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UNCOVERING NOVEL PROTEIN PARTNERS OF NUCLEOLAR PROTEIN 6 (NOP6) BY YEAST TWO-HYBRID ANALYSIS AND THEIR ROLE IN RIBOSOME BIOGENESIS

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LIST OF ABBREVIATIONS

Aa	Amino Acids
Amp	Ampicillin
DNA	Deoxyribonucleic acid
cDNA	Complementary deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
CC	Coiled-coil
dNTP	Deoxyribonucleic acid triphosphate
DTT	Dithiothréitol
EDTA	Ethylenediamine tetraacetate
ETS	External transcribed spacer
GST	Glutathione sulfo transferase
HRP	Horseradish peroxidase
ITS	Internal transcribed spacer
Kan	Kanamycin
MeOH	Methanol
LacZ	Gene of β-Galactocidase in yeast
LB	Luria Bertani
LiOac	Lithium Acetate
MeOH	Methanol

MBP	Maltose binding protein
$MgCl_2$	Magnesium Chloride
MSE	Middle sporulation element
NaCl	Sodium Chloride
NOR	Nucleolar organizer region
Oligo	Oligonucleotide
PCR	Polymerase chain reaction
PEG	Polyethylene Glycol
Pol I	Polymerase I
PVDF	Polyvinyldifluoride
RNase	Ribonuclease
SD	Synthetic Dropout
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
snoRNA	Small nucleolar ribonucleic acid
snoRNP	Small nucleolar ribonucleoprotein
TBS	Tris- Buffered Saline
TBS-T	TBS-Tween
TE	Tris-EDTA
TEMED	N,N,N, N'-Tetramethylethylenediamine
Tris	Tris (hydroxymethyl) aminomethane
Tween	Polyoxyethylene sorbitan monolaurate

UV	Ultraviolet
X-Gal	X-α-Gal 5-Bromo-4-chloro-3-indolyl alpha-D-galactopyranoside
YPD	Yeast Extract Peptone Dextrose

RÉSUMÉ

La biogenèse des ribosomes, localisée dans le nucléole, est un processus essentiel à la croissance des cellules et à leur prolifération. Les cellules cancéreuses présentent souvent de multiples nucléoles de grandes tailles, résultat d'une demande accrue en synthèse protéique nécessaire à une croissance cellulaire rapide. Afin de mieux comprendre les mécanismes qui régulent la biogenèse des ribosomes, nous avons utilisé la levure *Saccharomyces cerevisiae* comme modèle. snR30 (U17 chez l'humain) est une petite ribonucléoprotéine nucléolaire (snoRNP) essentielle à la maturation des ARN ribosomiques (ARNr). Nous avons purifié la snoRNP snR30 par chromatographie d'affinité et les protéines du complexe ont été identifiées par spectrométrie de masse. Une de ces protéines est Nop6, une protéine nucléolaire qui contient un domaine de liaison à l'ARN (RRM) et deux domaines « coiled-coil » (CC). La présence des CC suggère que Nop6 pourrait interagir avec d'autres protéines. Un partenaire possible de Nop6, Fir1, a été trouvée, au cours d'un criblage double hybride. De plus, des tests doubles hybrides indiquent que Nop6 interagit fortement avec Rrt5, une protéine associée à la transcription de l'ARNr. La délétion des deux domaines CC, n'a pas d'effet sur l'interaction entre Nop6 et Rrt5. Nos expériences in *vivo* et in *vitro* ont confirmé l'association entre Nop6 et Rrt5. Nos expériences d'immuno-microscopie ont montré la co-localisation de Nop6 et Rrt5 durant la sporulation. Nop6 semble avoir un rôle important dans la biogénèse des

ribosomes. Cependant, l'interaction entre Rrt5 et Nop6 durant la sporulation suggère une autre fonction inconnue de Nop6 dans la méiose.

CHAPTER 1: INTRODUCTION

1.1. The nucleolus

The nucleolus is the most distinctive sub-nuclear compartment and it is the site of both rRNA transcription and ribosome biogenesis. Ribosomal genes (r-genes) are transcribed in the form of Christmas trees on active chromatin. Electron microscopy images of these Christmas trees have depicted areas of the nucleolus with active r-genes with RNA Polymerase I (Pol I) transcription, or inactive r-genes onto nucleosomes, areas with condensed chromatin and no transcription (Brown & Dawid 1968, Gall 1968, Miller & Beatty 1969).

Different macromolecule localizations suggest the nucleolus has various roles in the cell. For instance, in addition to being involved in ribosome biogenesis, the nucleolus has been implicated in viral infection control, maturation of non-nucleolar RNAs, senescence and regulation of telomerase function (Raska et al 2006b). The best characterised of these other activities is the assembly of the signal recognition particle, an RNA-protein complex that targets translation of proteins to the endoplasmic reticulum.

Electron microscopy analysis of eukaryotic nucleoli identified several features of the nucleolus that allow it to be subdivided into its three sub-compartments, the fibrillar center (FC), dense fibrillar component (DFC) and granular component (GC). These compartments may differ in their arrangement and prevalence between species (Raska et al 2006a). The nucleolus is a very dynamic structure; models have depicted

the RNA and protein molecules to diffuse freely (Politz et al 1998). Ribosomal genes are primarily repeated and can be grouped into chromosomal regions called nucleolar organizer regions (NOR). Clusters of NORs organize to help form the nucleolus (Dammann et al 1993). During mitosis, NOR chromatin is present in two configurations: one favouring transcription and another, the inhibition of transcription. In higher organisms, the nucleus disassembles and the nucleolus disappears during the start of mitosis and is reformed at the end of mitosis. This pattern is not observed in *Saccharomyces cerevisiae*, where the nucleolus remains intact throughout mitosis (Dammann et al 1993, Dammann et al 1995).

Interestingly, the nucleolus is not a static structure. It changes its form prior to the start of the cell cycle from puffed and as it enters the cell cycle to threaded (Fuchs & Loidl 2004). This compartment is also involved in the sequestration of proteins involved in cell cycle regulation (Shou et al 1999). Pol I and its factors remain at the sites of rDNA repeats at the NORs of the chromosomes, while other pre-rRNA factors form a sheath around the condensed chromosomes, the perichromosomal space (Dundr et al 2000). These perichromosomal components are partitioned to the daughter nuclei together with the chromosomes and used for re-forming the nucleoli at telophase (Fuchs & Loidl 2004). In meiosis of plants and animals, nucleoli are resolved and only reform during telophase II (Loidl et al 1998). In *Saccharomyces cerevisiae*, the dynamics of the nucleolus differ from other organisms, and like other fungi, this is not well understood. Nucleoli maintain their integrity during both mitosis and meioisis which is linked to their endonuclear type of division (Loidl

2003). In meiosis in *S. cerevisiae*, nuclei do not completely separate at the end of the first division but form a dumbbell-shaped structure. The two meiosis II spindles are formed in the half-nuclei within the confines of the single parental-nuclear mass, and the haploid set chromatids migrate into four protrusions of the nucleus (Moens & Rapport 1971). A recent study viewed the dynamics of nucleolar division during meiosis. In meiosis II, the rDNA was present in one or two dots at the center of the cruciform tetrad until very late in anaphase when the majority of DNA had already entered the spores (Fuchs & Loidl 2004). Immunostaining for the nucleolar proteins Nop1, Nop5 and Nhp2, revealed the nucleoli to be attached to the NORs during the first meiotic division until anaphase II. At the end of the second meiotic division, nucleolar proteins remained within the cytoplasm of the ascus and no nucleolar material was detected in the prospores. It was only in late telophase or at the beginning of spore wall formation that low amounts of nucleolar proteins could be detected, probably due to the reformation of nucleolus (Moens & Rapport 1971).

1.2. Maturation of ribosomal RNA

The synthesis of ribosomes in eukaryotes involves the processing of precursor ribosomal RNA (pre-rRNA) and the sequential assembly of a large number of ribosomal proteins on the rRNAs. This is essential for both cell growth and proliferation, and is the most transcribed process at any given time (Martin et al 2004). Due to the energy expenditure and requirement for protein synthesis, there is

coordination between transcription, ribosome biogenesis and the formation of RNA-RNA and RNA-protein complexes (de la Cruz et al 1999).

Several diseases are caused by defects in ribosomal maturation factors including nucleolar proteins. For example, in the autosomal recessive disorder, Werner syndrome, there is a mutation in the gene encoding the nucleolar protein Wrn. Wrn appears to be involved in rRNA transcription by Pol I and its loss is hypothesized to result in premature aging, a phenotype of this disorder (Marciniak et al 1998, Shiratori et al 2002). Treacher Collins syndrome is another autosomal recessive disease caused by a mutation in the nucleolar protein Treacle which may affect mRNA and rRNA formation (Dixon et al 2007). In addition, Diamond-Blackfan anemia (DBA) is a disease characterized by abnormal pre-rRNA maturation. It includes mutations in ribosomal proteins essential for the correct assembly of ribosomal subunits. *RPS19* is one of the most frequently mutated genes (Ellis & Gleizes 2011). Thus, gaining a better understanding of the roles of these factors and characterizing these proteins may potentially contribute to the development of a treatment for diseases caused by defects in ribosome biogenesis (Louvet 2004).

Previously, there was little information regarding the dynamics of rRNA processing,, the development of affinity purification procedures and mass-spectrometry analyses has allowed the characterization of complexes, and hence the proteins likely involved in the various stages of ribosome biogenesis (Granneman & Baserga 2004).

In 1969, the Miller and Beaty analysis of electron micrographs identified actively transcribed rDNA genes, where the 5' ends of nascent pre-rRNA transcripts in *Xenopus laevis* oocytes were decorated with condensed terminal knobs. These terminal knobs represented large rRNA processing complexes that include the U3 snoRNP (Dragon et al 2002, Osheim et al 2004).

Ribosomal rRNA maturation and its assembly into ribosomal subunits involve several hundred proteins and small nucleolar ribonucleoprotein complexes (snoRNPs) (Kressler et al 1999, Venema & Tollervey 1999). First, Pol I generates a large pre-rRNA. This RNA contains the sequences for the mature ribosomal RNAs 18S, 5.8S and 25S, two external transcribed spacers (ETS) and two internal transcribed spacers (ITS). A major component of Pol I is its upstream activating factor (UAF). UAF acts as an activator of Pol I and an inhibitor of RNA polymerase II, a protein involved in the transcription of several ribosomal proteins (Siddiqi et al 2001). This primary transcript is chemically modified at numerous sites, and subsequently the subject of endo- and exonucleolytic cleavages to produce its mature forms. The fourth rRNA, 5S rRNA, is independently transcribed as a precursor by RNA polymerase III.

In yeast, the earliest detectable 35S pre-rRNA is cleaved at sites A0, A1 (5' ETS) and A2 (ITS1), which removes the 5' ETS and separates the 18S precursor (20S) from the 25S and 5.8S precursor (27SA2). The 20S pre-rRNA is then transported to the cytoplasm where it is cleaved at site D, yielding the mature 18S rRNA form. The 27SA2 pre-rRNA undergoes several processing steps to yield the mature 5.8S and 25S rRNAs (Granneman and Baserga 2004). About 85% of 27SA2

is cleaved at site A3 in ITS1 by the endonuclease MRP, followed by trimming to site B1S by Rat1 exonuclease; 15% is cleaved directly at site B1L by an unknown enzyme. Cleavage at the 3' end of the 25S (site B2) occurs alongside the cleavage at B1. The 27SB1 and 27SB2 are processed similarly at sites C1-C2 where they undergo 3' and 5' exonucleolytic digestion site E by the exosome complex (Fromont-Racine et al 2003, Granneman & Baserga 2004). The 35S RNA, non-ribosomal and ribosomal proteins form a large RNP complex that is rapidly converted into precursors of the 40S and 60S ribosomal subunits. The pre-40S particles are further processed in the cytoplasm, whereas the pre-60S mature in the nucleus before being transported to the cytoplasm. Thus, ribosome biogenesis is a process requiring several factors and the export of pre-ribosomal subunits to the cytoplasm for final maturation (Granneman & Baserga 2004).

1.3. Small nucleolar ribonucleoprotein complexes

Modification of residues on ribosomal precursors is essential for processing and maturation. The isomerization of uridines to pseudouridines and 2'-O-methylation of riboses are the most predominantly modified nucleotides in rRNAs (Fromont-Racine et al 2003). These modified residues include two key features: 1) they are not vital alone but globally play a role in RNA conformation, and 2) they are localized in functional regions of rRNAs (Green & Noller 1996); (Kowalak et al 1995); (Nissen et al 2000)).

SnoRNAs are short molecules of 60-600 nucleotides, but are mostly in the range of 70-200 nucleotides each (Dragon et al 2006). They can be divided into two major classes that contain evolutionarily conserved sequence elements (Kiss 2001). One major class of snoRNAs is the box C/D snoRNAs. Members of this class contain the motifs box C (PuUGAUGA) and D (CUGA), while the H/ACA snoRNAs contain box H (ANANNA) and the triplet ACA, a motif located exactly 3 nucleotides from the 3' end. Both classes of guide snoRNAs specify sites of modification by forming direct base pairing interactions with substrate RNA. The 2'-O-methylation guide snoRNAs establish long (10-21 bp) duplexes with the target sequence, while pseudourydilation guide snoRNAs form small duplexes (3-10 bp in length). In both classes, short stems bring the conserved boxes close to one another to form core structural motifs. In the box C/D snoRNAs, base pairing of 5' and 3' ends is common and favours the exposure of boxes C and D. Boxes C and D are required for snoRNA stability and accumulation. Base pairing between motifs box C and D leads to the formation of a K-turn motif that binds Snu13 (Dragon et al, 2006).

The C/D snoRNAs sometimess contain C and D-like motifs, C 'and D', which may reproduce the helix formed by boxes C and D. In addition, a conserved sequence specific for rRNA upstream of D or D' regions is present. Box H/ACA snoRNAs fold into a hairpin-hinge-haripin-tail structure; the hinge and tail are single-stranded regions that contain the H and ACA motifs (Figure 1. 1)

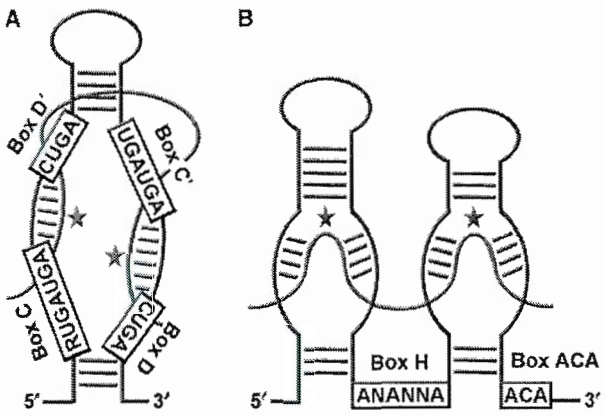

Figure 1. 1 Structure of snoRNAs.

Members of C/D box family contain short sequence elements RUGAUGA (box C) and CUGA (box D) positioned near the 5' and 3' termini. C' and D' boxes are also present. Guiding 2'-O-methylation involves base pairing of the 10-to-21-nucleotide-long sequence positioned upstream of box D (or D') to RNA. The H/ACA class consists of a hairpin-hinge-hairpin-tail secondary structure. One or both of the hairpins are interrupted by an internal loop, the pseudourydilation pocket, which contains two short (3-10 nts) nucleotide sequences complementary to sequences flanking the site of isomerisation. (Reichow et al 2007).

The 7-2/MRP RNA (component of RNase MRP) is another snoRNA although it is not classified as part of the two major groups of snoRNAs. This molecule has no known sequence motifs, however, its structure and certain sequences resemble the RNA component of RNase P, the endonuclease that cleaves the 5' extension of transfer RNA (tRNA) precursors. As a mutation in RNase P in yeast affects pre-

rRNA processing, a role in ribosome biogenesis is suggested (Chamberlain et al 1996).

Biogenesis of functional rRNAs, tRNAs and snRNAs includes the post-transcriptional covalent modification of many carefully selected ribonucleotides. These modifications are important for correct maturation, although these processes are not well understood (Kiss 2002). Although most snoRNAs participate in rRNA modification reactions, some members also take part in processing events. The box H/ACA snoRNA snR30 participates in 18S rRNA synthesis; however, its specific role remains to be characterized. Processing complexes form immediately after the initiation of pre-rRNA transcription. These complexes are viewed as terminal knobs at the edge of 'Christmas trees' in chromatin spreads of rRNA transcription units. Terminal knob formation requires a large complex that includes the U3 snoRNA (known as the small subunit (SSU) processome) (Dragon et al 2002).

1.4. Box H/ACA snoRNA associated proteins.

H/ACA RNAs are found in complexes with the following proteins: Cbf5 (dyskerin in humans), Gar1, Nhp2 and Nop10 (Kiss 2001). All four H/ACA proteins are required for cell viability and rRNA maturation. Cbf5 is a 55-kDa protein and a member of the TruB protein family of pseudouridine synthases (Koonin 1996). Pseudouridylation is catalyzed by Cbf5 (Hoang & Ferre-D'Amare 2001); (Zebarjadian et al 1999). Cbf5 was recently shown to interact directly with the guide

snoRNA and the remaining proteins (Youssef et al 2007) and contains a nuclear localization signal (NLS) and the acidic/lysine-rich domain (KKE/D) found in many nucleolar proteins. Mutations in dyskerin have been shown to cause a genetic disease called dyskeratosis congenita. This includes progressive bone marrow failure, abnormal skin pigmentation and mucosal leucoplakias.

Gar1, a 25 kDa nucleolar protein, is composed of a core domain containing all components needed for its localization and function. The core region is flanked by glycine and arginine-rich (GAR) domains notable for their RNA binding capacity (Girard et al 1992). Gar1 is associated with every H/ACA snoRNA (Girard et al 1992). *In vitro* binding assays showed the core domain to be inefficient in RNA binding, which may suggest a role for an accessory domain in mediating binding, including the GAR domain (Bagni & Lapeyre 1998).

Nop10 is a small nucleolar protein of 58 amino acids with no known motif. It is essential for 18S rRNA synthesis. It is thought that Nop10, along with Nhp2, are essential for uridine selection, association with pre-rRNA particles, and the stabilization of snoRNP complexes through snoRNA contacts (Henras et al 1998). Nop10 is an elongated protein that associates tightly with Cbf5 near the site of its catalytic domain and stabilizes its active site structure (Hamma et al 2005); (Manival et al 2006).

Nhp2 is a protein of 22 kDa and is similar to Snu13 (box C/D protein), suggesting they evolved from a common ancestor. In contrast to Snu13, Nhp2 does not bind specifically to RNA *in vitro*. However, it may do so when bound to another

H/ACA linked protein. Recently Nhp2 was shown to interact with RNAs containing irregular stem-loop structures, but only has weak affinity for double-stranded RNA. The central region of Nhp2p is believed to function as an RNA-binding domain, since it is related to motifs found in various RNA-binding proteins. Removal of two amino acids within the putative B-strand element of the central domain impairs its ability to interact with H/ACA snoRNAs (Henras et al 2001).

1.5. Nucleolar Protein 6 (Nop6)

The snoRNA snR30 is essential for the maturation of pre-rRNA. It was earlier demonstrated that snR30 was required for early processing events leading to 18S rRNA production (Morrissey & Tollervey 1993). We recently purified snR30 by affinity chromatography and identified proteins by mass spectrometry (Lemay et al 2011). One of the proteins was nucleolar protein 6, Nop6, a protein of 26 kDa implicated in ribosome biogenesis (Garcia-Gomez et al 2011). Interestingly, depletion of snR30 disrupts the association of Nop6 with U3, U14, snR4 and snR35 where Nop6 associates most strongly with snR35 (Lemay et al 2011). Nop6 was originally identified as a member of the hydrophillins, a group of proteins known for their high glycine content and hydrophilicity (Garay-Arroyo et al 2000a). A network-based algorithm of large scale data used to assign gene function predicted Nop6 to be a fungal-specific nucleolar protein involved in rRNA processing (Samanta & Liang 2003). Subsequent studies using GFP-tagged Nop6 confirmed its localization to the nucleolus (Huh et al 2003). There is also a genetic interaction between *NOP6* and

NEP1 as *the* deletion of *NOP6, SNR57 or TMA23* genes suppressed a mutation of Nep1 that affects ribosome biogenesis. This further highlights the role of Nop6 in this process (Buchhaupt et al 2007). Moreover, Nep1 is a factor that was shown to methylate pseudouridine residue 1191 of 18S rRNA, and is involved in the assembly of ribosomal protein S19 (Rps19) into pre-40S ribosome subunits. Nep1 is a highly conserved factor that has pseudo-N1-specific methyltransferase activity such that it can catalyze methylation at the N1 of pseudouridines (Piekna-Przybylska et al 2007, Thomas et al 2011). Loss of Nep1 results in a loss of cleavage at site A2, leading to an accumulation of 21S rRNA species. In fact, Nep1 is mutated in Bowen-Condradi syndrome, a lethal genetic disease characterized by low birth weight and a small head (Armistead et al 2009). A recent study on the role of Nop6 in ribosome biogenesis reported that deletion of Nop6 leads to a 20 % drop in 18S rRNA production and a drop in 40S subunit formation, which was suggested to be due to mild inhibition of pre-rRNA processing at cleavage site A2, and deletion did not affect snoRNA formation (Garcia-Gomez et al 2011). Tandem affinity purification followed by mass spectrometry and Northern blot analysis displayed that Nop6 is a component of the 90S pre-ribosomal complex. Localization of Nop6 was shown to be slightly dependant on the transcription of Pol I whereas a loss of the Pol I Rpa49 subunit led to a partial localization in the nucleus. Thus localization of Nop6 to the nucleolus is dependent on pre-rRNA transcription. The intracellular localization of Nop6-eGFP after *in vivo* shutdown of pre-rRNA transcription suggested that Nop6 binds to pre-rRNA early during transcription.

Our bioinformatics results suggest that Nop6 contains two Coiled-coil (CC) motifs, which flank an RNA Recognition Motif (RRM) (Figure 1. 2). Previous analysis also concluded Nop6 to be a basic protein as it is rich in both lysine and arginine, and contains monopartite and bipartite nuclear localization signal (NLS) (Garcia-Gomez et al 2011).

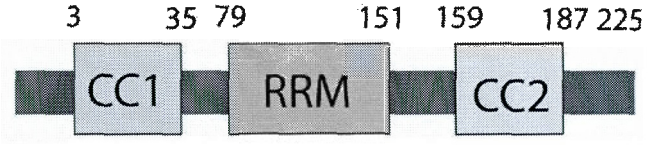

Figure 1. 2 Schematic representation of Nop6 domains.

Coiled-coil 1 (CC1), RNA-binding domain (RRM) and coiled-coil 2 (CC2) are indicated in the schematic representation. The amino acid position of each domain within Nop6 is also shown, as predicted from bioinformatics tools COILS and SMART.

1.6. The coiled-coil domains

Coiled-coil (CC) domains mediate protein-protein interactions and include a repetitive heptad sequence of (abcdefg) n. The first and fourth residues are usually hydrophobic and non-polar, together forming the hydrophobic core. In contrast, the charged and polar fifth and seventh residues form complementary side chains which can interact to form stabilizing salt-bridges. These domains contain two or five alpha helices that are wrapped in a superhelical fashion (Ulijn & Woolfson 2010). In

addition, there are several parallel and anti-parallel CC domains such as the parallel trimeric CC which is illustrated in a helical wheel diagram highlighting their different inter-chain interactions (Figure 1. 3). Dimeric coiled coils such as the anti-parallel MADS box transcription factor is frequently involved in gene regulation, either as activators or as other proteins that are involved in the DNA transcriptio(Changela et al 2003, Lavigne et al 1998, Santelli & Richmond 2000, Walters et al 1997). Using the two-hybrid system, one can verify whether these domains mediate specific protein-protein interactions. The probability of proteins containing CC domains can also be found through bioinformatics tools such as SMART or COILS (Lupas et al 1991, Newman et al 2000).

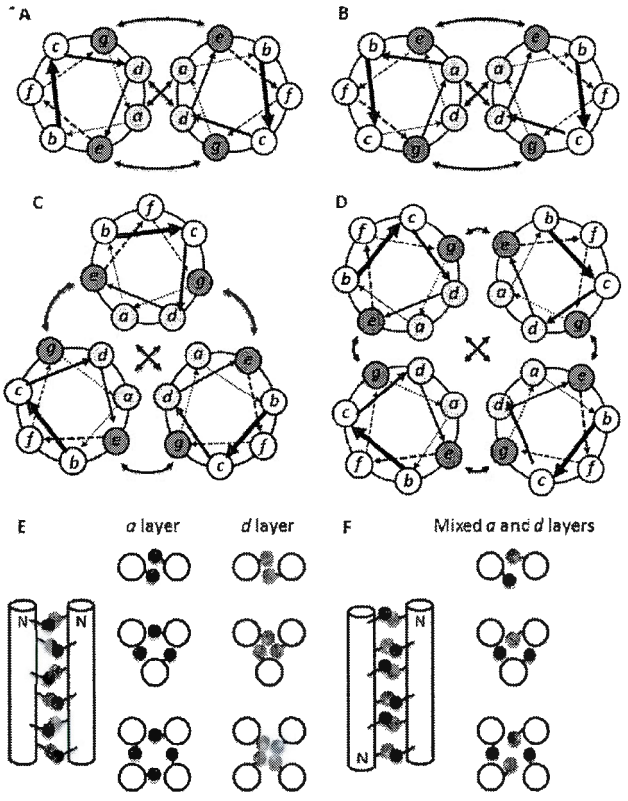

Figure 1. 3 Helical wheel diagram of coiled coil motifs.

(A) parallel dimeric coiled coil, (B) anti-parallel dimeric coiled coil, (C) parallel trimeric coiled coil, and a (D) parallel tetrameric coiled coil. The curved arrows indicate salt bridges while the crossed-arrows indicate hydrophobic interactions. The knobs-in-holes configuration are found in (E) parallel dimeric, trimeric and tetrameric coiled coils and (F) antiparallel dimeric, trimeric and tetrameric coiled coils. Taken from (Apostolovic et al 2010)

CHAPTER 2: HYPOTHESIS and OBJECTIVES

2.1 Hypothesis

Recent studies indicated that Nop6 is a nucleolar protein that is potentially involved in ribosome biogenesis. Moreover, bioinformatics searches have indicated that Nop6 contains two predicted CC motifs and an RNA recognition motif (RRM). The presence of the RRM would suggest that Nop6 may interact with snoRNAs, rRNA, or another RNA molecule. Given the presence of CC motifs in Nop6, which are sites of protein-protein interactions, Nop6 could regulate ribosome biogenesis through contacts with other proteins.

Our main hypothesis is that Nop6 associates with protein partners through its CC domains in complexes, and this helps to regulate ribosome assembly. In addition, due to the presence of an RRM, Nop6 may also affect ribosome maturation through contacts with RNA.

2.2. Objectives

The first objective was to identify partners of Nop6 using a yeast-two-hybrid screen with Nop6 as bait. The second objective was to confirm results of the two-hybrid screen by immunoprecipitation experiments (IPs). In order to conduct the IPs, strains expressing epitope-tagged protein partners of Nop6 were prepared by PCR amplification of modules containing the tag of interest and introduced through homologous recombination. The third objective was to determine if the interactions were direct using GST-pulldown experiments.

CHAPTER 3: RIBOSOME BIOGENESIS FACTOR NOP6 INTERACTS WITH RRT5

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Résumé

Le nucléole est le site de la biogenèse des ribosomes, un processus essentiel pour la survie et la prolifération cellulaire. L'assemblage et la maturation des ribosomes est complexe et comprend de nombreux facteurs impliqués dans le clivage des précurseurs d'ARN ribosomiques. Nous avons récemment découvert que la protéine Nop6 est associée à snR30, une petite RNP nucléolaire impliquée dans la maturation du pré-ARNr, ce qui suggère que Nop6 pourrait être impliquée dans la biogenèse des ribosomes. Des études bioinformatiques ont suggéré que Nop6, une protéine nucléolaire de 26 kDa, contient deux motifs coiled-coil (CC) qui flanquent un motif de liaison à l'ARN (RRM). Nous faisons l'hypothèse que Nop6 se lie à d'autres protéines à travers ses motifs CC, et que ces interactions participent à la maturation des pré-ARNr. Un partenaire potentiel de Nop6, Rrt5, a été retrouvé par une recherche de base de données. Nos résultats doubles hybrides chez la levure montrent que Nop6 interagit avec Rrt5 et que les CC de Nop6 ne sont pas nécessaires pour son association avec Rrt5. Ces résultats ont été confirmés par des tests de GST pulldown,

qui ont révélé que l'interaction Nop6/Rrt5 n'est pas dépendante d'ARN. En outre, nous avons confirmé par une analyse de type « western » que le gène *RRT5* est peu exprimée au cours de la croissance végétative et donc d'autres expériences ont été menées dans des conditions de sporulation. Des analyses par microscopie à immunofluorescence ont montré que Nop6 et Rrt5 sont localisées à la paroi cellulaire exclusivement pendant la sporulation, suggérant une fonction spécifique pour la sporulation.

Ribosome biogenesis factor Nop6 interacts with Rrt5

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3.1. Abstract

The nucleolus is the site of ribosome biogenesis, an essential process for cell survival and proliferation. The assembly and maturation of ribosomes is complex and includes many factors involved in the cleavage of ribosomal RNA precursors. We have recently found protein Nop6 in a complex with snR30, a small nucleolar RNP involved in pre-rRNA processing, which could implicate Nop6 in ribosome biogenesis. Bioinformatics studies have suggested that Nop6, a nucleolar protein of 26 kDa, contains two coiled-coil (CC) motifs which flank an RNA recognition motif (RRM). We hypothesize that Nop6 binds to other proteins through its CC motifs, and these interactions effectively participate in ribosomal subunit maturation. A potential partner of Nop6, Rrt5, was found by database mining. Our yeast two-hybrid results show that Nop6 interacts with Rrt5 and that the CC regions of Nop6 are not necessary for its association with Rrt5. These results were confirmed by GST pulldown assays, which revealed that the Nop6/Rrt5 interaction is not RNA dependent. In addition we confirmed using western blot analysis that Rrt5 is poorly expressed during vegetative growth and thus further experiments of Rrt5 were conducted under sporulating conditions. Immunofluorescence imaging localized Nop6 and Rrt5 to the cell wall exclusively during sporulation, suggestive of a sporulation-specific function for Nop6.

Key Words: Meiosis, nucleolus, Nop6, ribosome biogenesis, *Saccharomyces cerevisiae*

3.2. Introduction

The nucleolus is the most distinctive sub-nuclear compartment. It is the site of rRNA transcription and ribosome biogenesis (Gerbi et al 2001). Ribosomal genes are repeated genes that are grouped into chromosomal regions, nucleolar organizer regions (NOR), where many NORs organize to help form the nucleolus (Dammann et al 1993). This compartment is also involved in the sequestration of proteins involved in cell cycle regulation (Shou et al 1999). RNA polymerase I (Pol I) and its factors remain at the sites of rDNA repeats at the NORs of the chromosomes, while other pre-RNA factors form a sheath around the condensed chromosomes, the perichromosomal space (Dundr et al 2000). Maturation of ribosomal rRNA and its assembly into ribosomal subunits involves several hundred proteins and small nucleolar ribonucleoprotein complexes (snoRNPs) (Kressler et al 1999, Venema & Tollervey 1999).

Ribosome biogenesis begins with Pol I generating a large 35S pre-rRNA precursor that contains the sequences for mature ribosomal RNAs (18S, 5.8S, 25-28S), two external transcribed spacers (ETS) and two internal transcribed spacers (ITS). This primary transcript is subsequently chemically modified at numerous sites and subjected to endo- and exonucleolytic cleavages to produce its mature forms. The 35S pre-rRNA and non-ribosomal and ribosomal proteins form a large RNP complex that is rapidly converted into precursors of the 40S and 60S ribosomal subunits. The pre-40S particles are further processed in the cytoplasm, whereas the pre-60S particles mature in the nucleus prior to transport to the cytoplasm for final assembly.

The snoRNA snR30 is essential for the maturation of pre-rRNA. It was previously shown that snR30 is required for early processing events leading to 18S rRNA production (Morrissey & Tollervey 1993). We have recently purified snR30 by affinity chromatography and proteins were identified by mass spectrometry. One of the proteins was Nop6, a protein of 26 kDa. Interestingly, a depletion of snR30 disrupted the association of Nop6 with U3, U14, snR4 and snR35 snoRNAs; Nop6 associated most strongly with snR35 (Lemay et al 2011).

Nop6 was originally identified as a member of the group of proteins called hydrophillins due to its high glycine content and hydrophilicity (Garay-Arroyo et al 2000a). A network-based algorithm of large scale data used to assign gene function predicted Nop6 to be a fungal-specific nucleolar protein involved in rRNA processing (Samanta & Liang 2003). Studies using GFP-tagged Nop6 confirmed its localization to the nucleolus (Huh et al 2003) . A recent study on the role of Nop6 in ribosome biogenesis reported that the deletion of Nop6 leads to a 20 % decrease in 18S rRNA and a decrease in 40S subunit formation; it is suggested that this results from a mild inhibition of pre-rRNA processing at cleavage site A2 (Garcia-Gomez et al 2011). The intracellular localization of Nop6-eGFP after *in vivo* shutdown of pre-rRNA transcription suggested that Nop6 binds to pre-rRNA early during transcription (Garcia-Gomez et al 2011). These authors showed Nop6 to be a basic protein as it is rich in lysine and arginine, and contains both monopartite and bipartite nuclear localization signals (NLS). Our bioinformatics analyses suggest that Nop6 contains two coiled-coil (CC) motifs, which flank an RNA recognition motif (RRM) (Figure 3.

1A). This suggests Nop6 could mediate RNA maturation through its interactions with other proteins using its CC motifs as this structural motif is known for mediating such interactions (Ulijn & Woolfson 2010). Since Nop6 has already been implicated in ribosome biogenesis, identifying protein effectors for its function during this process is of interest. One interesting protein partner already identified in a genome-wide two-hybrid screen is Rrt5, a protein that is highly expressed during sporulation and possibly involved in regulating rDNA transcription (Hontz et al 2009, Naitou et al 1997).

We have determined that deletion of both Nop6 CC domains does not impede its association with Rrt5. In addition, Nop6 and Rrt5 co-localize only during sporulation, which suggests Nop6 could have a separate function from ribosome biogenesis.

3.3. Materials and Methods

3.3.1 Yeast strains and plasmids

Yeast strains that were used in this study are listed in Table 3.1. Rrt5-Myc was prepared through the integration of a 9xmyc epitope tag at the C-terminus using standard PCR amplification (Longtine et al 1998). The knockouts of Rrt5 and Nop6 were obtained by integrating a cassette of auxotrophic markers at the *NOP6* and *RRT5* loci. Strains expressing myc- or HA-tagged constructs under the control of their natural promoter were generated as described (Longtine et al 1998). Strains

YPH499, YPH500 were modified to express Ndt80 and Sum1 under the control of the GAL1 promoter (Longtine et al 1998).

The sequence corresponding to the open reading frame (ORF) of *NOP6* was amplified from genomic DNA extracted by standard procedures (Ausubel 1999) using oligonucleotides NOP6-For- (5'- CCC CCC GGG TAT GGG GTC CGA GGA AGA TAA AAA G-3') and NOP6-*Rev- (5'-* CGC CTC GAG TCA TTT AAG TAG TTT GGC TC-3'). The deletion mutant of Nop6 lacking the coiled-coil 1 domain was amplified using oligonucleotides NOP6 ΔCC1-For- (5'- CGC CCC GGG TAT GCC AGA AGG AAA AAG ACC C-3') and NOP6-*Rev- (5'-* CGC CTC GAG TCA TTT AAG TAG TTT GGC TC-3'). The deletion mutant lacking the CC2 domain was amplified using oligonucleotides NOP6-For- (5'- CCC CCC GGG TAT GGG GTC CGA GGA AGA TAA AAA G-3') and NOP6 ΔCC2-Rev- (5'- GCG CTC GAG CTA TCC GCC AAC AGT TAG CTC-3'). The double deletion mutant of Nop6 was amplified using oligonucleotides NOP6 ΔCC1-For- (5'- CGC CCC GGG TAT GCC AGA AGG AAA AAG ACC C-3') and NOP6 ΔCC2-Rev (5'- GCG CTC GAG CTA TCC GCC AAC AGT TAG CTC-3'). The PCR products were cloned between restriction sites XmaI and XhoI in pGBKT7 (Clontech) and pGEX4T1 (Amersham Pharmacia) (Table 3.3 and 3.5). A clone of NOP6 containing a mutation in the RNP1 motif was generated a two-step PCR mutagenesis strategy using a first amplification with oligonucleotides NOP6-For and NOP6-MutRNP1-R- (5'- GGC TGC CGC AGC TGC AGC AGC TGC GTC AGC TCT GAG GCG GAT CT-3') and separate amplification consisting of oligonucleotides NOP6-MutRNP1-For- (5'- GCA GCT

GCT GCA GCT GCG GCA GCC GAT GCT GAC AAA GAT CGC AC-3') and NOP6-*R*-. The two PCR products from the first amplification were further amplified together using oligonucleotides NOP6-F-and NOP6-*R*-.This RNP1 mutant was cloned between sites XmaI and XhoI in both pGBKT7 and pGEX4T1. Similarly, the ORF of *RRT5* was amplified using oligonucleotides RRT5-For- (5'- GCG GAA TTC ATG ACA GAA CAA GTT AAC AAT GAC-3') and RRT5-Rev- (5'- CGC GAA TTC CTA AGT AGC AGC CAC GGT-3') and cloned in the site EcoRI in pGADT7. The ORF of RRT5 was subcloned from pGADT7 into pMal-c5x (New England Biolabs). Two-hybrid plasmids, pGBKT7 containing Nop6 or mutant derivatives, and pGADT7 containing full length Rrt5 were transformed in AH109 The integrity of all constructs was verified by automated sequencing at the McGill University and Génome Québec Innovation Centre.

3.3.2. Sporulation of diploid cells

In order to verify whether deletion of *NOP6* and *RRT caused changes in sporulation*, strains YPH501, Nop6Δ/Nop6Δ, Rrt5Δ/Rrt5Δ, Nop6ΔRrt5Δ/Nop6ΔRrt5Δ, (Table 3. 1) were sporulated as previously described (Nickas & Neiman 2002a). Cells were sporulated after incubation in YPD (1% yeast extract, 2% peptone, 2% dextrose), grown to mid-log phase in YP acetate 0.1% and transferred to 2% potassium acetate at an optical density of 2 A600 units with rapid shaking at 30°C in liquid.

3.3.3. Immunoprecipitation experiments (IPs)

Cells expressing myc-tagged Rrt5 were grown in synthetic media to exponential phase (A_{600} ~ 0.5), sporulated as described (Nickas & Neiman 2002b), and harvested by centrifugation. Cells were stored at -80°c. The cell pellet was washed twice in ice-cold sterile water and resuspended in TMN100 buffer (25 mM Tris-HCl (pH 7.6), 10 mM $MgCl_2$, 100 mM NaCl, 1 mM 1,4-dithiothreitol (DTT), 0.1% NP-40) complemented with Complete protease inhibitor (Roche). The buffer to cell ratio was 100 µl/A_{600} unit. Whole cell extract was prepared with glass beads (Sigma). The lysate was cleared by centrifugation (5 min, 10000 x g). A volume of 0.5 ml of cell extract was incubated with 25 µl of ProteinA (Roche) beads coupled to myc mAb at 4°C for 2 h on a nutator. Beads were washed with 1 mL of TMN100 lacking NP40 and DTT several times. Elution from Protein A beads was carried out with SDS sample buffer. Samples were separated on polyacrylamide gels and analyzed by Western blotting.

3.3.4. GST-Pulldown

GST-Nop6 fusion constructs were expressed in Rozetta 2pLys (Novagen) grown to exponential phase in LB (1% tryptone, 0.5% NaCl, 0.5 % yeast extract) containing ampicillin (100 µg/ml). Fusion proteins were expressed by induction with 1mM IPTG for 2 hours. Rozetta 2pLys cells expressing MBP-Rrt5 were grown to exponential phase in LB broth and 0.2 % dextrose, and then induced with IPTG (0.3 mM) for 2 hours. Cells were harvested by centrifugation and frozen at -80 ° C. Cell

pellets were resuspended in PBS buffer (13.7 mM NaCl, 0.27 mM KCl, 10 mM Na_2HPO4, 0.2 mM KH_2PO4 pH 7.4). Total protein extracts were prepared by sonication and debris removed by centrifuging for 10 min. MBP-Rrt5 was bound to amylose column washed with NETN500 (0. 5% NP40, 0.1 mM EDTA, 20 mM Tris-HCl pH 7.4, 500 mM NaCl) and eluted with NETN200 (20 mM Tris-HCl pH7.4 , 200 mM NaCl, 0.1 mM EDTA) containing 10 mM maltose. GST-pulldown assays were carried out as described (Frangioni & Neel 1993). Essentially, GST or GST-Nop6 fusions were bound to 25 µL of GST coupled beads (GE healthcare), washed with NETN150 mM [0. 5% NP40, 0.1 mM EDTA, 20 mM Tris pH 7.4, 150 mM NaCl, Complete protease inhibitor cocktail (Roche)] and Rrt5-myc yeast extracts or MBP-Rrt5 were incubated with GST columns followed by elution with SDS 2X sample buffer. Samples were migrated in a polyacrylamide gel and analyzed by Western blotting. Rrt5 was detected through Western blotting with anti-myc (9E10) and anti-MBP (New England Biolabs) antibodies.

3.3.5 Immunofluorescence microscopy

Yeast cells were processed as described to detect changes in protein expression during sporulation (Nickas & Neiman 2002a). Purified anti-Nop6 rabbit polyclonal antibodies, and 9E10 anti-myc mouse monoclonal antibody were used to recognize Nop6 and Rrt5-myc, while mouse monoclonal 17C12 anti-fibrillarin antibody was used to identify the nucleolar marker Nop1 (Yang et al 2001). Antibodies were diluted 1/500 in blocking buffer (0.5% BSA, 0.5% Tween-20 in

PBS) and incubated overnight at 4°C. Samples were washed in blocking buffer several times and incubated for 30 minutes at room temperature with secondary antibodies Alexa Fluor 488 goat anti-rabbit IgG and Alexa Fluor 555 goat anti-mouse IgG (Invitrogen) diluted 1/1000 in blocking buffer. Nuclear DNA was stained for 15 min with 1 µg/ml of 4'-6-diamidino-2-phenylindole (DAPI). Coverslips were washed with blocking buffer and slides were mounted with ProLong Gold antifade reagent (Invitrogen). FITC-concanavalin A staining was used to determine the cell wall association of Nop6 and Rrt5, and Alexafluor 555 was used to detect Nop6 and Rrt5 (Nickas & Neiman 2002a). Specimens were observed with an Eclipse Ti inverted microscope (Nikon) using an Apochromat objective (numerical aperture 1.4). Images were acquired with a Monochrome CCD camera (CFW1312 from Scion Corporation) and processed with ImageJ version 1.34s (Wayne Rasband, National Institutes of Health, USA).

3.4. Results and Discussion:

3.4.1 Nop6 interacts with Rrt5 in the two-hybrid system

After conducting a literature search for potential partners of Nop6 we have found that a previous array screen showed that Nop6 interacts with Yfr032c, a protein of unknown function. However, further studies have not confirmed this interaction (Uetz et al 2000). Northern analyses studying the expression levels of several genes of chromosome VI indicated that *YFR032C* is highly expressed during sporulation and mRNA was undetectable in vegetative growth (Naitou et al 1997). Yfr032c was also identified as a modulator of transcription of ribosomal DNA (rDNA). It has been re-named Rrt5 for "Regulator of ribosomal DNA transcription" (Hontz, Niederer et al. 2009).

To examine the interaction between Nop6 and Rrt5 in the yeast two-hybrid system, Nop6 was cloned in the bait vector pGBKT7 and Rrt5 was cloned into the prey vector pGADT7. These proteins showed a strong interaction with the ability to bind as cells can grow on media containing a high concentration of 3-aminotriosol (15 mM), an inhibitor of the product of the *HIS3* reporter gene. Nop6 contains two coil-coiled domains and an RNA- recognition motif (RRM) (Figure 3. 1). The RNA-recognition motif (RRM), also known as RBD (RNA-binding domain) or RNP (ribonucleoprotein domain) is the most frequent RNA binding domain in higher vertebrates and most studied (Clery et al 2008). We created deletion mutants of Nop6 lacking one or both of the CC motifs (Figure 3. 1). The loss of both motifs, leaving

just the RRM and a portion of flanking sequences intact, still produced a strong association with Rrt5 (Figure 3. 1D). This suggests that the CC motifs of Nop6 do not mediate its interaction with Rrt5. Rrt5 also contains a RBD domain; however we were unable to express Rrt5 deletion mutants lacking the RRM. In addition NOP6-RNP1 mutant cloned into pGBKT7 was unstable and could not be expressed in AH109 cells.

3.4.2. Nop6 and Rrt5 may be involved in the regulation of sporulation

RRT5 mRNA expression was previously shown to be strongly expressed during sporulation (Naitou et al, 1997). Rrt5 protein bearing a 9-myc tag was barely detectable under vegetative growth and highly expressed during sporulation (Figure 3. 2, lanes 3 and 5). Rrt5 was slightly immunoprecipitated under vegetative conditions, and since it is highly expressed during sporulation a large amount was immunoprecipitated under these conditions (Figure 3. 2, lanes 4 and 6). Naitou et al, (1997) indicated that *RRT5* does not contain an URS1 regulatory site found in most early sporulation genes, and thus, is most likely a middle or late-meiotic sporulation gene. The loss of *RRT5* does not affect sporulation, however loss of *NOP6* alone lowers sporulation (Table 3. 1). Interestingly the double knockout of *RRT5* and *NOP6* shows normal levels of sporulation compared to single *NOP6* KO. There appears to be a global loss in sporulation in *NOP6* KO cells, however this is just due to less cells being counted. This indicates that the effects of *NOP6* loss could be alleviated by the

loss of *RRT5* and Rrt5 mediates the effects of Nop6 during sporulation. Ectopic overexpression of two regulators of middle sporulation genes, *NDT80* and *SUM1* under the inducible GAL1 promoter did not affect the levels of *RRT5* transcript (data not shown).

RRT5 gene appears to also contain a MSE element in its promoter region, which contains the consensus sequence of an MSE (Table 3. 2) MSEs of genes regulated by global middle sporulation gene activator Ndt80are indicated in Table 3. 2A and genes with MSEs and not regulated by Ndt80 are indicated in Table 3.2B. Many middle sporulation genes are activated by the global activator Ndt80, which also positively regulates its own expression. Ndt80 is a transcriptional activator that binds to the conserved middle sporulation element that is upstream of middle sporulation genes. These MSE elements could have dual function as during sporulation they function as activator sites and during vegetative growth as repressors sites. The fact that overexpression of Ndt80 does not increase the levels of *RRT5* would indicate that *RRT5* may be a mid-late sporulation gene (data not shown). In contrast to RRT5, the loss of *NOP6* alone lowers sporulation, which suggests that this normally, nucleolar protein could have a function during sporulation (Table 3. 1).

In *Saccharomyces cerevisiae*, the dynamics of the nucleolus are different from other organisms and like other fungi, this is not well understood. Nucleoli maintain their integrity during both mitosis and meiosis which is linked to their endonuclear type of division (Loidl 2003). In meiosis, nuclei do not completely separate at the end

of the first division but form a dumb-bell shaped structure. Immunostaining against nucleolar proteins Nop1, Nop5 and Nhp2 revealed that the nucleoli are attached to the NORs during the first meiotic division until anaphase II. At the end of the second meiotic division, nucleolar proteins remained with the cytoplasm of the ascus and no nucleolar material was detected in the prospores (Fuchs & Loidl 2004). This may indicate that Nop6 as a nucleolar protein may have a separate function during meiosis due to possible changes in localization.

3.4.3. The interaction between Nop6, and Rrt5 is not RNA-mediated

As the yeast two-hybrid system can sometimes produce artefacts, we used standard *in vitro* methods to confirm the association of Nop6 with Rrt5. Myc tagged Rrt5 was used in pull-down assays with GST-Nop6 or its mutant derivatives immobilized on glutathione Sepharose.

In order to verify the association of Nop6 and Rrt5, Nop6 was immunoprecipitated from whole cell extracts expressing Rrt5-myc, by coupling it to Agarose protein-A beads, which were bound to anti-Nop6 rabbit polyclonal antibodies. Nop6 was able to co-immunoprecipitate Rrt5 (Figure 3. 3A). In addition, the association of GST-Nop6 expressed from *E. coli* with Rrt5-myc expressed from yeast whole cell extracts was tested to confirm that the association of Nop6 with Rrt5 was direct. Glutathione Sepharose beads were coupled to bacterial extracts that expressed GST alone, GST-Nop6 or GST-Nop6 mutant derivatives followed by

incubation with yeast whole cell extracts expressing Rrt5-myc. Rrt5 was able to associate with GST-Nop6 and its mutant derivatives, but not GST alone (Figure 3. 3B). Finally, to confirm the direct interaction of Nop6 and Rrt5, GST and GST-Nop6 constructs expressed from bacteria were coupled to glutathione beads followed by the addition of Rrt5 expressed in fusion with MBP. MBP-Rrt5 could be pulled down with GST-Nop6 constructs, but not GST alone (Figure 3.3C). We confirmed that the association of Nop6 with Rrt5 was not mediated through its coiled-coil (CC) domains as observed in the two-hybrid tests as loss of both CCs did not disrupt this association (Figure 3.3 B and C). To examine if the association between Nop6 and Rrt5 is RNA-mediated, Nop6 was immunoprecipitated from whole cell extracts expressing Rrt5-myc in the presence or absence of RNase A. The association between Nop6 and Rrt5 was not disrupted in the presence of RNase A, which indicates a protein-protein interaction with Nop6 (Figure 3.3A). The RNP1 motif in the RRM is known to be vital for RNA recognition and its disruption was hypothesized to affect the interaction with Nop6 via an RNA intermediate. The mutation of RNP1 did not alter the stability of the association of Nop6 and Rrt5 (Figure 3.3B and C), which further indicates this interaction is not RNA-mediated.

3.4.4. Nop6 and Rrt5 co-localize to the cell wall in spores

Nop6 was previously demonstrated to be a potential ribosome biogenesis factor. It was localized to the nucleolus, the site of ribosome biogenesis ((Huh et al 2003);Garcia-Gomez et al, 2011; Lemay et al, 2011). Rrt5-myc cells were grown under vegetative conditions or sporulated in 2 % acetate solution in order to conduct immunolocalization. During vegetative growth, Nop6 co-localized with the nucleolar marker Nop1, while Rrt5 localized to the cell periphery (Figure 3. 4A and 3B). After induction of sporulation, Rrt5 and Nop6 both co-localized to the cell membrane of asci (Figure 3. 4A). Unlike Nop6, the nucleolar marker Nop1 remained in discrete points after sporulation (Figure 3. 4A). In order to confirm this localization to the cell wall, cells were stained with the cell wall marker concanavalin A (ConA). Indeed, Nop6 and Rrt5 co-localized with ConA to the cell wall (Figure 3. 4C). This result could indicate an unexpected function of Nop6 during sporulation.

Ribosome biogenesis is a complex process involving several hundred factors. Nop6 is a factor that is involved in ribosomal maturation whose function is still not well understood. We have shown that Nop6 interacts physically with Rrt5 and they co-localizes to the cell wall of spores. Thus Nop6 seems to be an interesting factor that may be altered after sporulation.

3.5. Tables

Table 3. 1. Nop6 deletion alone lowers sporulation frequency.

Strain	Sporulated	Unsporulated	Percent Sporulated
YPH501	494	1309	27 %
rrt5Δ/rrt5Δ	568	2130	20.9 %
nop6Δ/nop6Δ	66	468	11.7 %
nop6Δrrt5ΔY/nop6Δrrt5Δ	345	1193	22.2 %

Table 3. 2. Mid-sporulation elements and their regulation.

(A)			gNCRCAAAA/T
SMK1	-69	←	GTCACAAAAT
SPS1	-370	←	GACACAAAAT

(B)			gNCRCAAAA/T
SPR6	-273	→	TACACAAAAT
SPS2	-30	←	GCCACAAAAT
RRT5	-889	→	GCCACAAAAT

Table 3. 3 Yeast strains used in study

Strain	Alias	Genotype	Source
YPH499	-	MATa *ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*	(Sikorski & Hieter 1989)
YPH500	-	*MATα ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*	(Sikorski & Hieter 1989)
YPH501	-	YPH499/YPH500	(Sikorski & Hieter 1989)
AHY1	NOP6ΔYPH499	YPH499, *NOP6Δ::HIS1*	(Lemay et al, 2011)
AHY2	NOP6ΔYPH500	YPH500, *NOP6Δ::TRP1*	(Lemay et al, 2011)
AHY3	RRT5-9-Myc/RRT5-3-Myc	YPH499, RRT5-9-Myc ::TRP1/YPH500, RRT5-3-Myc ::HIS1	This work
AHY4	NOP6ΔYPH499/ NOP6ΔYPH500	YPH499, *NOP6Δ::HIS1*/ YPH500, *NOP6Δ::TRP1*	This work
AHY5	RRT5ΔYPH499/ RRT5ΔYPH500	YPH499, *RRT5Δ::TRP1*/ YPH500, *RRT5Δ::HIS1*	This work
AHY6	GAL::NDT80	YPH499, P_{GAL1}-*NDT80::TRP1*/ YPH499, P_{GAL1}-*NDT80::TRP1*/ YPH500, P_{GAL1}-*NDT80::HIS1*	This work
AHY7	RRT5ΔYPH499/ NOP6ΔYPH500	YPH499, *RRT5Δ::TRP1*/ YPH500, *NOP6Δ::HIS1*	This work
AH109	-	MATa, trp1-901, leu2–3, 112, ura3-52, his3-200, gal4Δ, gal80Δ, LYS2::GAL1UAS-$GAL1_{TATA}$-HIS3, $GAL2_{UAS}$-$GAL2_{TATA}$-ADE2, URA3::$MEL1_{UAS}$-$MEL1_{TATA}$-lacZ, MEL1	Clontech

AHY8	pGBKT7+pGADT7-Rrt5 (AH109)	Same as AH109 except has pGBKT7 and pGADT7-Rrt5	This work
AHY9	pGBKT7-Nop6+pGADT7-Rrt5 (AH109)	Sames as AH109 except has pGBKT7-Nop6 and pGADT7-Rrt5	This work
AHY10	pGBKT7-Nop6+pGADT7(AH109)	Same as AH109 except has pGBKT7-Nop6 and pGADT7	This work
AHY11	pGBKT7-Nop6 ΔCC1+pGADT7	Same as AH109 except has pGBKT7-Nop6 ΔCC1and pGADT7	This work
AHY12	pGBKT7-Nop6 ΔCC1+pGADT7-Rrt5	Same as AH109 except has pGBKT7-Nop6 ΔCC1and pGADT7-Rrt5	This work
AHY13	pGBKT7-Nop6 ΔCC2+pGADT7	Same as AH109 except has pGBKT7-Nop6 ΔCC2 and pGADT7	This work
AHY14	pGBKT7-Nop6 ΔCC2+pGADT7-Rrt5	Same as AH109 except has pGBKT7-Nop6 ΔCC2 and pGADT7-Rrt5	This work
AHY15	pGBKT7-Nop6 ΔCC1/ΔCC2+pGADT7	Same as AH109 except has pGBKT7-Nop6 ΔCC1/ΔCC2 and pGADT7	This work
AHY16	pGBKT7-Nop6 ΔCC1/ΔCC2+pGADT7-Rrt5	Same as AH109 except has pGBKT7-Nop6 ΔCC1/ΔCC2+pGADT7-Rrt5	This work

Table 3. 4 Oligonucleotides used in study

	Sequence
pGEX-NOP6 For	5'–GCGGGATCCGGGTCCGAGGAAGATAAAAAG–3'
pGEX-NOP6 Rev	5'–CGCCTCGAGTCATTTAAGTAGTTTGGCTC–3'
NOP6 KO F1	GTA ATC TAA TGT AGA GGA CAG GAT AGA GAT TGA AGA CGT CTA CAG CTA AAC GGA TCC CCG GGT TAA TTA A
NOP6 KO R1	TTT ATT TTC TTT TTG TCT AAG ATA CAT GGC GTA TAA AAT TAA ATT TGT ACG AAT TCG AGC TCG TTT AAA
NOP6-BamHI-For	GCG GGA TCC GGG TCC GAG GAA GAT AAA AAG
NOP6-XhoI-Rev	CGC CTC GAG TCA TTT AAG TAG TTT GGC TC
DeltaCC1 nop6-For (XmaI)	CGC CCC GGG TAT GCC AGA AGG AAA AAG ACC C
DeltaCC2 nop6-Rev (XhoI)	GCG CTC GAG CTA TCC GCC AAC AGT TAG CTC
Nop6 mut RNP1-for	GCA GCT GCT GCA GCT GCG GCA GCC GAT GCT GAC AAA GAT CGC AC
Nop6 mut RNP1-rev	GGC TGC CGC AGC TGC AGC AGC TGC GTC AGC TCT GAG GCG GAT CT
Nop6-Xma-2H	CCC CCC GGG TAT GGG GTC CGA GGA AGA TAA AAA G
YFR032C-EcoRI-For	GCG GAA TTC ATG ACA GAA CAA GTT AAC AAT GAC
YFR032C-EcoRI-Rev	CGC GAA TTC CTA AGT AGC AGC CAC GGT
S3-YFR032C	TCC CAC CTC CGC CTG CTT CGT CTT CCG ATC GTC CAA CCG TGG CTG CTA CTC GTA CGC TGC AGG TCG AC

S2-YFR032C	TTT ATG TTA AGT TCT CAC TGT TTA TAC CCC CTT TTC AAT TTT ATA AAG ATA TCG ATG AAT TCG AGC TCG
YFR032CcheckCtFOR	CGC GAC CCA CCT CCA GCA GC
YFR032CcheckCtREV	CTT AAA AGG GGT TCC CCC AGC CGC
F4-YFR032C	TCT TTT TCC TCC TTA TGT GCC ACA AAA TGG AAG CGT CAC AAA TTA ATA ACG AAT TCG AGC TCG TTT AAA C
R2-YFR032C	ATG GTA GTG GTA GTG TCA CTG GTA GTG TCA TTG TTA ACT TGT TCT GTC ATT TTG AGA TCC GGG TTT T
YFR032CcheckNtFOR	TTT ATC TCA ATA TCC AGT ACC GTT TTC
YFR032CcheckNtREV	CGC CAT AGT TGT TTA GGA AGG C
Yfr032c Mut delta RRM NdeI-For	GAC CAT ATG ACA CGT TCC TTA CGA GGC
Yfr032c Mut delta RRM c-XmaI-	AAC CCC GGG GTA GAG TCT GCC TCG TAA GGA ACG TGT AAC TTT AGA AAT AGT TTC TGA TTT
RRT5-Check-KO-For	CCA CAA GCA TGC CCT ACA CGG CAC
R1-RRT5-KO	TTT ATG TTA AGT TCT CAC TGT TTA TAC CCC CTT TTC AAT TTT ATA AAG ATG AAT TCG AGC TCG TTT AAA C
F1-RRT5-KO	AAC ACA TAG AGA AGT ATA AGG CCT CAC ATA AGC ATA CAA ACA AGC CCG CAC GGA TCC CCG GGT TAA TTA A
F4-NDT80	GAC AAA GCT CCA GAA CGG TTG TCT TTT GTT TCG AAA AGC CAA GGT CCC TTG AAT TCG AGC TCG TTT AAA C
R3-NDT80	TTG GAT ACG AGA TCA TCC TGT AAT ACT GGA TCT GTG TTT TCC ATT TCA TTA GCA CCA CCG CAC TGA GCA GCG TAA TCT G
R2-NDT80	GAT ACG AGA TCA TCC TGT AAT ACT GGA TCT

	GTG TTT TCC ATT TCA TTC ATC ATT TTG AGA TCC GGG TTT T
NDT80-CheckNt-For	TAC TTC CGC GGC TAT TTG ACG TTT TCT
NDT80-CheckNt-Rev	TCT TTT ATA ACC TAC CCA CTC TTC ATC AAT ATG
F4-SUM1	ACA GGC ATA TTT TAT CAA AAG TGT CAG CAA ACA GAG CAC AAG GGA CTT GTG AAT TCG AGC TCG TTT AAA C
R3-SUM1	AGT CTC TGT TCA TTG GTT ATG TTA TCA GAA GGG GCT GTG GTG TTC TCA GAA GCA CCA CCG CAC TGA GCA GCG TAA TCT G
R2-SUM1	CTC TGT TCA TTG GTT ATG TTA TCA GAA GGG GCT GTG GTG TTC TCA GAC ATC ATT TTG AGA TCC GGG TTT T
anti-ySRP	CCC ACC AGA AAG CCA TTA CAG CC
anti-18S	CAT GGC TTA ATC TTT GAG AC

Table 3. 5 Plasmids used in study

Name	*Source*
pGBKT7	Clonetech Matchmaker
pGADT7	Clonetech Matchmaker
pGBKT7-XmaI For-Nop6 fulllength-XhoI-Rev	This work
pGBKT7-XmaI For-Nop6mutdeltaCC1-XhoI-Rev	This work
pGBKT7-XmaI For-Nop6mutdeltaCC2-XhoI-Rev	This work
pGADT7-XmaI For-Rrt5 fulllength-XhoI-Rev	This work
pGADT7-NdeI-Rrt5mutdeltaRRM-EcorI-Rev	This work
pGADT7-EcorI-For-Rrt5mutdeltaRRM-XmaI-Rev	This work
pFA6-TRP1	Longtine et al, 1998
pFA6-HISMX6	Longtine et al, 1998
pFA6-TRP1-PGAL1	Longtine et al, 1998
pFA6-kanMX6-PGAL1	Longtine et al, 1998
pFA6-HIS3MX6-PGAL1	Longtine et al, 1998
pMalc5x-Rrt5	This work
pGEX4T1-XmaI For-Nop6MutRNP1-XhoI-Rev	This work
pGEX4T1-XmaI For-Nop6 fulllength-XhoI-Rev	This work
pGEX4T1-XmaI For-Nop6mutdeltaCC1-XhoI-Rev	This work
pGEX4T1-XmaI For-Nop6mutdeltaCC2-XhoI-Rev	This work

3.6. Figure and Table Legends

Table 3. 6. Nop6 deletion alone lowers sporulation frequency.

YPH501 cells containing double KO of *RRT5* and/or *NOP6* were sporulated as described (Nickas & Neiman 2002a). The number of sporulated cells for each individual strain was counted by standard methods and percentage was marked.

Strain *nop6*

Figure 3.1. Two-hybrid interaction of Nop6 and Rrt5 does not require the CC domains.

AH109 cells expressing either empty vectors or pGBKT7-Nop6 and pGADT7-Rrt5 were used. In (A) the structure of Nop6 is illustrated. Nop6 contains two CC domains CC1 (3-35), and CC2 (159-187), and a RRM (79-151). (B) Rrt5 contains a RRM (19-101) and another nucleotide binding motif, αβ plait. Cells were plated on control medium lacking leucine and tryptophan (C) to select for bait (pGBKT7) and prey (pGADT7) plasmids or selective medium also lacking histidine (D) to test for activation of the reporter gene *HIS3* in the presence of 15 mM 3-AT. Nop6 is expressed from the bait vector pGBKT7, and Rrt5 is expressed from the prey vector pGADT7. Nop6 lacking both CC1 and CC2 is named Nop6 Δ CC1/CC2

Figure 3.2. Rrt5 is highly expressed during sporulation.

Rrt5 was immunoprecipitated during sporulation (S) or vegetative growth (V) (lanes P). Cells were sporulated by being grown in rich media followed by transfer to 2% acetate solution. The mouse IgG heavy chain (HC) runs at 55 kDa. The untagged strain used as a control was YPH501. Total extracts (T) and pellets from IPs (P) are shown. Additional breakdown products of Rrt5 were present in total extracts of Rrt5-myc during sporulation (lane 5).

Figure 3.3 The interaction of Rrt5 and Nop6 is not RNA-mediated.

Whole cell extracts were untreated (-) and treated (+) with RNase A 1mg/ml. (B) Whole cell extract of Rrt5-myc was pulled down with GST-Nop6, its mutant derivative (Lanes 3-7). GST alone was used as a control (lane 2). (C) Whole cell extract containing MBP-Rrt5 was pulled down with GST-Nop6 (lanes 3-6). GST alone was used as a negative control (lane 2). Total extract (T) and pellets (P0 are shown.

Figure 3.1 Nop6 and Rrt5 co-localize to the cell wall spores.

Fluorescence from YPH501 cells expressing Rrt5-9-myc. Cells were grown in 2% potassium acetate and processed for visualization of fluorescent proteins as described in materials and methods. Nop6 was detected with anti-Nop6 rabbit polyclonal antibodies. Rrt5 was detected using anti-myc monoclonal mouse antibodies. Nop1 was detected by anti-Nop1 monoclonal antibodies. Cells were visualized during vegetative conditions or under sporulation (A) Nop6 (green) co-localizes with nucleolar protein Nop1 (red) during vegetative growth, but not during sporulation. Nuclear DNA was stained with DAPI (merged images during vegetative growth). (B) Nop6 (green) co-localizes with with 9myc-tagged Rrt5 (red) during sporulation. (C) Nop6 (red in top panel) and Rrt5 (red in bottom panel) localize with cell wall staining substrate concanvalin A (Con A; green) in spores.

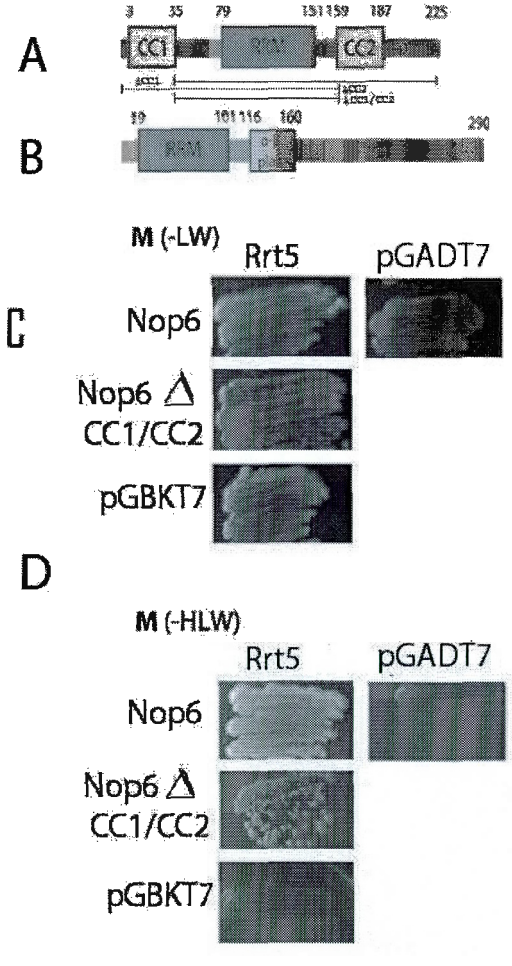
A
B
C
M (-LW)
Rrt5
pGADT7
Nop6
Nop6 Δ CC1/CC2
pGBKT7
D
M (-HLW)
Rrt5
pGADT7
Nop6
Nop6 Δ CC1/CC2
pGBKT7

Figure 3.1

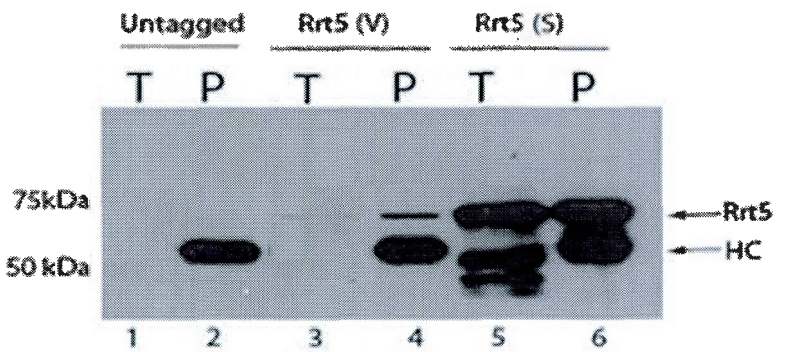

Figure 3.2

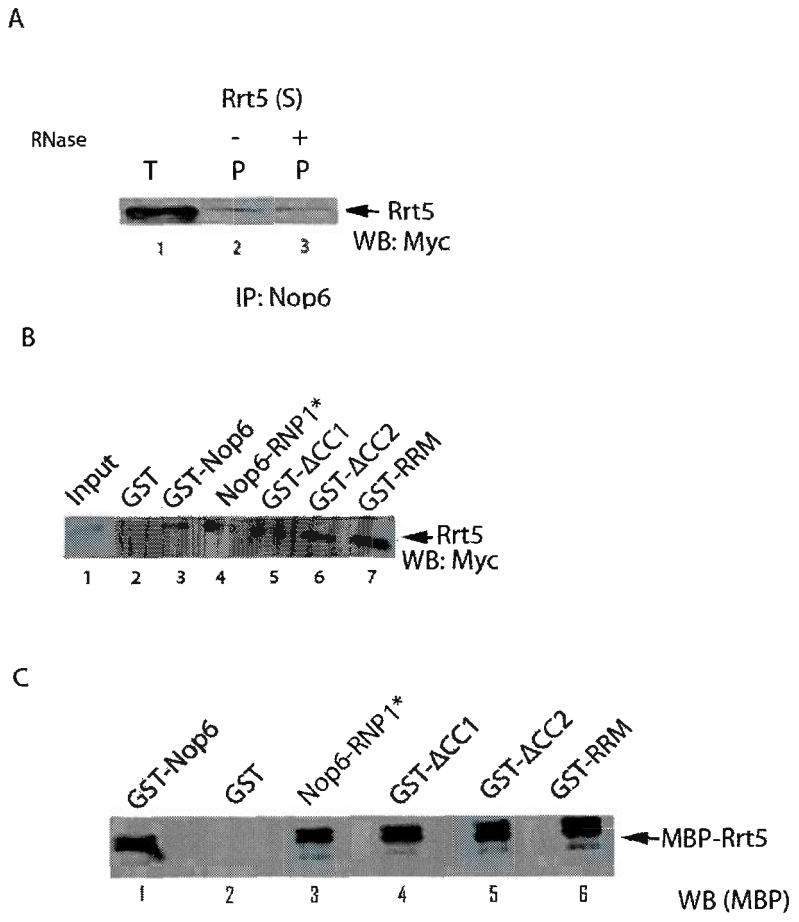
A
Rrt5 (S)
RNase
-
+
T
P
P
Rrt5
WB: Myc
1
2
3
IP: Nop6
B
Input
GST
GST-Nop6
Nop6-RNP1*
GST-ΔCC1
GST-ΔCC2
GST-RRM
Rrt5
WB: Myc
1
2
3
4
5
6
7
C
GST-Nop6
GST
Nop6-RNP1*
GST-ΔCC1
GST-ΔCC2
GST-RRM
MBP-Rrt5
WB (MBP)
1
2
3
4
5
6

Figure 3.3

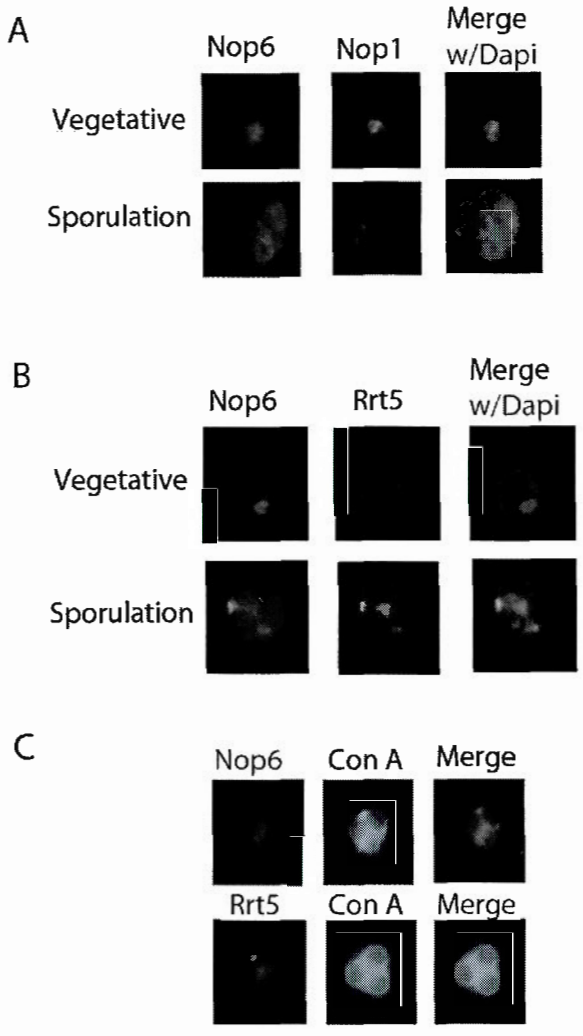
A
Nop6
Nop1
Merge w/Dapi
Vegetative
Sporulation
B
Nop6
Rrt5
Merge w/Dapi
Vegetative
Sporulation
C
Nop6
Con A
Merge
Rrt5
Con A
Merge

Figure 3.4

CHAPTER 4: GENERAL DISCUSSION AND PERSPECTIVES

Nop6 is a non-essential protein that was originally identified as a member of a group of proteins called hydrophillins due to their high glycine content and hydrophilicity (Garay-Arroyo et al 2000b). A network based algorithm of large scale data to assign gene function predicted Nop6 to be a fungal-specific nucleolar protein involved in rRNA processing (Samanta & Liang 2003). Previous analysis has also concluded that Nop6 is a basic protein since it is rich in lysine and arginine and contains monopartite and bipartite nuclear localization signal (NLS) (Garcia-Gomez et al 2011). Recently Nop6 was shown to associate with the small nucleolar RNA snR30, a snoRNA involved in 18S rRNA cleavage, although it seems to interact with other snoRNAs as well (Lemay et al 2011).

Our bioinformatics results suggest that Nop6 contains two CC motifs, which flank an RNA reconition motif (RRM) (Figure 1.2). The presence of CC motifs is indicative of associations with other proteins and we have hypothesized that Nop6 regulates ribosome biogenesis through its association with other proteins. In order to find possible partners of Nop6, we did a vast literature search and used the yeast two-hybrid system. After screening three genomic libraries (C1, C2 and C3; James et al, 1996), using full-length Nop6 as bait, we have found 2 positive clones corresponding to fragments of Fir1 in the C3 library. Unlike the C1 and C3 libraries, we were unable to screen a sufficient amount of transformants with the C2 library, as one needs to screen 2, 000, 000 transformants to cover 99 % of the library. We could have used an

alternative to the two-hybrid screen to find potential partners by doing a GST-pulldown with columns containing GST-Nop6 and trapping partners from a whole cell extract and analyzing samples by mass spectrometry. Fir1 protein localized to the bud neck and is upregulated during mitosis, but it does not contain a CC motifs or any particular structural motif. We have confirmed our results from the two-hybrid screen where Fir1 associates with Nop6 through GST-pulldown. However, due to the low abundance of Fir1, we were unable to show a co-immunoprecipitation of Fir1.

A previous large-scale study has shown that Nop6 interacts with Rrt5, a factor involved in rDNA transcription (Uetz, Giot et al. 2000; Hontz et al 2009). Using the yeast two-hybrid system we have confirmed this interaction. Also, through *in vitro* experiments such as GST-pulldown we have shown that Nop6 and Rrt5 interact. Rrt5 has been shown to be up regulated during meiosis and is thought to be a middle or mid-late sporulation gene (Hontz et al 2009). We have shown using immunofluorescence microscopy that Nop6 and Rrt5 co-localize specifically during meiosis, indicating a possible meiosis specific function. In addition, deletion of *NOP6* causes a decrease in sporulation, but this effect is reversed with the deletion of *RRT5*. The fact that Nop6 co-localizes with a protein that is highly expressed during sporulation and causes a loss in sporulation indicates it may have a sporulation specific effect. Identifying direct interacting partners of Nop6 is important to more fully understand its role during ribosome biogenesis, as this process is vital for protein synthesis. There are also several diseases caused by defects in ribosome biogenesis, thus understanding better how factors regulate vital steps will allow the

development of further treatments.

Perspectives:

Interestingly, we have shown that Nop6, a protein involved in ribosome biogenesis, interacts with Rrt5 although this interaction does not appear to involve the CC domains of Nop6. In order to further examine which region of Nop6 interact with Rrt5, future experiments could involve further deletion constructs of Nop6 and Rrt5 in order to determine the interacting regions of these proteins. We have shown that this interaction is not RNA-dependent as RNase treatment did not inhibit this interaction; thus, this interaction could be mediated by regions flanking the RRM region of Nop6. Once the region of interaction between Nop6 and Rrt5 is determined future work could focus on using BLAST analysis to determine the structural motifs mediating this interaction. One could also do pulldown experiment using GST-Nop6 with whole cell extracts from cells expressing Rrt5-myc during vegetative growth or sporulation, followed by SDS-PAGE and mass-spectrometry to determine proteins associated with Nop6 and Rrt5. Moreover, future experiments could involve two-hybrid analyses of proteins associated with Nop6 and Rrt5.

Our studies have shown that Nop6 and Rrt5 appear to co-localize specifically during sporulation to the cells wall of spores, and *NOP6* KO appears to lower sporulation. Further experiments to study the dynamics during sporulation could involve FRET analysis of Nop6, Rrt5 and spore wall specific proteins to determine proteins interacting with Nop6 and Rrt5 during sporulation. Furthermore, Rrt5

appears to contain a MSE element so another study could focus on mutating the MSE to determine if cells could still enter meiosis. Finally one could study the dynamics of ribosome biogenesis during sporulation involving Nop6 and Rrt5. One could do polysome profiling with *NOP6* KO *and RRT5* KO cells during sporulation to determine if there is a change in the ribosomal profile. In addition, snoRNAs that are important for ribosomal maturation could be detected using northern blot analyses to determine their profile during sporulation of these KOs.

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Appendice

6.1 Prediction of Nop6 coiled-coil domain

Coiled-coil (CC) domains are important for mediating the association between many protein factors as they function in their proper cellular environments' and they are one of the most important domains for mediating interactions. These domains contain autonomous repetitive heptad sequences of (abcdefg)n (Ulijn & Woolfson 2010). In order to identify the presence of possible CC domains within Nop6, the bioinformatics tool COILs was used. This analysis enabled us to identify the presence of two CC domains in the N and C-terminal portions of Nop6 (Figure 6. 1A). The first CC domain had a prediction of 80% whereas the second had a probability of 100 %. Nop6 is also predicted to contain an RRM (RNA recognition motif, Figure 6. 1B). This analysis allowed for the construction of deletion mutants of Nop6 which lack the CC1, CC2 or both domains in bait vector pGBKT7 for further analysis in the two hybrid system. For the two-hybrid screen only the full-length ORF of Nop6 cloned in pGBKT7 was used to screen for potential partners.

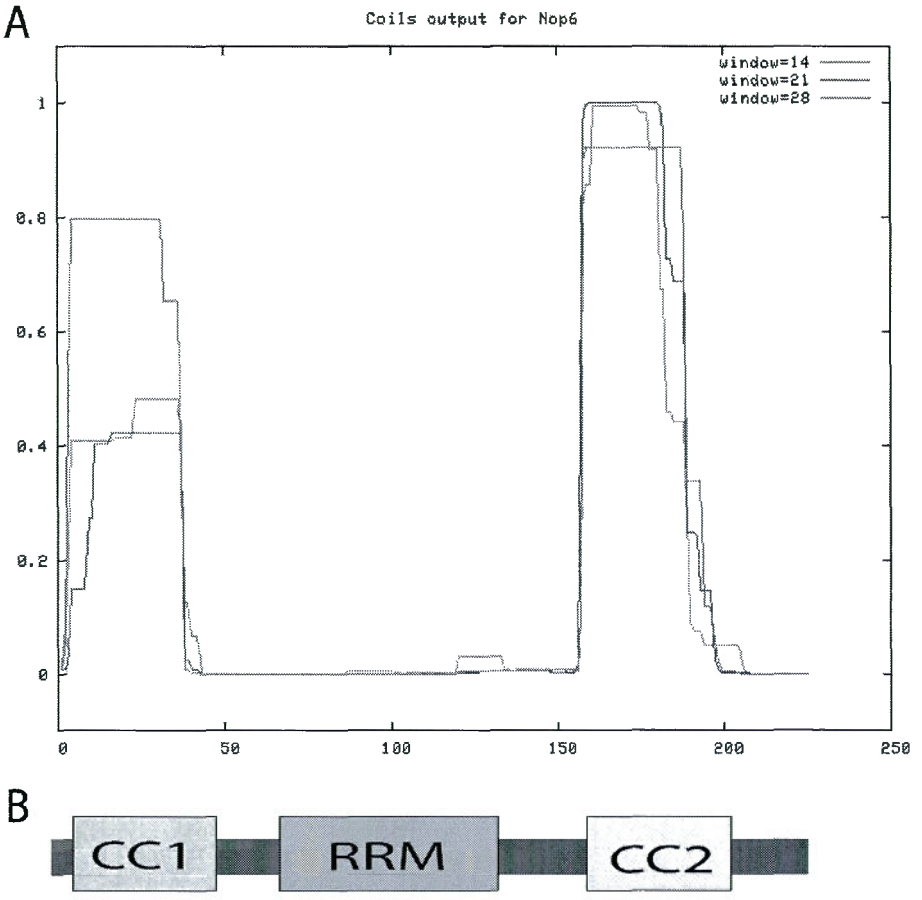

Figure 6. 1 Prediction of coiled-coil domains of Nop6

(A) Prediction of CC domains of Nop6 with probability of 80 and 100 % in the N-terminal and C- terminal regions. The x-axis represents the amino acid residue of Nop6 (225 amino acids) and the y-axis represents the percent probability of the presence of these CC domains. The three curves represent the three p frames of 14, 21 and 28 on COILS (Lupas et al, 1991).

(B) Schematic representation of Nop6 Coiled-Coil domains CC1 (position 3-52), CC2 (159-187) and RRM (65-130).

6.2. Cloning possible prey partners of Nop6 into prey vector pGADT7

The yeast two-hybrid system is a valuable tool to screen for protein partners which interact *in vivo* with a protein of interest. PCR amplification of prey was conducted (Table 6.4). PCR fragments (10% of intial reaction) were migrated on 1% agarose gel to confirm amplification followed by gel extraction (Feldan). An example of confirmation of PCR was the presence of RDH54 and FIR1 fragments after amplification (Figure 6. 2A). Potential prey were cloned into pGADT7 which contains a HA epitope tag (Figure 6. 2.B) with RDH54 cloned between XmaI and XhoI restriction sites (Figure 6. 2B).

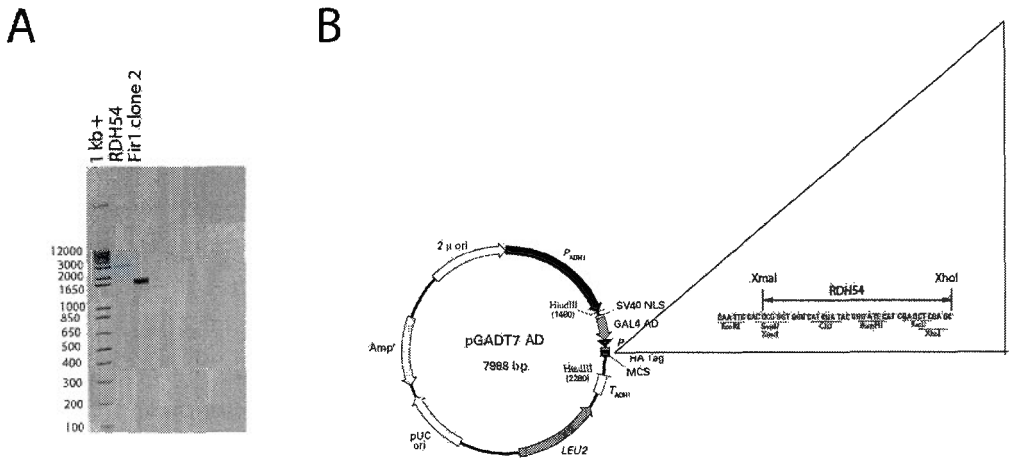

Figure 6. 2 Example of PCR amplification and cloning of possible Nop6 partners into prey vector pGADT7.

(A) 1 % agarose gel of RDH54 and Fir1 clone 2 found in two-hybrid screen. The marker 1 kb+ (Invitrogen) and molecular weight (base pairs) are represented. (B) Schematic of RDH54 open reading frame cloned between sites XmaI and XhoI in pGADT7 and fused to AD (Activation domain)(Clonetech Matchmaker).

6.3. Screen of genomic DNA libraries C1, C2, and C3 with pGBKT7-Nop6 bait

The three genomic libraries of pGAD consisting of different reading frames were screened with Nop6 as bait for potential partners using the yeast two-hybrid system. The number of transformants obtained from plating cells containing both bait and library vector on non-selective media of SD-Trp-Leu was 2 340 000, 250 000 and 2 334 000 for C1, C2 and C3 libraries Positive transformants were selected from selective media of SD-Trp-Leu-His+ 2 mM 3-AT and re-streaked on plates containing SD-Trp-Leu-His+ 2 mM 3-AT and X-α-gal to select for clones activating both *HIS3* and *MEL1* reporter genes on master plates (Table 6. 2)..

6.4 Analysis of plasmid DNA from positive clones of two-hybrid screen

AH109 colonies which activated both *MEL1* and *HIS3*, indicating an interaction of Nop6 with a potential partner, were selected and yeast plasmid preparations were conducted using Feldan DNA minipreparation kit (Feldan). Plasmids were isolated from yeast cells, transformed into *E. coli* DH10B, and four clones of bacteria were selected from each original yeast transformant for plasmid

preparation. Four bacterial colonies were selected because often yeast transformants may contain more than one library plasmid (James et al 1996b) and this can be verified by digestion with restriction enzymes which should produce a different digestion pattern if there is a difference between plasmids. Library prey vectors were digested with PstI and BglII (NEB) and digests were separated on a 1 % agarose gel (Figure 6. 3). Clones 31, 12 and 14 from library C1 showed similar profiles so just one preparation was chosen to be retransformed into AH109 with pGBKT7 alone or pGBKT7-Nop6 to choose transformants which were not auto-activators. Due to the size of each inserts certain inserts could adopt a conformation enabling auto-activation of reporter genes without the prescence of Nop6 (Bartel et al 1993). Positive yeast clones, including C1 library Clone 7, a yeast clone which was positive for MEL1 and HIS3 activity and whose DNA was extracted and retransformed into *E. coli*. Plasmids from four of these bacterial colonies were extracted and digested with PstI and BglII and separated on a 1% agarose gel (Figure 6.3). DNA preparations from Clone 7, lanes 1 and 2-4 differentiated in migration pattern after digestion thus indicating they contained different plasmids, and these preparations were transformed into AH109 with pGBKT7 or pGBKT7-Nop6 to confirm if these library plasmids were autoactivators. The two-hybrid interaction between Nop6 and these retransformed library plasmids was confirmed by their ability of growth on SD-Trp-Leu-His- +2 mM 3-AT only in the presence of Nop6 and a library plasmid and not with the empty vector pGBKT7. The pattern of digest reveals several bands in which could indicate PstI and BglII restrictions sites within each clone (Figure 6. 4).

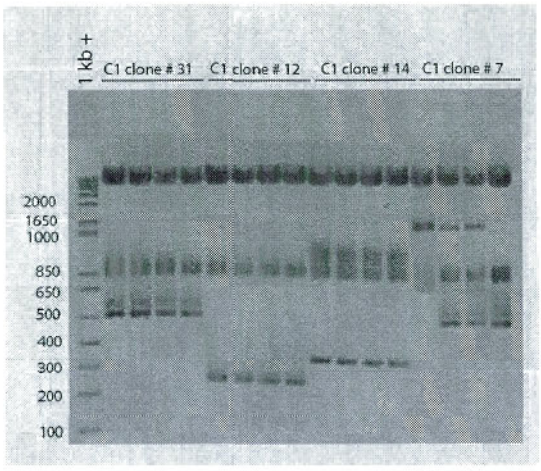

Figure 6. 3 Analysis of positive clones by digestion with PstI and BglII.

Plasmid DNA minipreps of positive clones #31, 12, 14 and 7 from library C1 were digested with PstI and BglII and migrated on a 1 % agarose gel. Ladder 1 kb+ (Invitrogen) was migrated with digests. Molecular weights of marker are represented in base pairs. Four plasmids isolated from four positive yeast clones are shown.

6.4 BLAST analysis of plasmids from positive clones and identification of prey proteins

Library plasmids retrieved from positive clones were sequenced using a forward primer hybridizing in the activation domain sequence of the construct before the ClaI site and a reverse primer after the PstI site (Table 6. 2). In order to analyze the sequence of plasmids from positive clones, a portion of the sequence after the ClaI site (James et al 1996b) were used to conduct BLAST analysis and compared to known sequences from the Saccharomyces Genome Database (SGD). Figure 6. 4 (A) shows the BLAST alignment of an Open reading frame (ORF) found in the library prey vector from a positive clone. The sequence in was used to search for potential homologous sequences matching it from a clone from the C3 library. The sequence matched a region of Fir1 (Figure 6.4 B), a protein involved in polyadenylation ((Mangus et al 2004). The ORF translation of this sequence is shown in Figure 6. 4C. In order to deduce the length of each clone, a reverse primer after the PstI site was used to verify the end of each insert. After screening each genomic library for possible partners of Nop6 one plasmid from the C1 library and four from the C3 library appeared to be true positives, all of which corresponded to fragments of Fir1 (Table 6. 1). Four clones retrieved from the C3 library corresponded to two fragments of Fir1.

Table 6. 1 Sequenced plasmids from potential positive clones

Library	Clone	Protein	Function	Localization
C1	C1 #1	False positive which includes bottom strand/ DYN2	Cytoplasmic light chain dynein,	Unknown
C1	C1#19 clone 1	False positive out of frame/MON2	Peripheral membrane protein in endocytosis	Endosome
C1	C1#19 clone 2	False positive producing short peptide/CAD1	AP-1-like basic leucine zipper (bZIP) transcriptional activator involved in stress responses	Cytoplasm and nucleus
C1	C1#33 clone 1	False positive producing short peptide/OGG1	Mitochondrial glycosylase	Mitochondria
C1	C1#33 clone 2	False positive producing short peptide/PCl1	Cyclin involved in the regulation of polarized growth	Nucleus and site of polarized buds
C1	C1# 36 clone 1	False positive with short peptide/CAD1		Cytoplasm and nucleus
C1	C1#50	False positive with short peptide/CAD1		Nucleus and site of polarized buds
				Cytoplasm and nucleus
C1	C1#10	False positive short peptide/DYN2		Unknown
C1	C1#62 clone 2	False positive with reverse complement/MAG1	3-methyl-adenine DNA glycosylase involved in protecting DNA against alkylating agents	Nucleus
C1	C1 #42 clone 1	False positive		
C1	C1#42 clone 2	False positive with short peptide/PDC1	Key enzyme in alcoholic	Cytoplasm and nucleus

			fermentation	
C1	C1#12 clone 3	False positive/PDC1		Cytoplasm and nucleus
C1	C1 #9 clone 1	False positive/DYN2		Unknown
C1	C1 #69	False positive		
C1	C1 #71 clone 1	False positive/BDF1	Protein involved in transcription initiation at TATA-containing promoters	Nuclear chromatin
C1	C1#63 clone 2	False positive/SWR1	Swi2/Snf2-related ATPase that is the structural component of the SWR1 complex, which exchanges histone variant H2AZ (Htz1p) for chromatin-bound histone H2A	Nucleus
C1	C1#10 clone 3	False positive/RDH54	DNA-dependent ATPas involved in recombinational repair of DNA double-strand breaks during mitosis and meiosis	Nucleus
C1	C1#8	False positive/MSA2	Putative transcriptional activator, that interacts with G1-specific transcription factors	Cytoplasm
C1	C1# 20	False positive/short peptide		
C1	C1#21	Fir1/produces a short peptide	Protein involved in 3' mRNA processing	Bud neck
C1	C1 #4	Fir1/produces a	Protein involved	Bud neck

		short peptide	in 3' mRNA processing	
C1	C1 # 7	False positive producing reverse complement/THI2	Transcriptional activator of thiamine biosynthetic genes	Nucleus
C1	C1 # 13	False positive producing reverse complement/SGS1	Nucleolar DNA helicase involved in chromosome synapsis	Nucleolus
C3	C3# 18	Fir1		Bud neck
C3	C3# 13	Fir1		Bud neck
C3	C3#19	Fir1		Bud neck
C3	C3#20	Fir1		Bud neck
C3	C3#4	False positive/DYN2		
C3	C3#33	False positive/DYN2		
C3	C3#9 clone 1	False positive with reverse complement/ATP3	Gamma subunit of the F1 sector of mitochondrial F1F0 ATP synthase	Mitochondria

Several of the clones produced false positives such as DYN2 and CAD1, which formed short peptides or reverse complements of these open reading frames. Four potentially interesting clones, which produced false positives as it contained intervening stop codons when translated, were cloned into pGADT7 in order to confirm if these were real candidates. There is a chance that certain constructs do produce fusion proteins, but in some cases inserts may be out of frame due to the inclusion of portions of the 5' UTR which disrupts the reading frame and proteins may have a real interaction with Nop6 in the normal cellular environment. Four potentially interesting clones which appeared as false positives included BDF1, a protein involved in transcription initiation of TATA-containing promoters, SWR1, a Swi2/Snf2-related ATPase that is the structural component of the SWR1 complex, RDH54, a DNA-dependent ATPase involved in recombination repair of DNA double-strand breaks during mitosis and meiosis and MSA2, putative transcriptional activator, that interacts with G1-specific transcription factors (Table 6. 1). RDH54, SWR1 and BDF1 associate in a complex and control chromatin remodelling, transcription and meiosis thus they could potentially interact with Nop6 and Rrt5, however after cloning these ORFs into pGADT7 we were unable to obtain viable transformants of AH109 containing both bait and prey constructs most likely due to the toxicity associated with overexpressing the full-length proteins. In addition, MSA2 interacts with G1-specific transcription factors and could be a protein linking Fir1 and Nop6 during mitosis, but unfortunately the fusion protein of MSA2 cloned in pGADT7 was not expressed. Fir1 was chosen for further analysis because it was

the only true positive clone and it is involved in 3' end formation of mRNAs and could indicate a role for nop6 in mRNA processing through its interaction with Fir1.

A

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1   NNNNNNcNNN NNNNNNNNNN NNcNatctat tcgatgatga agatacccca ccaaacccaa
61  aaaaagagat cgaatttccg ggggatccat cg[illegible]                    aggagtagct
121 tggtttacga tcgggatggc tatagccagt ttcaagaagg taacacctca tcgtcaactg
181 aatcttctct agaacagccc tcatggtcgt attcagatgc agatgattct attccatatg
241 ccccaccatga tgtgtctttc gagttaaata atgcagatgc tgataagctt cttggaattg
    atgagaaccc caacgtcaat ctgaagatac aaaggagaaa tctcaaaaac ccacaacata
361 ttaagagtaa aacagataag gaatgtgagg [illegible]  tgaaaaaaat gtttcgttaa
421 aaattcigtc aacaccaaat aaactgattg acatcccttg ctcagagcac atgaaatccc
481 cacaagcac aggtttaaat gggacattaa aatttttcca gcagtttgaa cctagcgagg
541 agccaacttc tccaactcgc caagttaacc ctgaatcact ggacaaactc gatatgtcat
601 tcaaatttcc ctccagtact acgaacaata acgtggatac ggtacctgaa aatcgaaact
661 ctggagcaac gaacaatgaa gatttcctga aagttgatac tccgcttgtg aatcaatcat
721 ttgaatccag aagacgatg ttaatagact tgnaaaaaca tccgacgaat aataatccga
    ggatacataa acatcggaga acacaggagcg ttcataaNat tgatgacatt tccttNNNtt
841 tcgaggcaac ctccacacca ccagctccga cctctgctcN Ntcaattcct gttgaacatt
901 ctaacccatN NNNNNNNNNN NaaataccNN NNNNNNNNNc cNNacNNNNc acNNNNNNtc
961 NaNNNNcNNN tgatNN
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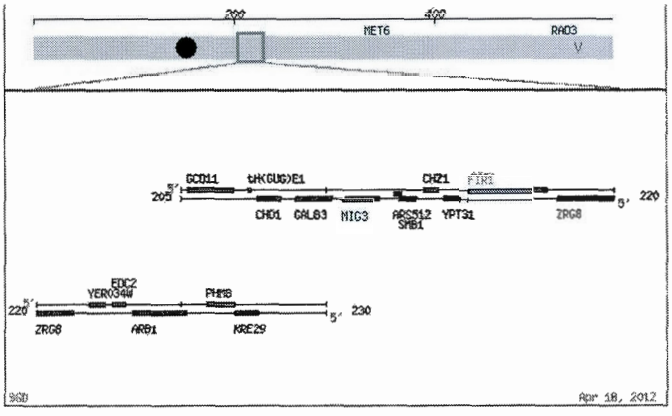
B



C

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QNKDKKRSSLVYDGDGYSQFQEGNTSSSTESSLEQPSSSYSDEDDSIPYAHHDV
SFELNNADADKLLGIDENANVNLKIQRRNLKNPQHIKSKTDRECEEKNTEKNVSLKILST
PNKLIDIPDLEHMKSPPSTGLNGTLKFFQQPEPSEEPTSPTRQVNPESLDKLDMSFKFPS
STTNNNVDKVH
```

Figure 6. 4 An example of bioinformatics analysis using BLAST of positive clones from two-hybrid screen from *S. cerevisiae.*

(A) Part of sequence of insert obtained from genomic library clone. Sequence after the ClaI restriction site was used for search and must be in frame with EcorI restriction site in order to have the correct reading frame. (B) Open reading frame map representing the resulting ORF obtained (Fir1 clone 1) from Saccharomyces

Genome Database, SGD. This region is that starting from aa 247 within Fir1 on chromosome V.

(C) Amino acid sequence of insert of Fir1.

Table 6. 2 Yeast two-hybrid screen of Nop6 partners

Genomic DNA library	Total transformants	#False positives	#Interactors
C1	2 340 000	21	1
C2	250 000	0	0
C3	2 334 000	3	4

6.5 Analysis of fusion protein products from baits and prey proteins

In order to confirm the interaction between Nop6 and its prey, constructs corresponding to potential partners of Nop6 were cloned into pGADT7 and their expression level was checked. One example of a prey construct that was an interesting candidate for interacting with Nop6, Krr1, was found after a literature search. Large scale tandem affinity purification recently showed that Nop6 appears in the same complex as Krr1 (Krogan et al 2006). A large RNP required for the processing of the small-ribosomal-subunit rRNA, called the small subunit (SSU) processome has been purified and shown to contain U3 and 28 other proteins (Dragon et al 2002). Krr1 has been shown to be part of this complex according to criteria established in a recent study where it is a nucleolar protein, co-immunoprecipitates with U3 snoRNA and MPP10 and is involved in 18S rRNA processing (Bernstein et al 2004). In order to study if there is a direct interaction between Nop6 and Krr1, AH109 cells were transformed with pGBKT7-Nop6 and pGADT7-Krr1 to analyse their two-hybrid interaction. In addition the empty vector, pGBKT7 and pGADT7 were transformed with pGADT7-Krr1 and pGBKT7-Nop6 and yeast cells expressing both vectors were selected on media containing SD-Leu-Trp. The analysis of the expression of fusion proteins was conducted according to (Kushnirov 2000) and proteins were resolved on 10% resolving polyacrylamide gels and transferred to PVDF membranes. In order to detect fusion protein expression, western blots were conducted using anti-myc (9E10) for pGBKT7 which expresses a Myc epitope and

anti-HA for pGADT7 that includes a HA epitope tag (Clontech matchmaker). The empty vectors of pGBKT7 and pGADT7 expressed DNA binding domains and activation domains that migrated at 22 kDa, (Figure 6. 5A (lanes 3, 4) and b (lane 6). We were able to detect both Nop6 fusion proteins from the bait vector and Krr1 from the prey vector from two independent clones (Figure 6. 5A (lanes 5, 6) and B (lanes 2-5). A positive control, which includes Bfr2 under the galactose inducible promoter with a N-terminal 3-HA tag and expressing a C-terminal 9-myc tag on Enp2, was also loaded. After plating cells on selective media AH109 cells containing pGBKT7-Nop6 and pGADT7-Krr1 did not show any interaction as they were unable to grow on SD-HLW+2 mM 3-AT. Other possible candidates such as Rdh54, Swr1 and Bdf1 were found in the two-hybrid screen (Table 6. 1), but appeared as false positives due to the fact that the retrieved sequences contain stop codons. These were interesting candidates for interacting with Nop6 due to their function during meiosis, however probably due to their toxicity, transformants could not be retrieved and fusion proteins could not be measured. Since the full-length sequence of Rdh54, Swr1 and Bdf1 appear to be toxic for cells, one could clone functional motifs from these proteins into bait and prey vectors of the two-hybrid system to confirm their interaction with Nop6. For example, Swr1 contains DEXDc DEAD box motif and HELICc motif (Simple Modular Architecture Research Tool or SMART tool) so these could be cloned to see if they mediate the interaction between Nop6 and Swr1.

A portion of Fir1 was shown to interact with Nop6 when expressed from a library vector and was thus selected for further studies (Table 6. 1). The full-length

construct of Fir1 appeared to be toxic for cells as no yeast transformants could be obtained when it was expressed from pGADT7. In addition after cloning the portion of Fir1 corresponding to clone 2, no expression of Fir1 could be detected. Thus, confirmation of its interaction with Nop6 could not be conducted with pGADT7. Conversely, Rrt5, which appears to be involved in rDNA transcription and is overexpressed during meiosis, was cloned into the two-hybrid vector pGADT7, and it was shown to be a stable partner of Nop6.

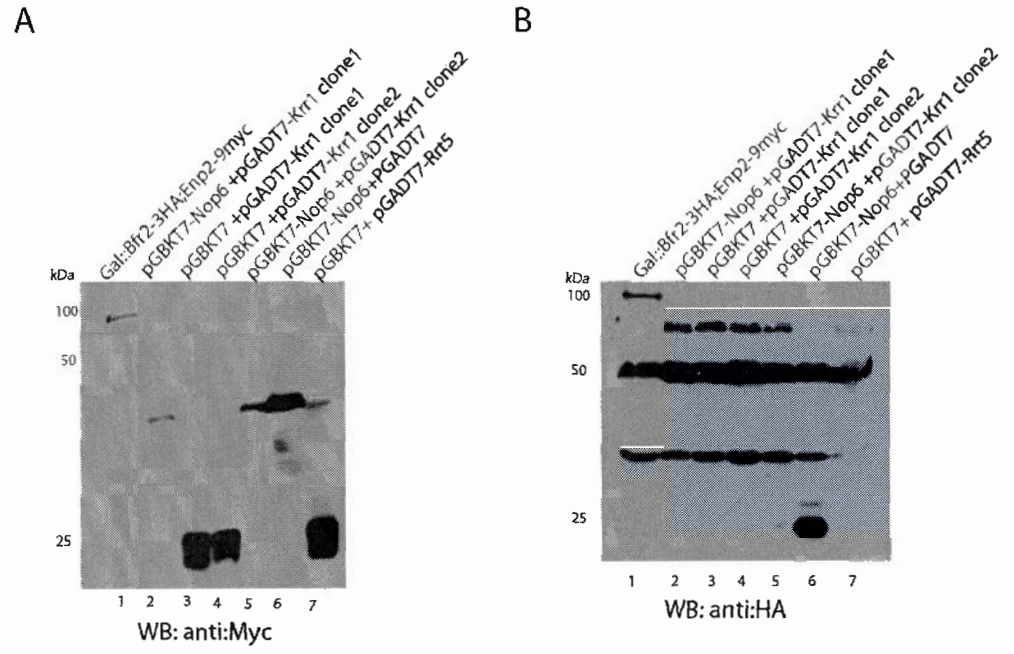

Figure 6. 5 Example of analysis on polyacrylamide gel of protein extractions of expression of bait and prey proteins to confirm two-hybrid interactions.

10% polyacrylamide gel was used to enhance separation of the DNA binding domain (A) and Activation domain (B) reflected by empty vectors pGBKT7 or pGADT7. Proteins were extracted according to (Kushnirov 2000) and for each well 10 µl of sample was loaded with SDS 2X sample buffer.

6.6 Nop6 interacts with Fir1 in the yeast two-hybrid system

We confirmed that two fragments of Fir1 obtained from positive clones interact with Nop6, while expressing just the empty vector did not cause auto-activation.

The positive clones of Fir1 code for the region of Fir1 encompassing amino acids 247-807 and 247-842. We have also cloned full-length Fir1 into pGADT7 in order to confirm this interaction with Nop6, however this was toxic for cells.

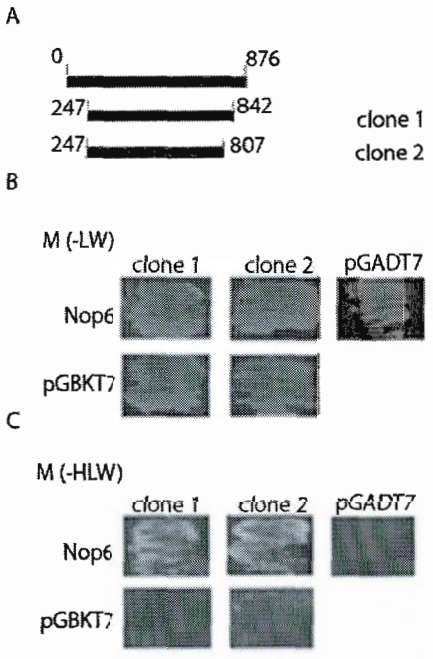

Figure 6. 6 Two-hybrid interaction of Nop6 and Fir1 fragments retrieved from a genomic DNA screen includes two regions.

(A) Fir1 fragments encompassing regions: Clone 1 (AA 247-842) and clone 2 (247-807). Cells were plated on medium lacking leucine and tryptophan (B) or medium lacking leucine tryptophan and histidine (C). Nop6 was expressed from the bait vector pGBKT7, and Fir1 was expressed from the library vector pGAD C3 (James et al 1996a).

6.7. The interaction between Nop6, and Fir1 is not RNA-mediated

As the yeast two-hybrid system can sometimes produce artefacts, we used standard *in vitro* methods to confirm the association of Nop6 with Fir1. We used a Fir1-TAP in pull-down assays with GST-Nop6 mutants immobilized on glutathione sepharose. The RRM is also a well-recognized motif in eukaryotes and its structure is characterized by the packing of two alpha-helices on a four-stranded beta-sheet (Clery et al 2008). Our previous finding that the interaction between Nop6 and Rrt5 does not require the CC motifs displayed a possible role for the RRM motif (and flanking regions) and we tested if this was the case with Fir1 as well.

The association of Nop6 and Fir1 does not require the CC1 and CC2 domains of Nop6 (Figure 6. 7A). The association of Nop6 and Fir1 is not RNA-mediated as mutations in RNP1 motif or the presence of RNase A does not inhibit this interaction (Figure 6.7 A and B.). Fir1-TAP bound to GST-Nop6 constructs, but not GST alone (Figure 6.7.).

The association between Nop6 and Fir1 was tested in GST pulldowns. These experiments indicated that Nop6 and Fir1 could interact under certain cellular conditions. Since Fir1 is in low abundance, we used a higher amount of whole cell extract for experiments with Fir1-TAP; however, Fir1-TAP was still unable to co-immunoprecipitate Nop6 (data not shown). Fir1 was previously shown to interact with Pap1, a protein important for mRNA polyadenylation (del Olmo et al 1997). Future experiments could use knockouts of Fir1 and Nop6 and examine if there is a

decrease in polyadenylation. Also, immunoprecipitations of Pap1 could be conducted, and one could examine if Nop6 interacts with Pap1 and study the relationship of Nop6, Pap1 and Fir1.

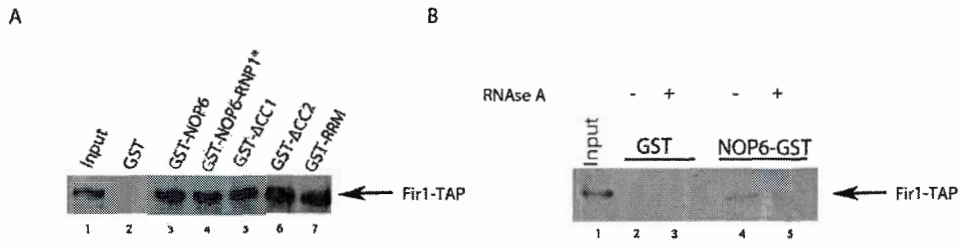

Figure 6. 7 The interaction of Fir1 and Nop6 is not RNA-mediated.

(A) Total extract of Fir1-TAP was co-immunoprecipitated with GST-Nop6 full length and mutants (lanes 3-7), but not GST alone (lane 2). (B) Yeast cellular extracts were untreated (2, 4), or treated with RNase A 1mg/ml (3, 5). Fir1-TAP was co-immunoprecipitated with Nop6 bound GST beads. 5% of total extracts were used as input with cellular extracts from 50 ODs of cells loaded on beads.