* suppresses a *CTR9* deletion (Collins et al, 2007). Ctr9p is a component of the Paf1 transcription elongation complex, and the suppression shows that Rtt107p could play a negative role in transcription elongation. Here, we have shown that the deletion of *RTT107* increased the occupancy of Rpb4p at the *ZDS1* ORF and the transcription of the *ZDS1* gene. This suggested that Rtt107p might inhibit transcription of *ZDS1* by preventing Rpb4p-containing RNA Pol II from transcribing *ZDS1*. The Rpb4p-lacking RNA Pol II that transcribed *ZDS1* did so less efficiently as elongation was impaired. However, Rtt107p was not found at the *ZDS1* promoter, indicating that it might have exerted its effect on transcription elongation off DNA, possibly via Med3p.

Srp14p, whose interaction with Nhp6p was confirmed *in vitro*, is a component of the signal recognition particle, which targets proteins to membranes. Srp14p, like Nhp6p, activates RNA Polymerase III transcription and was also detected in the nucleolus (Grosshans et al, 2001). The reverse recruitment hypothesis postulates

that the association of GEMs with nuclear pores regulates gene expression (Santangelo, 2006), and it would be interesting to see if Srp14p played a role in the sub-nuclear localization of GEMs to NPCs (Nuclear Pore Complexes). We noted that the Split-Ubiquitin assay showed *in vivo* protein interactions of Nhp6p with Rap1p and Mlp1p, which are components of GEMs and NPCs, respectively. Rap1p and Mlp1p play an important role in the recruitment of active genes to GEMs that are associated with NPCs (Casolari et al, 2004; Luthra et al, 2007). However, we were not able to confirm these interactions *in vitro*. Mlp1p was not detected in the yeast extract. It is possible that the protein had precipitated together with the cellular membranes. Rap1p was detected in the yeast extract but could not be precipitated with GST-Nhp6bp. It is possible that the protein interaction occurred inside intact nuclei only.

Tfb1p and Tfb4p, whose interactions with Nhp6p were confirmed by GST pull-down experiments, are components of TFIIH. Both proteins were detected at the *ZDS1* locus, but their effect on *ZDS1* transcription was not determined as the two respective deletion strains are inviable. TFIIH also plays an important role in DNA repair (Bardwell et al, 1994). Furthermore, Nhp6p has also been shown to have interactions with histones H2A, H2B, H3 and H4. The strong interaction of Nhp6p with the histones was not surprising, as Nhp6p is a chromatin component. Histones play important roles in transcription and DNA repair (Matsubara et al, 2007).

5.3 Conclusion

Nhp6p is an architectural chromatin component, and the results presented here are not generally applicable to the expression of all *S. cerevisae* genes. Nhp6p affects the expression of about 10% of the *S. cerevisiae* genes only, repressing about half of these and activating the other half (Kerkmann, 2000). The specific effect Nhp6p has on the transcription of a particular gene presumably depends upon the promoter architecture and the other proteins bound to the gene. Nhp6p, on the other hand, generally supports transcription elongation, as suggested by the 6-AU phenotype of the *NHP6* deletion strain. The deletion of *RPB4* reduced growth on 6-AU plates and the over-expression of Rpb4p suppressed the 6-AU phenotype of the *NHP6* deletion strain, indicating that Nhp6p generally supported transcription elongation via Rpb4p. Nhp6p, Med3p and the essential RNA Pol II subunit Rpb2p were found at the entire *ZDS1* locus, while Rpb4p was only detected at the *ZDS1* promoter, suggesting that the RNA Pol II that transcribed *ZDS1* was lacking the dissociable Rpb4p subunit. In addition, the deletion of *NHP6* reduced binding of Rpb4p to the *ZDS1* promoter, while the deletion of *MED3* recruited Rpb4p to the *ZDS1* ORF. This indicated that Nhp6p loaded Rpb4p onto RNA Pol II at the *ZDS1* promoter, while Med3p prevent *ZDS1* promoter clearance of RNA Pol II that contained Rpb4p. Taken together, Nhp6p and Med3p repressed transcription of *ZDS1* by controlling the local subunit composition of RNA Pol II.

5.4 Future work

S. cerevisiae cells lacking *NHP6* displayed reduced growth on 6-AU plates, consisted with previous reports that Nhp6p supports the elongation of transcription. Our observation that the deletion of *RPB4* increased sensitivity to 6-AU indicated that RNA Pol II lacking Rpb4p was defective for transcription elongation. Previously published observation that the deletion of *RTT107* suppressed the deletion of the transcription elongation factor *CTR9* indicates that Rtt107p is a general inhibitor of transcription elongation (Collins et al, 2007). *S. cerevisiae* cells lacking *RTT107* and *MED3* were not sensitive to 6-AU, however, transcription of *ZDS1* was increased in the *RTT107* and *MED3* deletion strains. Since the deletion of *RTT107* and *MED3* increased the occupancy of Rpb4p at the *ZDS1* ORF, it is possible that Rtt107p and Med3p might inhibit transcription elongation by preventing Rpb4p-containing RNA Pol II from clearing the promoters. The triple deletion strains *JD52ΔNHP6ΔRTT107* and *JD52ΔNHP6ΔMED3* and the double deletion strains *JD52ΔRPB4ΔRTT107* and *JD52ΔRPB4ΔRTT107* could be generated. If the deletion of *RTT107* and/or *MED3* suppressed the 6-AU sensitivity of cells lacking *NHP6* and/or *RPB4*, Rtt107p and Med3p would be revealed as general inhibitions of transcription elongation.

In addition, we concluded that Nhp6p and Med3p repressed transcription of *ZDS1* by controlling the local subunit composition of RNA Pol II. We stated that Nhp6p might have loaded Rpb4p onto RNA Pol II at the *ZDS1* promoter, while Med3p

might have prevented *ZDS1* promoter clearance of RNA Pol II that contained Rpb4p. Next, I plan to determine whether this regulation can be found at other promoters as well. Based on Kerkmann's microarray results (Kerkmann, 2000), seven genes whose transcription was repressed more then three-fold in the *NHP6* deletion strain were selected to test if Nhp6p was present at the respective chromosomal locations. Nhp6p was found at the promoters as well as in the open reading frames of all seven genes. The seven genes were *PUR5*(*YDR454C*), *RPL7B*(*YRL198W*), *COS8*(*YHL048W*), *ZDS1*(*YMR273C*), *YHL049C*, *TEL2*(*YGR099W*) and *UME6*(*YDR027C*). Audrau and his colleagues (2006) explored the genome-wide location of Mediator and published a list for Mediator-bound genes. Besides at the *ZDS1* gene, Mediator was found at *PUR5*, *YHL049C* and *UME6*. Real-time PCR results indicated that the transcription levels of *YHL049C* gene in cells lacking *NHP6* and in cells lacking *MED3* were increased approximately 2.5-fold and 2-fold, respectively. The deletion of *NHP6* and *MED3* increased *UME6* mRNA transcription approximately two-fold as well. Real-time PCR further showed that the transcription of *PUR5* was increased approximately four-fold in cells lacking *NHP6*, however, the deletion of *MED3* repressed *PUR5* transcription. Next, I can perform ChIP assays to test whether Rpb4p and RNA Pol II are present at these three genes in wild-type strain, the *NHP6* deletion strain and in the *MED3* deletion strain. This might help to find more examples of genes where Nhp6p and Med3p repress gene transcription by controlling the local subunit composition of RNA Pol II.

CHAPTER 6

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6. References

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CHAPTER 7

APPENDICES

7. Appendices

Appendix 1: Average scores between the interactions of the C_{ub} fusion Nhp6ap and the 34 N_{ub} fusions isolated from library screen in *JD52*

JD52	Nhp6a-Cub-RUra3p				Nhp6a-Cub-RUra3p		
	raw score				relative score		
LS	WL	UWL	FWL		UWL	FWL	average
A1.1	5	4	6		1	7	4
A2.1	6	4	6		2	6	4
A3.1	6	4	6		2	6	4
A4.1	5	1	5		4	6	5
A5.1	6	4	5		2	5	3.5
A6.2	6	0	6		6	6	6
A9.1	6	1	5		5	5	5
A10.1	6	0	6		6	6	6
A11.1	6	4	6		2	6	4
A12.1	6	0	6		6	6	6
A14.1	6	0	6		6	6	6
A14.2	6	6	4		0	4	2
A15.1	6	3	6		3	6	4.5
A16.1	6	1	5		5	5	5
A16.2	6	0	6		6	6	6
A17.4	6	0	6		6	6	6
A19.2	6	0	6		6	6	6
A20.1	6	0	6		6	6	6
A26.3	6	6	1		0	1	0.5
A38.1	6	6	6		0	6	3
B1.1	6	6	6		0	6	3
B3.1	6	1	6		5	6	5.5
B4.1	5	1	6		4	7	5.5
B4.4	6	0	4		6	4	5
B6.1	6	0	6		6	6	6
B6.2	6	0	5		6	5	5.5
B8.1	6	6	6		0	6	3
B15.1	6	1	6		5	6	5.5
B23.2	6	4	6		2	6	4
B29.1	6	2	6		4	6	5
B30.2	6	0	3		6	3	4.5
B32.1	6	2	6		4	6	5
B32.2	6	3	6		3	6	4.5
B47.1	6	6	5		0	5	2.5
Nub	6	6	0		0	0	0

Appendix 2: Average scores between the interactions of the C_{ub} fusion Nhp6bp and the 34 N_{ub} fusions isolated from library screen in *JD52*

JD52	Nhp6b-Cub-RUra3p raw score				Nhp6b-Cub-RUra3p relative score		
LS	WL	UWL	FWL		UWL	FWL	average
A1.1	6	0	6		6	6	6
A2.1	6	1	6		5	6	5.5
A3.1	6	1	6		5	6	5.5
A4.1	6	0	6		6	6	6
A5.1	6	2	6		4	6	5
A6.2	6	0	6		6	6	6
A9.1	6	6	2		0	2	1
A10.1	6	0	6		6	6	6
A11.1	6	1	6		5	6	5.5
A12.1	6	0	6		6	6	6
A14.1	6	0	6		6	6	6
A14.2	6	4	5		2	5	3.5
A15.1	6	1	6		5	6	5.5
A16.1	6	0	5		6	5	5.5
A16.2	6	0	6		6	6	6
A17.4	6	0	6		6	6	6
A19.2	6	0	4		6	4	5
A20.1	6	3	6		3	6	4.5
A26.3	6	6	2		0	2	1
A38.1	6	5	5		1	5	3
B1.1	6	2	6		4	6	5
B3.1	6	1	6		5	6	5.5
B4.1	5	0	5		5	6	5.5
B4.4	6	2	6		4	6	5
B6.1	6	0	6		6	6	6
B6.2	6	0	6		6	6	6
B8.1	6	3	6		3	6	4.5
B15.1	6	1	6		5	6	5.5
B23.2	6	2	6		4	6	5
B29.1	6	0	6		6	6	6
B30.2	6	1	5		5	5	5
B32.1	6	5	5		1	5	3
B32.2	6	0	6		6	6	6
B47.1	6	2	6		4	6	5
Nub	6	6	0		0	0	0

Appendix 3: Average scores between the interactions of the C_{ub} fusion Nhp6ap and the N_{ub} fused transcription factors in *JD52*

JD52	Nhp6a-Cub-RUra3p raw score				Nhp6a-Cub-RUra3p relative score		
Ada1	5	5	1		-2	2	0
Ada2	6	5	1		-1	1	0
Ada3	6	4	2		0	2	1
Ada4	5	3	2		0	3	1.5
Ada5	6	4	1		0	1	0.5
Ahc1	6	4	2		0	2	1
Arp7	5	4	2		-1	3	1
Arp9	5	4	3		-1	4	1.5
Ccl1	6	4	3		0	3	1.5
Hho1	5	2	3		1	4	2.5
hta1	5	2	4		1	5	3
Htb1	5	0	4		3	5	4
Hht1	5	0	4		3	5	4
Kin28	6	5	3		-1	3	1
Med1	6	5	3		-1	3	1
Med2	5	6	2		-3	3	0
Med3	4	0	3		2	5	3.5
Med4	5	5	2		-2	3	0.5
Med6	6	4	4		0	4	2
Med7	5	4	3		-1	4	1.5
Med8	4	2	1		0	3	1.5
Med9	4	5	2		-3	4	0.5
Med10	5	4	2		-1	3	1
Med11	5	5	2		-2	3	0.5
Met18	5	5	3		-2	4	1
Orc5	6	6	2		-2	2	0
Rad2	6	5	2		-1	2	0.5
Rap1	5	0	3		3	4	3.5
Rgr1	5	5	2		-2	3	0.5
Rox3	5	2	3		1	4	2.5
Rpb2	4	5	2		-3	4	0.5
Rpb3	6	4	3		0	3	1.5
Rpb4	6	5	2		-1	2	0.5
Rpb5	6	5	6		-1	6	2.5
Rpb6	5	1	4		2	5	3.5
Rpb7	6	6	2		-2	2	0
Rpb8	5	6	3		-3	4	0.5
Rpb10	6	3	4		1	4	2.5

Rpb11	5	5	2		-2	3	0.5
Rpb12	6	4	5		0	5	2.5
Sir1	5	5	1		-2	2	0
Sir2	5	5	1		-2	2	0
Spt3	6	3	2		1	2	1.5
Spt7	4	3	1		-1	3	1
Spt8	4	3	3		-1	5	2
Srb2	5	5	2		-2	3	0.5
Srb4	6	5	2		-1	2	0.5
Srb5	5	0	2		3	3	3
Srb6	5	2	4		1	5	3
Srb8	5	5	2		-2	3	0.5
Srb10	5	5	3		-2	4	1
Srb11	4	3	3		-1	5	2
Ssn6	6	4	2		0	2	1
Ssl1	5	3	3		0	4	2
Ssl2	5	3	2		0	3	1.5
Swi2	5	5	3		-2	4	1
Swi3	6	4	5		0	5	2.5
Suf5	5	5	3		-2	4	1
Suf6	6	5	3		-1	3	1
Suf11	5	5	5		-2	6	2
Suf12	6	6	2		-2	2	0
Taf1	5	1	4		2	5	3.5
Taf17	6	4	4		0	4	2
Taf19	6	3	5		1	5	3
Taf25	6	5	2		-1	2	0.5
Taf30	6	3	6		1	6	3.5
Taf40	6	5	3		-1	3	1
Taf47	6	5	2		-1	2	0.5
Taf48	5	4	4		-1	5	2
Taf60	5	5	2		-2	3	0.5
Taf61	4	3	1		-1	3	1
Taf65	5	5	2		-2	3	0.5
Taf67	5	3	3		0	4	2
Taf90	5	5	2		-2	3	0.5
Taf145	5	5	2		-2	3	0.5
Tfa1	5	0	4		3	5	4
Tfa2	5	1	4		2	5	3.5
Tfb1	5	2	2		1	3	2
Tfb2	5	5	2		-2	3	0.5
Tfb3	5	5	3		-2	4	1
Tfb4	5	1	4		2	5	3.5

Tfg2	6	2	4		2	4	3
Tfg3	5	3	4		0	5	2.5
Tup1	5	5	2		-2	3	0.5
YCTD	6	3	4		1	4	2.5
Nub	6	4	0		0	0	0